Increased Plasma Lipid-Poor Apolipoprotein A-I in Patients with Coronary Artery Disease

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Background: Preβ1-HDL participates in a cyclic process involved in the retrieval of cholesterol from peripheral tissues. Although preβ1-HDL can be measured by two-dimensional electrophoresis or crossed immunoelectrophoresis, these methods are time-consuming and require technical expertise. In this study, we separated plasma lipid-poor apolipoprotein A-I (apo A-I) by high-performance size-exclusion chromatography.

Methods: We measured plasma lipid-poor apo A-I in 20 male patients with coronary artery disease [CAD; mean (SD) age, 64.0 (18) years] and 15 male controls [54.7 (17) years] and in 7 female CAD patients [70.3 (7.7) years] and 9 female controls [65.1 (4.7) years].

Results: Lipid-poor apo A-I was most stable when stored at −80°C in the presence of aprotinin (final concentration, 50 kIU/L). The lipid-poor apo A-I concentration decreased during incubation at 37°C, and this was not prevented by the addition of 2 mmol/L of the lecithin:cholesterol acyltransferase (LCAT) inhibitor, 5,5'-dithiobis(2-nitrobenzoic acid). Lipid-poor apo A-I was significantly higher in CAD patients than in controls [38.3 (7.9) mg/L for male CAD patients vs 29.3 (7.3) mg/L for male controls; 43.3 (11) mg/L for female CAD patients vs 27.1 (7.4) mg/L for female controls (P <0.01 for both)]. There were no significant differences in LCAT activity or cholesteryl ester transfer protein (CETP) concentration between patients and controls. Moreover, the plasma lipid-poor apo A-I concentration was not significantly correlated with LCAT or CETP activities.

Conclusions: Although the production of lipid-poor apo A-I in plasma is not fully understood, our results indicate that lipid-poor apo A-I could be used as a marker for arteriosclerosis and demonstrate that it is not identical to the preβ1-HDL measured by other methods.

HDLs play a very important role in reverse cholesterol transport (1–3), and HDL particles are heterogeneous in terms of particle size, lipid content, and apolipoprotein composition. It is well known that HDLs can be divided into major subfractions: those with α mobility and the preβ-migrating HDL (4, 5). In HDL subfractions, apolipoprotein A-I (apo A-I)4-containing lipoproteins with preβ electrophoretic mobility are called preβ1-HDL (6), small lipid-poor-apo A-I-containing lipoprotein (7), or preβ apo A-I (8), which have attracted attention as the first acceptors of cellular free cholesterol and are regulated by lecithin:cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP), phospholipid transfer protein, lipoprotein lipase, and hepatic triacylglycerol (9). At present, apo A-I concentrations are measured by various methods, including crossed immunoelectrophoresis (10), two-dimensional electrophoresis (6, 11) and sandwich enzyme immunoassays (12). It is thus uncertain whether preβ1-HDL, small lipid-poor-apo A-I-containing lipoprotein, and preβ apo A-I are identical, and interpretation of the data is controversial. Indeed, in one report, the plasma preβ1-HDL concentrations measured by two-dimensional electrophoresis were increased in coronary artery disease (CAD) (13), whereas in another report, the preβ1-HDL concentration measured by crossed immunoelectrophoresis was decreased in patients with CAD (14). Recently, Nanjee and Briton (7) separated very small apo

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Nonstandard abbreviations: apo A-I, apolipoprotein A-I; LCAT, lecithin: cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; CAD, coronary artery disease; HP-SEC, high-performance size-exclusion chromatography; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TG, triglyceride; DTNB, 5,5’-dithiobis(2-nitrobenzoic acid); and ABC1, ATP-binding cassette transporter 1.

The purpose of the present study was to measure lipid-poor apo A-I fractionated by HP-SEC in patients with CAD and to evaluate the effect of LCAT and CETP on the concentrations of lipid-poor apo A-I.

