Semiquantitative Analysis of Apolipoprotein A-I Modified by Advanced Glycation End Products in Diabetes Mellitus

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Background: Apolipoprotein A-I (Apo A-I), the major component of high-density lipoprotein (HDL), is modified by reactive α -oxoaldehydes, such as methylglyoxal (MG) and glycolaldehyde (GA), and these modifications affect the function of Apo A-I. GA- and MG-modified Apo A-I serum levels were semiquantitatively evaluated in diabetic patients to elucidate the association of each protein with diabetes and to determine its appropriateness as a serum marker of diabetes. **Methods:** We enrolled 44 subjects in this study (diabetic subjects, $n = 24$; nondiabetic subjects, $n = 20$). GA- and MG-modified Apo A-I levels in serum were determined by sandwich enzyme-linked immunosorbent assay (ELISA) by using anti-GA or anti-MG an-

tibody and anti-Apo A-I antibody. **Results:** The GA-modified Apo A-I levels did not significantly differ between the diabetic and nondiabetic subjects (1.00 ± 0.38 vs. 0.96 ± 0.22). However, the MG-modified Apo A-I levels in the diabetic subjects were significantly higher than those in the nondiabetic subjects (1.33 ± 0.52 vs. 0.90 ± 0.20). In addition, MG-modified Apo A-I levels correlated with the glycated hemoglobin (HbA1c) levels, HDL-cholesterol levels, and the homeostasis model assessments of insulin resistance, which are indicators of insulin resistance. **Conclusion:** The MG-modified Apo A-I level may be an indicator of diabetic dyslipidemia and insulin resistance. J. Clin. Lab. Anal. 27:231–236, 2013. © 2013 Wiley Periodicals, Inc.

Key words: apolipoprotein A-I; advanced glycation end products; HDL cholesterol; diabetes mellitus; enzyme-linked immunosorbent assay

INTRODUCTION

Hyperglycemia is the most definitive characteristic of diabetes mellitus. However, the fasting plasma glucose (FPG) level is strongly influenced by daily meals, and

the measurement of glycated hemoglobin (HbA1c) is recommended as an indicator of the patient's blood glucose state in the previous 1–2 months (1). HbA1c is an early product of Amadori rearrangement after a Schiff base

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is formed when an aldehyde group reacts with an *N*-terminal amino group or a lysine residue ϵ -amino group of the hemoglobin molecule. These Amadori rearrangement products are then converted to advanced glycation end products (AGEs) via complex reactions, including oxidation, dehydration, and aggregation (2). AGE modification is known to occur not only with hemoglobin, but also with various proteins, thus affecting their functions (3).

Chronic hyperglycemia leads to the production of highly reactive and toxic α -oxoaldehydes such as methylglyoxal (MG) and glycolaldehyde (GA). It has recently been reported that these oxoaldehydes affect the function of apolipoprotein A-I (Apo A-I), the major protein component of high-density lipoprotein (HDL) (4, 5). In type 2 diabetes patients, Apo A-I is modified by GA and MG derived from the reduction of sugars, and AGE modification of Apo A-I decreases lecithin-cholesterol acyltransferase activity (6, 7) and cholesterol removal from cells via ATP-binding cassette transporter A1 (8–10). These findings suggest that AGE modification of Apo A-I causes the dyslipidemia associated with type 2 diabetes mellitus. A decrease in the HDL cholesterol (HDL-C) level is observed in the early stage of diabetes progression (11). HDL plays an important role in the reverse cholesterol transport pathway, which relies on the function of Apo A-I (12, 13); therefore, lower HDL-C levels are associated with an increased risk of acute coronary syndrome.

By measuring AGE-modified Apo A-I levels in the serum, it is possible to make a precise assessment of the complications in diabetic patients, such as dyslipidemia. In this study, GA- and MG-modified Apo A-I were semiquantitatively evaluated in diabetic subjects to elucidate the association of each protein with diabetes and to determine its appropriateness as a serum marker of diabetes.

