Absence of Correlation Between Glycated Hemoglobin and Lipid Composition of Erythrocyte Membrane in Type 2 Diabetic Patients

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Correlation of glycated hemoglobin (HbA_{1c}) level with degrees of certain peroxidative changes in erythrocyte membrane lipids in diabetic patients have been reported. In the present study, peroxidation of erythrocyte lipids was assessed by changes in tocopherols (Toc), phospholipids (PL), and malondialdehyde (MDA). Membrane cholesterol, Toc, and PL were determined from the same lipid extract. Toc and cholesterol were measured simultaneously by high-performance liquid chromatography (HPLC), and each PL class was determined by a single HPLC elution with ultraviolet light (UV) detection. The detection of PL with UV depends primarily on double bonds in fatty acids and shows a decrease in fatty acids by peroxidation. Changes in Toc and each PL were calculated on the basis of cholesterol and SM, respectively, since cholesterol and sphingomyelin (SM) in the cell membrane are not prone to peroxidation. MDA was measured by an HPLC method with fluorescence detection. These methods for assessment for peroxidation of membrane lipids in intact erythrocytes were validated by experiments with 2, 2-azobis(2-amidinopropane)dihydrochloride (AAPH) and *tert*-butylhydroperoxide (tBHP); nevertheless, significant differences in the levels of Toc, each PL class, and MDA between a high-HbA_{1c} group and a low-HbA_{1c} group were not detected.

HYPERGLYCEMIA has been identified as a risk factor in the development of diabetic complications.[1] Hyperglycemia may produce oxidative stress by the generation of oxygen-derived free radicals and oxidative stress may be a pathogenesis of diabetic complications.[2,3] Peroxidative changes in lipid composition of erythrocyte membrane lipids may cause hemorrheologic disturbances, which can play an important role in the impairment of microvascular flow and can cause complications in diabetic patients.[2-4] Several abnormalities of erythrocyte membrane lipids due to oxidative stress in diabetic patients have been reported, including an increase in malondiadehyde (MDA), an end product of fatty acid peroxidation,[5-7] changes in fatty acid composition of phospholipids (PL),[8-11] changes in PL,[7,12-16] a decrease in α-tocopherol (α-Toc),[17] an increase in 7-oxocholesterol,[18] and an increases in glycated phosphatidylethanolamine.[19] Some of these abnormalities in erythrocyte lipids are reported to correlate positively with glycated hemoglobin (HbA_{1c}) level.[7,12,18] However, there are some discrepancies among these reported abnormalities of lipid composition of erythrocytes. Furthermore, no differences in erythrocyte α-Toc level and fatty acid composition between healthy subjects and diabetic patients have been reported.[20,21] Thus, it can be said that peroxidative changes in erythrocyte membrane lipids in diabetic patients have not been clearly settled.

Some PL classes in human erythrocytes contain a high amount of polyunsaturated fatty acids that are prone to peroxidation,[22-24] and detection of PL with ultraviolet light (UV) primarily depends on double bonds in unsaturated fatty acids.[25] We previously reported a method employing high-performance liquid chromatography (HPLC) that can separate all of the major PL of erythrocyte membranes by means of a single chromatographic elution with UV detection.[26-28] Furthermore, we measured both Toc and cholesterol in erythrocyte membranes in a total lipid extract simultaneously by an HPLC method.[27,28] In the present study, we applied these methods to the assessment of changes in erythrocyte membrane lipid of diabetic patients, and found no significant differences in PL, Toc, or MDA levels between a high-HbA_{1c} group and a low-HbA_{1c} group.