**Materials and Methods**

**Participants**

Blood samples were obtained after an overnight fast from 20 male [mean (SD) age, 64.0 (18) years] and 7 female [70.3 (7.7) years] patients with CAD that had been confirmed by coronary angiography. Fifteen males [54.7 (17) years] and 9 females [65.1 (4.7) years] without CAD or hyperlipidemia were also included as age-matched controls. In addition, 17 healthy normolipidemic males were recruited for a preliminary study. Informed consent was obtained from all participants, and the protocol was approved by the Ethics Committee of Gifu University School of Medicine.

**HP-SEC**

Blood was drawn into tubes containing dipotassium EDTA (final concentration, 1.0 g/L). Plasma was immediately separated and was subjected to HP-SEC according to the method of Nanjee and Briton (7). Briefly, HP-SEC was performed with a 30 cm × 10 mm (i.d.) Superdex 200 HR 10/30 column (Pharmacia) connected in series to a 30 cm × 10 mm (i.d.) Superdex 75 HR 10/30 column (Pharmacia). Immediately after separation, 100 µL of plasma or serum was subjected to HP-SEC. The flow rate was 0.5 mL/min, and the buffer was 50 mmol/L Tris-HCl (pH 7.4) containing 150 mmol/L NaCl.

After the fractionation of plasma by HP-SEC, the apo A-I concentration in 0.5 mL of each fraction was determined by two-step sandwich ELISA as reported previously (15). Each well of a Microtest plate (Nunc A/S) was coated with 50 µL of primary monoclonal antibody [anti-apo A-I; 8.0 g/L, diluted with phosphate-buffered saline (PBS), pH 7.2] and incubated overnight at 4 °C. After incubation, the plates were washed twice with washing solution (PBS, pH 7.2, containing 0.5 mL/L Tween 20 and 1 g/L Na3VO4) and then blocked with 300 µL of blocking reagent [PBS, pH 7.2, containing 0.05 mL/L Tween 20 and 5 g/L bovine serum albumin (BSA)]. After blocking for 2 h at 37 °C, the plates were washed three times with washing solution. Appropriately diluted calibrators or 50 µL of plasma sample and 100 µL of reaction reagents (PBS containing 5 g/L BSA, 0.5 mL/L Tween 20, 10 mmol/L MgCl2, and 1 g/L Na3VO4) were incubated in the plate for 2 h at room temperature. After incubation, the plates were washed three times with washing solution and then incubated for 2 h at room temperature with β-galactosidase-labeled secondary antibody. β-Galactosidase activity was measured with o-nitrophenol-β-d-galactopyranoside solution as substrate. Reaction mixtures were incubated for 1.5 h at room temperature, and the reaction was terminated by the addition of 0.1 mol/L Na2CO3. The plates were read at 405 nm on a Behring ELISA processor II, and the readings were corrected for absorbance at 650 nm. Plasma apo A-I concentrations were also measured in appropriately diluted samples.

**Two-dimensional electrophoresis**

Plasma and lipid-poor apo A-I fractionated by HP-SEC were subjected to two-dimensional agarose–polyacrylamide gel electrophoresis according to the method of Castro and Fielding (4). Briefly, electrophoresis in the first dimension was performed in 7.5% agarose gels in 50 mmol/L barbital buffer (pH 8.6). Electrophoresis in the second dimension was performed with the agarose strip inlaid on a gradient (2–15%) polyacrylamide gel and run in 0.025 mol/L Tris-glycine buffer (pH 8.3) at 1900 V-h. Plasma proteins were electrotransferred at 30 V for 10 h to a nitrocellulose membrane, and the membrane was incubated with biotinylated anti-human apo A-I and streptavidin-horseradish peroxidase at room temperature for 1 h. Immunodetection was performed by the luminescence method (ECL; Amershams Life Science). The reagents were incubated for 1 min with the nitrocellulose membrane, which was then exposed to hyperfilm ECL.

