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Serum amyloid A does not affect high-density lipoprotein cholesterol measurement by a homogeneous assay

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ABSTRACT

Background: Serum amyloid A (SAA), which is one of the acute phase proteins, alters the structure of HDL by associating with it during circulation. We focused on whether SAA influences the values of HDL-cholesterol (HDL-C) measurements when using a homogeneous assay.

Methods: HDLs were isolated by ultracentrifugation from serum samples of 248 patients that were stratified into three groups based on their serum SAA concentrations (low: SAA ≤ 8 μg/mL; middle: 8 < SAA ≤ 100 μg/mL; and high: SAA > 100 μg/mL). HDL-C concentrations of the serum samples measured by the homogeneous assay were compared with the total cholesterol concentrations of HDL fractions isolated by ultracentrifugation.

Results: HDLs obtained from patients with low SAA concentrations were separated into their general particle sizes and classified as HDL_2 and HDL_3 by native-gel electrophoresis. On the other hand, HDLs obtained from patients with high SAA concentrations occasionally showed distributions different from the typical sizes of HDL_2 and HDL_3, such as extremely small or large particles. Nevertheless, HDL-C concentrations measured using the homogeneous assay were strongly correlated with those measured using the ultracentrifugation method, regardless of the SAA concentrations. However, the ratios of HDL-C concentrations obtained by the homogeneous assay to those obtained by the ultracentrifugation method for patients with high SAA concentrations were significantly lower than those of patients with low SAA concentrations.

Conclusions: A large amount of SAA attached to HDL altered the HDL particle size but did not essentially affect HDL-C measurement by homogeneous assay.

1. Introduction

HDL is known as the anti-atherogenic lipoprotein. Epidemiological studies have shown that low plasma concentrations of HDL are associated with an increased risk of cardiovascular events [1]. HDL-cholesterol (HDL-C) is the only standardized parameter available to estimate the plasma HDL concentration. Currently, HDL-C concentrations are measured by homogeneous assay methods using automatic analyzers in clinical laboratories. Although these methods enable quick and easy HDL-C measurement, it has been reported that the measured values differ depending on the principles of the methods, including different reactivities toward apolipoprotein *E*-containing HDL [2,3].

Serum amyloid A (SAA) is a highly-conserved, acute phase protein that is predominantly synthesized by the liver. During acute inflammation, SAA concentrations in the serum can increase to up to 1000-fold of the basal levels [4–6]. Under these conditions, SAA displaces apolipoprotein A-I (apoA-I) in HDL and becomes the major apolipoprotein of circulating HDL [6–8]. In addition, some researchers have reported that group IIA secretory phospholipase A2 and endothelial lipase increase during the acute phase response (APR) and markedly change HDL lipids and apolipoprotein contents [9,10]. We also previously showed that the HDLs in patients with inflammation were characterized by a relatively decreased cholesterol content and reduced negative charge [8]. These data indicate that during the APR,

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HDL is different in terms of its structure and other properties, compared with normal HDL; however, it is not known whether the HDL-C of a patient with inflammation can be accurately measured by homogeneous assays commonly used in clinical laboratories.

In the present study, we assessed the effect of SAA attachment to HDL on HDL-C values measured by a homogeneous assay in comparison with HDL-C concentrations obtained by an ultracentrifugation method, reference method.

2. Material and methods

2.1. Patients and serum samples

Anonymized serum samples with various C-reactive protein (CRP) concentrations were obtained from 248 patients without clinically apparent liver disease, who were treated at The University of Tokyo Hospital. The SAA concentration of the serum samples were measured by latex agglutination turbidimetric immunoassay, and the serum samples were stratified into three groups, based on their SAA concentrations: low SAA (SAA ≤ 8 μg/mL, n = 94), middle SAA (8 < SAA ≤ 100 μg/mL, n = 37), and high SAA (SAA > 100 μg/mL, n = 117).

