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The Susceptibility of Red Blood Cells to Autoxidation in Type 2 Diabetic Patients With Angiopathy

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We examined the in vitro susceptibility of red blood cell (RBC) lipids to oxidation in type 2 diabetic patients with or without angiopathy. Lipid peroxidation was assessed by quantifying thiobarbituric acid (TBA) reactivity as malondialdehyde (MDA). We also examined the RBC antioxidant status by determining glutathione (GSH) levels. Before in vitro oxidation, RBC MDA levels were significantly higher in both diabetic groups than in the controls ($P < .001$), and a significant difference was found between the two diabetic groups ($P < .05$). After in vitro treatment of RBCs with hydrogen peroxide, the degree of lipid peroxidative damage was significantly higher in diabetic patients with angiopathy versus diabetics without angiopathy ($P < .001$). Diabetic patients have low RBC GSH levels compared with controls, and after in vitro oxidation, the levels were significantly decreased in diabetics ($P < .001$). There was not a significant correlation between RBC MDA levels and glycated hemoglobin (GHb), plasma cholesterol, and triglyceride. The correlation between RBC MDA and GSH was weak ($P < .001$). We suggest that the results of this study might help to clarify the role of oxidative mechanisms as an in vitro model of degenerative damage in type 2 diabetic angiopathic complications.

ATHEROSCLEROSIS is a multifactorial disease, and several risk factors contribute to its development. These factors are abnormal plasma lipoprotein levels, thrombosis, hypertension, obesity, and smoking. Although some of the factors seem to cluster to a greater extent in diabetics than in nondiabetic patients, on their own, they do not completely explain the increased incidence of atherosclerosis in diabetes.[1]

Numerous investigators have searched for other factors that may explain the development of early and severe forms of atherosclerosis in diabetes mellitus. One factor commonly considered is the increased level of lipid peroxides in diabetes.[2] Lipid peroxidation, the oxidative alteration of polyunsaturated fatty acids, is an important mechanism in cellular damage in a number of pathological conditions.[3] Therefore, oxidative injury of endothelial cell membranes may be an important mechanism.[4] Deleterious effects of free radicals, the unstable, highly reactive chemical species with an unpaired electron in their structure, have also been implicated in the pathogenesis of atherosclerosis.[5] Some studies have demonstrated a direct cytotoxic effect of free radicals on the vascular endothelium.[2,4,6] Another factor contributing to the complications is hemorrheologic disturbances, which may play an important role in the impairment of diabetic microvascular flow and diabetic complications.[7,8] It has been shown that red blood cells (RBCs) from diabetic patients exhibit reduced membrane deformability, increased viscosity, and abnormal adherence to endothelial cells.[9] Normal RBCs are resistant to oxidative damage through their antioxidant enzyme systems. However, both enhanced free-radical generation and decreased antioxidants may increase the lipid peroxidation of RBCs in diabetic patients.[10]

Malondialdehyde (MDA), an index of lipid peroxidation,[11] may be present in RBC membranes of diabetic patients during conditions of increased oxidative stress.[12] RBC glutathione (GSH) is a physiological constituent of the intracellular antioxidant defense system and is present in all mammalian cells. GSH protects the cell against free radicals, hydrogen peroxide, and organic peroxides.[13] GSH peroxidase (EC 1.11.1.9) eliminates organic peroxides in the presence of GSH. During the reaction, GSH is oxidized to GSH disulfide (GSSH). GSSH is then reduced to GSH by NADPH. NADPH is regenerated from NAD by the pentose phosphate shunt. Therefore, depressed GSH levels have been associated with enhanced lipid peroxidation as a result of decreased scavenging of free radicals.[14]

The aim of the present study is to examine the in vitro RBC lipid susceptibility to oxidation and RBC GSH levels in patients with or without angiopathy.

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SUBJECTS AND METHODS

Diabetics

The study was performed with 30 type 2 diabetic patients with angiopathy (17 females and 13 males; age, 50.5 ± 8.4 years; body mass index [BMI], 26.5 ± 0.6 kg/m^2; duration of disease, 13.4 ± 3.5 years [mean ± SD]) and 30 type 2 diabetic patients without angiopathy (17 females and 13 males; age, 48 ± 5.2 years; BMI, 25.8 ± 0.7 kg/m^2; duration of disease, 9.5 ± 3.2 years).