MATERIALS AND METHODS

Subjects

Forty-four subjects were enrolled (diabetic subjects, $n = 24$; nondiabetic subjects, $n = 20$). All subjects provided informed consent, and the protocol was approved by the Kitasato University Medical Ethics Committee (B09–81). We excluded patients with hemoglobinopathy and those who were undergoing insulin therapy. Furthermore, we excluded patients with serious systemic diseases, such as acute/chronic inflammation and malignancies. The nondiabetic subjects had normal FPG (<7.0 mmol/l) and HbA1c ($<6.5\%$) levels, and they were not on any medications. For each subject, the serum levels of the following biochemical markers were measured after an overnight fasting period: FPG, HbA1c, fasting insulin (FIRI), total cholesterol (TC), triglyceride (TG), HDL-C, and low-density lipoprotein cholesterol (LDL-C; Table 1). The

TABLE 1. Clinical Characteristic of Study Subjects

	Nondiabetic subjects	Diabetic subjects
<i>n</i> (female)	20 (11)	24 (3)
Age	60.0 $\pm$ 12.9	57.3 $\pm$ 12.1
FPG (mmol/l)	5.6 $\pm$ 0.5	10.1 $\pm$ 2.4***
HbA1c (%)	5.7 $\pm$ 0.3	8.7 $\pm$ 1.3***
FIRI	4.3 $\pm$ 2.7	18.9 $\pm$ 25.7*
HOMA-IR index	1.1 $\pm$ 0.7	9.6 $\pm$ 15.3*
TG (mmol/l)	1.0 $\pm$ 0.4	2.0 $\pm$ 1.4**
TC (mmol/l)	5.5 $\pm$ 0.9	5.0 $\pm$ 1.4
HDL-C (mmol/l)	2.1 $\pm$ 0.6	1.4 $\pm$ 0.3***
LDL-C (mmol/l)	3.1 $\pm$ 0.8	3.2 $\pm$ 0.7

FPG, fasting plasma glucose; FIRI, fasting immunoreactive insulin; HOMA-IR, homeostasis model assessment of insulin resistance; TG, triglyceride; TC, total cholesterol; HDL-C, HDL-cholesterol; LDL-C, LDL cholesterol.

Results are given as mean $\pm$ SD.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus nondiabetes group.

Student's unpaired *t*-test or Mann–Whitney *U*-test, as appropriate.

subjects' sera were analyzed using a Hitachi Autoanalyzer 7600 (Hitachi, Tokyo, Japan). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated from FPG and FIRI levels. Serum samples from six healthy volunteers in our laboratory were used to evaluate reproducibility and preservation stability.

Semiquantification of GA- and MG-Modified Apo A-I by Enzyme-Linked Immunosorbent Assay (ELISA)

To prevent interference by a nonspecific response with glycated albumin in GA- (GA-A-I) and MG-modified Apo A-I (MG-A-I) measurements, albumin-free serum that had been prepared in advance was used (14). To remove serum albumin, we used Blue Sepharose CL-6B (GE Healthcare UK Ltd., England). An 800 μ l aliquot of the blue sepharose solution (25 g Blue Sepharose CL-6B in 20 ml of 50 mmol/l NaCl containing Tris-HCl buffer at pH 7.0) was centrifuged at $10,000 \times g$ for 10 min, and the supernatant was removed. To the precipitate blue sepharose, 100 μ l of serum was added and incubated for 15 min. Subsequently, 100 μ l of 50 mmol/l NaCl containing Tris-HCl buffer (pH 7.0) was added and centrifuged at $10,000 \times g$ for 10 min, and the supernatant was removed. Approximately 90% of the albumin was removed by this method when subjected to sodium dodecyl sulfate-poly acrylamide gel electrophoresis. Furthermore, the sera were diluted with phosphate buffered saline (PBS) containing Tween-20 to expose the antigenic sites of Apo A-I (15). A 360- μ l solution of 0.15% Tween-20 and 4.5% skim milk in PBS (pH 7.2) was added to 180 μ l of albumin-free serum.

