〈Original Article〉

Effect of lecithin: cholesterol acyltransferase on the formation of cross-linked apolipoprotein A-I

Yoshifumi Kurosaki[1)], Hitoshi Ikeya[2)], Hisao Haniu[3)] and Zensuke Ogawa[1)]

Summary It is reported that self-associated apolipoprotein A-I (A-I) is in HDL. In the present study we found that these polymers were produced by the action of lecithin-cholesterol acyltransferase (LCAT). A-I that was 280 kDa in size (A-I-280), thought to be polymers of 10 molecules, was found after plasma incubation for 72 h at 37°C.

Production of A-I-280 was then decreased when LCAT was inhibited. There was also a positive correlation between level of A-I-280 production and LCAT activity in plasma ($r = 0.578$, $p = 0.039$). The amount of A-I-280 formed was not affected by incubating with a glycation promoter, antioxidants, or a cross-linking inhibitor. These indicated that the cross-linking of A-I by glycation and oxidation were not associated with the formation of A-I-280.

A cross-linked A-I, A-I-280, detected in this study was suggested to be formed in the normal HDL metabolism containing LCAT.

Key words: Apolipoprotein A-I, HDL, RCT, Cross-linking, LCAT

1. Introduction

A number of studies have reported an inverse correlation between cholesterol in high density lipoprotein (HDL-C) in plasma and coronary artery disease[1-4)]. HDL transports cholesterol in peripheral tissues to the liver and plays an important role in the reverse cholesterol transport (RCT) pathway. It thus has an anti-arteriosclerosis action. The function of lipoprotein is attributed to apolipoprotein, and it is thought that measurement of the apolipoprotein concentration in lipoprotein and observation of qualitative changes would be useful in helping to diagnose coronary artery disease.

Apolipoprotein A-I (A-I) is the most important apolipoprotein component in HDL, and it plays an important role in lipid metabolism. A-I contributes to the reverse transport of cholesterol accumulated in

[1)]Department of Molecular Medical Biology, the Graduate School of Medical Sciences, Kitasato University, Sagamihara, Japan
[2)]Kanagawa Rehabilitation Hospital, Atsugi, Japan
[3)]Department of Integrative Physiology & Bio-System Control, School of Medicine, Shinshu University, Matsumoto, Japan

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Corresponding Author: Yoshifumi Kurosaki, Department of Molecular Medical Biology, the Graduate School of Medical Sciences, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa, NA 228-8555, Japan

peripheral tissue[5-7] in three steps: It recovers free cholesterol (FC) in blood vessel walls via ATP-binding cassette, subfamily A, member 1 (ABCA-1)[8]; esterifies FC by activating lecithin-cholesterol acyltransferase (LCAT; EC 2.3.1.43)[9]; and intake the esterified cholesterol (EC) into the liver via scavenger receptor class B type I (SR-BI)[10-12]. A-I has several high-order structures, and interconversion between lipid-free/poor and lipidated states occurs in the process of metabolism. The lipid-free form of A-I is in a labile state, but it is rapidly lipidized and so almost all A-I is in a lipid-bound form in plasma. Lipid-free/poor A-I, on the other hand, is reported to be the first acceptor in recovering FC from peripheral tissue[13]. Structurally, A-I consists of two helical domains. The N-terminal helical domain in particular is important in stabilizing the lipid-free conformation of A-I and binding with HDL, ABCA-1 and SR-BI[14].

The structure and function of A-I have been well studied as described above; however, there are not many reports on self-association of A-I in vivo. There have been some studies for self-association in vitro. In those studies, it is reported that cross-linked A-I is produced with dependence on pH, A-I concentration, ion strength, and other factors[15-19]. A-I is also reported to be polymerized by oxidation[20, 21] or glycation[22-26] in vitro, and glycated A-I has been shown to have reduced ability to activate LCAT[27]. To our knowledge there have been no investigations of whether A-I self-association occurs in the normal lipid metabolism process. In the present experiment, we found that A-I with molecular weight of 280 kDa (A-I-280) was produced when plasma was incubated at 37℃. We then investigated the relationship between A-I-280 formation and cholesterol esterification by LCAT in plasma.

