Erythrocyte Membrane Lipid Peroxidation and Glycosylated Hemoglobin in Diabetes

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Erythrocytes of diabetic patients have abnormal membrane properties. We examined in vivo membrane lipid peroxidation in erythrocytes of diabetic subjects and its possible relationship with hyperglycemia. Lipid peroxidation was assessed in fresh, untreated erythrocytes by quantitating thiobarbituric acid reactivity and an adduct of phospholipids and malonyldialdehyde (MDA), an end product of lipid peroxidation, with thin-layer chromatography of lipid extract of diabetic erythrocytes. There was a significantly increased membrane lipid peroxidation in diabetic erythrocytes compared with nondiabetic erythrocytes. The degree of membrane lipid peroxidative damage in erythrocytes was significantly correlated with the level of glycosylated hemoglobin, an index of mean glucose level for the preceding 3–4 mo. This suggests that peroxidation of membrane lipids and accumulation of MDA occurs in erythrocytes of diabetic patients. *Diabetes* 38:1539–43, 1989

It has been emphasized that hemorheologic disturbances can play an important role in the impairment of diabetic microvascular flow and diabetes complications (1). Several abnormalities have been identified in the erythrocytes of diabetic patients as reviewed by Jones and Peterson (1). In particular, erythrocytes of diabetic patients have a reduced life span (2,3), excessive aggregation (4,5), altered membrane phospholipid asymmetry (6), and an increased tendency to adhere to endothelial cells (6,7). Other studies of human erythrocytes in vitro and rat and rabbit erythrocytes in vivo have documented that membrane lipid peroxidation can result in decreased cell survival (8), altered membrane phospholipid asymmetry (9–11), hypercoagulability (10,12), and increased adhesion to endothelial cells (11).

This study was undertaken to determine whether diabetic erythrocytes show membrane lipid peroxidative damage in vivo and to examine the cause of this membrane damage. We quantitated membrane lipid peroxidation in washed, untreated erythrocytes obtained from fresh blood of diabetic patients and examined its correlation with glycosylated hemoglobin (GHb) levels, an index of the mean blood glucose level for the preceding 3–4 mo.

RESEARCH DESIGN AND METHODS

Permission from the Institutional Human Experiments Committee to use blood of diabetic patients was obtained before starting this study. Diabetic patients were recruited at random from the University Hospital pediatric clinic, criterion for inclusion being a request by the patient's physician for a GHb value. Physicians did not know of this criterion or whether any given patient's blood was included in the study. All diabetic subjects in this study had insulin-dependent diabetes, except one who had non-insulin-dependent diabetes with a GHb value of 5%. Control subjects were nondiabetic age-matched pediatric volunteers.

Blood was collected into tubes containing EDTA. Part of the blood was sent to Roche Clinical Laboratories for GHb assay; the remaining blood was then centrifuged at our laboratory at 2000 rpm for 7 min in a refrigerated centrifuge. Plasma and buffy coat were discarded. Erythrocyte suspension was filtered through cotton wool to remove any remaining leukocytes (13). The cells were washed with a cold 0.15 M NaCl solution two times after 1–10 dilutions. A complete blood count by electronic counter was done on washed cell samples to check for contamination by leukocytes. Preparation of washed erythrocytes and biochemical analyses were done immediately after blood collection from nondiabetic volunteers and diabetic patients. Membrane lipid peroxidation was determined by the following methods.

Thiobarbituric acid (TBA) reactivity. Malonyldialdehyde (MDA), an end product of fatty acid peroxidation, can react with TBA to form a colored complex that has maximum ab-