In diabetic patients with angiopathy, four had background retinopathy defined as the presence of microaneurysms by indirect ophthalmoscopy and fluorescein angiography; 18 had hypertension defined as measured blood pressure repeatedly above 160/95 mm Hg for at least 6 months; 10 had angina with effort (five of them also had hypertension); and three had nephropathy as evaluated by microalbuminurea defined as an albumin excretion rate greater than 20 μg/min but less than 200 μg/min on at least two overnight collections. Twenty-two patients in this group were treated with oral hypoglycemic agents (sulfonylurea) and eight with NPH insulin. Diabetic patients with hypertension were treated with diuretics (n = 10), alpha-methyldopa (n = 2), and propranolol (n = 6). In this group, all patients were on a dietary regimen (1,400 to 1,600 kcal/d, with 50% carbohydrate, 30% fat, and 20% protein).

In diabetic patients without angiopathy, 23 were treated with oral hypoglycemic agents (sulfonylurea) and seven were on a dietary regimen only. They had normal renal function. They did not have retinopathy, effort angina, or hypertension.

In both diabetic groups, none of the subjects smoked or drank alcohol and none were on medications such as heparin, hypolipidemics, and antioxidants (vitamin E, ascorbic acid, GSH, and probucol).

Controls

Twenty-five nondiabetic healthy controls (10 females and 15 males; age, 49.8 ± 9.2 years; BMI, 26.4 ± 1.5 kg/m^2) were chosen from medical and laboratory staff. Five control subjects smoked fewer than five cigarettes per day. None of the control subjects were receiving any medication, and they had normal liver and renal function. None of the controls had a family history of diabetes.

Written consent was obtained from all subjects.

Blood Collection

Blood samples were collected from the patients (before they received their medication) and controls after an overnight fast, into tubes containing EDTA. Part of the blood was analyzed for glycated hemoglobin (GHb); the remaining blood was then centrifuged at 2,000 rpm for 10 minutes in a refrigerated centrifuge. After removing the plasma and discarding the buffy coat, the erythrocytes were washed with a cold 9-g/L sodium chloride solution three times after five dilutions. A complete blood cell count by electronic counter was performed on washed cell samples to check for contamination by leukocytes.

Measurement of RBC Lipid Peroxidation

In vitro peroxidation was measured using a modification of the method of Stocks and Dormandy[15] in which RBC suspensions were prepared as described before and incubated with hydrogen peroxide for 2 hours at 37°C with constant shaking. The final composition of the incubation mixture was 5 mmol/L hydrogen peroxide and 2 mmol/L sodium azide. The time 0 sample was obtained by terminating the reaction immediately after hydrogen peroxide was added. For termination of peroxidation, trichloroacetic acid/0.13% sodium arsenite solution was added.

Lipid peroxidation was determined by measurement of MDA generation. MDA was assayed as thiobarbituric acid (TBA)-reactive material forming a complex with absorption at 532 nm. MDA values were determined by the molar absorption coefficient of the MDA-TBA complex at 532 nm = 1.56×10^5 L · mol^{-1} · cm^{-1}. The intraassay and interassay coefficient of variation for the MDA test was 4.7% and 4.9%, respectively. Values are expressed as nanomoles of MDA per gram of hemoglobin (Hb). The Hb concentration was measured by Drabkin's reagent.

Measurement of RBC GSH

RBC GSH levels were measured by the method of Beutler et al,[16] and the values are expressed as micromoles of GSH per gram of Hb. Washed RBCs (0.2 ml) were added to distilled water (1.8 mL). The hemolysate was deproteinized and centrifuged at 3,000 rpm for 20 minutes. A volume of 0.5 mL supernatant was added to 2 mL sodium phosphate (0.3 mol/L). After 0.2 mL dithiobis-nitrobenzoate (0.4 mg/mL) was added, absorbency at 412 nm was measured immediately after mixing. The GSH concentration was calculated using 1.36×10^4 L · mol^{-1} · cm^{-1} as a molar absorption coefficient. The intraassay and interassay coefficient of variation for GSH was 3.8% and 3.9%, respectively.

Plasma Analysis

Plasma glucose levels were determined by a glucose oxidase method (Sigma catalog no. 315-500; Sigma, St Louis, MO). Plasma total cholesterol and triglyceride levels were determined by commercial enzymatic kits (Sigma catalog no. 352-100 and 336-50, respectively). GHb levels were measured by column chromatographic methods.[17]

Statistical Analysis

Data are expressed as the mean ± SD. Differences between groups were considered significant at a P level less than .05. Data were compared using the Student t test for paired data or ANOVA followed by the Tukey-Kramer multiple-comparison test. Pearson's correlation coefficient was used for correlation analysis.

