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Erythrocyte Susceptibility to Lipid Peroxidation in Patients with Coronary Atherosclerosis

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In recent years it has been reported that free oxygen radicals play an important role in the pathogenesis of degenerative diseases and that antioxidant vitamins such as vitamins E or C prevent their harmful effects. In this study, we evaluated the following: Erythrocyte susceptibility to lipid peroxidation; the role of erythrocyte glutathione (GSH) as an antioxidant; plasma lipid fractions; and the relationship between plasma lipid peroxides and antioxidant vitamin levels. Thiobarbituric acid-reactive substance (TBARS) levels were measured to determine the levels of plasma lipid peroxides and the susceptibility to lipid peroxidation when erythrocytes were stressed by hydrogen peroxide for 2 h *in vitro*. Erythrocyte TBARS production was significantly higher in patients with coronary atherosclerosis than in the controls. On the other hand, the levels of plasma high-density lipoproteins, vitamin C, vitamin E and erythrocyte GSH were significantly lower, and the levels of plasma total cholesterol, triglycerides, low-density lipoproteins and TBARS were significantly higher in the patients with coronary atherosclerosis than in the controls. In conclusion, our results indicate that erythrocytes from patients with coronary atherosclerosis are more susceptible to oxidation than those of controls and that these patients have lowered antioxidant capacity as revealed by decreased plasma levels of vitamins C and E.

Key words: atherosclerosis, vitamin C, vitamin E, malondialdehyde, glutathione

Polyunsaturated lipids are oxidized by free radicals in a process known as lipid peroxidation. Free radicals are an unstable, highly reactive chemical species containing unpaired electrons, that can easily oxidize macromolecules. They exert their effects on tissues by the peroxidation of polyunsaturated fatty acids of membrane phospholipids, a process that can result in increased membrane fluidity, increased membrane permeability and a loss of membrane integrity (1). Free radicals cannot be measured directly in humans. Consequently, indirect markers of their activity must be used. Malondialdehyde is an end product of lipid peroxidation and a thiobarbituric acid-reactive substance. Thiobarbituric acid reactive substance (TBARS), which include malondialdehyde, are widely used as indicators of lipid peroxidation (2).

Lipid peroxides, which may arise as a consequence of tissue damage, can themselves lead to the initiation of atherosclerosis. Atherosclerosis is initiated not only by lipid peroxidation of membrane phospholipids but also by low-density lipoprotein (LDL) oxidation and by promotion of thrombin generation in the platelets by the inhibition of antithrombin (3). In addition, it has been shown that lipid peroxides inhibit arterial prostacyclin synthesis *in vitro* (4), and erythrocyte membrane structure and function are known to be influenced by alterations in circulating lipids and lipid peroxides (5). It has been suggested that patients with coronary arterial disease have increased serum levels of TBARS (6, 7).

However, cells possess several defense systems to guard against free radicals, including antioxidants (small molecules such as α-tocopherol, ascorbic acid, β carotene, glutathione) and antioxidant enzymes (such as glutathione peroxidase, glutathione reductase, glutathione S-transferase, catalase and superoxide dismutase). In particular α-tocopherol and glutathione peroxidases are the most effective antioxidants to protect LDL from oxidative modification (8). Subjects with low levels of β carotene and vitamin C are at increased risk for ischemic

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heart disease (9).

In past studies of ischemic heart diseases, increased lipid peroxidation and low plasma levels of antioxidant vitamins such as vitamins C and E have been reported (6, 7, 9, 10). However, the relationship between erythrocyte susceptibility to lipid peroxidation and decreased plasma antioxidant capacity and increased lipid peroxidation in plasma has not been studied. In the present study, we investigated this relationship.

Materials and Methods

Subjects and design of study. The declaration of Helsinki was adhered to in this study. Informed consent was obtained from the subjects. We studied patients with coronary arterial disease who were admitted to the Coronary Care Unit at Cerrahpasa University Hospital and met the following criteria: a) Age between 50–60 years; b) No chronic liver, renal, lung or malignant disease; c) No usage of vitamin supplements (outside of this study); and d) No tobacco consumption. The patient group consisted of 27 men and 20 women (mean age: 58 years). Atherosclerosis was diagnosed by angiography in 22 patients, by electrocardiographic evidence of myocardial ischemia during a bicycle exercise test in 10, and by a history of myocardial infarction in 15. As for the patients who were diagnosed by angiography, a luminal narrowing of at least > 50% of the diameter in at least one of the major epicardial coronary arteries, and ischemia verified by the exercise test within the previous 6 months were regarded as sufficient for participation in this study. Eight patients had arterial hypertension. These patients were put on a low-salt diet. Six patients were taking angiotensin-converting enzyme inhibitors, 17 were taking aspirin and 12 were taking β blockers and long-acting nitrates. None had diabetes mellitus.