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sorbance at 532 nm (14). For this purpose, 0.2 ml of packed cells were suspended in 0.8 ml phosphate-buffered saline (8.1 g NaCl, 2.302 g Na_2HPO_4, and 0.194 g NaH_2PO_4/L, pH 7.4) and 0.025 ml of butylated hydroxytoluene ([BHT] 88 mg/10 ml absolute alcohol). Thirty percent trichloroacetic acid (0.5 ml) was then added. Tubes were vortexed and allowed to stand in ice for at least 2 h. Tubes were centrifuged at 2000 rpm for 15 min. One milliliter each of the supernatant was transferred to another tube. To this we added 0.075 ml 0.1 M EDTA and 0.25 ml of 1% TBA in 0.05 N NaOH. Tubes were mixed and kept in a boiling-water bath for 15 min. Absorbance was read at 532 and 600 nm after tubes were cooled to room temperature in a double-beam Lambda 3B Perkin-Elmer spectrophotometer. BHT, an antioxidant, was added to prevent MDA formation during the assay, which could result in falsely elevated TBA reactivity. The addition of BHT to standard MDA did not affect the color development with TBA. Absorbance at 600 nm was subtracted from absorbance at 532 nm. MDA values in nanomoles per milliliter of packed cells were determined with the extinction coefficient of MDA-TBA complex at 532 nm = $1.56 \times 10^5 \cdot cm^{-1} \cdot M^{-1}$. Packed-cell volume of erythrocytes was determined with an Autocrit centrifuge, and complete blood counts were determined with a Coulter counter. TBA reactivity was also expressed per number of cells and per erythrocyte phospholipid. Details of erythrocyte phospholipid determination are given in the lipid-extraction method. TBA has been used to measure sialic acid (15). However, sialic acid should not interfere in MDA assay because maximum absorbance of sialic acid–TBA complex is at 549 nm in contrast to 532 nm for MDA-TBA complex. Second, sialic acid needs to be treated with a strong oxidizing agent periodate before its assay in contrast to MDA assay, in which no oxidizing agent is added; instead, our assay procedure contained BHT, an antioxidant. Third, sialic acid–TBA complex is unstable in basic conditions used for MDA-TBA complex formation (15).

Measurement of MDA by TBA reactivity is the most widely used method for assessing lipid peroxidation (16). We also used an additional new method, thin-layer chromatography (TLC), to assess membrane lipid peroxidation in erythrocytes.

Phospholipid-MDA adduct measurement. Peroxidative membrane damage was also assessed by determining the amount of heterologous phospholipid-MDA adduct separated by TLC of erythrocyte lipid extracts as described earlier (17,18). The quantitation of phospholipid-MDA adduct by TLC gives the amount of MDA cross-linked between phosphatidylethanolamine and phosphatidylserine and has also been used to assess lipid peroxidative damage by other investigators (19).

Lipid extraction, drying, and washing were performed as described by Rose and Oklander (20). Separation of various phospholipid classes in the lipid extract was accomplished by TLC on silica gel H glass plates (silica 60, 0.25-mm thickness, Brinkman, Westbury, NY) with solvent system chloroform/methanol/glacial acetic acid/water (50:25:8:4 vol/vol). Different phospholipid spots on the TLC plate were visualized by exposing the plate to iodine vapors and were encircled with a fine needle. Localization of various phospholipids on the TLC plate was confirmed by authentic standards and specific sprays on the plate (21). The amount of phospholipid was determined by quantitating phospholipid-phosphorus from the silica gel after scraping it into Pyrex glass tubes (22).

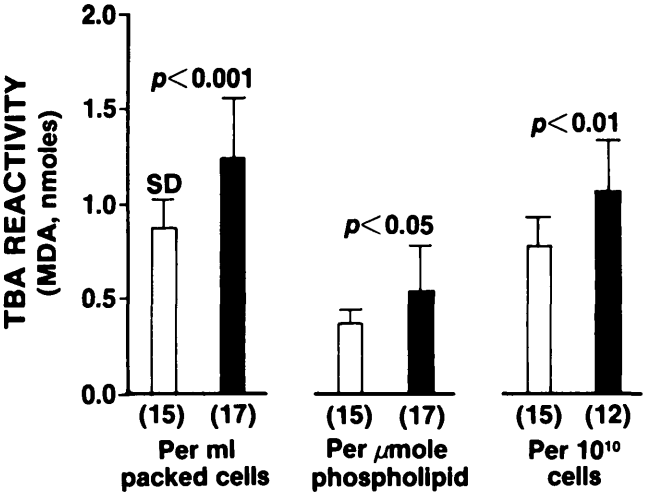

FIG. 1. Thiobarbituric acid (TBA) reactivity between erythrocytes of nondiabetic (*open bars*) and diabetic (*solid bars*) subjects. Differences in TBA reactivity between erythrocytes of nondiabetic and diabetic subjects were significant regardless of whether TBA reactivity was expressed per milliliter of packed cells, per micromole erythrocyte phospholipid, or per number of cells. Values are means ± SD. Numbers of patients are given in parentheses.

