• BRIEF REPORTS •

Lipid peroxidation and antioxidant status in colorectal cancer

Elzbieta Skrzydlewska, Stanislaw Sulkowski, Mariusz Koda, Bogdan Zalewski, Luiza Kanczuga-Koda, Mariola Sulkowska

Stanislaw Sulkowski, Mariusz Koda, Luiza Kanczuga-Koda, Mariola Sulkowska, Department of Pathology, Medical University of Bialystok, Poland
Elzbieta Skrzydlewska, Department of Analytical Chemistry, Medical University of Bialystok, Poland
Bogdan Zalewski, Department of General Surgery, Medical University of Bialystok, Poland
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Correspondence to: Elzbieta Skrzydlewska, Department of Analytical Chemistry, Medical University of Bialystok, Mickiewicza 2, 15-230 Bialystok, Poland. skrzydle@amb.edu.pl
Telephone: +48-85-7485707   **Fax:** +48-85-7485707
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Abstract

AIM: Reactive oxygen species (ROS) can induce carcinogenesis via DNA injury. Both enzymatic and non-enzymatic parameters participate in cell protection against harmful influence of oxidative stress. The aim of the present study was to assess the levels of final lipid peroxidation products like malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) in primary colorectal cancer. Moreover, we analysed the activity of main antioxidative enzymes, superoxide dismutase (Cu, Zn-SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GSSRG-R) and the level of non-enzymatic antioxidants (glutathione, vitamins C and E).

METHODS: Investigations were conducted in 81 primary colorectal cancers. As a control, the same amount of sample was collected from macroscopically unchanged colon regions of the most distant location to the cancer. Homogenisation of specimens provided 10% homogenates for our evaluations. Activity of antioxidant enzymes and level of glutathione were determined by spectrophotometry. HPLC revealed levels of vitamins C and E and served as a method to detect terminal products of lipid peroxidation in colorectal cancer.

RESULTS: Our studies demonstrated a statistically significant increase in the level of lipid peroxidation products (MDA-Adc. muc.-2.65±0.48 nmol/g, Adc.G3-2.15±0.44 nmol/g, clinical IV stage 4.04±0.47 nmol/g, $P<0.001$ and 4-HNE-Adc.muc. -0.44±0.07 nmol/g, Adc.G3-0.44±0.10 nmol/g, clinical IV stage 0.52±0.11 nmol/g, $P<0.001$) as well as increase of Cu,Zn-SOD (Adc.muc.-363±72 U/g, Adc.G3-318±48 U/g, clinical IV stage 421±58 U/g, $P<0.001$), GSH-Px (Adc.muc. -2143±623 U/g, Adc.G3-2005±591 U/g, clinical IV stage 2467±368 U/g, $P<0.001$) and GSSG-R (Adc.muc.-880±194 U/g, Adc.G3-795±228 U/g, clinical IV stage 951±243 U/g, $P<0.001$) in primary tumour comparison with normal colon (MDA-1.39±0.15 nmol/g, HNE-0.29±0.03 nmol/g, Cu, Zn-SOD-117±25 U/g, GSH-Px-1723±189 U/g, GSSG-R-625±112 U/g) especially in mucinous and G3-grade adenocarcinomas as well as clinical IV stage of colorectal cancer. We also observed a decrease of CAT activity (Adc.muc. -40±14 U/g, clinical IV stage 33±18 U/g vs 84±17 U/g, $P<0.001$) as well as a decreased level of reduced glutathione (clinical

IV stage 150±48 nmol/g vs 167±15 nmol/g, $P<0.05$) and vitamins C and E (vit. C-clinical IV stage 325±92 nmol/g vs 513±64 nmol/g, $P<0.001$; vit. E-clinical IV stage 13.3±10.3 nmol/g vs 37.5±5.2 nmol/g).

CONCLUSION: Colorectal carcinogenesis is associated with serious oxidative stress and confirms that gradual advancement of oxidative-antioxidative disorders is followed by progression of colorectal cancer.

