

Nonproteic Antioxidant Status in Plasma of Subjects with Colon Cancer

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Reactive oxygen species (ROS) could be important causative agents of a number of human diseases, including cancer. Thus, antioxidants, which control the oxidative stress state, represent a major line of defense regulating overall health. Human plasma contains many different nonenzymatic antioxidants. Because of their number, it is difficult to measure each of these different antioxidants separately. In addition, the antioxidant status in human plasma is dynamic and may be affected by many factors. Thus, the relationship between nonenzymatic antioxidant capacity of plasma and levels of well-known markers of oxidative stress (oxidized proteins, lipid hydroperoxides, decreases in thiol groups) better reflects health status. The present study considers antioxidant capacity and oxidative stress in human plasma of patients with colon cancer or precancerous lesions, as well as before and after surgical removal of tumors and/or chemo/radiation therapy. Healthy blood donors were used as controls. Colon cancer patients demonstrated a significant decrease in nonproteic antioxidant status and in total thiol groups with respect to healthy controls, whereas oxidized proteins and lipid hydroperoxide levels were significantly increased. In patients with precancerous lesions, the only unmodified parameter was the thiol group level. After surgery, the levels of oxidized proteins, lipid hydroperoxides, and total thiol groups were restored to those seen in healthy subjects, whereas nonproteic antioxidant capacity remained unmodified from that determined before surgery. Conversely, chemo/radiation therapy increased both nonproteic antioxidant capacity and levels of oxidized proteins and lipid hydroperoxides and significantly decreased total thiol groups. These results further support the hypothesis that oxidative stress correlates to the risk of some forms of cancer, not only in the initial stages but also during progression. *Exp Biol Med* 228:525–528, 2003

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Colon cancer remains today an important cause of death, especially in Western countries. Improvements in screening programs and the encouraging results of surgery have prolonged the lifespan of patients with this pathology, but mortality is still very high. It has been demonstrated that the factors able to influence the prognosis are: grading, staging, nodal involvement, adjacent tissue involvement, and hepatic recurrences (1, 2). Several studies have been undertaken to find new oncological markers able to identify a tumor before its macroscopic development.

Evidence suggests the pathological role of free radicals in a variety of diseases, among which the most important are atherosclerosis, chronic inflammation, and cancer (3, 4). Free radicals are inevitable byproducts of biological redox reactions. In fact, reactive oxygen species, such as $\cdot\text{OH}$, H_2O_2 , and other chemical forms, are produced as part of many normal and essential biological processes (4, 5). Plasma and other biological fluids are rich in antioxidant molecules, which can be subdivided into two major groups: those that prevent initiation and those that slow down the progression of a peroxidative chain reaction (6–8). The former includes primary antioxidants such as ceruloplasmin and transferrin, which act by binding metal ions; the latter group includes vitamins A, E, and C and reduced glutathione, which act by reducing the propagation and amplification chain. However, a great majority of antioxidants have multiple antioxidant properties and can thus act by binding metal ions, as well as by directly scavenging oxidizing species or by regenerating other oxidized antioxidants. In addition, plasma also contains noncharacterized antioxidants, which may contribute to counteract oxidative stress. The multiform nature of the primary antioxidant renders its quantitative analysis extremely vague; thus, a “battery” of measurements is necessary to adequately assess oxidative stress in biological systems.

Because the antioxidant status of human plasma is dynamic and can be affected by various factors, including diet, physical exercise, injury, and disease, the relationship be-

tween nonproteic antioxidant capacity (NPAC) of plasma and oxidized protein, lipid hydroperoxides and total thiol groups better reflects real oxidative stress and health status. To verify the involvement of free radical damage in tumor progression, the present study was directed at evaluating oxidized proteins, lipid hydroperoxide levels, total thiol groups, and NPAC in plasma of human subjects with colon cancer or precancerous lesions (ulcerative colitis, polyposis). In addition, in some cancerous patients the same experimental parameters were assayed before and after surgical removal of tumor and/or chemo/radiation therapy.