2. Materials and methods

1. Samples

Blood was collected by venous puncture from normolipidemic healthy volunteers obtained the approval for this study in our laboratory. Plasma specimens were prepared by drawing blood into tubes containing EDTA. The blood was clarified by centrifugation at 1000 × g for 15 min at 4℃.

2. Isolation of proteins in HDL with ultracentrifugation

Plasma specimens from ten healthy volunteers were pooled. Pooled plasma was adjusted to a density of 1.063-1.21 g/ml with sodium bromide solution[28]. A Hitachi RP50T-2 rotor was used. HDL obtained from ultracentrifugation was processed to remove lipids with the use of organic solvent, tri-n-butylphosphate: acetone: methanol (0.5: 6: 0.5)[29]. The precipitate was dried and 0.1 ml of 0.325 mol/l DTT, and 4% CHAPS in 0.045 mol/l Tris-HCl buffer was added. It was then heated at 100℃ and left at room temperature to cool. After that, 1.4 ml of 8 mol/l urea, 4% CHAPS, and 0.1 mol/l DTT in 0.045 mol/l Tris-HCl buffer were added and incubated for 15 min at 37℃ to prepare samples for electrophoresis.

3. Protein identification by two-dimensional electrophoresis and peptide mass fingerprinting

Proteins in HDL processed as described above were electrophoresed on pH range 3-10 agarose gels (agar GEL A-M310; ATTO, Tokyo, Japan) for the first separation by charge. Individual gel strips were replaced along polyacrylamide gel (PAGEL NPU-D10L, ATTO, Tokyo, Japan) and electrophoresed again for separation by size. PMF was done following electrophoresis. Target spots were cut out from the gel and 1.5 ml of 100 mmol/l ammonium carbonate in 50% acetonitrile was added. Decoloration was done at 4℃ overnight. After decoloration, 0.1 mg/ml of sequencing grade trypsin 2 μl was added directly to the gel and left to sit for 15 min. Then 0.5 mmol/l ammonium carbonate 20 μl was added and reacted at 37℃ overnight. After the supernatant was collected, 5% trifluoroacetic acid in 50% acetonitrile 25 μl was again added to the precipitated gel, and after voltexing for 30 min the supernatant was collected. After the dried peptide was again dissolved in 0.2% trifluoroacetic acid 5 μl, it was mixed with 10 mg/mL α-cyano-4-hydroxyl-cinnamic acid in 50% acetonitrile 1 μl and peptide solution 1 μl, and the peptide was measured with a mass spectrometer (Applied Biosystems; Voyager ElitePro). Using the peak value of the measured peptide, a MASCOT search (Peptide

mass fingerprinting; Matrix Science) was done (database: NCBInr, taxonomy: Mus musculus, peptide tolerance: ±0.5 Da). Probability based MOWSE score[30] was confirmed to be above the threshold (= 67), and protein was identified.

4. Measurement of cholesterol in lipoproteins separated by HPLC using gel filtration columns

HPLC was conducted following the methods of Hara et al.[31]. The HPLC system consisted of two pumps, CCPM II and CCPE (Tosoh, Tokyo, Japan), and a UV-VIS Detector/S-3702 (Soma, Tokyo, Japan). Two gel filtration columns, Protein KW-804 (Shodex, Tokyo, Japan) columns, were used to separate lipoprotein, and eluent LP-01F (Tosoh, Tokyo, Japan) was sent to the columns at a rate of 0.7 ml/min by the CCPMII pump. Cholesterol enzymatic reagent was sent at a rate of 0.4 ml/min. One hundred μl of sample was injected to HPLC and reacted with enzymatic reagent in a reactor coil at 37℃ for 5 min, after which absorbance at a measured wavelength of 555 nm was continuously monitored. Next, the enzymatic reagent was change to eluent, and each fraction was collected every minute for detection of cross-linked A-I in each lipoproteins.