Measurement of GHb. The human erythrocyte is freely permeable to glucose, and within each erythrocyte, GHb is

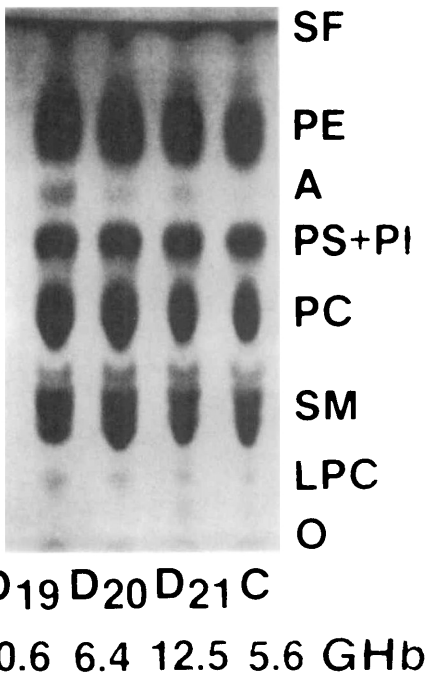

FIG. 2. Thin-layer chromatography of erythrocyte phospholipids. Phospholipids were visualized by exposing plate to iodine vapors. Note increased intensity of A in diabetic subject with elevated glycosylated hemoglobin (GHb) value compared with diabetic subject with lower GHb value and control subject. C, control; D, diabetic; PE, phosphatidylethanolamine; A, phospholipid-malonyldialdehyde adduct; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin; LPC, lysophosphatidylcholine; O, origin; SF, solvent front.

TABLE 1
Phospholipid classes in erythrocytes of nondiabetic and diabetic subjects

Subjects	*n*	Lysophosphatidyl-choline	Sphingomyelin	Phosphatidyl-choline	Phosphatidylineserine plus phosphatidylinositol	Phospholipid-malonyldialdehyde adduct	Phosphatidyl-ethanolamine
Nondiabetic	17	0.9 ± 0.3	25.2 ± 1.3	33.5 ± 1.4	9.1 ± 0.5	0.2 ± 0.2	31.3 ± 1.3
Diabetic	21	1.4 ± 0.5*	23.2 ± 1.7*	33.4 ± 2.5	8.4 ± 1.3	0.7 ± 0.4*	32.9 ± 1.6*

Values are percentages of totals in means ± SD. *n*, Number of samples in each group.
*$P < .002$ vs. nondiabetic subjects.

formed continuously from hemoglobin A at a rate dependent on the ambient glucose concentration. The formation of GHb is nonenzymatic, slow, and irreversible. It is now widely accepted that measurement of GHb in a single blood sample provides an index of the mean blood glucose level during the preceding 3–4 mo (23,24). GHb values of the patients were obtained from the clinical laboratory after biochemical analyses of the blood had been completed. GHb was measured by Roche with Bio-Rad columns and kit.

Data were analyzed statistically with the unpaired Student's *t* test and regressional analyses with EPISTAT statistical software for the IBM PC/XT.

RESULTS

Figure 1 shows TBA reactivity in fresh, untreated erythrocytes of nondiabetic and diabetic subjects. There was significantly more TBA reactivity in the erythrocytes of diabetic subjects compared with nondiabetic subjects regardless of whether the TBA reactivity was calculated per milliliter of packed cells, per micromole erythrocyte phospholipid, or per number of cells. This suggests that erythrocytes in diabetic patients in vivo have significantly more membrane lipid peroxidative damage compared with erythrocytes of nondiabetic subjects.