2.2. Biochemical measurements

All assays were performed using an automatic analyzer, JCA-BM8000 series (JEOL), by following the manufacturer's protocol. SAA and CRP were measured by the latex agglutination turbidimetric immunoassay using the LZ test 'Eiken' SAA (Eiken Chemical) and CRP-latexX2 (Denka Seiken), respectively. HDL-C, low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC) and triglyceride (TG) levels were determined using the corresponding commercial kits: MetaboLead HDL-C, Determiner L LDL-C, Determiner L TCII and Determiner L TGII (Kyowa Medex), respectively, following the manufacturer's protocol.

HDL-C was also measured by homogeneous method. The reaction between cholesterol in non-HDL lipoproteins (chylomicron (CM), very low-density lipoprotein (VLDL) and LDL) and the enzyme used for measuring cholesterol is suppressed by electrostatic interactions between polyanion and a cationic compounds. After converting cholesterol esters in HDL to free cholesterol with cholesterol esterase, hydrogen peroxide is formed when free cholesterol contained in HDL reacts with cholesterol oxidase. Finally, the quantity of hydrogen peroxide was determined in the presence of peroxidase with N-ethyl-N-(3-methylphenyl)-N'succinyl-ethylenediamine and 4-aminoantipyrine using a colorimetric assay.

LDL-C was analyzed by the homogeneous method based on the principle of selective solubilization method. The unique surfactant allows only LDL to solubilize and inhibits other reactions with non-LDL lipoproteins (CM, VLDL and HDL) following a reaction with cholesterol esterase, cholesterol oxidase and peroxidase. TC levels were determined by colorimetric enzymatic method, which utilizes cholesterol esterase, cholesterol oxidase and peroxidase. TG was measured enzymatically with lipoprotein lipase, glycerol kinase, glycerol triphosphate oxidase and peroxidase. Albumin (Alb) was measured by the bromocresol purple (BCP)-improved method (Wako Pure Chemical Industries).

2.3. Evaluation of HDL particle size

Whole lipoproteins (d ≤ 1.210 g/mL) isolated by ultracentrifugation from randomly selected serum samples of patients with low or high SAA concentrations were dialyzed against PBS [11], and subjected to native-gel electrophoresis (native-PAGE) using 8% polyacrylamide gels. Separated proteins were stained with Coomassie brilliant blue (CBB) or transferred to polyvinylidene fluoride membrane (Millipore) for immunoblotting. ApoA-I was probed with a goat anti-apoA-I polyclonal antibody (Academy Bio-Medical Company), followed by a peroxidase-conjugated rabbit anti-goat IgG (Medical & Biological Laboratories). Finally, the bands containing apoA-I were visualized using 3,3′-diaminobenzidine-4HCl and hydrogen peroxide. SAA was probed with a rabbit anti-SAA polyclonal antibody (ASSAYPRO), followed by a peroxidase-conjugated goat anti-rabbit IgG (Beckman Coulter). The bands containing SAA were visualized using the ECL Prime Western Blotting Detection Reagent (GE Healthcare).

2.4. Measurement of HDL-C by ultracentrifugation method

Serum samples were ultracentrifuged at a density of 1.063 g/mL for 37 h. After centrifugation, the top fraction, containing CM, VLDL and LDL, was removed, and the bottom layer containing only HDL as a lipoprotein was used to measure HDL-C. The volume error was compensated by using the ratio of the Alb concentration in the bottom layer to that in the serum.

2.5. Statistical analysis

Results are expressed as the mean ± SD. Comparison among the three stratified groups was performed using the Kruskal–Wallis test, followed by a post-hoc Dunn's multiple comparison test using Prism 5 for Mac OS X Version 5.0c. Differences were considered significant at $P < 0.05$. Individual P values are included in the text and figures. Comparison between the homogeneous assay and ultracentrifugation method was performed by Passing-Bablok regression using RStudio ver. 1.1.442 loaded with the 'mcr' package ver. 1.2.1. The Passing-Bablok regression line for each analysis was plotted along with its regression equation as well as the 95% confidence interval of both coefficients.

Table 1
Biochemical characteristics of patients.