5. Change of A-I structure by plasma incubation

Plasma was obtained from a healthy volunteer. Plasma was incubated for 0-72 h at 37℃. When the incubations were complete, the mixtures were analyzed with SDS-PAGE and immunoblotting. The amount of cross-linked A-I formed was determined by calculating cross-linked A-I/A-I monomer using intensity of the band obtained with densitograph software. In additional experiments plasma was incubated in the presence of LCAT inhibitor (1.7 mmol/l DTNB or 10 mmol/l N-ethylmaleimide (NEM)), antioxidant (250 μmol/l mannitol or 250 μmol/l dibuthylhydroxytoluene (BHT))[32], 3 mmol/l methylglyoxal (MG) and 3 mmol/l aminoguanidine (AG)[24] (final concentrations). DTNB, MG, and AG were purchased from Sigma-Aldrich. For the amount of cross-linked A-I formed, the plasma incubated in the absence of each reagent was taken to be 100 and the relative concentrations were shown using intensity of the band obtained with densitograph software. The same experiment was conducted with six other plasma.

6. Lipoprotein isolation using polyethylene glycol (PEG)

HDL_3 and HDL_2 were isolated using PEG in plasma. The method followed the method of Kurt et al.[33]. One hundred μl of plasma was mixed with 200 μl of the solution, 150 g/l PEG 20,000 (Wako pure chemical industries, Ltd., Tokyo Japan) in a 0.1 mol/L PBS, pH 7.5. After 10 min at room temperature, the reactants were centrifuged at 400 × g for 15 min.

7. SDS-PAGE and immunoblotting

Using 5-20% gradient gel (PAGEL, ATTO Co. Ltd., Tokyo, Japan), SDS-PAGE was conducted for detection of cross-linked A-I in samples. Electrophoresed proteins were transferred to polyvinylidene difluoride membrane, and then reacted with mouse anti-human apolipoprotein A-I monoclonal antibody (mAb) (Calbiochem, Cat. No. 178472: diluted to 1/1000 in skim milk-PBS) solution and peroxidase conjugated goat anti-mouse IgG antibody (Calbiochem: diluted to 1/1000 in skim milk-PBS). 3,3',5,5'-tetramethylbenzidine (Ez West Blue, ATTO, Tokyo, Japan) was used for staining, and band analysis was conducted with densitograph software (CS Analyzer ver. 2.0, ATTO, Tokyo, Japan).

8. Determination of LCAT activity

Plasma specimens were collected from 13 fasting healthy volunteers who had normal cholesterol and triglyceride levels. LCAT activity was measured using Anasolve LCAT (Sekisui Medical Co., Ltd., Tokyo, Japan)[34].

9. Statistical Analysis

Results are expressed as means and standard deviations (mean ± SD). Differences of $p < 0.05$ were considered significant. The Student's *t* test was used for normally distributed data. The correlation between LCAT activity and the formation of A-I-280 was made by using analysis of variance and Pearson's test.

Table 1 MALDI-TOFMS identification of the apo A-I antibody reactive proteins in HDL

Spot No	Identified protein name	MOWSE score
1	Chain C, Crystal structure of lipid-free human apolipoprotein A-I	181
2	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antitrypsin)	64
3	Chain C, Crystal structure of lipid-free human apolipoprotein A-I	123
4	Chain C, Crystal structure of lipid-free human apolipoprotein A-I	238
5	Apolipoprotein A-IV	199
6	Chain C, Crystal structure of lipid-free human apolipoprotein A-I	129
7	Chain C, Crystal structure of lipid-free human apolipoprotein A-I	173
8	Apolipoprotein E precursor	139
9	Apolipoprotein E precursor	86

3. Results

1. Analysis of A-I in HDL fractions purified by ultracentrifugation with matrix assisted laser desorption ionization-time of flignt mass spectrometry (MALDI-TOFMS)

Two-dimensional electrophoresis and immunoblotting was performed with proteins in HDL fractionated with ultracentrifugation (Fig. 1a). In addition to A-I monomer, 5-6 types of protein that react with A-I antibody were detected in HDL fractions. Those proteins had almost the same isoelectric point, suggesting that they were A-I polymers.

Protein in HDL was analyzed with MALDI-TOFMS. An analysis was conducted for a total of 9 proteins including those that reacted with A-I antibody and nearby proteins (Fig. 1b). Among the 9 kinds of protein, a total of 6 (spot No. 1, 3, 4, 6, 7, and 9) were A-I, including A-I monomers (Table 1). Peaks of peptide chains derived from protein other than A-I were not detected in six proteins (data not shown).