A control group (20 men and 20 women, mean age: 54 years) was composed of healthy volunteers not suffering from metabolic disorders or cardiac ischemia. The absence of cardiac ischemia was verified by angiography. None of the control subjects used antioxidants or hypolipemic drugs, and none smoked. Their liver and kidney function test results were normal. The main characteristics of the subjects and their plasma lipid fractions are shown in Table 1.

Experimental procedure. Blood samples were drawn into glass tubes containing ethylenediamine tetraacetic acid (EDTA) and the plasma was separated by centrifugation at 2,000 × g for 10 min. Erythrocytes separated from the plasma were washed 3 times with 0.9 g/100 ml saline solution at 4 °C. Both plasma and erythrocytes were assayed immediately.

Table 1 The mean characteristics and plasma lipid fractions (means ± SD) of controls and patients with coronary atherosclerosis

	Patients	Controls
n	47	40
Male/Female	27/20	20/20
Mean age (years)	58 ± 2	54 ± 3
Total cholesterol (mg/dl)	*240 ± 23	186 ± 29
Triglyceride (mg/dl)	*169 ± 45	127 ± 26
High density lipoprotein cholesterol (mg/dl)	*30 ± 9	47 ± 10
Low density lipoprotein cholesterol (mg/dl)	*176 ± 46	114 ± 26

*As compared with controls $P < 0.001$.

Biochemical analysis. Plasma total cholesterol, high-density lipoprotein (HDL), and triglyceride levels were measured with commercial kits from the Sigma Chemical Company, MO, USA (Cat nos: 352-20, 352-3, 339-10, respectively), and the LDL levels were calculated (11).

Assay of malonaldehyde. The plasma TBARS levels were measured according to the Stocks and Dormandy (12) and Jain (13) with slight modifications. Briefly, 0.2 ml plasma was added to 0.8 ml phosphate-buffered saline (pH 7.4). To this, 0.5 ml 30% (w/v) trichloroacetic acid (TCA) was added. The tube was vortexed and allowed to stand in ice for about 1 h and then centrifuged at 1000 × g for 15 min. A 1-ml portion of the supernatant was transferred another glass tube, and this was added 0.075 ml EDTA (2 mM) and 0.25 ml 2% (w/v) thiobarbituric acid (TBA) in NaOH (0.05 nmol/l). The mixture was vortexed and placed in a water bath at 95 °C for 15 min. Absorbance was read against a blank at 532 nm after the tube was cooled to room temperature. 1, 1, 3, 3-Tetramethoxypropane was used as a standard. The plasma TBARS levels were expressed as nmol/ml.

The TBARS levels after oxidant stress were used as a measure of erythrocyte susceptibility to lipid peroxidation. The TBARS levels were measured after the erythrocyte suspension of standardized hemoglobin was exposed to hydrogen peroxide according to the method of Stocks *et al.* (5). A 5-ml portion of azide buffer (1,000 ml phosphate buffer including 0.9% NaCl + 10 ml 0.4 M sodium azide, pH: 7.0) was added to the packed cells and the cell suspension was mixed. The hemoglobin (Hb)

concentration was measured by transferring 20 μl of the suspension to 5 ml Drabkin reagent which containing 50 mg KCN, 1 g Na_2CO_3, 200 mg $K(FeCN_6)$ per liter. A sufficient amount of buffered saline was added to the suspension mixture to bring the final Hb concentration to exactly 3 g/100 ml. A 5-ml portion of the suspension was transferred to a glass boiling tube and equilibrated in a 37 °C shaking water-bath for 10 min. A 5-ml portion of H_2O_2 solution was added as an oxidative agent, and the mixture was then incubated at 37 °C for 2 h. Next, 3 ml of the cell suspension was added to 2 ml TCA-arsenite solution (280 g TCA was added to 500 ml deionized water, followed by 13 g of sodium arsenite). The mixture was centrifuged, 0.2 ml of the supernatant was transferred into a tube and was studied by the method described above. The Erythrocyte TBARS levels were expressed as μmol/g Hb.