The reagents were purchased from Sigma and were of analytical grade.

RESULTS

Characteristics of the Patients

Characteristics of the patients are shown in Table 1. The patients had higher plasma total cholesterol and triglyceride levels than the healthy controls ($P < .001$). There was no

Table 1. Characteristics and Plasma Levels of Cholesterol, Triglyceride, GHb, and Glucose in Healthy Controls, Type 2 Diabetic Patients Without Angiopathy, and Type 2 Diabetic Patients With Angiopathy (mean ± SD)

Parameter	Controls	Type 2 Diabetics	
		Without Angiopathy	With Angiopathy
No. of subjects	25	30	30
Sex (female/male)	10/15	17/13	17/13
Age (yr)	49.8 ± 9.2	48 ± 5.2	50.5 ± 8.4
BMI (kg/m^2)	24.6 ± 1.5	25.8 ± 0.7	26.5 ± 0.6
Plasma cholesterol (mmol/L)	6.9 ± 1.2	7.3 ± 0.8*	7.5 ± 1.0*
Plasma triglyceride (mmol/L)	2.2 ± 0.9	2.4 ± 0.90*	2.5 ± 1.1*
GHb (%)	5.8 ± 0.4	8.2 ± 0.5*	8.5 ± 0.7*
Plasma glucose (mmol/L)	5.2 ± 0.5	8.7 ± 0.5*	8.8 ± 0.3*

*$P < .001$ v controls.

significant difference in plasma total cholesterol, triglyceride, and GHb levels between the two diabetic groups.

Susceptibility of RBCs to In Vitro Oxidation

At time 0, RBC MDA levels were significantly higher in both diabetic groups than in the controls ($P < .001$). There was a significant difference in RBC MDA levels between diabetic patients with angiopathy and diabetic patients without angiopathy ($P < .05$). After in vitro peroxidation, RBC MDA production was elevated from 150 ± 50, 440 ± 65, and 400 ± 63 nmol/g Hb to 195 ± 35, 870 ± 50, and 607 ± 75 nmol/g Hb in controls, diabetic patients with angiopathy, and diabetic patients without angiopathy, respectively ($P < .001$; Table 2). The increase in RBC MDA after in vitro oxidation was significantly greater in diabetic patients with angiopathy (90% to 95%) than in diabetic patients without angiopathy (45% to 75%; $P < .001$). In controls, the increase in RBC MDA levels was 13% to 15%.

RBC GSH Levels

RBC GSH levels were significantly lower in diabetic patients with angiopathy and without angiopathy compared with the controls ($P < .001$). There was no significant difference in RBC GSH levels between the patient groups. After in vitro peroxidation, RBC GSH levels were significantly decreased from the values at time 0 in the patient groups ($P < .001$). There was no significant difference in RBC GSH levels between the patient groups (Table 2). In the controls, the group difference in RBC GSH was not statistically significant.

Correlation Analysis

In all groups, no correlation was found between RBC MDA levels (at time 0) and total cholesterol, triglyceride, and GHb. There was a weakly significant negative correlation between RBC MDA and GSH ($r = -.390$, $P < .005$). The correlation between RBC GSH and cholesterol, triglyceride, and GHb was not statistically significant in any group.

DISCUSSION

Diabetes mellitus is associated with a high prevalence of microvascular and atherosclerotic complications. The term "diabetic angiopathy" suggests that the pathogenic mechanism responsible for localized disturbances in the metabolic supply of nervous or muscular cutaneous tissues resides in the vessel wall. The cause of these complications is not understood, although there is increasing evidence that hemorheological factors directly or indirectly contribute to the pathogenesis of vascular complications in diabetes mellitus.[18] In particular, the erythrocytes of diabetic patients have a reduced life span, an excessive aggregation, an altered membrane phospholipid asymmetry, and an increased tendency to adhere to endothelial cells.[7,18] The erythrocyte redox state is of utmost importance in maintaining cellular homeostasis. Oxidative stress may occur in the cell from increased production of free radicals and/or impaired antioxidant defense systems and result in oxidative attack of the polyunsaturated fatty acid components of lipidic macromolecules, followed by functional and structural cell impairment.[19]