2. Cross-linked A-I in HDL fractionated with gel filtration column

Cross-linked A-I was observed in plasma lipoprotein fractions isolated with HPLC with gel filtration columns. With HPLC, HDL was fractionated in approximately 26-36 min. Cross-linked A-I were in HDL fractionated at 26-30 min (Fig. 2).

3. Changes of cross-linked A-I with plasma incubation

Observations were made for the kind of changes that occurred in cross-linked A-I when plasma

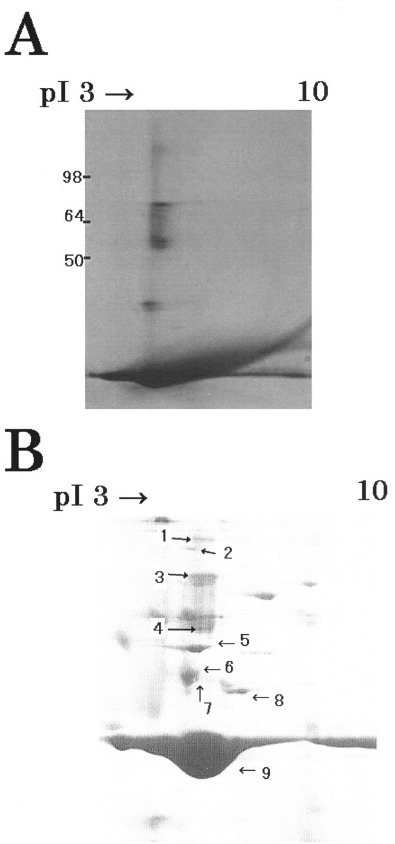

Fig. 1 A-I antibody-reactive proteins in HDL fraction ated with two dimensional electrophoresis. HDL fraction obtained ultra-centrifugation was performed two-dimensional electrophoresis and visualized with immunoblotting with anti A-I mAb (A) or CBB (B). Proteins (No. 1-9) in the image B were analyzed with MALDI-TOFMS (Table.1).

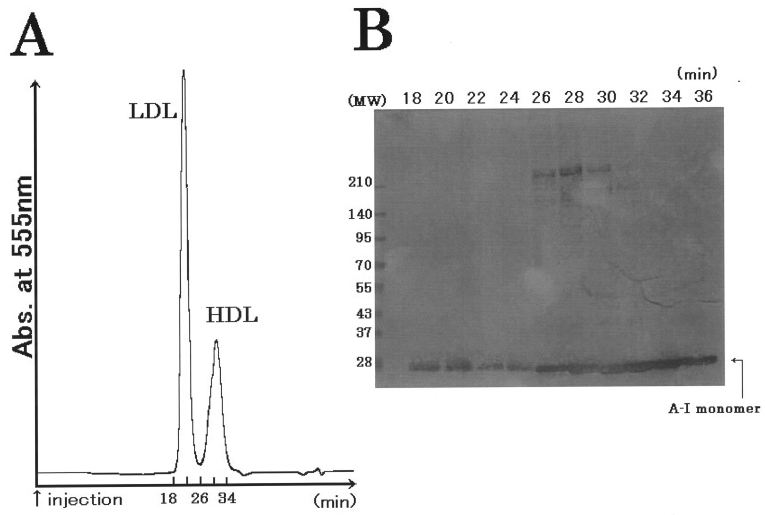

Fig. 2 Cross-linked A-I in HDL fractionated with gel-filtration column.
Lipoproteins in healthy plasma were separated with HPLC using gel-filtration columns and determined cholesterol (A). Separated lipoproteins were collected each two min. Those fractions were delipidated and performed immunoblotting with anti A-I mAb (B).