INTRODUCTION

Colorectal cancer is one of the most frequent neoplastic diseases in human population and one of the most frequent causes of death. There are a lot of pathological factors, including reactive oxygen species (ROS) involved in the process of cancer initiation and progression[1]. Damages to DNA, protein, cell membrane and mitochondria are involved in carcinogenesis, although no specific biochemical marker has been identified yet. In addition, information on the biochemical alterations in tissue and blood, particularly of antioxidant status, and its correlation with the clinical staging of the disease, is lacking.

It is known that ROS are formed in excess in chronic diseases of the gastrointestinal tract[2] but the precise mechanisms of oxidative stress being induced in cancer cells and the role of ROS in colorectal cancer progression are still not exactly understood. Changes in some parameters of antioxidative system in colorectal cancer were found in our earlier studies[3]. The current researches, comprising a more extensive group of patients, dealt with the analysis of connections between the degree of lipid peroxidation as well as the parameters of antioxidative system and selected anatomico-clinical features of carcinoma.

The aim of the present study was to assess the levels of lipid peroxidation products like malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) in primary colorectal cancer. Moreover, we analysed the non-enzymatic antioxidants (glutathione, vitamins C and E) and the activity of antioxidative enzymes, particularly superoxide dismutase (Cu, Zn-SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GSSRG-R).

MATERIALS AND METHODS

Investigations were conducted in 81 primary colorectal cancers in II, III and IV clinical stage[4]. As a control, the same amount of samples was collected from macroscopically unchanged colon regions of the most distant location to the cancer.

Conventional histopathological parameters, including AJCC/UICC TNM stage, tumor type, and grade of differentiation, were assessed by two independent pathologists. Differentiation and histological type of the cancer were determined following the World Health Organization guidelines[4].

Preparation of tissue samples

Tissues were removed quickly and placed in iced 0.15 mol/L NaCl solution, perfused with the same solution to remove blood cells. Next, tissue samples were blotted on filter paper, weighed and homogenized in 9 mL ice-cold 0.25 mol/L sucrose and 0.15 mol/L NaCl with addition of 6 μL 250 mmol/L butylated hydroxytoluene in ethanol, to prevent formation of new peroxides during the assay. Homogenization procedure was performed under standardized conditions; 10% homogenates were centrifuged at 10 000 g for 15 min at 4 ℃ and the supernatant was kept on ice until assayed.

Biochemical assays

Superoxide dismutase (Cu, Zn-SOD; EC.1.15.1.1) activity was measured after rehomogenization of the initial supernatant and centrifugation at 10 000 g for 30 min at 40 ℃ as described by Sykes et al[5]. This method could measure the activity of cytosolic SOD. Mn-SOD of the mitochondria was known to be removed during the separation procedure. A standard curve for SOD activity was constructed using SOD from bovine erythrocytes (Sigma, Biochemicals, St. Louis, MO, USA). One unit of SOD activity was defined as the amount of the enzyme required inhibiting the oxidation of epinephrine to adrenochrome by 50%. The activity was expressed in universal units. Glutathione peroxidase (GSH-Px; EC.1.11.1.6) activity was measured spectrophotometrically using a technique based on the procedure of Paglia and Valentine[6], whereas GSH formation was monitored by measurement of oxidation of reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) to oxidized form of nicotinamide adenine (NADP). The final concentration of GSH was 0.2 mmol/L and of H_2O_2 was 0.3 mmol/L. The activity was expressed in universal units. Glutathione reductase (GSSG-R; EC.1.6.4.2) activity was determined with the method of Mize and Langdon[7] by monitoring the NADPH at 340 nm. The reaction mixture contained 0.2 mol/L KCl, 1 mmol/L EDTA and 1 mmol/L oxidized GSH (GSSG) in 0.1 mol/L potassium phosphate buffer, pH 7.1. The activity was expressed in universal units. Catalase (CAT; EC.1.11.1.9) activity was measured in supernatant of the brain or liver homogenates with 1% Triton X-100. The decrease in absorbance of hydrogen peroxide at 240 nm was measured[8]. The rates were determined

at 25 ℃ using 10 mmol/L hydrogen peroxide. The activity was expressed in universal units. Glutathione (GSH) concentration was measured using the Bioxytech GSH-400 test. The method proceeded in two steps. The first step led to the formation of substitution products between a patented reagent and all mercaptans (GSH) which ware present in the sample. The second step transformed specifically the obtained with GSH substitution products into a chromophoric thione whose maximal absorbency wavelength was 400 nm.