	SAA			*P*		
	Low (n = 94)	Middle (n = 37)	High (n = 117)	Low vs. Middle	Low vs. High	Middle vs. High
SAA [μg/mL]	3.4 ± 1.8	20.4 ± 18.3	1377.3 ± 1086.2	< 0.001	< 0.001	< 0.001
Alb [g/dL]	4.1 ± 0.4	3.7 ± 0.7	2.8 ± 0.6	< 0.05	< 0.001	< 0.001
CRP [mg/dL]	0.04 ± 0.07	0.91 ± 2.27	12.97 ± 7.53	< 0.05	< 0.001	< 0.001
HDL-C [mg/dL] (Ultracentrifugation method)	62 ± 20	59 ± 28	41 ± 15	n.s.	< 0.001	< 0.001
HDL-C [mg/dL] (Homogeneous method)	67 ± 21	65 ± 31	43 ± 16	n.s.	< 0.001	< 0.001
LDL-C [mg/dL]	110 ± 33	105 ± 42	86 ± 35	n.s.	< 0.001	< 0.05
TC [mg/dL]	193 ± 41	191 ± 63	151 ± 43	n.s.	< 0.001	< 0.001
TG [mg/dL]	125 ± 73	135 ± 79	107 ± 52	n.s.	n.s.	n.s.

Data is presented as mean ± S.D. Comparison among the three groups was performed using Kruskal–Wallis test. Significant differences between individual groups were identified post hoc by Dunn's Multiple Comparison Test. n.s.: not significant.

3. Results

3.1. Biochemical characteristics of the subjects

Biochemical data of three groups divided by the SAA concentrations is shown in Table 1. As expected, significant differences in inflammatory marker CRP levels were observed among the three groups. In contrast, increasing tertiles of SAA were inversely correlated with the Alb, HDL-C, LDL-C, and TC concentrations, but not correlated with the TG concentrations.

3.2. Particle size of the patients' HDLs

Whole lipoproteins, isolated from the serum samples with the low, middle and high SAA concentrations (low; SAA ≤ 8 μg/mL, n = 15, middle; 8 < SAA ≤ 100 μg/mL, n = 9, high; SAA > 100 μg/mL, n = 25) were subjected to native-PAGE and analysis was performed on particle sizes of HDLs (apoA-I) and SAA distribution. HDLs obtained from the serum samples with low SAA concentrations produced two distinct HDL subparticles; HDL_2 and HDL_3. On the other hand, HDLs obtained from the serum samples with the high SAA concentrations exhibited two different patterns; one was characterized as an intermediate particle size between HDL_2 and HDL_3 (Fig. 1B, Lane 3), and the other was characterized by the existence of an extremely large and small HDL particles (Fig. 1B, Lane 4). SAA was found to be present in this intermediary particle for some patients (Fig. 1C, Lane 3) and in the extremely large particles for others (Fig. 1C, Lane 4). In samples where SAA levels are low, intermediate particle sized HDL was observed, but neither HDL with larger particle size than HDL_2 nor smaller particle size than HDL_3 was observed (Fig. 1B, Lane 1).

3.3. Correlation of the HDL-C between two methods

HDL-C concentrations in the serum samples obtained from the patients with various SAA concentrations (low: SAA ≤ 8 μg/mL, n = 94; middle: 8 < SAA ≤ 100 μg/mL, n = 37; high: SAA > 100 μg/mL, n = 117) were measured by both the homogeneous assay generally used in clinical laboratories and the ultracentrifugation method described above. The results of the homogeneous assay were strongly correlated with those of the ultracentrifugation method in all of the patients ($r = 0.994$), however when these two methods were compared using Passing-Bablok regression (non-parametric regression analysis to assess analytical accuracy between two methods), a 95% confidence interval of the intercept ($\beta_0 = 0.175$) and slope ($\beta_1 = 1.080$) values were [−0.434, 0.930] and [1.066, 1.097] respectively. It suggests that while there was no systematic difference between these two methods, a small proportional difference exist between these measurements (Fig. 2A). No distinct difference was observed in the distributions among the data when measurements of both methods were subdivided into the three groups (low, middle and high SAA) ($r = 0.994$, 0.996 and 0.986, respectively) (Fig. 2B, C, and D), indicating that the homogeneous assay possesses the sufficient ability to measure HDL-C in patients with different levels of SAA for clinical purpose. The only regression coefficient that produced an intercept ($\beta_0 = 1.546$) that was not within its 95% CI [0.604, 2.242] was from HDL-C measurements of patients with high SAA. Furthermore, the ratios of HDL-C values in low, middle and high SAA obtained by the homogeneous assay to those by the ultracentrifugation method were 0.93 ± 0.06, 0.92 ± 0.11, and 0.88 ± 0.08, respectively (mean ± S.D.). The ratios in the high SAA were slightly but significantly lower than those in the low SAA ($P < 0.001$) and in the middle SAA ($P < 0.01$).

