Glycated Hemoglobin and Lipid Peroxidation in Erythrocytes of Diabetic Patients

Masayuki Inouye, Takaya Mio, and Kimiaki Sumino

In diabetes, glycation and subsequent browning (or glycoxidation) reactions are enhanced by elevated glucose concentrations. It is unclear whether the diabetic state per se also induces an increase in the generation of oxygen-derived free radicals (OFRs). However, there is some evidence that glycation itself may induce the formation of OFRs. OFRs cause oxidative damage to endogenous molecules, including cholesterol. 7-Oxocholesterol is known to be one of the major products of cholesterol oxidation. The level of cholesterol peroxidation products was assessed in erythrocyte membrane lipid by monitoring the peak height ratio of 7-oxocholesterol, one of the products of cholesterol peroxidation, to cholesterol with gas chromatography/mass spectrometry (GC/MS). The peak height ratio of 7-oxocholesterol to cholesterol was used as a biomarker of lipid peroxidation. The hemoglobin A1c (HbA1c) value, an index of glycemic stress, was measured by high-performance liquid chromatography. We examined the relationship between the levels of cholesterol peroxidation products and HbA1c in erythrocytes of diabetic and healthy subjects. There was a significantly increased ratio of 7-oxocholesterol to cholesterol in diabetic erythrocytes compared with control erythrocytes. The ratio of 7-oxocholesterol to cholesterol was significantly correlated with the level of HbA1c. This suggests that glycation of hemoglobin via chronic hyperglycemia is linked to cholesterol peroxidation in erythrocytes of both diabetic and healthy subjects.

INTEREST HAS GROWN as to the role of oxygen-derived free radicals (OFRs) as mediators of tissue damage in many disease processes, including diabetes.[1-5] It has also been proposed that the OFR attack on low-density lipoprotein (LDL) may initiate a chain of reactions resulting in a modified, oxidized LDL that is more atherogenic than native LDL.[6-8] Lipid peroxidation is an important result of OFR stress. To determine the degree of OFR stress, one approach is to measure the concentration of compounds thought to be products of the OFR attack on endogenous molecules. Zhang et al[9] found 7-oxocholesterol to be the most abundant oxysterol after copper oxidation of LDL. However, they identified cholest-3,5-dien-7-one as a dehydration product of 7-oxocholesterol in a gas chromatographic analysis. They showed that this compound was generated from 7-oxocholesterol during analysis of underivatized samples, but it disappeared when the samples were silylated before analysis. Bhadra et al[10] also found cholest-3,5-dien-7-one to be the dominant oxysterol after copper oxidation of LDL. It is likely that 7-oxocholesterol was one of the major cholesterol oxidation products in oxidized LDL, but it was detected as cholest-3,5-dien-7-one because cholest-3,5-dien-7-one was artifactually formed from 7-oxocholesterol by dehydration in the gas chromatographic analysis. We could also detect cholest-3,5-dien-7-one, a dehydration product of 7-oxocholesterol, in methylated lipids of diabetic erythrocyte membranes using gas chromatography/mass spectrometry (GC/MS).

One possible source of OFRs in diabetes is autoxidation of glucose.[11] Glucose is the source of glycated proteins. In the presence of metal ion, they can be a source of OFRs.[12,13] The glycation of hemoglobins may generate OFRs, and OFRs formed in excess of the scavenging capacity of erythrocytes would cause oxidative damage to phospholipid in the inner membrane bilayer of erythrocytes. Another possible source of OFRs in diabetes is an increased polyol pathway flux caused by hyperglycemia.[14] In the first half of the polyol pathway, the decrease in the NADPH/NADP ratio resulting from conversion of glucose to sorbitol can reduce the effectiveness of the glutathione redox cycle in scavending OFRs. In the second half of the polyol pathway resulting from conversion of sorbitol to fructose, fructose is a 10-fold better substrate than glucose for glycation; therefore, the formation of OFRs would be accelerated by increased polyol pathway flux.