The HPLC methods were used to determine the level of vitamin C[9] and vitamin E[10]. Three hundred μL of liver homogenates mixed with an equal volume of metaphosphoric acid (100 g/L) stabilized the ascorbic acid. Before analysis, samples were centrifuged (3 500 g, 4 min) to remove precipitated protein and then the samples were assayed immediately. However, vitamin E was extracted from liver homogenates with hexane containing 0.025% butylated hydroxytoluene. The hexane phase was removed and dried with sodium sulfate, and 50 μL of the hexane extract was injected on the column. Lipid peroxidation was assayed by HPLC measuring malondialdehyde (MDA) as a malondialdehyde-thiobarbituric acid adduct[11] and 4-hydroxynonenal (4-HNE) as a fluorimetric derivative[12].

Statystical analysis

The results were expressed as mean±SD. Statistical analysis was performed using Mann-Whitney's test and $P<0.05$ was considered statistically significant.

RESULTS

Considerable parameters of antioxidative system and the level of lipid peroxidation products matched the analysed anatomo-clinical features of colorectal cancer in a statistically significant manner. The lipid peroxidation products and the activity of antioxidative enzymes increased ($P<0.001$) during progression of colorectal cancer. The levels of MDA and 4-HNE (Table 1) as well as superoxide dismutase (Cu, Zn-SOD), glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-R) (Table 2) were significantly increased ($P<0.001$) in cancer tissue compared to control group and were the highest in G3-grade adenocarcinoma and mucinous adenocarcinoma and clinical IV stage of colorectal cancer. Statistically significant differences $P<0.05$, $P<0.001$ between levels of lipid peroxidation products as well as enzyme activity among all clinical stages (II-III, II-IV and III-IV) of colorectal cancer as well as between activity of Cu, Zn-SOD, GSH-Px and GSSRG-R in G2-grade and G3-grade adenocarcinoma and mucinous adenocarcinoma were observed. On the other hand

Table 1 Levels of final lipid peroxidation products – malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in the colorectal cancer and normal colon mucosae (mean±SD)

| | Variable | n | Levels of lipid peroxidation products (nmol/g tissue) | |
			MDA	4-HNE
Histological type/grade of	Adc.G2	60	1.97±0.39[a]	0.40±0.08[b]
colorectal cancer	Adc.G3	6	2.15±0.44[d]	0.44±0.10[d]
	Adc.muc	15	2.65±0.48[f,i,k]	0.44±0.07[f,i]
Clinical stage of colorectal cancer	II	46	1.7±0.39[a]	0.37±0.07[a]
	III	29	2.25±0.43[d,g]	0.45±0.09[d,g]
	IV	6	4.04±0.47[f,i,k]	0.52±0.11[f,i,k]
Control (normal colon mucosa)			1.39±0.15	0.29±0.03

[a]$P<0.05$, [b]$P<0.001$-comparison between Adc G2 or stage II cancers and control group. [c]$P<0.05$, [d]$P<0.001$-comparison between Adc G3 or stage III cancers and control group. [e]$P<0.05$, [f]$P<0.001$-comparison between mucinous adc or stage IV cancers and control group. [g]$P<0.05$-comparison between Adc G2 or stage II cancers and Adc G3 or stage III cancers. [i]$P<0.05$-comparison between Adc G2 or stage II cancers and mucinous adc or stage IV cancers. [k]$P<0.05$-comparison between Adc G3 or stage III cancers and mucinous adc or stage IV cancers.