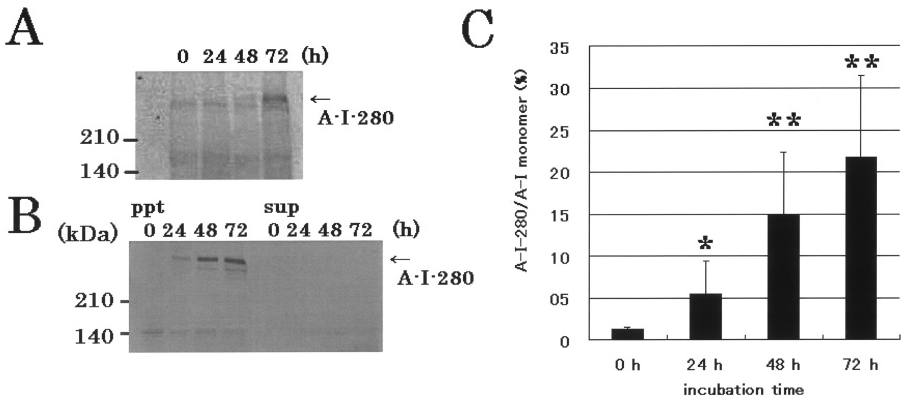

Fig. 3 Changes of cross linked A-I in plasma incubation at 37℃.
Plasma was incubated at 37℃ for 0-72 hour and performed immunoblotting (A). The same experiment was conducted with six other plasma. Additionally, incubated plasma specimens were added 10% PEG and centrifuged after incubation at room temperature for five minutes. Each supernatant and precipitation were performed immunoblotting with anti A-I mAb (B). The result of A was analyzed with densitometric soft, CS Analyzer ver 2.0 (ATTO), and A-I-280/A-I monomer ratio were calculated (C). Results are expressed as the mean ± S.D. *: $P < 0.05$ vs. 0 h, **: $P < 0.01$ vs. 0 h, statistical comparison by student's *t*-test.

obtained a healthy donor was incubated at 37℃ for 24-72 h. At incubation for 24 h, 280 kDa cross-linked A-I (A-I-280) was newly detected, and the level of cross-linked A-I increased until at least 72 h (Fig. 3a). In six plasma specimens, similar findings were obtained.

HDL_2 and HDL_3 were isolated using PEG and the same procedures were carried out. Production of A-I-280 was confirmed in precipitates containing HDL_2, LDL, and VLDL, but was not seen in supernatant including HDL_3 (Fig. 3b). When the amounts of A-I-280 produced in precipitates of 6 plasma

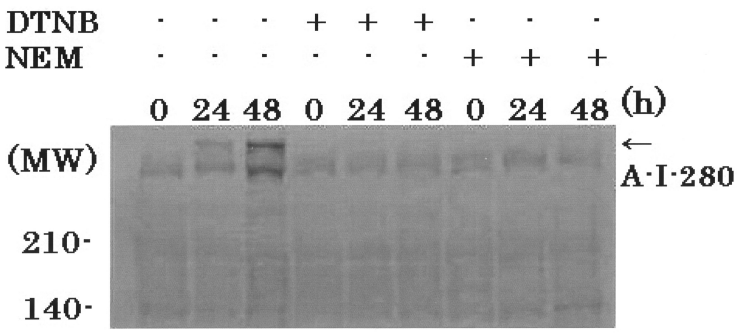

Fig. 4 Effect of LCAT inhibitors on A-I-280 formation.
Plasma was incubated in the presence or absence of 1.7 mmol/l DTNB or 10 mmol/l NEM for 48 h at 37℃. Samples were performed immunoblotting with anti A-I mAb.

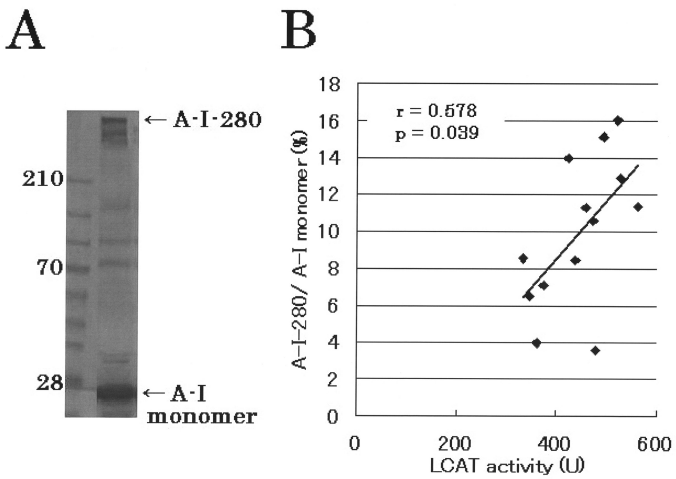

Fig. 5 Correlation between LCAT activity and A-I-280 concentration.
LCAT activity and A-I-280 formation in plasma obtained from 13 healthy volunteers were compared. Plasma specimens were incubated for 48 h at 37℃ and performed immunoblotting with anti A-I mAb (A). The amount of A-I-280 formed was calculated as A-I-280/A-I monomers from the band intensity in immunoblotting (B).

spesimens were measured, the level of A-I-280 had increased significantly (Fig. 3c).