This study was undertaken to determine whether the ratio of 7-oxocholesterol to cholesterol correlates with the degree of glycation of hemoglobin in erythrocytes of diabetic and healthy subjects.

SUBJECTS AND METHODS

Subjects

Informed consent for study participation was obtained from 27 non–insulin-dependent diabetes mellitus (NIDDM) patients and 29 healthy control subjects. Clinical characteristics of the subjects are listed in Table 1.

Lipid Extraction

Blood (7 mL) was collected from each subject and anticoagulated with EDTA disodium. A 1.5-mL aliquot of each sample was transferred to determine the hemoglobin A1c (HbA1c) level. Lipid extraction from erythrocyte membrane and methanolysis was quickly performed by a previously described method[15] to prevent lipid peroxidation in the air. Antioxidants were not used in our experiment because they interfered in the gas chromatographic peaks. Erythrocytes were separated by centrifugation at 1,500 × *g* for 10 minutes to discard the plasma and buffy coat. The erythrocytes were hemolyzed by adding 30 mL distilled water and separated by centrifugation at 480,000 × *g* for 10 minutes to discard the supernatant. After repeating this procedure three times, the erythrocyte membranes were dried. The pellet was redissolved in chloroform followed by ultrasonication and extraction. The extract was transferred to a Reacti-Vial (Pierce Chemical, Rockford, IL) and evaporated to dryness under a gentle nitrogen stream. Five percent hydrogen chloride methanol solution was added to the extract. The vial was capped tightly and heated at 100°C for 60 minutes for methanolysis. Fatty acids were converted to methylesters of fatty acids. After cooling, the contents

From the Department of Internal Medicine, Hyogo Rehabilitation Center Hospital, Kobe; and Department of Public Health, Kobe University School of Medicine, Kobe, Japan.

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Address reprint requests to Masayuki Inouye, MD, Department of Internal Medicine, Hyogo Rehabilitation Center Hospital, Akebono-cho 1070, Nishi-ku, Kobe 651-2181, Japan.

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Table 1. Clinical and Biochemical Characteristics of NIDDM and Healthy Subjects

Characteristic	Healthy (n = 29)	NIDDM (n = 27)	P
Age (yr)*	59.3 ± 14.5	56.4 ± 12.0	—
Sex ratio (male:female)	15:14	14:13	—
Smokers (n)	13	14	—
Duration of treatment (yr)*	—	5.6 ± 3.2	—
Treatment ratio (insulin:oral agent)	—	7:20	—
Antihypertensive therapy (n)	—	5	—
Total cholesterol (mg/dL)*	222.3 ± 33.2	233.3 ± 22.7	<.05
HDL cholesterol (mg/dL)*	45.8 ± 10.2	41.1 ± 10.2	<.05
Triglyceride (mg/dL)*	157.6 ± 32.8	231.6 ± 65.8	<.001
HbA1c (%)*	4.6 ± 0.8	7.5 ± 1.9	<.001
7-Oxocholesterol/cholesterol (%)*	3.1 ± 1.9	20.9 ± 16.1	<.001

Abbreviation: HDL, high-density lipoprotein.
*Mean ± SD.

were evaporated to dryness under nitrogen and the residue was dissolved in 100 μL benzene. A 1-μL aliquot of the solution was used for injection into the GC/MS apparatus.

GC/MS Conditions

A JEOL DX-300 GC/MS (JEOL, Tokyo, Japan) was connected on-line with a JEOL DA-5000 mass data analyzer. A 30-m × 0.53 mm (film thickness, 1.5 μm) fused silica capillary column (DB-1; J & W Scientific, Folsom, CA) was used for separation. The carrier gas was helium and the flow rate was 15 mL/min. The column temperature was programed for 140°C to 300°C (4°C/min). Mass spectrometry was performed at an ion source temperature of 250°C, ionization voltage of 70 eV, ionization current of 30 μA, and acceleration energy of 3.0 kV. An electron-impact mass spectrum was obtained with scans of 3 seconds over the range 50 to 500 *m/z*.