Assay of vitamin C. The plasma vitamin C levels were determined with a diagnostic kit from the Boehringer Mannheim Biochemicals, Mannheim, Germany (Cat no: 409 677) and was expressed as nmol/ml.

Assay of vitamin E. The plasma vitamin E levels were measured by Hansen's micro method (14). The glass-stoppered, 5-ml centrifuge tubes were prewashed with an inorganic cleansing agent as described by Udenfriend (15), and rinsed 4 times with tap water then 5 times with distilled water. The micro cuvets were washed with concentrated nitric acid for 15 min, rinsed with tap water, and then rinsed with distilled water. A 0.1-ml portion of plasma was placed in a centrifuge tube and 1 ml distilled water was added. A glass stopper was inserted, and the tube was shaken for 1 min. A 1-ml portion of absolute ethanol was then added to the tube, which was again shaken for several min to ensure precipitation of the protein present. The tube was tightly sealed with a glass stopper during shaking to prevent evaporation and contamination. A 2-ml portion of reagent-grade hexane was then added, and the tube was shaken for 20 min. The tube was then removed from the shaker and centrifuged at 900 × g for 5 min. A small aliquot of the top clear organic phase was removed with a disposable pipet and placed in a micro cuvet. Against a blank, with activation at 295 nm, the fluorescence of the samples was read at 340 nm. The working standard used for the measurements was 0.125 μmol/ml of α-tocopherol in distilled ethanol. Results are expressed as nmol/ml.

Assay of glutathione. The erythrocyte glutathione (GSH) levels were measured by Beutler *et al.'s* method (16). First, 0.2 ml of the erythrocytes was added to 1.8 ml of distilled water, and 3 ml of the precipitating solution (1.67 g glacial meta phosphoric acid + 0.2 g EDTA + 30 g NaCl per 100 ml solution) was mixed with hemolysate. The mixture was allowed to stand for approximately 5 min and then filtered. A 2-ml portion of the filtrate was added to 8 ml of the phosphate buffer (pH: 7), and then another 1 ml of the DTNB solution (40 mg 5, 5 dithiobis 2-nitro benzoic acid per 100 ml of 1% sodium citrate) was added. The optical density was measured at 432 nm with a spectrophotometer. Results were calculated using the standard GSH calibration curve and are expressed as μmol/g Hb.

All reagents were obtained from the Sigma Chemical Company. The data were analyzed using the Student's t- test, and the correlation analysis was performed using the Pearson-Pravais test. Significance was established at the $P < 0.05$ level. Data are expressed as means ± SD.

Results

In the patient group, plasma total cholesterol, LDL and triglyceride levels were significantly higher ($P < 0.001$) but the HDL levels were significantly lower ($P < 0.001$) than those in the control group (Table 1).

Plasma TBARS levels were significantly higher in males (2.88 ± 0.31 nmol/ml) than in females (2.36 ± 0.26 nmol/ml) ($P < 0.001$) in the control group, but no such significant differences existed in the patient group. The other parameters did not differ significantly between males and females in either of the groups. Plasma TBARS levels in the patient group (5.75 ± 0.63 nmol/ml) were significantly higher than those in the control group (2.62 ± 0.39 nmol/ml) ($P < 0.001$). There was a significant positive correlation between plasma TBARS and both LDL and triglyceride levels (r: 0.32, $P < 0.001$ and r: 0.38, $P < 0.001$, respectively). In addition, there was a significant negative correlation between plasma TBARS and HDL levels (r: −0.52, $P < 0.001$), but no correlation was found between plasma TBARS and total cholesterol levels.

Plasma vitamin C levels (33.21 ± 10.9 nmol/ml) and plasma vitamin E levels (22.60 ± 6.10 nmol/ml) were significantly lower in patients than in the controls (62.31 ± 14.42 nmol/ml, 34.24 ± 8.20 nmol/ml, $P < 0.001$ and $P < 0.001$, respectively). The results are shown in Fig. 1. There was a negative correlation between plasma TBARS levels and both vitamin C (r: −

0.71, $P < 0.001$) and vitamin E levels (r: -0.80, $P < 0.001$).