Materials and Methods

Patient Selection. Fifty-four patients (32 males and 22 females) with colorectal cancer, having an average age of 60 years (range 36–84), were randomly selected for this study, as were 32 patients with precancerous lesions (ulcerative colitis, polyposis) and 20 healthy subjects. Determinations were performed before and after the surgical removal of a tumor in 18 of the 54 colon cancer patients and before and after chemo/radiation therapy in 22 of the 54 patients.

Sample Preparation. Heparinized venous blood was collected after overnight fasting. Plasma was separated by centrifugation at 800g for 20 min. Plasma samples were immediately analyzed for total thiol groups and lipid hydroperoxide. Aliquots of plasma destined for oxidized protein and NPAC assays were frozen at -80°C and analyzed within 3 days.

Determination of Lipid Hydroperoxide Levels. Lipid hydroperoxide levels were evaluated by oxidation of Fe^{2+} to Fe^{3+} in the presence of xylenol orange (FOX assay) at $\lambda = 560 \text{ nm}$ (9). The assay mixture contained, in a total volume of 1 ml: 100 μl of plasma, 100 μM xylenol orange, 250 μM ammonium ferrous sulfate, 90% methanol, 4 mM butylated hydroxytoluene, and 25 mM H_2SO_4 . After a 30-min incubation at room temperature, the absorbance at $\lambda = 560 \text{ nm}$ was measured using a U-2000 Hitachi spectrophotometer. Calibration was obtained using hydrogen peroxide (0.2–20 μM). Results are expressed as nmol/ml plasma.

NPAC. NPAC was evaluated measuring its free radical scavenging ability. Superoxide anion was generated *in vitro* as described by Russo *et al.* (10). The assay mixture contained, in a total volume of 1 ml: 100 mM triethanolamine-diethanolamine buffer (pH 7.4), 3 mM NADH, 25 mM/12.5 mM EDTA/ MnCl_2 , 10 mM β -mercapto-ethanol, and ethanolic extract of plasma. After a 20-min incubation at 25°C , the decrease in absorbance at $\lambda = 340 \text{ nm}$ was measured spectrophotometrically. Results are expressed as percentage of inhibition of NADH oxidation.

Oxidized Protein Assay. Oxidized proteins were measured in 1 ml of plasma by a spectrophotometric method for carbonyl assay in the presence of dinitrophenylhydrazine at $\lambda = 370 \text{ nm}$ (11). A blank without dinitrophenylhydrazine was used through the procedure and its absor-

bance was subtracted. Carbonyl content was calculated using $\epsilon_M = 22,000$. Results are expressed as nmol/ml plasma.

Total Thiol Group Determination. Total thiol groups were measured in 200 μl of plasma using a spectrophotometric assay based on the reaction of thiols with 2,2-dithio-bis-nitrobenzoic acid at $\lambda = 412 \text{ nm}$ (12). Results are expressed as $\mu\text{mol/ml}$ plasma.

Results

Patients with Colon Cancer and Precancerous Lesions. Data obtained in this study demonstrated a significant decrease in NPAC and in total thiol groups in subjects with colon cancer compared with healthy subjects, whereas oxidized proteins and lipid hydroperoxide levels were significantly increased (Fig. 1). In patients with precancerous lesions (ulcerative colitis, polyposis), a decrease in NPAC accompanied by a significant increase in oxidized proteins and lipid hydroperoxides was observed, whereas no significant modification in total thiol group levels was found (Fig. 1).

Effect of Surgery and Chemo/radiation Therapy. To determine the effect of surgical removal of tumors or chemo/radiation therapy in colon cancer patients, the same experimental parameters were evaluated before and after treatment. As can be seen in Figure 2, surgery restored levels of oxidized proteins, lipid hydroperoxides, and total thiol groups to those seen in healthy subjects. NPAC was unmodified with respect to that determined before surgery and was still significantly lower than that in healthy controls. In contrast, chemo/radiation therapy increased both NPAC and levels of oxidized proteins and lipid hydroperoxides and led to a significant decrease in total thiol groups.

Discussion

Over recent years, researchers have focused on the pathologic role of free radicals in a variety of diseases, among which the most important are atherosclerosis and cancer (4, 13). It has been proposed that oxygen free radicals mediate the detrimental effects of malignancy and that removing them results in a survival advantage (13–15). Colon cancer is a major health problem, particularly because of the number of patients affected each year. It has been demonstrated that the 5-year survival period is increased when the disease is discovered early and the tumor is not yet fully developed (16). It is, therefore, important to find new, reliable markers enabling an early diagnosis of this pathology.