MATERIALS AND METHODS

Subjects

Informed consent was obtained from 39 patients with type 2 diabetes mellitus. The blood samples were drawn from the patients at random. The patients were divided into 2 groups according to their HbA_{1c} level. The HbA_{1c} level of the low-HbA_{1c} group ($n = 20$) was $6.17\% \pm 0.45\%$ and that of the high-HbA_{1c} group ($n = 19$) was $9.50\% \pm 0.92\%$. The range of HbA_{1c} levels in normal adults is 4.3% to 5.8% in the laboratory. Some clinical features of the patients are listed in Table 1. Vitamin E was not prescribed to any of the patients, but self-supplementation of the vitamin was not checked. Blood from 5 nondiabetic healthy volunteers, aged 21 to 24, (healthy group) was also obtained, but levels of HbA_{1c} for these persons were not measured.

Preparation of Washed Erythrocytes

Venous blood from the patients and the healthy volunteers who had fasted overnight was collected into tubes containing citric acid and immediately cooled in an ice bath. Serum and buffy coats were removed after centrifugation at $1,000 \times g$ for 5 minutes and erythrocytes were washed 4 times in Hank's balanced buffer with centrifugation at $1,000 \times g$ for 10 minutes at 4°C. A small portion of the top layer was removed at each washing. The washed erythrocytes were resuspended in the isotonic buffer so that the final concentration was a hematocrit (Hct) of 5%. Hct value was determined with an automated blood counter (Sysmex F-80, Kobe, Japan).

Extraction of Lipids From Erythrocytes

Ten milliliters of the erythrocyte suspension was centrifuged at $1,000 \times g$ for 10 minutes and the packed erythrocytes were used for

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Table 1. Characteristic of Patients With Type 2 Diabetes

	Low-HbA_{1c} Group	High-HbA_{1c} Group
No. of patients	20	19
Male	10	Male 10
Female	10	Female 9
Age (yr)	56.2 ± 5.9	55.7 ± 6.4
HbA_{1c} (%)	6.17 ± 0.45	9.50 ± 0.92
Total cholesterol	215.2 ± 34.9	235.6 ± 47.8
HDL-cholesterol	57.0 ± 14.9	63.1 ± 26.9
LDL-cholesterol	129.4 ± 34.9	134.1 ± 34.7

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein.

lipid analysis. The extraction of the total lipids including Toc from the packed erythrocytes was done immediately after the preparation of the washed erythrocytes as described in our previous reports.[27] Briefly, the packed erythrocytes were hemolyzed by addition of 1 mL of 5-mmol/L phosphate buffer (pH 7.4) containing 5 mmol/L ascorbic acid, which was followed by addition of 1 mL 80-mmol/L sodium dodecyl sulfate. Then, 2 mL of ethanol containing 1.2 mmol/L BHT was added, and the mixture was left at room temperature for 60 minutes. Next, 2 mL of *n*-hexane containing 1.2 mmol/L BHT was added to the mixture and it was mixed vigorously. After centrifugation at 1,000 × *g* for 5 minutes, the *n*-hexane layer was transferred to another tube. The remaining aqueous layer was washed again with 2 mL *n*-hexane. This hexane layer was combined with the first extract, and the combined hexane layer was dried under a stream of nitrogen gas.

Measurement of Cholesterol and Tocopherols

The dried total lipid extract was dissolved in 200 μL of *n*-hexane/isopropanol (3:1, vol/vol), and an aliquot (usually 5 μL) was injected into an HPLC system. Cholesterol and Toc were measured simultaneously by the HPLC method as described in our previous reports.[27,28] The column was a TSK-GEL ODS-80Ts, 250 × 4.6 mm (Tosoh, Tokyo, Japan). The HPLC system (HP-1100, Yokogawa Electric Co, Tokyo, Japan) was connected in series to a fluorescence detector (FS-8020, Tosoh) and an evaporative light scattering detector (Dedex-55, Vitry sur Seine, France).

Determination of Each Phospholipid Class

The separation and detection of PL classes were done by the HPLC method reported previously.[26-28] The column was a Wakosil 5 NH2, 150 × 4.6 mm (Wako Pure Chemical Co, Osaka, Japan) and guard column was a μ-Bondapak NH2, 20 × 4 mm) (Waters Co, Tokyo, Japan). An aliquot (5 μL) of the lipid extract, which was used for the detection of Toc and cholesterol, was injected into the HPLC system. Each PL was detected at 205 nm UV.