In the patient group, the erythrocyte GSH levels (9.9 ± 1.3 μmol/g Hb) were significantly lower than those in the control group (11.2 ± 1.1 μmol/g Hb) ($P < 0.001$), but the erythrocyte TBARS levels after *in vitro* lipid peroxidation were significantly higher (0.46 ± 0.16 μmol/g Hb) than in the control group (0.34 ± 0.03 μmol/g Hb) ($P < 0.001$) as shown in Figs. 2 and 3. There were significant but weak correlations between erythrocyte GSH levels and erythrocyte TBARS production (r: -0.40, $P < 0.001$), and between erythrocyte GSH levels and plasma TBARS levels (r: -0.36, $P < 0.001$). In addition, positive correlations between erythrocyte GSH levels and plasma vitamin C and vitamin E levels were found (r: 0.42 $P < 0.001$, r: 0.50 $P < 0.001$, respectively). A significant positive correlation between erythrocyte TBARS production and plasma total cholesterol and LDL levels was found (r: 0.48, $P < 0.001$, r: 0.52, $P < 0.001$, respectively). No correlation was found between erythrocyte TBARS production and the plasma levels of triglycerides and HDL.

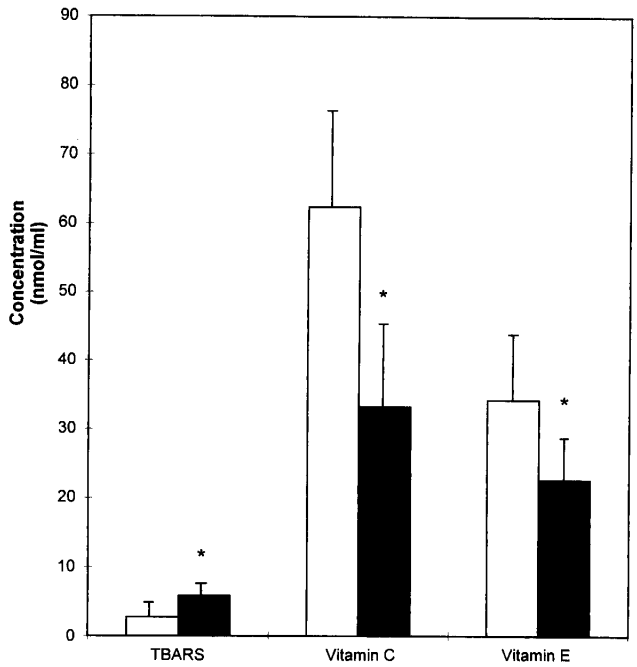

Fig. 1 Plasma thiobarbituric acid-reactive substance (TBARS), vitamin C and vitamin E concentrations (means ± SD) in controls (n: 40) and patients with coronary atherosclerosis (n:47).
*As compared with controls $P < 0.001$.
□:Controls; ■:Patients.

Discussion

Lipid peroxides formed by the peroxidation of unsaturated fatty acids were first detected in atherosclerotic human aortas more than 30 years ago (17). A significant but weak correlation between plasma TBARS and LDL levels and a significant negative correlation between

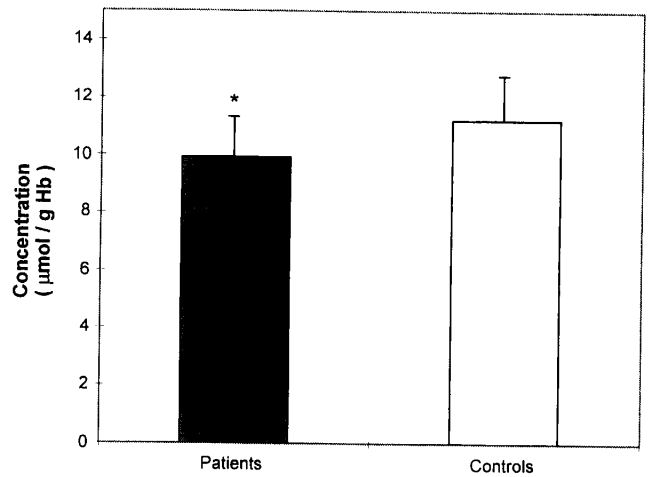

Fig. 2 Erythrocyte glutathione concentrations (means ± SD) in controls (n:40) and patients with coronary atherosclerosis (n:47).
*As compared with controls $P < 0.001$.

