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An investigation of the relationship between the levels of oxidative stress in mesenteric and peripheral serum and clinicopathological variables in colorectal cancer

ABSTRACT

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

Material and Methods: 150 patients with colorectal cancer who underwent surgery between May 2005 and March 2010 were prospectively analyzed. The differences between the 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.

Introduction

Colorectal cancer is one of the most frequently occurring types of cancer in human populations and 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 the 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

30 oxidative nucleoside damage (4). Damage to DNA, proteins, cell membranes, and mitochondria is involved
31 in carcinogenesis, although no specific biochemical marker has yet been confirmed.

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

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

41 **Materials and Methods**

42 The patients were volunteers and were treated in accordance with the protocol approved by the research
43 ethics committee of the local institution. In our study, 150 patients with colorectal cancer were prospectively
44 analyzed. These patients were surgically treated by General Surgery, Haseki Education and Research
45 Hospital, Istanbul and Yuzuncu yıl University Faculty of Medicine, Van. The operations were performed
46 between May 2005 and September 2010. Surgical resection was performed on 130 patients, while the
47 tumors were considered unresectable in 20 patients.

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

52 The variables analyzed included the staging of the colorectal cancer by means of TNM classification, the
53 degree of cell differentiation, the diameter of the tumor, and the presence or absence of lymphatic invasion.
54 According to the TNM classification, 35 patients were in Stage I, 40 were in Stage II, 42 were in Stage III,
55 and 33 were in Stage IV. With regard to the degree of cell differentiation, there were 50 patients with well-
56 differentiated (WD) tumors, 60 patients with moderately-differentiated (MD) tumors, and 40 patients with
57 poorly-differentiated (PD) tumors. Regarding the diameter of the tumor, 41 patients had tumors \leq 3.9 cm in
58 diameter, 65 patients had tumors 4.0-7.9 cm in diameter, and 44 patients had tumors \geq 8.0 cm in diameter.
59 The presence of venous invasion was identified in the lesions of 37 patients, while lymphatic invasion was

60 identified in 113 patients. Demographics and other selected characteristics of the cases are presented in
61 Table 1.

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

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

70 *TBARS Assay*

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

75 Plasma (400 µl) prepared by the hydrolysis of 1,1,3,3-tetramethoxypropane (Sigma Chemical Co.) was
76 mixed with 400 µl orthophosphoric acid (0.2 mol/l) (Sigma Chemical Co.) and 50 µl BHT (2 mmol/l)
77 (Sigma Chemical Co.) in 12 x 72 mm tubes. A total of 50 µl TBA reagent (0.11 mol/l in 0.1 mol/l NaOH)
78 (Fluka Chem.) was then added, and the contents were mixed. Subsequently, the contents were incubated at
79 90°C for 45 min in a water bath. The tubes were then kept on ice in order to prevent further reaction.
80 TBARS were extracted once with 1000 µl n-butanol (Sigma Chemical Co.). The upper butanol phase was
81 read at 535 nm and 572 nm in order to correct for baseline absorption in the Shimadzu UV-1601 (Shimadzu)
82 UV-spectrophotometer. MDA equivalents (TBARS) were calculated using the difference in absorption at
83 these two wavelengths, and quantification was performed with a calibration curve (10).

84 *4-HNE Assay*

85 4-HNE was measured by enzyme-linked immunosorbent assay (ELISA).

86 *Statistical Analysis*

87 Data are presented as means ± SD. The comparison of the groups was performed using the Kruskal-Wallis
88 one-way analysis of variance. P<0.05 was taken as significant. Binary (post hoc) comparisons and a
89 Bonferroni-corrected Mann-Whitney U test (significance limit was taken as P <0.0033) were made.

90 Analyses were performed using the SPSS 17.0 statistical package program. P values < 0.05 were considered
91 statistically significant.

92 **Results**

93 Two statistical analysis methods were performed, one numerical and the other categorical, and each of the
94 markers was analyzed in relation to its peripheral and mesenteric concentrations. With regard to the
95 numerical, descriptive measurements of the MDA levels, the mean for MDA (M) was 2.76 nmol/L \pm 2.12
96 nmol/L and the mean for MDA (P) was 2.64 nmol/L \pm 2.27 nmol/L, with a statistically significant difference
97 ($p < 0.05$). The comparison between the proportions of positive rates of mesenteric and peripheral MDA was
98 performed by means of a marginal homogeneity test. No statistical difference was found.