It has been suggested that oxygen and organic free radical intermediates are involved in the initiation, promotion, and/or progression stages of carcinogenesis (17, 18). Increased production of reactive species may result in a decrease in total antioxidant capacity *in vivo* but, as a measure of the extent of systemic oxidative stress, NPAC alone cannot be the tool of choice. For these reasons, this study

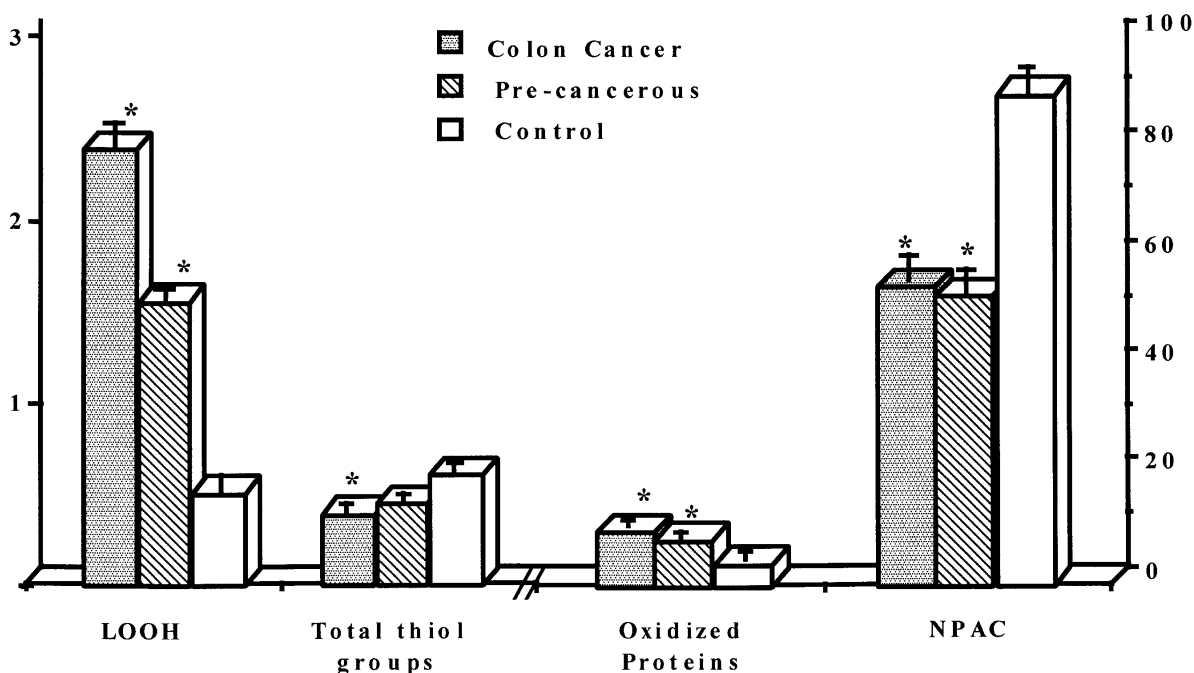


Figure 1. NPAC, oxidized proteins, lipid hydroperoxides, and total thiol groups in plasma of healthy subjects (control), patients with precancerous lesions, and colon cancer patients. Results are mean \pm SD of three determinations per patient. Statistical analysis by Student *t* test. **P* < 0.001 compared with healthy subjects.