Measurement of Erythrocyte Malondialdehyde

The washed erythrocytes were re-suspended in the isotonic buffer so that the concentration was Hct of 9%. After addition of 25 μL of 1% BHT, 0.5 mL of 30% trichloroacetic acid (TCA) was added to the erythrocyte suspension. After vigorous mixing with a vortex mixer, the tube was centrifuged at 1,000 × *g* for 5 minutes, and the supernatant was transferred to another tube and stored at −30°C until use. MDA was measured by the HPLC method described previously.[27] An aliquot (50 μL) of the supernatant was mixed with 0.5 ml of 0.2% thiobarbituric acid (TBA) in 2 mol/L sodium acetate containing 1 mmol/L diethyltriaminopentaacetic acid and 10 μL of 5 % BHT. The mixture was incubated at 95°C for 45 minutes. After cooling on ice, the mixture was filtered through a 0.2-μm filter, and an aliquot was injected in to

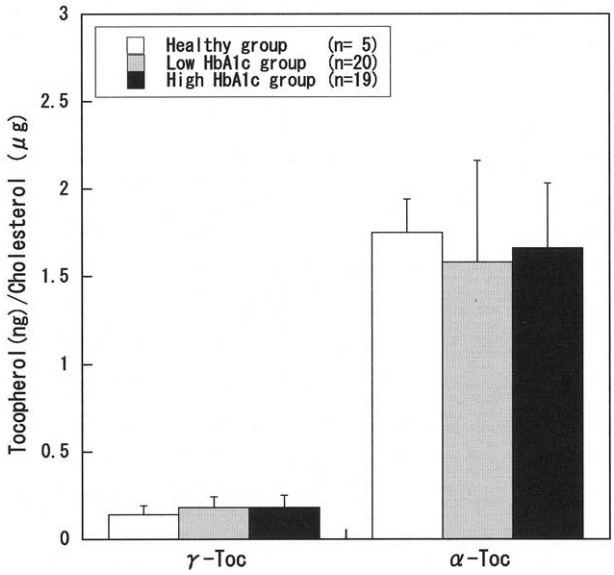

Fig 1. Toc levels of erythrocytes were not significantly different between the low-HbA_{1c} group, the high-HbA_{1c} group, and the young healthy persons. Toc and cholesterol were determined simultaneously by HPLC. Toc were detected with a fluorescence detector and cholesterol with an evaporative light-scattering detector. Each bar represents the mean ± SD.

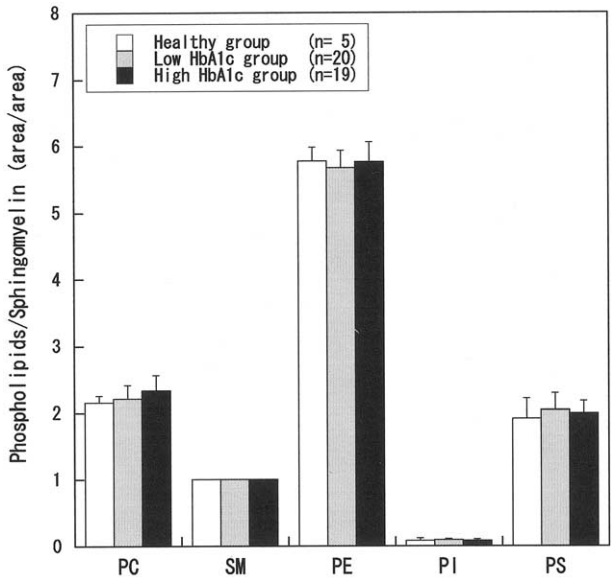

Fig 2. PL composition of erythrocytes was not significantly different between the low-HbA_{1c} group, the high-HbA_{1c} group, and the healthy persons. An aliquot of the same lipid extract used for the determination of cholesterol and Toc was injected into the HPLC system. Each PL was detected with UV at 205 nm. Each bar represents the mean ± SD. PC, phosphatidylcholine; PE, phosphatidylethanoamine; SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine.