**Measurement of PreB1-HDL**

Preβ1-HDL was measured by ELISA (Daiichi Pure Chemicals) as reported previously (12). Purified human apo A-I (used as a calibrator) and sample plasmas diluted 2121-fold with BSA-PBS were added to each well of a 96-well plate coated with a monoclonal antibody to preβ1-HDL. After incubation of the plate for 1 h at room temperature, the wells were washed three times with 1 g/L BSA in PBS, and the adsorbed preβ1-HDL was incubated with a horseradish peroxidase-coupled secondary antibody (goat anti-human apo A-I polyclonal antibody). The rest of the procedure was identical to that for the apo A-I ELISA described above. The intraassay CV was 3.1–5.3%, and the interassay CV was 4.9–9.1%.

**Measurement of LCAT activity and CETP concentration**

LCAT activity in serum was measured by an Anasolv LCAT assay (Daiichi Chemicals, Co., Ltd.). For the colorimetric measurement of cholesterol in this assay, a 500 nmol/L recrystallized cholesterol solution in isopropanol was used as the calibrator, and the intra- and interassay CVs were both <4%. Serum CETP was measured by an assay from Daiichi Chemicals, and the calibration curve was constructed with use of recombinant human CETP. The intra- and interassay CVs were <8.0% and 12%, respectively. Serum cholesterol, triglycerides, and HDL-cholesterol were measured by enzymatic assays from...
International Reagent Corp.; Eiken Chemical Co., Ltd.; and Daiichi Pure Chemicals, respectively.

STATISTICAL ANALYSIS
The data are expressed as the mean (SD) unless otherwise indicated, and the statistical analysis was performed by the unpaired Student t-test or Wilcoxon test. Correlation coefficients were obtained by linear regression analysis.

Results
SEPARATION OF LIPID-POOR APO A-I
A representative distribution of apo A-I in plasma profiled by HP-SEC is shown in Fig. 1. The apo A-I distribution profiles from 12 plasma samples consisted of a major peak at 40 min and a minor peak at 53 min with a clear separation between them at 50 min. The apo A-I distributions in HDL2 (1.063 < d < 1.125 kg/L), HDL3 (1.125 < d < 1.210 kg/L), and the bottom fraction (d >1.210 kg/L) isolated by ultracentrifugation are shown in Fig. 2. The peak elution time of the bottom fraction is almost the same as that of the minor peak described above. The apo A-I in the minor peak fractions that eluted between 51 and 56 min was considered to be lipid-poor apo A-I based on its mobility in two-dimensional electrophoresis (Fig. 3) and its molecular weight of ~55 000, which was measured by HPLC calibrated with the following proteins: α₁-antitrypsin (M₉ 54 000), albumin (M₉ 67 000), transferrin (M₉ 76 000), and IgG (M₉ 160 000). Fractions eluted between 51 and 56 min were thus collected in one tube, and the apo A-I concentration of this sample was considered the lipid-poor apo A-I concentration after volume correction.

We examined the effect of postprandial hyperlipidemia on the concentration of lipid-poor apo A-I in plasma. Plasma was drawn from five healthy male volunteers after an 11-to 13-h fast and at 2.5 h after a meal. All volunteers received a meal (15 kcal/kg of body weight) that provided 45% of calories as carbohydrate, 30% as fat, and 25% as protein. Although triglyceride (TG) concentrations increased significantly after the meal (P <0.05 by paired t-test), there was no significant difference in lipid-poor apo A-I concentration between the fasting and postprandial states (P = 0.543, Wilcoxon test).

IN VITRO STUDY
We examined the effects of freezing and storage on lipid-poor apo A-I. Venous blood was drawn into evacuated glass tubes containing dipotassium EDTA (final concentration, 1 g/L of blood) and aprotinin (final concentration, 50 kIU/L). Plasma samples were stored at 4 or −80 °C after separation. When plasma supplemented with aprotinin was immediately subjected to HP-SEC and the fractions were stored at −80 °C, the concentration of lipid-poor apo A-I was stable for 1 week (Fig. 4). The intra- and interassay CVs for lipid-poor apo A-I were 6.8% and 7.2%, respectively, under the above conditions.