Peak Height of Ratio of 7-Oxocholesterol to Cholesterol

To estimate the degree of lipid peroxidation, the relative peak heights of cholest-3,5-dien-7-one and cholesterol were measured in each chromatogram with the aid of MS software. The peak height ratio of cholest-3,5-dien-7-one to cholesterol was calculated as the peak height ratio of 7-oxocholesterol to cholesterol.

HbA1c Estimation

HbA1c values were measured by high-performance liquid chromatography (Hi-AUTOA1c, HA-8121 analyzer; Kyoto Dalichi Kagaku, Kyoto, Japan).[16] The normal range for HbA1c is 3.4% to 5.8%.

Chemicals

7-Oxocholesterol and cholest-3,5-dien-7-one were purchased from Steraloids (Wilton, NH). Free fatty acid methylester and sterols were from Sigma (St. Louis, MO). Five percent hydrogen chloride methanol solution and solvent were from Nacalai Tesque (Kyoto, Japan).

Statistical Analysis

The data were analyzed statistically with the unpaired Student's *t* test and regression analyses with Stat Mate (Nankodo, Japan) statistical software. Differences were considered significant at a *P* level less than .05.

RESULTS

Table 1 lists biochemical data for the diabetic and healthy subjects. The total cholesterol level and triglycerides were significantly higher in diabetics compared with healthy subjects. High-density lipoprotein cholesterol levels were significantly lower in diabetic subjects versus healthy subjects.

Figure 1 shows the representative total ion chromatogram of methylated lipid from the erythrocyte membranes of a diabetic patient whose HbA1c value was 7.4%. The peaks were identified on the basis of their retention time and fragment ion