Fig. 1. Change in GA-A-I and MG-A-I levels following preservation at 4°C. Sera of the healthy volunteers ($n = 6$) were preserved at 4°C (0–14 days), and changes in GA-A-I and MG-A-I serum levels were analyzed (A: GA-A-I, C: MG-A-I). In addition, 5.6–27.8 mmol/l glucose was added to the healthy volunteers' pooled sera, and the changes in GA-A-I and MG-A-I levels following preservation at 4°C were evaluated (B: GA-A-I, D: MG-A-I). Significant differences in the serum levels after preservation were determined by one-way ANOVA, and no significant differences were observed between the levels of GA-A-I and MG-A-I.

The GA-A-I and MG-A-I levels were determined by sandwich ELISA by using an anti-GA or anti-MG antibody and anti-Apo A-I antibody. The anti-GA antibody (9D8; Trans-Genic, Inc., Japan) or anti-MG antibody (14B5; Trans-Genic, Inc.) was diluted with a carbonate buffer (pH 9.6) to 0.25 $\mu\text{g}/\text{ml}$, and 100 μl of the antibody solution was solid-phased on plates by incubating overnight at room temperature. After washing the plate with a sufficient amount of PBS (pH 7.4), 300 μl of carbonate buffer (pH 9.6) containing 3% skim milk was added to the plate for 3 hr at room temperature. After washing the plate with PBS (pH 7.4) containing 0.1% Tween 20, 100 μl of the prepared serum was added to the plate and incubated at 37°C for 1 hr. After washing, it was allowed to react with rabbit anti-Apo A-I antibody (178422; Calbiochem, Germany) diluted 1:1,000 with 3% skim milk in PBS (pH 7.4) for 1 hr at 37°C. The plate was washed again, and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (401353, Calbiochem, Germany) diluted 1:10,000 was added to the plate and incubated for 1 hr at 37°C. After washing, color was developed with tetramethylbenzidine (KPL, Inc., Gaithersburg, Maryland) as a sub-

strate, and the absorbance was measured at 450 nm after adding 1 mol/l HCl to the plate. The ELISA data for GA-A-I and MG-A-I were expressed as relative concentrations (absorbance from the diabetic or nondiabetic sample divided by absorbance from the pooled serum of the six healthy volunteers). The inter- and intra-assay coefficients of variation (CVs) for GA-A-I were 3.0–10.9% and 5.1–10.2%, respectively. The inter- and intra-assay CV for MG-A-I were 2.3–3.8% and 6.3–7.8%, respectively.

Statistical Analysis

Significant differences between the diabetic and nondiabetic groups were determined by the Student unpaired t -test or Mann-Whitney U test. Significant differences within the serum preservation samples were determined by one-way analysis of variance (ANOVA). The relationships between the different variables were analyzed using the Pearson correlation coefficient. Ky Plot 5.0 (Kyens Lab, Inc., Tokyo, Japan) was used for all the analyses. Significance was set at $P < 0.05$. The results are expressed as mean $\pm$ SD.

RESULTS

Semiquantification of GA-A-I and MG-A-I Levels in Diabetic Subjects

In the assay for GA-A-I and MG-A-I levels, the preservation stability at 4°C was evaluated with sera of the healthy volunteers (Fig. 1). The GA-A-I and MG-A-I serum levels determined by the present method were unchanged after storing the sera for 14 days at 4°C. At a total glucose concentration of 27.8 mmol/l, the pooled healthy sera did not affect the GA-A-I and MG-A-I levels after 14 days.

The GA-A-I and MG-A-I levels in the pooled sera were compared between the diabetic and nondiabetic subjects. The GA-A-I levels showed no significant differences between the diabetic and nondiabetic subjects (1.00 ± 0.38 vs. 0.96 ± 0.22 ; Fig. 2A). However, the MG-A-I serum levels in the diabetic subjects were significantly higher than those in the nondiabetic subjects (1.33 ± 0.52 vs. 0.90 ± 0.20 ; Fig. 2B).