4. Effect of LCAT inhibitors on the formation of A-I-280

To confirm that LCAT contributes to A-I-280 formation, LCAT inhibitors, DTNB or NEM, were added to plasma, which was then incubated at 37℃ for 48 h (Fig. 4). Both DTNB and NEM inhibited the formation of A-I-280.

5. Relation between LCAT activation and A-I-280 concentration

The relation between LCAT activity and A-I-280 concentration after incubation at 37℃ for 72 h was investigated in plasma obtained from 13 healthy volunteers. The A-I-280/A-I monomer ratio in the plasma from healthy volunteers was calculated. The amount of A-I-280 was positively correlated with LCAT activity, with a correlation coefficient of 0.578 (Fig. 5).

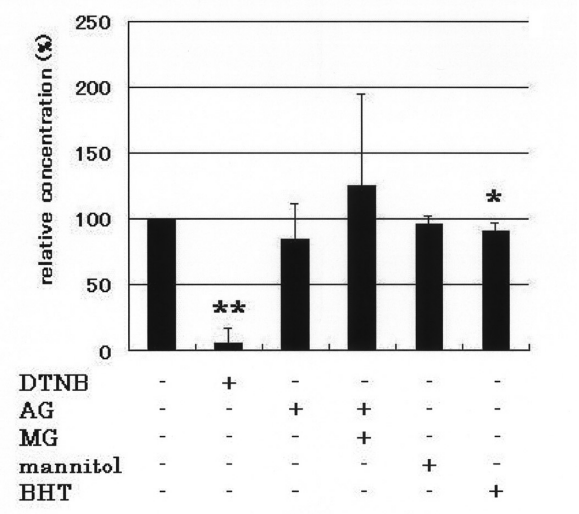

Fig. 6 Impact of cross-link inhibitor (aminoguanidine; AG), glycation promoter (methylglyoxal; MG) and antioxidants (mannitol or BHT) on the formation of A-I-280.
Six plasma specimens were incubated in the presence or absence of 3 mmol/L aminoguanidine, 3 mmol/L methyl glyoxal, 250 μ mol/l mannitol, and 250 μ mol/l BHT for 48 h at 37℃. Samples were performed and immunoblotting with anti A-I mAb. Results are expressed as the mean ± S.D. *: $P < 0.05$ vs. control, **: $P < 0.01$ vs. control, statistical comparison by student's *t*-test.

6. Effects of antioxidant and cross-linking inhibitor on the formation of A-I-280

It was investigated in six healthy plasma whether or not antioxidant or cross-linking inhibitor affected A-I-280 (Fig. 6). Cross-linking inhibitor, AG, was added to plasma and the amount of A-I-280 formed was quantified. The formation of A-I-280 was not affected by the addition of AG. Additionally, AG and MG, which promotes the glycation and cross-linking of protein, were added to plasma. AG had not inhibited the formation of A-I-280, but the formation of MG-derived cross-linking was suppressed (data not shown).

Antioxidants, mannitol and BHT, were added to plasma and incubated for 48 h, and the amounts of A-I-280 were compared. No significant difference in A-I-280 production was seen with adding mannitol. There was a significant decrease when BHT was added. However, the amount of that decrease was small compared with plasma added DTNB.

4. Discussion

A-I is the major apolipoprotein in HDL, and plays a crucial role in the RCT[35]. Most previous investigations of A-I polymers were related to oxidation or glycation in vitro, and there has been little discussion of the polymerization of A-I in the process of normal lipid metabolism. We detected polymerization of A-I in plasma in the process of HDL metabolism via LCAT.