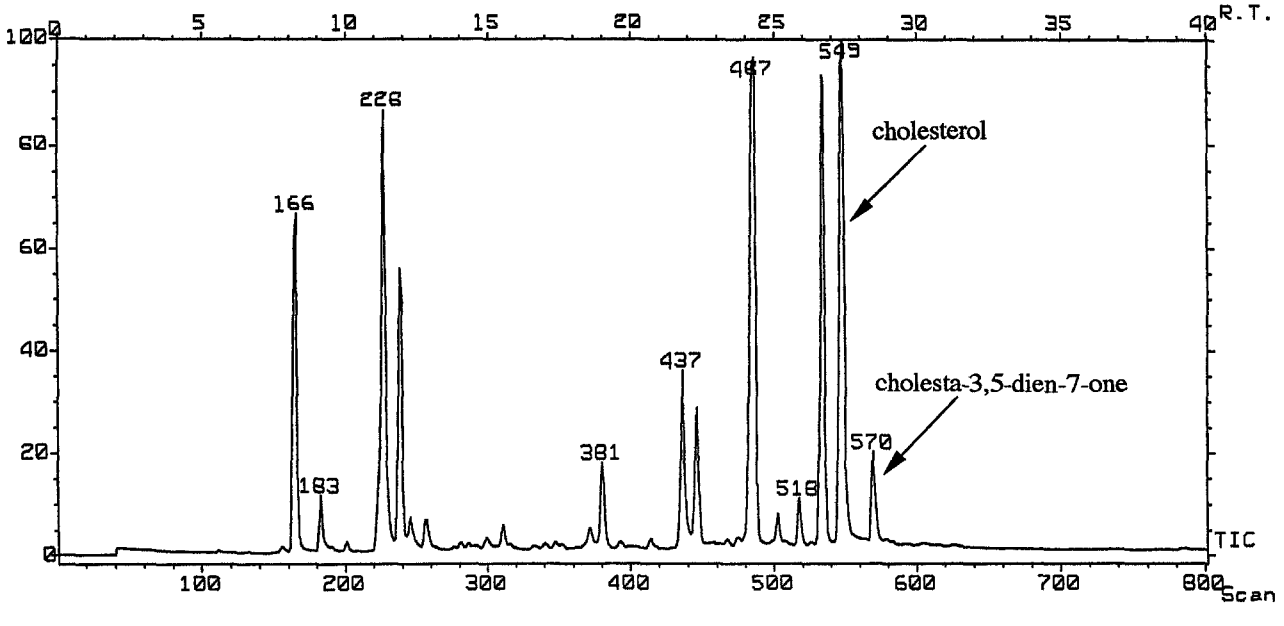

Fig 1. Representative total ion chromatogram of erythrocyte membrane lipid extract from a diabetic subject. Peak components at scans 166, 228, 238, 281, 381, 437, 487, 534, 549, and 570 were C16:0, C18:1, C18:0, C20:4, C22:0, C24:1, cholest-3,5-dien, cholesteryl methylester, cholesterol, and cholest-3,5-dien-7-one, respectively.

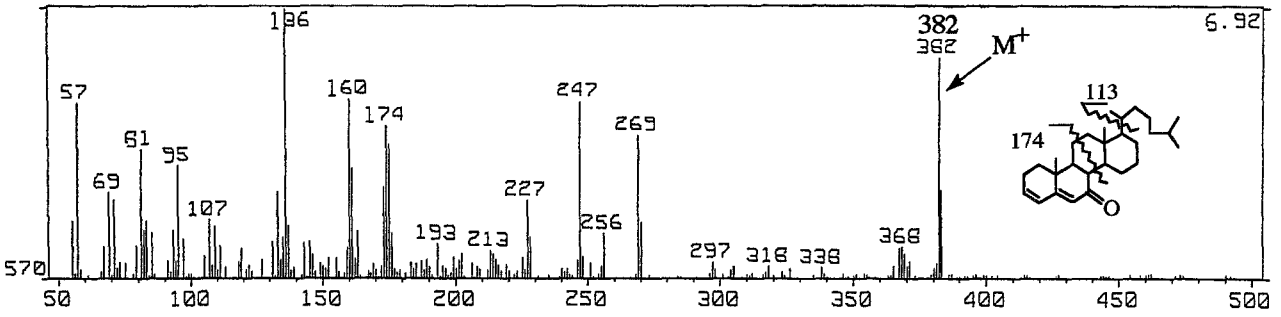

Fig 2. Mass spectrum of cholest-3,5-dien-7-one at scan 570 in Fig 1.

intensity ratio being the same as those of the synthesized standard samples. The peak component at scan 570 was identified as cholest-3,5-dien-7-one, a dehydration product of 7-oxocholesterol via methanolysis. Figure 2 shows the mass spectrum of cholest-3,5-dien-7-one at scan 570 in Fig 1. Cholest-3,5-dien-7-one produced molecule-ion (M^+) peaks at *m/z* 382. It is difficult to clarify the origin of individual fragment ions. However, *m/z* 367, 269, and 174 indicate the fragment ions of $[M\text{-}CH_3]^+$, $[M\text{-}C_6H_{17}]^+$, and $[C_{11}H_{10}O]^+$ (Fig 2). Using GC/MS, it was confirmed that the methanolysis of standard 7-oxocholesterol resulted in the production of cholest-3,5-dien-7-one (data not shown). Since 7-oxocholesterol was not detected in our study, it was surmised that 7-oxocholesterol in each sample is completely converted to cholest-3,5-dien-7-one via methanolysis.

These peak height ratios of 7-oxocholesterol to cholesterol and HbA1c values are presented in Table 1. There was a significantly increased peak height ratio in erythrocytes of diabetic patients compared with healthy subjects. Naturally, there was a significantly elevated HbA1c in erythrocytes of diabetic patients compared with healthy subjects. Linear regression analysis of the peak height ratio of 7-oxocholesterol to cholesterol and HbA1c values in diabetic and healthy erythrocytes showed a significant positive correlation (regression equation, $y = 5.8\times - 23.0$, $r = .82$, $P < .001$; Fig 3).