To study whether LCAT activity affects the concentration of lipid-poor apo A-I, we incubated plasma at 37 °C with or without LCAT inhibitor. Although plasma total apo A-I was not changed during incubation, lipid-poor apo A-I decreased within 30 min (Fig. 5). This decrease, however, was not prevented by 2 mmol/L of the LCAT inhibitor 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).

LIPID-POOR APO A-I CONCENTRATIONS IN PATIENTS WITH CAD
Plasma lipid-poor apo A-I concentrations were significantly higher in patients with CAD than in controls in both males and females (P <0.01 for both; Table 1). In
contrast, there were no significant differences in preβ1-HDL concentrations between patients and controls. Additionally, we observed no significant correlation between preβ1-HDL and lipid-free HDL concentrations. Serum CETP concentrations and LCAT activity were not significant different between patients and controls. Plasma lipid-poor apo A-I concentrations were correlated positively with TG concentrations ($P < 0.01$) and negatively with HDL-C concentrations ($P < 0.01$).

**Discussion**

We measured plasma lipid-poor apo A-I fractionated by HP-SEC according to the method of Nanjee and Briton (7)}

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**Fig. 3.** Two-dimensional gel electrophoresis.

(Left), whole plasma; (right), lipid-poor apo A-I. 1D, first dimension; 2D, second dimension.

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**Fig. 4.** Effect of the storage of plasma with or without aprotinin on lipid-poor apo A-I concentration.

( ), $4^\circ C$ without aprotinin; ( ), $4^\circ C$ with aprotinin; ( ), $-80^\circ C$ without aprotinin; ( ), $-80^\circ C$ with aprotinin.

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**Fig. 5.** Effect of LCAT activity on lipid-poor apo A-I concentrations in plasma.

( ), no DTNB added; ( ), DTNB added.
with minor modifications. The molecular weight of lipid-poor apo A-I in our study was almost 55,000, which is somewhat smaller than the 67,000–75,000 reported previously (8), and the two-dimensional electrophoretic mobility of lipid-poor apo A-I was similar to that of preβ1-HDL (8,11). Plasma lipid-poor apo A-I was not stable, and its concentration increased with time during storage at 4 °C. Instability has also been reported for preβ1-HDL measured by two-dimensional electrophoresis (10) and ELISA (16). In the present study, aprotinin-supplemented (final concentration, 50 KIU/L) plasma lipid-poor apo A-I obtained by HP-SEC was stable for up to 7 days at −80 °C.

The plasma lipid-poor apo A-I concentration was significantly higher in patients with CAD than in controls, which is consistent with a previous report showing that preβ1-HDL determined by two-dimensional electrophoresis is increased in CAD (13). However, the preβ1-HDL concentration measured by ELISA in this study was not increased in CAD. More interestingly, the plasma preβ1-HDL concentration in CAD is entirely controversial: it has been reported to be increased (13), unchanged (17), and decreased (14) in CAD. Hattori et al. (14) suggested that this discrepancy may be attributable to the heterogeneous patient group in each study and the different methods or assay conditions used. Actually, the monoclonal antibody used in the ELISA does not react with apo A-I in α-HDL, but only with the apo A-I in preβ1-HDL (12). In contrast, lipid-poor apo A-I in this study was measured by use of a polyclonal antibody that also reacted with apo A-I in α-HDL. Because lipid-poor apo A-I is isolated based on particle size alone, it is different from the preβ1-HDL measured by ELISA. Indeed, in the present study, we observed no correlation between them.