Table 2 Activity of antioxidative enzymes and levels of non-enzymatic antioxidants in colorectal cancersand normal colon mucosae

Variable		n	Activity of antioxidative enzymes (U/g tissue)				Level of non-enzymatic antioxidants (nmol/g tissue)		
			Cu, Zn-SOD	CAT	GSH-Px	GSSG-R	GSH	Vit.C	Vit.E
Histological	Adc.G2	60	304±38[b]	73±15[a]	1 892±583[a]	759±242[a]	151±37	439±85[a]	31.9±9.5[a]
type/grade of	Adc.G3	6	318±48[d]	62±18[c]	2 005±591[c]	795±228[c]	143±35	407±92[d]	29.7±9.2[c]
colorectal cancer	Adc. muc	15	363±72[f,i]	40±14[f,i,k]	2 143±623[f]	880±194[f]	144±43	398±104[f]	33.5±8.5[e]
Clinical stage of	II	46	237±42[b]	76±14[a]	1 854±552[a]	737±228[a]	174±36[a]	458±88[a]	33.1±9.1[a]
colorectal cancer	III	29	289±47[d,g]	57±16[c,g]	1 987±699[c]	824±236[c]	156±39[c]	399±90[d,g]	29.1±9.4[c]
	IV	6	421±58[f,i,k]	33±18[f,i,k]	2 467±368[f,i]	951±243[f,i]	150±48[e]	325±92[f,i]	13.3±10.3[f,i,k]
Control (normal colon mucosa)			117±25	84±17	1 723±189	625±112	167±15	513±64	37.5±5.2

Statistical differences: see Table 1.

catalase (CAT) activity was significantly decreased ($P<0.001$) in cancer tissue and was the lowest in mucinous adenocarcinoma and clinical IV stage cancer group (Table 2). The decreased levels of vitamin C and vitamin E as well as reduced glutathione in cancers were also observed ($P<0.001$, $P<0.001$ and $P<0.05$ respectively).

DISCUSSION

Formation of reactive oxygen species is a normal consequence of a variety of essential biochemical reactions. It is also known that oxygen radicals could be formed in excess in chronic diseases of the gastrointestinal tract[2]. The main source of oxidants in the gut is probably phagocytes, which are accumulated in the mucus of patients with bowel diseases and could generate oxidants upon activation, which might contribute to the increased risk of cancer[13]. Therefore, an adequate range of antioxidative defences within and outside the cells, has also been considered to be very important to offer protection against oxidative damages of cell components including membrane phospholipids[14].

Oxygen radical production, which increases with clinical progression of diseases, involves increased lipid peroxidation, as a result of which there are cellular membrane degeneration and DNA damage. Extent of lipid peroxidation cloud be determined by estimation of the final lipid peroxidation products - malondialdehyde and 4-hydroxynonenal, compounds known to produce protein cross-linking through Schiff's base with DNA and DNA damage[15]. In the present study, the levels of malondialdehyde and 4-hydroxynonenal in colorectal cancer tissue were significantly increased with clinical staging of the disease. This finding is in accordance with previous work that reported increased plasma and tissue MDA concentrations in colorectal cancer patients[16,17]. It has been reported that malondialdehyde is a well-characterized mutagen[18] that reacts with deoxyguanosine to form a major endogenous adduct with DNA in human livers[19]. DNA from colon biopsies had also a significantly increased level of 8-OHdG, 2-hydroxyadenine and 8-hydroxyadenine[13]. These lesions caused by hydroxyl radical attack could signify the increase in DNA damage and/or decrease in their repair. However, 4-hydroxynonenal was found to be genotoxic in primary cultures of rat hepatocytes at low concentrations, which might occur under *in vivo* conditions of oxidative stress[18]. These α,β-unsaturated aldehydes have also the ability to increase oxidative stress by promoting the cellular consumption of glutathione and by inactivating selenium-dependent glutathione peroxidase[20]. It is not known, however, if colonocytes exposed to 4-hydroxy-2-nonenal in *in vivo* conditions could undergo a similar oxidative stress. Moreover, reaction of aldehydes produced during lipid peroxidation with amino acid residues of proteins might lead to oxidative modification of them[21]. In this process, the final products of lipid peroxidation, such as malondialdehyde and 4-hydroxynonenal as well as other products resulting from polyunsaturated fatty

acid damage could cause protein breakdown[22]. In recent years, several groups have focused their studies on structural protein modifications by free radicals[23,24]. Recently it has been demonstrated that superoxide anion as well as alkoxyl peroxyl and radicals could inactivate one of the antioxidant enzymes - catalase and reduce the effectiveness of cells to defend against free radical damage[25]. In this paper, it was shown that during colorectal cancer development the activity of catalase decreased. Catalase is used by cells to defend against the toxic effects of hydrogen peroxide, which is generated by various reactions and/or environmental agents or by the action of superoxide dismutase, enzymes while detoxifying superoxide anion[26].