DISCUSSION

The glycation of hemoglobin appears to be a slow nonenzymatic posttranscriptional event occurring over the approximately 120-day life span of the red blood cell.[17] HbA1c comprises 3.4% to 5.8% of total hemoglobin in normal human red blood cells, but is increased in patients with overt diabetes mellitus.[18-20] The HbA1c level is monitored as a reliable index of glycemic control in diabetes.[21,22]

Most methods available for the measurement of OFRs are unfortunately indirect and nonspecific.[23,24] Traditional methods of estimating lipid peroxidation have most frequently used the thiobarbituric acid (TBA) and conjugated-diene assay. Both of these assays lack specificity (false-positive absorbance by noninterest chromogens) and, in the case of conjugated dienes, sensitivity (low extinction coefficient), particularly with in vivo samples.[25,26] GC/MS, on the other hand, represents a sensitive and specific method to identify and quantify lipid peroxidation products.[27-29] It was confirmed that 7-oxocholesterol is methanolyzed to produce cholest-3,5-dien-7-one in our study. Accordingly, it is not cholest-3,5-dien-7-one but 7-oxocholesterol that exists in erythrocyte membranes in vivo.

Peroxides and hydroperoxides of cholesterol were not detected in this study, since they may be heat-instable in gas chromatography. We measured the peak height ratio of 7-oxocholesterol to cholesterol and the HbA1c level in erythrocytes obtained from diabetic and healthy subjects and examined the correlation between them. Our results showed that the ratio of 7-oxocholesterol to cholesterol, a marker of lipid peroxidation, is significantly related to the HbA1c level, an index of glycemic stress. This could suggest that increased glycation of hemoglobin via chronic hyperglycemia may predispose phospholipid in the inner membrane bilayer of erythrocytes to oxidative damage. Our findings are supported by a study by Jain,[30] who reported that erythrocytes incubated with elevated levels of glucose showed significantly increased membrane lipid peroxidation compared with control erythrocytes. In contrast to our study, Requena et al[31] reported no indication of increased oxidative modification of either the lipid or protein components of red blood cell membranes in a group of poorly controlled diabetic and control subjects. They did not mention any differences in plasma lipid status when comparing diabetic and control subjects.

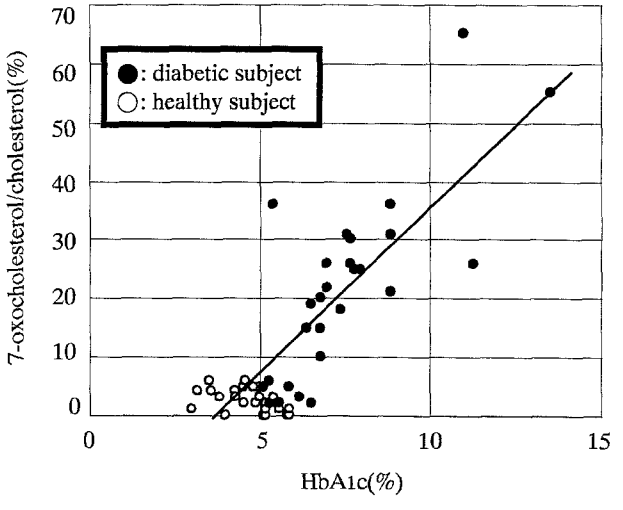

Fig 3. Linear regression analysis of peak height ratio of 7-oxocholesterol to cholesterol and Hb A1c in both diabetic and healthy subjects ($r = .82$, $P < .001$). Regression equation, $y = 5.8\times - 23.0$.

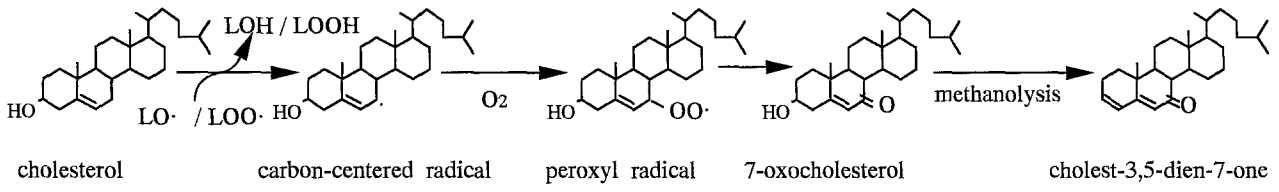

Fig 4. Hypothetical reactions in cholesterol peroxidation. LO , lipid alkolyl radical; LOO , lipid paroxyl radical.