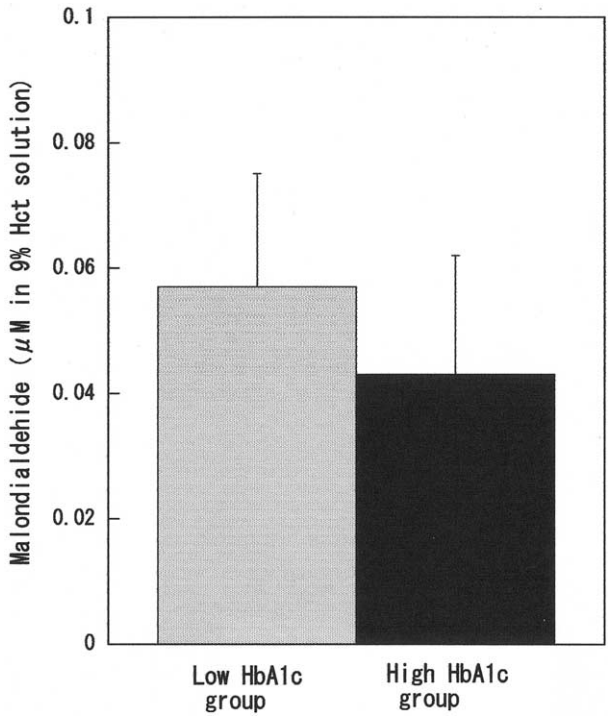

Fig 3. MDA level of erythrocytes was not significantly different between the low-HbA_{1c} group and the high-HbA_{1c} group. MDA was determined by HPLC with a fluorescence detector. Each bar represents the mean ± SD.

the HPLC system. The column used was a Cosmosil 5 C18-AR-II, 150 × 4.6 mm (Nakarai Tesque, Kyoto, Japan). The mobile phase was acetonitrile/water (60:40, vol/vol) and the flow rate was 1 mL/min at 35°C column temperature. The MDA was detected by the fluorescence detector at 515 nm (excitation) and 553 nm (emission).

Peroxidation of Normal Erythrocytes With tBHP and AAPH

The total volume of the reaction mixture for peroxidation was brought up to 5 mL by phosphate-buffered saline (PBS), and the final concentration of erythrocytes was 5 % Hct. After preincubation at 37°C for 5 minutes, reactions were started by addition of *tert*-butylhydroperoxide (tBHP) and 2,2-azobis(2-amidinopropane)dihydrochloride (AAPH). The final concentrations of tBHP and AAPH were 1 mmol/L and 50 mmol/L, respectively. The reactions were terminated by cooling the tubes in an ice bath, and the erythrocytes were pelleted by centrifugation. The determination of PL and Toc from the packed erythrocytes were performed as described above. PL classes were collected from the HPLC system and were subjected to fatty acid analysis. Fatty acid analysis of each PL class was done as described previously.[26,28]

Statistics

Statistical analyses were performed by a paired *t* test using Microsoft Excel 2000 (Microsoft Co, Japan). Differences were considered significant at $P < .05$.