Another important finding of this study was a significant increase in the activity of Cu, Zn-SOD, GSH-Px and GSSRG-R in all clinical stages of cancer patients as compared to the control group, the maximum was at G3-grade adenocarcinoma and mucinous adenocarcinoma as well as in clinical IV stage of colorectal cancers. It was reported that increase in activity of Cu Zn-SOD, GSH-Px and GSSRG-R might occur on the way of induction of genetic expression[27]. Increased superoxide dismutase activity could augment superoxide radical dismutation, thus leading to intensification of hydrogen peroxide generation. Since catalase activity reduced, the level of hydrogen peroxide increased in cancer tissue. It may correspond with the report, which showed that some human cancer lines produced a large amount of hydrogen peroxide[28]. At the same time, oxygen radicals might increase secretion of the matrix metalloproteinase and collagenase as well as production of angiogenic factors (e.g., VEGF and IL-8). These factors could promote not only the local growth of neoplasm but also metastasis[29]. This agrees with the results of our present study since the highest increase in activity of Cu, Zn-SOD, GSH-Px and GSSRG-R as well as decrease in CAT activity ($P<0.001$) were observed in G3-grade adenocarcinoma and mucinous adenocarcinoma as well as in clinical IV stage of colorectal cancer. The highest level of MDA and 4-HNE was also found in clinical IV-stage cancer group.

Oxidants, including hydrogen peroxide, have been found to be able to induce expression of genes coding enzymes of antioxidative system. This kind of induction of antioxidative endogenic enzymes caused by hydrogen peroxide was found in human fibroblast cultures[27]. This increase might be caused by more extensive accessibility of enzymatic cofactors such as transient metal ions[30]. As a result of oxidative stress, iron and copper ions would become more accessible to antioxidative enzymes. Induction of genes coding supraperoxide dismutase activity has also been revealed in cases of burns and skin infections[31]. The above changes in dismutase activity, under genetic control, could make up a defensive mechanism, which enables organism adaptation to various environmental stress. Other studies showed a similar direction of changes in superoxide dismutase and glutathione peroxidase activities in cases of colorectal cancer[33], while GSH level increased or

decreased depending on studies[32,33]. Our result pointed to the increased activity of glutathione peroxidase in cancerogenesis process; however, the effective activity of this enzyme might take place only in the presence of GSH cofactor, whose level was, however decreased. Therefore, the effectiveness of GSH-Px activity was restricted, as manifested by the intensification of lipid peroxidation and the increased level of final products of their peroxidation.

As increased oxidative stress coupled with membrane damage due to lipid peroxidation antioxidant enzymes, a scavenger of oxygen radicals, might have increased as a compensatory mechanism; an antioxidant enzyme could enhance the cytotoxic ability of macrophages to scavenge free radicals. They are present mainly intracellularly, whereas non-enzymatic antioxidants are present both extracellularly and intracellularly, thus presenting at the site of generation and also at the site of action of ROS in an adequate amount. As chemical scavengers, they prevent radical-induced cellular damage. It has been observed that ascorbic acid deficiency could result in accumulation of lipid peroxide, which is an intermediate product of lipid peroxidation[34]. Moreover, it is known that vitamin C plays an important role in the synthesis of connective tissue proteins such as collagen, and deficiency of it, therefore affects the integrity of intracellular matrix and has a permissive effect on tumour growth. Deficiency of vitamin C could hinder tumo encapsulation. Our results have proved that non-enzymatic level of antioxidants, such as vitamins C and E as well as GSH decreased in colorectal cancer. In such a situation non-enzymatic antioxidants are not able to prevent oxidative modifications of cell components. Their levels decrease with progression of colorectal cancer and are therefore higher in clinical stage II and lower in stage IV of colorectal cancer. The present results and the findings of previous studies show that colorectal carcinogenesis is associated with serious oxidative stress and that advancement of oxidative-antioxidative disorders is followed by progression of colorectal cancer.

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