Figure 2 illustrates TLC of lipid extracts of fresh, untreated erythrocytes from nondiabetic and diabetic subjects. Erythrocytes from diabetic patients showed the presence of a new lipid termed *A*. This lipid spot was found to be phosphorus positive, ninhydrin negative, and sugar negative when specific spray reagents were used. Cochromatography with standards ruled out this new spot being glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, NADPH, NADP, or sorbitol. Similar new lipid can also be formed in vitro by treating erythrocytes from nondiabetic patients with exogenous standard MDA and has been characterized as an adduct of phosphotidylserine, phosphatidylethanolamine, and MDA (17,18). This may suggest that erythrocytes of diabetic patients have undergone significant in vivo membrane lipid peroxidation.

The phospholipid composition of erythrocytes of nondiabetic and diabetic subjects is shown in Table 1. There is significantly more phospholipid-MDA adduct and lysophosphatidylcholine and lower sphingomyelin erythrocytes of diabetic patients compared with erythrocytes of nondiabetic patients. Proportion of other phospholipid fractions (phosphatidylcholine and phosphatidylserine plus phosphatidylinositol) was not significantly different between erythrocytes of nondiabetic and diabetic subjects.

Significant positive correlations between GHb and the TBA reactivity of diabetic erythrocytes were $r = .67$ ($P < .01$), $r = .63$ ($P < .05$), and $r = .69$ ($P < .05$) when the TBA reactivity of erythrocytes was expressed per milliliter of packed cells, per micromole erythrocyte phospholipid, and per number of cells, respectively (Fig. 3). This suggests that the degree of membrane lipid peroxidation in erythrocyte is related to the extent of hyperglycemia in diabetic patients.

Linear regression analyses of GHb value and phospholipid-MDA adduct formation in diabetic erythrocytes showed a significant positive correlation ($r = .48$, $P < .05$; Fig. 4).

Figures 3 and 4 suggest that the degree of blood glucose

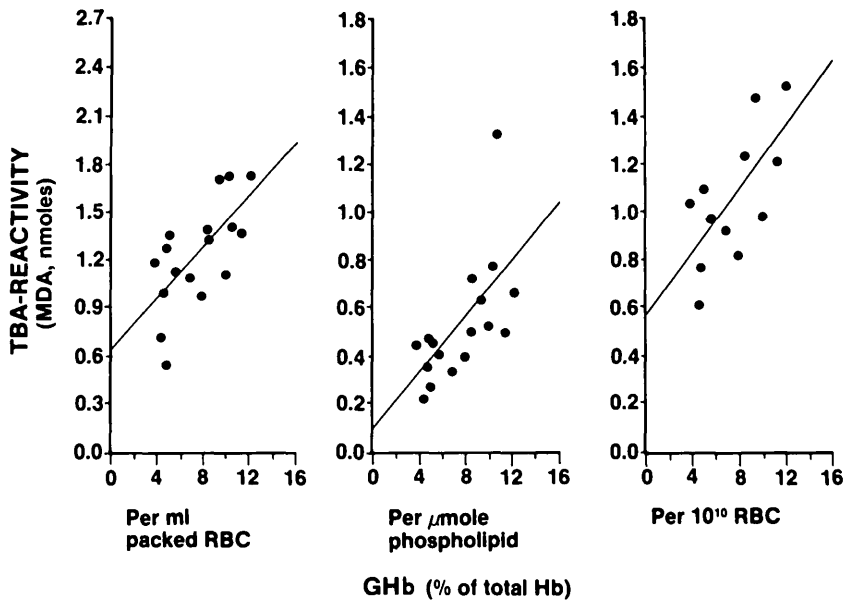

FIG. 3. Linear regression analyses of thiobarbituric acid (TBA) reactivity between erythrocytes and glycosylated hemoglobin (GHb). Regression equation was $y = 0.08x + 0.63$, $y = 0.058x + 0.094$, and $y = 0.066x + 0.57$ when TBA reactivity was expressed as per milliliter packed erythrocyte ($r = .67$, $P < .01$); per micromole phospholipid ($r = .63$, $P < .05$); and per number of cells ($r = .69$, $P < .05$); respectively. Note significant positive correlation between increase in TBA reactivity of erythrocytes with higher GHb levels in diabetic patients.