Proteins in HDL fractions were immunoblotted with A-I mAb. Several proteins were reacted with A-I mAb. These proteins had almost the same isoelectric point, suggesting that they may be polymers of A-I. In an analysis of proteins that reacted with A-I mAb by MALDI-TOFMS, these proteins were identified as A-I. Moreover, peptide chains derived from proteins other than A-I were not detected in these proteins. These results strongly suggest that the proteins detected were cross-linked A-I. Since A-I monomers have a molecular weight of 28 kDa, if these proteins were cross-linked A-I they should have molecular weights that are integral multiples of 28 kDa. Among the molecular weights estimated from electrophoresis images, however, are some that do not appear to be integral multiples of 28

kDa. Elucidation of this problem will require detailed analysis of the structure and molecular weight of cross-linked A-I.

There have been reports on cross-linked A-I from several groups. These reports are related to self-association that is dependent on A-I concentration, ion strength, pressure, and pH in the sample. There are no reports on polymerization of A-I within the body. James et al. isolated an A-I group in which there was self-association of monomer, dimer, tetramer, and octamer and an A-I group in which there was no polymerization at all, and reported that the A-I group with no polymerization was incompetent in vivo[36]. This shows that A-I self-association is important in RCT in vivo.

Lipoproteins in plasma were isolated with gel filtration columns. In these lipoproteins, it was found that the cross-linked A-I was contained in HDL with large particle sizes. This indicates the possibility that cross-linked A-I is formed in the maturation process of HDL. To confirm whether or not A-I is polymerized in HDL metabolism, the change of A-I conformation was observed in the plasma incubated at 37℃ for 0-72 h. When plasma was incubated, cross-linked A-I with a molecular weight of 280 kDa was formed. It is reported that HDL_3 contained much free cholesterol is converted to EC-rich HDL_2 by the action of LCAT when serum is preserved at 4℃[37]. Then, by adding 10% PEG to plasma, HDL_3 fraction and HDL_2 fraction containing other lipoproteins were isolated. A-I-280 was found to be present in the HDL_2 fraction. This indicated that A-I-280 was in mature HDL_2 that precipitated with 10% PEG. A-I in mature HDL is catabolized in the liver, but part of that is reused in the RCT. Moreover, it is reported that remnant HDL from which EC was catabolized via SR-BI is divided into that catabolized in the kidneys and that reused in the RCT from the liver[38, 39]. The A-I-280 discovered in the present study in vitro was not detected in plasma immediately after blood collection. This suggested that HDL including A-I-280 might be rapidly catabolized. It is unclear how the selection is made as to whether A-I will be catabolized or whether it will be returned to the HDL metabolic pathway. A-I-280 may contribute to the catabolism of HDL and A-I and be important in the RCT.

To confirm that the action of LCAT contributes to formation of A-I-280, LCAT inhibitor was added to plasma and incubated at 37℃. It was found that production of A-I-280 was suppressed by inhibiting LCAT. Additionally, a positive correlation was observed between LCAT activity and A-I-280 concentration in the plasma obtained from the healthy volunteers. This indicated that LCAT is important in A-I-280 formation. As in previous reports, much cross-linked A-I was probably self-associated non-enzymatically. However, A-I-280, the focus of the present study, was suggested to be formed during normal lipid metabolism in the body.

It has been reported from several studies that A-I oxidation and glycation contributes to self-association of A-I[22, 23]. It was investigated whether oxidation and glycation of A-I contributes to the formation of A-I-280. When plasma specimens were incubated in the presence of antioxidants, mannitol and BHT, it was found that the amount of A-I-280 decreased with BHT. However, that decrease was slight compared to when LCAT inhibitor was added. In comparison with LCAT, it seemed that oxidation has almost no effect on the formation of A-I-280. When plasma specimens were incubated with glycation promoter, MG, there was no change in the formation of A-I-280 compared with a control in which no MG was added. A cross-linking inhibitor, AG, did not affect the amount of A-I-280 formed, but suppressed the formation of MG-derived cross-linked A-I. These findings indicated that the mechanism of A-I-280 formation in this study differed from that of cross-linked A-I formed in vitro with the influence of oxidation and glycation in previous reports.

This study suggests the possibility that the formation of cross-linked A-I changes in the process in which LCAT plays on HDL metabolism. Observation of the kinetics in A-I self-association should be important to identify the qualitative changes in HDL in diabetes mellitus or hyperlipidemia.

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