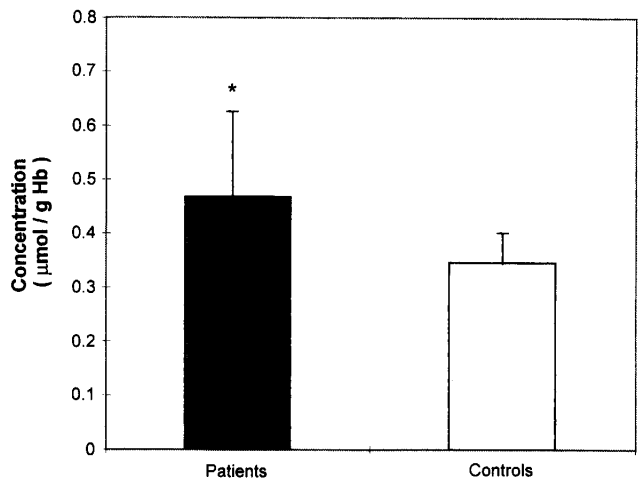

Fig. 3 *In vitro* erythrocyte thiobarbituric acid-reactive substances concentrations (means ± SD) in controls (n:40) and patients with coronary atherosclerosis (n:47).
*As compared with controls $P < 0.001$.

plasma TBARS and HDL levels were found in the present study. Of human plasma lipoproteins, LDL is the most susceptible to peroxidation. The oxidized LDL is recognized by scavenger receptors, leading to an increased uptake by endothelial cells and macrophages and an accumulation of cholesterol esters in the monocyte or macrophage. LDL uptake by the scavenger receptors pathway is not regulated, and the cell therefore continues to acquire lipids (18). HDL does not share this property, and experiments suggest that it may even protect LDL from peroxidation (7).

Vitamin E, despite its low molar concentration in the body, effectively serves as the major lipid-soluble, chain-breaking antioxidant. Vitamin E can be reduced by vitamin C (ascorbic acid) and glutathione (19). Vitamin C is a water-soluble free radical scavenger. In the present study, it was observed that plasma vitamin C and E levels were significantly lower in patients than in the controls, which may have been because these antioxidants in the patients were depleted due to their utilization against increased lipid peroxidation. A significant inverse relationship between coronary heart disease mortality and plasma levels of vitamin E and vitamin A has been reported by Gey *et al.* (10, 20). In contrast, Panczenko *et al.* (21), in their study of the effects of antioxidant vitamin supplements on peroxidation in patients during the 21-day period following coronary bypass surgery, have determined that daily oral supplements of vitamins C, E and A decrease plasma lipid peroxide concentrations.

Erythrocyte membrane structure and function may be influenced by increased lipid peroxidation and decreased plasma antioxidant capacity in patients with atherosclerosis. Red blood cells contain a large number of antioxidant compounds, one of them being glutathione. Glutathione is involved in a wide variety of biological reactions such as the maintenance of protein thiol groups (-SH) in the reduced state. In our subjects with coronary atherosclerosis, we observed a significant decrease in erythrocyte GSH concentration. This was likely due to low plasma levels of antioxidants and increased utilization against free radicals. This conclusion is supported by our discovery of a positive correlation between erythrocyte GSH levels and plasma levels of vitamins C and E, and a negative correlation between erythrocyte GSH levels and plasma TBARS levels. Decreased GSH levels in erythrocytes make them more susceptible to lipid peroxidation and the negative correlation between erythrocyte GSH levels and erythrocyte TBARS production is the best evidence of this susceptibility. On the other hand, the erythrocyte membrane lipid composition is influenced by circulating lipids (5). Increased plasma total cholesterol levels may increase the erythrocyte membrane cholesterol content and membrane fluidity. Thus, erythrocytes become more sensitive to oxidative stress. In support of this notion, we found a positive correlation between total plasma cholesterol and erythrocyte TBARS levels after *in vitro* lipid peroxidation.

In conclusion, the level of plasma TBARS is the most sensitive parameter in estimating the oxidative condition of plasma. In patients with coronary atherosclerosis, there is a negative correlation between lipid peroxidation and levels of plasma antioxidants such as vitamin C and vitamin E. Furthermore, a decrease in plasma antioxidants and in cellular antioxidants such as GSH, and an increase in plasma total cholesterol can cause a decrease in erythrocyte resistance to oxidative stress, allowing structural and functional defects to occur.

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