RESULTS AND DISCUSSION

Reportedly, the MDA level of erythrocytes and the HbA_{1c} level in diabetic patients were significantly correlated.[7] It is also reported that the ratio of 7-oxocholesterol to cholesterol as well as the ratio of conjugated linoleic acid to linoleic acid was

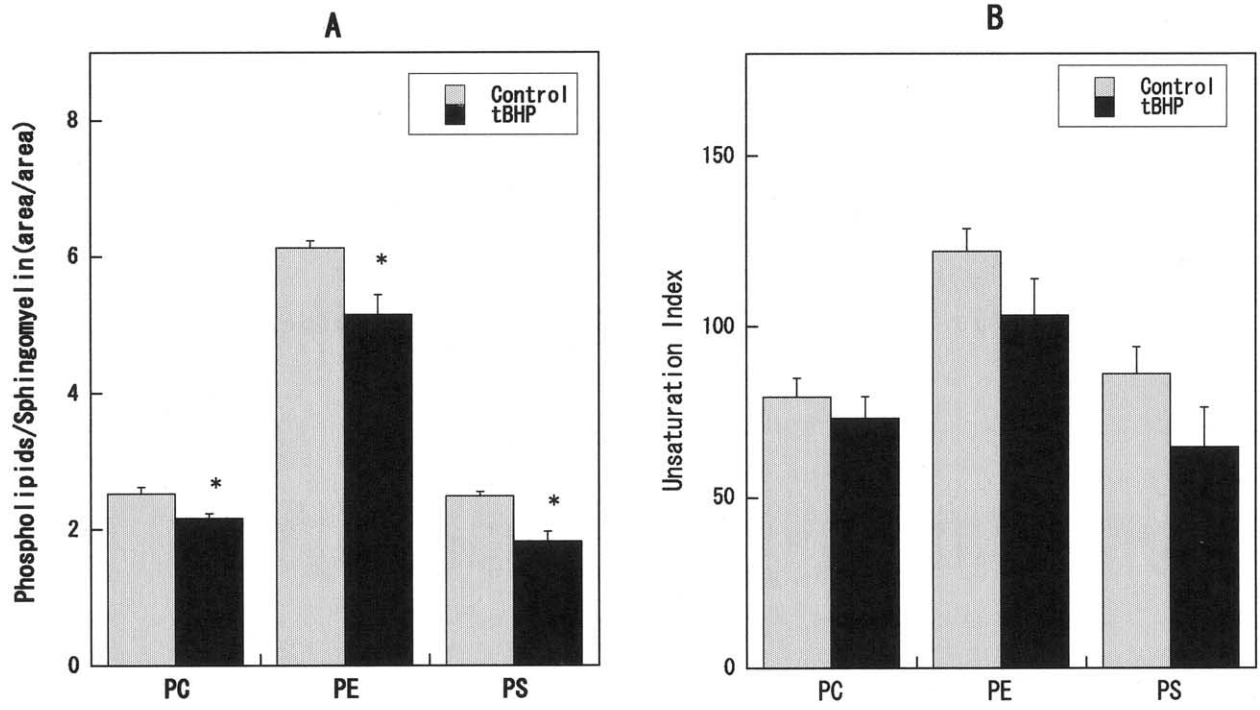

Fig 4. Changes in each PL after incubation of intact erythrocytes with 1 mmol/L tBHP for 10 minutes (A) correlated with the changes in the unsaturation index of fatty acids of each PL (B). Each PL was collected directly from the HPLC system used in (A) and was subjected to determination of fatty acid composition. Each data point represents the mean ± SD of 3 determinations of the same erythrocytes from a healthy volunteer. *$P < .05$ by paired *t* test.