What then is the difference between lipid-poor apo A-I and preβ1-HDL measured by two-dimensional electrophoresis? It is well known that the preβ1-HDL concentration in plasma is decreased by LCAT (13,18,19) and increased by CETP (19,20). In the present study, however, LCAT and CETP concentrations in CAD patients were not different from those in controls, and they were not correlated with plasma lipid-poor apo A-I concentrations. In addition, LCAT activity did not directly affect plasma lipid-poor apo A-I in an in vitro study of an LCAT inhibitor, DTNB. These results indicate that the lipid-poor apo A-I concentration is not regulated by LCAT and CETP, unlike preβ1-HDL. With regard to the LCAT assay, however, the fractional esterification rate of cholesterol in HDL may be more suitable for this study because only the free cholesterol on HDL is a substrate for LCAT as measured by the fractional esterification rate (21). On the other hand, lipid-poor apo A-I was negatively correlated with HDL-C and positively correlated with TG concentrations, as reported previously for preβ1-HDL. Because preβ1-HDL is also modified by hepatic lipase (22) and lipoprotein lipase (23) activity, there is a possibility that the concentration of lipid-poor apo A-I is also affected by these enzymes. Furthermore, ATP-binding cassette transporter (ABC1) activity may also regulate the lipid-poor apo A-I concentration because it is known that decreased ABC1 activity inhibits formation of spherical mature HDL, possibly leading to the accumulation of lipid-poor apo A-I in plasma (2,24–26). In other words, although lipid-free apo A-I molecules might be very rapidly lipidated by ABC1 and transformed into α-lipoprotein A-I-like particles (27), lipid-poor apo A-I could increase when the lipid translocase activity of ABC1 is decreased even if LCAT activity is not decreased. In this study, the concentration of lipid-poor apo A-I and the ratio of lipid-poor apo A-I to total apo A-I in plasma were 28 (7) mg/L and 2.6 (0.8)%, respectively, which are lower than the 60–80 mg/L and 4–8%, respectively, for preβ1-HDL (8,9). We therefore conclude that lipid-poor apo A-I is not identical to preβ1-HDL but may constitute a part of preβ1-HDL.

In conclusion, plasma lipid-poor apo A-I, as separated by HP-SEC, increases in CAD, and this increase is not associated with changes in LCAT activity or CETP concentration. Although regulation of the production of lipid-poor apo A-I is not fully understood, the accumulation of lipid-poor apo A-I in plasma may represent a defect in some step of this process that promotes the unidirectional removal of excess peripheral tissue cholesterol, as does preβ1-HDL (28,29). The measurement of lipid-poor apo A-I concentrations in various pathologic conditions could further our understanding of the overall functions and roles of the HDL subpopulations.

| Table 1. Concentrations of lipid-poor apo A-I, preβ1-HDL, and other lipids. a |
|---|---|---|---|---|---|---|---|
|    | n | Age, years | TC, mmol/L | TG, mmol/L | HDL-C, mmol/L | LDL-C, mmol/L | Lipid-poor apo A-I, mg/L | Lipid-poor apo A-I/total apo A-I, % | Preβ1-HDL, mg/L |
| Males | Controls | 15 | 54.7 (17) | 4.59 (0.62) | 1.53 (0.81) | 1.30 (0.23) | 2.56 (0.70) | 29.3 (7.3) | 2.7 (0.8) | 58.2 (18) |
|       | CAD | 20 | 64.0 (18) | 5.03 (0.62) | 1.90 (1.15) | 1.17 (0.34) | 3.01 (0.75) | 38.3 (7.9) | 4.1 (1.1) | 56.8 (14) |
| Females | Controls | 9 | 65.1 (4.7) | 5.13 (0.7) | 1.14 (0.52) | 1.42 (0.39) | 3.21 (0.67) | 27.1 (7.4) | 2.4 (0.8) | 67.8 (19) |
|       | CAD | 7 | 70.3 (7.7) | 5.13 (0.52) | 2.24 (1.15) | 1.32 (0.47) | 2.8 (0.54) | 43.3 (11) | 4.1 (1.6) | 75.1 (10) |

a Values are the mean (SD).

b TC, total cholesterol; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol.
c-d Compared with controls: a P < 0.05; b P < 0.01; c P < 0.001.

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