Relationships Between GA-A-I or MG-A-I Levels and Parameters Related to Glycemic Control

The relationship between the GA-A-I or MG-A-I levels and parameters related to glycemic control were analyzed (Fig. 3). GA-A-I levels showed no correlation with HbA1c levels, HDL-C levels, or the HOMA-IR index. In contrast, MG-A-I levels positively correlated with HbA1c levels and the HOMA-IR index. Furthermore, MG-A-I negatively correlated with HDL-C levels.

DISCUSSION

It has been reported that GA and MG affect Apo A-I function and have a role in the progression of dyslipidemia in diabetic patients. Some researchers have reported a relationship between nonenzymatic glycation and Apo A-I function. However, quantitative analyses of serum GA-A-I and MG-A-I levels in diabetic patients have not been reported. In the present study, serum levels of GA-A-I and MG-A-I in diabetic subjects were semiquantitatively evaluated using sandwich ELISA.

GA-A-I levels did not significantly differ between the diabetic and nondiabetic groups. However, serum MG-A-I levels were significantly elevated in the diabetic subjects. In patients with diabetes mellitus, hypo- α -lipoproteinemia, a risk factor for cardiovascular disease (CVD), often develops. One cause of its development is the decrease in Apo A-I expression in the liver (16, 17). On the other hand, α -oxoaldehydes, including MG, generated in chronic hyperglycemia also contribute to the decrease in reverse cholesterol transport by HDL, because of decreased HDL-C levels (18, 19). Previous reports have suggested that the in-

Fig. 2. Semiquantitative analyses of GA-A-I and MG-A-I levels in diabetes mellitus. GA-A-I and MG-A-I serum levels were semiquantitatively determined by ELISA. The data for GA-A-I and MG-A-I were expressed as relative concentrations compared with those in the pooled sera obtained from healthy volunteers. The open circles represent the mean $\pm$ SD (ns = not significant, *** P < 0.001 with the unpaired Student t -test).

crease in serum MG-A-I levels leads to a decrease in HDL-C level in diabetic patients. In our study, we found that MG-A-I levels inversely correlated with HDL-C levels. Additionally, it has been reported that lipid-poor Apo A-I inhibits neutrophil infiltration into the artery wall and the expression of intercellular adhesion molecule-1 and vascular adhesion molecule-1 in rabbit carotid sections, and that MG modification impairs these anti-inflammatory properties of Apo A-I (20). The impaired functioning of

Fig. 3. Correlation between serum GA-A-I or MG-A-I levels and diabetes-related parameters in all subjects. Correlations between serum GA-A-I (A–C) or MG-A-I (D–F) levels and diabetes-related parameters were determined. Nondiabetic subjects are represented with open circles, and diabetic subjects are represented with closed circles. The relationships between the different variables were analyzed using the Pearson correlation test (R , correlation coefficient).

AGE-modified Apo A-I may significantly contribute to the increased risk of atherosclerosis associated with diabetes. The sera of some of the study subjects had high MG-A-I levels even though their HDL-C levels were within the normal range. The combination assay of HDL-C and MG-A-I may make it possible to more sensitively detect dyslipidemia in diabetes. Premature CVD is the most common cause of morbidity and mortality in diabetic patients; therefore, it is important to accurately monitor the progression of dyslipidemia in order to prevent development of CVD in diabetic patients.

Our results showed that MG-A-I levels correlated with the HOMA-IR indices. A previous report suggested that AGEs inhibit insulin action and lead to insulin resistance in the skeletal muscle of diabetic patients (21). Another report showed that MG impairs insulin signaling and glucose-induced insulin secretion from pancreatic β -cells (22). Insulin resistance is an important parameter for observing the progression of type 2 diabetes (23, 24). It has been suggested that the determination of MG-A-I lev-

els reflects insulin resistance in the progression of type 2 diabetes.

In conclusion, the serum MG-A-I levels were elevated, but GA-A-I levels were maintained, in diabetic subjects examined in our study. MG-A-I levels may be a measurable index in the management of diabetic dyslipidemia and insulin resistance.

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