However, hypertriglyceridemia was observed in our group of diabetic subjects. The discrepancy could be partly attributed to hypertriglyceridemia, which can be a source of oxidizable lipid substrate in plasma. There is a possibility that nonenzymatic glycation of proteins in plasma may also cause oxidation of the outer-membrane lipid of erythrocytes. Hunt et al[32] reported that human low-density lipoprotein (LDL) incubated with high glucose and cupricion yielded higher levels of TBA reactivity and lipid peroxide formation than LDL exposed to copper without glucose. Since there were, in fact, comparable increases in both lipemia (triglycerides) and glycemia in the diabetic subjects, the increased OFRs may originate from an increase in oxidizable lipid substrate in plasma, as well as an increase in glycated hemoglobin or plasma proteins. Increased OFRs may attack both sides of the membrane lipid of erythrocytes and convert the resident cholesterol to 7-oxocholesterol. It is surmised that the altered phospholipid organization would cause decreased deformability of erythrocytes, which may lead to diabetic microangiopathy. Reduced erythrocyte deformability has been reported in diabetic patients with microvascular disease.[33] No consensus has been reached as to the mechanism underlying the reduced erythrocyte deformability observed in diabetic patients. Previously suggested mechanisms include increased internal viscosity[34] and increased erythrocyte membrane rigidity.[35] Membrane lipid peroxidation is also suggested as a possible cause of increased erythrocyte rigidity in diabetic patients.[36] The oxidative damage may be anticipated to occur in endothelial or other cells of diabetic subjects. Thus, the peroxidation of membrane cholesterol may also play an important role in the increased erythrocyte membrane rigidity.

The exact mechanism by which elevated blood glucose leads to membrane lipid peroxidation in erythrocytes of diabetic subjects is not clear. Nonenzymatic glycation of proteins has been postulated to explain the relationship between hyperglycemia and lipid peroxidation.[37] This hypothesis is based on the observation that glucose can attach to the amino groups of proteins using a nonenzymatic process that forms a Schiff-base compound. This Schiff-base adduct will convert to form stable glycation products such as glycated hemoglobins. After several days and weeks of increased formation of glycated products, these products will begin to oxidize, which can generate OFRs that could cause lipid peroxidation. This process is accelerated when there is an increased amount of glucose present. In brief, chronic hyperglycemia results in autoxidative glycation/oxidation and lipid peroxidation.[38] The glycation of hemoglobin via hyperglycemia may also generate OFRs. OFRs formed in excess of the detoxifying capacity of erythrocytes would cause oxidative damage to the membrane lipid in erythrocytes. Most unsaturated fatty acids in the inner-membrane bilayer of erythrocytes are susceptible to peroxidation.[30] Cholesterol peroxidation is postulated to occur as follows (Fig 4): lipid alkoxyl or peroxyl radicals arising from peroxidation of unsaturated fatty acids would initially abstract a reactive allylic 7-hydrogen atom from cholesterol, yielding the carbon-centered radical, which may react with oxygen (O_2) to yield peroxyl radical. 7-Oxocholesterol is proposed to form by bimolecular decomposition of the peroxyl radicals. Cholest-3,5-dien-7-one may form by dehydration of 7-oxocholesterol via methanolysis.

The erythrocyte is a suitable target to investigate the relationship between glycation and lipid peroxidation via hyperglycemia in diabetic subjects. In conclusion, it is suggested that glycation via chronic hyperglycemia links cholesterol peroxidation in erythrocytes. Furthermore, the ratio of 7-oxocholesterol to cholesterol is an indicator of peroxidative damage in the erythrocyte and other tissue membranes of diabetic patients.

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