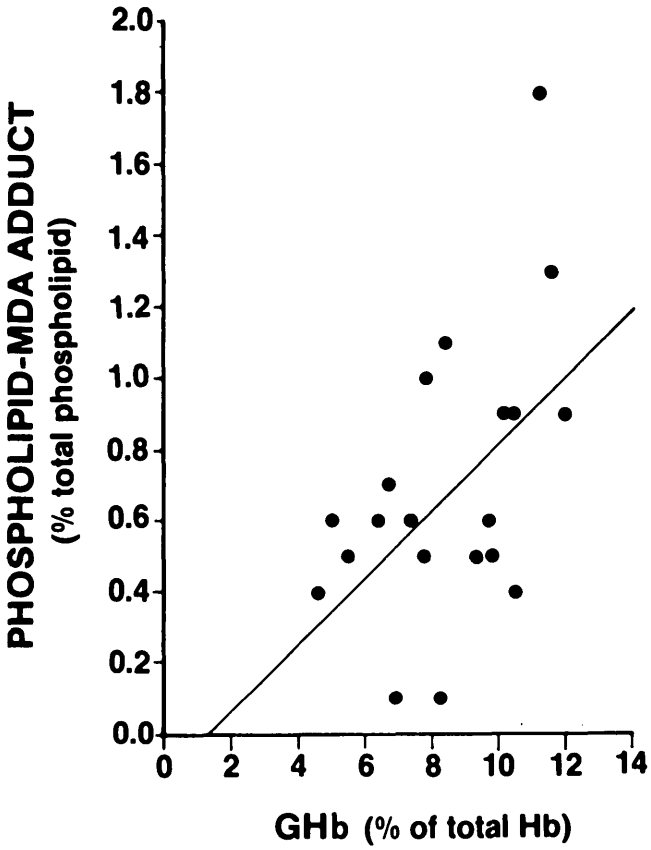

FIG. 4. Linear regression analyses of amount of phospholipid-malonyldialdehyde (MDA) adduct in erythrocytes and glycosylated hemoglobin (GHb) level in diabetic patients (r = .48, P < .05). Regression equation was $y = 0.094x + -0.13$. Note increase in phospholipid-MDA adduct formation in erythrocytes of patients with higher GHb values.

level elevation correlates with the accumulation of MDA and therefore with membrane lipid peroxidation in erythrocytes of diabetic patients.

DISCUSSION

Several studies have shown elevated levels of lipid peroxidation products in plasma of diabetic subjects and rats (25–30). Previous studies have also shown that erythrocytes of diabetic patients are more susceptible to lipid peroxidation when treated with hydrogen peroxide in vitro (26,30). This study documents the occurrence of lipid peroxidation in the membranes of fresh, untreated erythrocytes of diabetic patients. Furthermore, it was shown that the amount of membrane lipid peroxidation is significantly correlated with the amount of GHb, an index of elevated blood glucose levels. MDA is relatively water soluble, and erythrocytes were washed before MDA assay; thus, the origin of MDA (TBA reactivity) that has been measured needs comment. Most unsaturated fatty acids susceptible to oxidation are present in the inner membrane bilayer (18), and it is possible that MDA formed in the inner bilayer is not removed during washing of erythrocytes; MDA and other TBA reactants could also be formed from lipid hydroperoxides, intermediate products of the lipid peroxidation process, which are unlikely to be washed by saline but can be broken down to MDA and other TBA reactants during assay.

The exact mechanism by which elevated blood glucose leads to membrane lipid peroxidation in erythrocytes of diabetic subjects is not known. Recent in vitro studies in a cell-free buffer have shown that glucose can enolize and thereby reduce molecular oxygen under physiological conditions, yielding α-keto aldehydes, hydrogen peroxide, and free radical intermediates (31,32). Hydrogen peroxide formed by superoxide dismutation regenerates the catalytic metal oxidation state and produces hydroxyl radicals (33). Oxygen radicals formed over and above the detoxifying capacity of erythrocytes can cause peroxidative breakdown of phospholipid fatty acids and accumulation of MDA (34–37).

Our finding of membrane lipid peroxidation suggests that peroxidative lipid damage in the membrane may have a role in the increased coagulability, altered phospholipid organization, and reduced ^{51}Cr-labeled cell survival of erythrocytes and cellular damage known to occur in other tissues of diabetic patients.

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