In this study, type 2 diabetic patients had significantly higher RBC MDA levels and significantly lower RBC GSH levels than the controls. In vitro studies have shown that glucose can enolize and thereby reduce molecular oxygen under physiological conditions, yielding alpha-ketoaldehydes, hydrogen peroxide, and free-radical intermediates.[20] Hydrogen peroxide formed by superoxide dismutation produces hydroxyl radicals. Radical levels above the detoxifying capacity of RBCs can cause lipid peroxidation and accumulation of MDA.[21] Reports on the state of antioxidant levels in diabetic patients are contradictory: both increases and decreases of antioxidant activity are reported.[21-24] The decrease in RBC GSH levels may be a result of decreased activity of enzymes involved in the pentose phosphate pathway.[8] Also, lower GSH levels may be a result of oxidised GSH passing through RBC membranes due to lipid peroxidation–induced membrane damage.[25]

We have found a significant difference in the RBC oxidant status of type 2 diabetic patients with and without angiopathy. It has been suggested that peroxidized lipids provide an index of the severity of atherosclerosis,[25] and plasma lipid peroxide levels were significantly higher in type 2 diabetic patients with peripheral vascular disease or coronary artery disease.[26] Lipid peroxides are also directly cytotoxic to vascular endothelium. On the other hand, we have not found any difference in RBC GSH levels between diabetic patients with and without angiopathy. It has been suggested that the levels of RBC GSH and lipid peroxides show an inverse correlation with both glycemic control (GHb) and the presence of complications.[27] Therefore, we selected the patients from well-controlled, age-matched type 2 diabetics. Our results may be due to the heterogeneous patient groups selected (different degrees of angiopathy or type of therapy).

In this study, we demonstrated that RBCs pretreated with

Table 2. RBC MDA and GSH in Healthy Controls, Type 2 Diabetic Patients Without Angiopathy, and Type 2 Diabetic Patients With Angiopathy at Time 0 and 120 Minutes After In Vitro Treatment of RBCs With Hydrogen Peroxide (mean ± SD)

			Type 2 Diabetics			
	Controls (n = 25)		Without Angiopathy (n = 30)		With Angiopathy (n = 30)	
Parameter	0 min	120 min	0 min	120 min	0 min	120 min
MDA (nmol/g Hb)	150 ± 50	195 ± 35§	400 ± 63*	607 ± 75§	440 ± 65*†	870 ± 50‡§
GSH (μmol/g Hb)	11.8 ± 0.5	11.5 ± 1.2	9.5 ± 0.4*	8.7 ± 0.3§	9.7 ± 0.3*	8.5 ± 0.5§

*$P < .001$ *v* controls.

†$P < .05$ *v* group without angiopathy at time 0.

‡$P < .001$ *v* group without angiopathy at 120 min.

§$P < .001$ *v* time 0.

hydrogen peroxide from diabetic patients with angiopathy were more sensitive than RBCs from diabetic patients without angiopathy. Hydrogen peroxide is known to exert various effects on cells. It influences the membrane phospholipid distribution between the external and internal membrane surfaces. In endothelial cells, it inhibits cyclooxygenase.[28] Under experimental conditions, peroxide treatment can be regarded as a model for the stress effects produced by oxidants.[19] Two factors could account for an abnormal increase in the 120-minute MDA level. Firstly, a high 120-minute MDA level could be an expression of high initial substrate (polyunsaturated lipid) concentrations. Secondly, a true increase in susceptibility to autoxidation could reflect an impaired antioxidant mechanism.[12] Since the antioxidant status was similar in our diabetic patient groups, the first factor could account for an abnormal increase in the lipid peroxidation levels in type 2 diabetic patients with angiopathy after in vitro peroxidation. It has been demonstrated previously that membrane polyunsaturated fatty acids are elevated in atherosclerotic patients.[29]

Although Jain and McVie[30] found a significant negative correlation between hyperglycemia and GSH in erythrocytes of diabetic patients, we found only a negative correlation between RBC MDA and RBC GSH levels. This finding suggests that the decrease in antioxidant levels is the result of elevated oxidation levels.

Finally, our results indicate that not only increased free-radical production but also reduced degradation of hydrogen peroxide in the elevated oxidative condition may deteriorate the RBC membrane. Additionally, the results of this study may help to clarify the role of the oxidative mechanism as an in vitro model of degenerative damage in diabetic angiopathic complications.

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