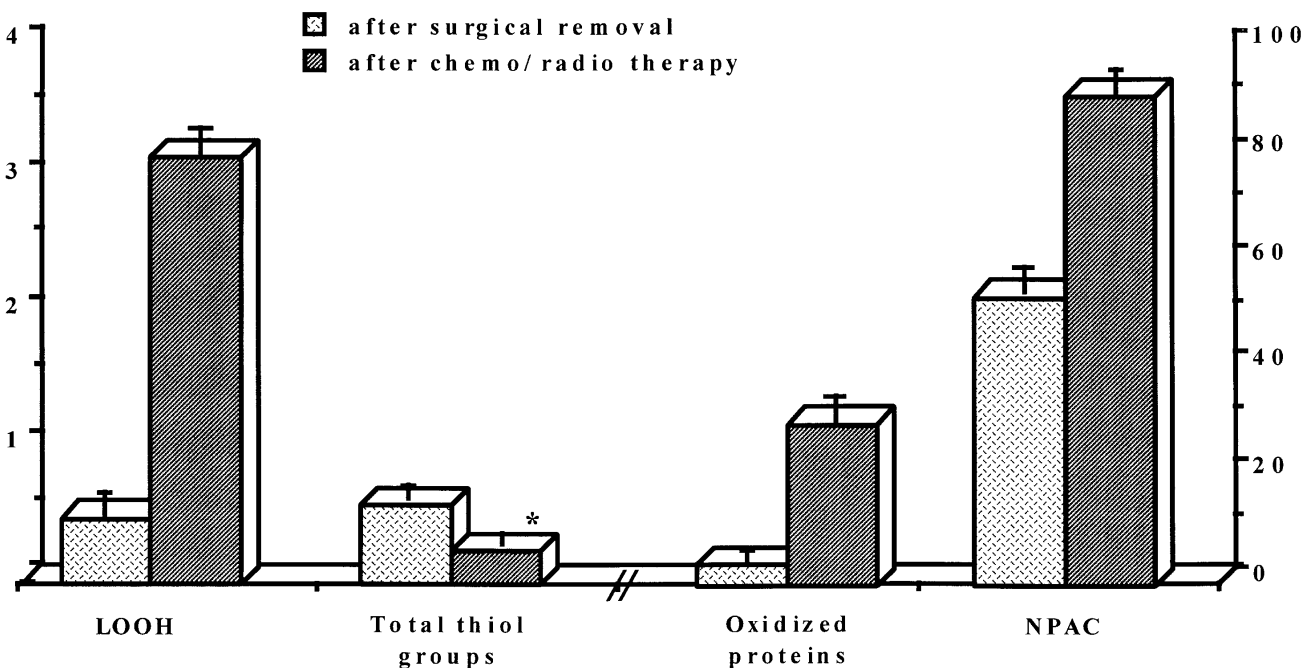


Figure 2. NPAC, oxidized proteins, lipid hydroperoxides, and total thiol groups in plasma of patients with colon cancer after surgical removal or chemo/radiation therapy. Results are mean \pm SD of three determinations per patient. Statistical analysis by Student *t* test. **P* < 0.001 compared with healthy subjects.

reports a combined evaluation of antioxidant capacity and oxidative stress markers.

Results obtained here confirm that oxidative stress is involved in carcinogenesis and that precancerous lesions may result in neoplasia when antioxidant defenses are unable to counteract free radicals. In fact, it has been demonstrated that inflammatory cells are particularly effective in

generating oxygen-derived oxidants (19). The possibility that chronic inflammation poses a risk for cancer in men is inferred from considerable clinical experience indicating human malignancies often occur at sites of ongoing chronic inflammation as well as from a number of recent experimental observations (20).

Our results also demonstrate that changes in the plasma

of patients are related to neoplasia. In fact, the results reported in Figure 2 show that surgical tumor removal restored levels of oxidized proteins, lipid hydroperoxides, and total thiol groups to those of healthy subjects. In contrast, chemo/radiation therapy increased both NPAC and levels of oxidized proteins and lipid hydroperoxides whereas a significant decrease in total thiol groups was evident. The increased oxidative stress induced by this therapy may be caused by the mechanism of action of the antitumor agents. In fact, it has been reported that chemotherapy and radiation therapy are associated with increased formation of ROS and depletion of critical plasma antioxidants (21–23). Plasma thiol groups are critical endogenous antioxidants that act concurrently in scavenging and/or reducing free radicals, thus breaking the peroxidative chain and allowing the repair of oxidatively damaged molecules. Thus, the reduction of total thiol groups observed after chemo/radiation therapy confirms a mechanism of action of these treatments that might evoke an adaptive response resulting in an increased NPAC.

Other evidence emerging from these data suggest that, in addition to their positive effect on general health, antioxidants may also exert specific beneficial effects on tumor progression and may represent a valid therapeutic support during treatment.

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