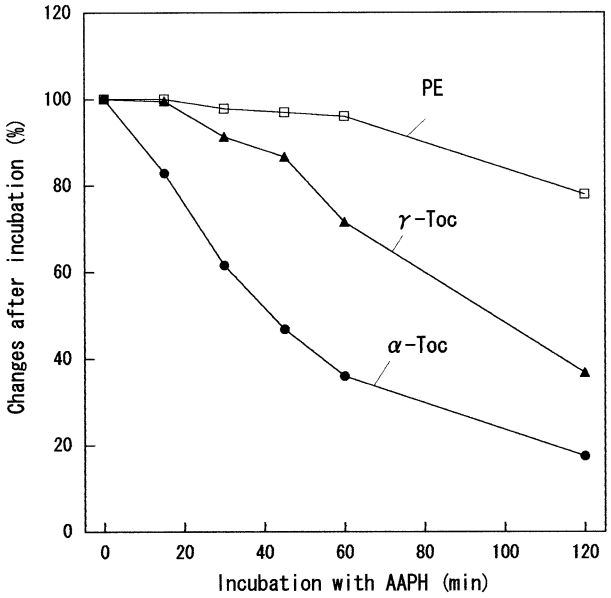

Fig 5. Time course of the decrease in PE and Toc of intact erythrocytes after incubation with 50 mmol/L AAPH at 37°C. α-Toc decreased rapidly followed by the decrease of γ-Toc. There was no decrease of PE until the level of α-Toc reached < 50% of the initial level. Each data point represents the mean of duplicated experiments on the same erythrocytes from a healthy person.

significantly correlated with the level of HbA_{1c}.[10,18] These data suggest that chronic hyperglycemia is linked to peroxidation of erythrocyte lipids. However, in the present study of erythrocytes from type 2 diabetic patients, no significant differences in either α-Toc or PL were observed between the high-HbA_{1c} group and the low-HbA_{1c} group (Figs 1 and 2), and the MDA level of erythrocytes was not correlated with the HbA_{1c} (Fig 3).

In this study, the membrane cholesterol, Toc, and PL were determined from the same lipid extract. The cholesterol and Toc were measured simultaneously by HPLC, and cholesterol was regarded as an internal standard for HPLC. Each PL class was detected by a single chromatographic elution with UV detection. Because free cholesterol and sphingomyelin (SM) in the cell membrane do not contain fatty acid and polyunsaturated fatty acids, respectively, the substances are assumed to be not prone to peroxidation as compared to Toc and other PL. Therefore, the changes in Toc and each PL were calculated on the basis of cholesterol and SM, respectively. Thus, the variability of values of Toc and each PL class after long processes of determination calculated on some blood counts were diminished.

The detection of PL with 200 to 210 nm UV depends primarily on the double bond in fatty acids of PL; therefore, the decrease in membrane PL detected by the HPLC method reflects mainly the decrease in polyunsaturated fatty acids.[25,26] In fact, Fig 4 shows that the decrease in each PL class of the intact erythrocytes by incubation with tBHP correlates well with the decrease in the unsaturation index of fatty acids (sum of unsaturated fatty acids [%] × no. of double bonds) in each PL. The correlation of the changes (increase or decrease) in each PL class measured by the HPLC method with the changes in the unsaturation index of fatty acids was also shown in our previous report.[28] Figure 4 shows that the standard deviations of each PL class determined by the HPLC method are much smaller than those of the unsaturation index, because the procedure for the determination of each PL class is much simpler as compared to the procedure for the determination of the fatty acid composition of each PL class. The fatty acid analysis showed preferential decrease in the polyunsaturated fatty acids; the average extent of the decrease of arachidonic acid was 14% in phosphatidylethanoamine (PE) and 22% in phosphatidylserine (PS), and that of docosahexanoic acid was 20% in PE and 25% in PS. Figure 5 shows the time course of the decreases in PE, α-Toc, and γ-Toc after incubation of intact erythrocytes with 50 mmol/L AAPH, a water-soluble radical initiator, for 120 minutes at 37°C. The decrease in α-Toc occurred rapidly after the incubation with AAPH followed by the decrease in γ-Toc. The decrease in PE did not occur until the concentration of α-Toc became less than 50% of the initial level. The same pattern of changes in Toc and PE by the peroxidation of erythrocytes with tBHP was observed previously.[27] These results indicate that peroxidation of fatty acids in membrane PL do not occur without a decrease in membrane Toc. The present methodology for assessment of peroxidation of membrane lipids in intact erythrocytes was validated by the experiments with the radical initiators; however, differences in the levels of Toc, PL class, and MDA between the high-HbA_{1c} group and the low-HbA_{1c} group were not detected.

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