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

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

106

107 **Discussion**

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

118 The formation of ROS is a normal event in primal biochemical reactions. Oxygen radicals can be formed in
119 elderly patients with chronic diseases of the gastrointestinal system (5). The primary source of oxidants in

120 the gut is presumably phagocytes, which are accumulated in the mucus of patients with bowel diseases and
121 could affect oxidants upon activation. This might contribute to the increased risk of cancer (13).

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

133 In colorectal cancers, the local cytokine network and the levels of nitric oxide (NO) and ROS are known to
134 be closely related to cancer progression and metastasis (15). Similar to previous studies, we found that the
135 levels of ROS in blood were higher in cases of advanced colorectal cancers. The levels of MDA and 4-HNE
136 in colorectal cancer samples were significantly increased with the clinical staging of the disease. Our
137 findings were in accordance with previous work that reported increased plasma MDA concentrations in
138 colorectal cancer patients (16, 17). 4-HNE was found to be genotoxic in primary cultures of rat hepatocytes
139 at low concentrations, which might occur in in vivo conditions of oxidative stress (18). However, colonocytes
140 exposed to 4-hydroxy-2-nonenal in in vivo conditions could undergo a similar oxidative stress. Moreover,
141 the reaction of aldehydes produced during lipid peroxidation with amino acid residues of proteins might lead
142 to their oxidative modification (19). In this process, the final products of lipid peroxidation, such as MDA
143 and 4-HNE as well as other products resulting from polyunsaturated fatty acid damage, could cause protein
144 breakdown (20).

145 In our study, we showed significant differences between peripheral and mesenteric MDA levels. In the first
146 investigation, the sample was composed of 250 patients who were analyzed retrospectively. All of them
147 underwent the peripheral and mesenteric assaying of their oxidative stress marker levels, which were
148 evaluated in relation to histopathological variables. Both of these markers had high levels in TNM Stage IV,
149 both in mesenteric and peripheral blood. Thus, the markers had significantly higher levels when the

150 neoplastic disease was no longer limited to the colon. These results probably indicate the presence of liver
151 metastasis or occult lymph node metastasis. In our results, the mesenteric and peripheral oxidative stress
152 levels were also higher in the presence of venous invasion. This may corroborate our hypothesis that
153 drainage via the portal vein system is an important component of the distribution of these markers. These
154 markers in the peripheral blood have still not been studied clearly. They could be distributed via the portal
155 vein system, the lymphatic systems, or both. Our results showed that there is a strong association between
156 mesenteric and peripheral oxidative stress markers and the extent of venous invasion and the grade of
157 invasion into the colorectal wall. These biomarkers have demonstrated usefulness in following up patients
158 who have undergone surgery with curative intent, with increases in their levels in the event of potential
159 tumor recurrence or the development of metastases. In this study, there were associations between peripheral
160 and mesenteric oxidative stress levels.

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

165
166 There are no conflict of interest.

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217 **Table 1.** Demographics and other selected characteristics of the patients.

		218
Mean age (years)	58.5 (21-80)	219
Woman, n(%)	89 (59.4)	220
Tumor stage, n(%) [*]		221
Stage I	35 (23.4)	222
Stage II	40 (26.6)	223
Stage III	42 (28)	224
Stage IV	33 (22)	225
Cell differentiate, n(%)		226
Well	50 (33.4)	227
Moderately	60 (40)	228
Poorly	40 (26.6)	229
Tumor diameter (cm), n(%)		230
≤ 3.9	41 (27.3)	231
4.0-7.9	65 (43.3)	232
≥ 8.0	44 (29.4)	233
Lymphatic invasion, n(%)	113 (75.3)	234
Venous invasion, n(%)	37 (24.7)	235
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239 ^{*}Tumor stage was obtained according to the TNM classification criteria

240 **Table 2.** Descriptive measurements of the oxidative stress markers and the histopathological variables and
 241 staging of the colorectal cancer (mean \pm SD).
 242

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

243
 244 * According to the TNM classification criteria