4. Discussion

In a clinical setting, HDL-C is a strong marker used to estimate the risk of atherosclerotic disease development in cardiovascular disease. Homogeneous assays for HDL-C are simple and convenient methods; they are widely used in clinical laboratories in Japan. However, the heterogeneity of HDL particles occasionally makes it difficult to obtain an accurate HDL-C concentration [2,3]. It is well known that most of the SAA induced by inflammation circulates in a complex with HDL [8]. However, it is not clear whether the HDL-C concentration in serum obtained from a subject with inflammation is accurately measured by homogeneous assays. In this study, we evaluated the influence of SAA on HDL-C values measured by homogeneous assay.

From the subfraction analysis, HDL remodeling was observed in the patients with high SAA concentrations. The HDL particles containing SAA molecules were detected as an intermediary band (between HDL_2 and HDL_3) or as an extremely large band. The extremely small HDL particles, which did not contain SAA, were concomitantly detected with the extremely large HDL particles. These small particles might be newly-constructed nascent-HDL from the release of apoA-I from mature HDL [12]. Although similar remodeling has been observed in many patients with severe inflammation, no definite relevance between the serum SAA concentration and HDL particle size was observed. This is consistent with our previous report [8]. It means that the HDL particle size observed during inflammation would be determined by the structure of the typical HDL in each individual but not the SAA concentration. Further, it is well known that SAA increases by 1000-fold of the baseline level for 2–3 days due to acute inflammation and usually returns to the baseline level within 1–2 weeks [4]. Therefore, the HDL particle size might be affected if the blood sample was collected during the increased or decreased stages of SAA concentration. The HDL profile of patient 4 (SAA: 832.4 μg/mL), who had a high SAA concentration, included extremely small particles, such that it would be obtained at an early stage of inflammation. In contrast, the HDL profile of patient 3 (SAA: 2238.4 μg/mL) would be obtained at a late stage of inflammation, after metabolizing and/or catabolizing the extreme-sized HDL particles.

In clinical laboratories, it is important to know whether the HDL-C

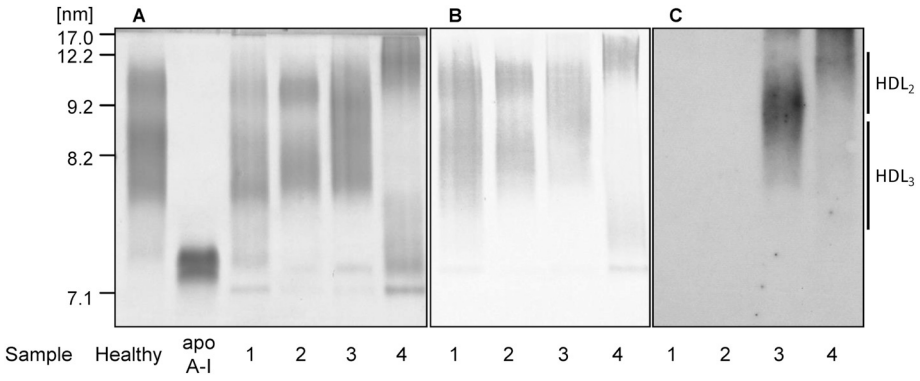

Fig. 1. Particle size of the patients' HDL.
The profile of HDL obtained from 49 patients (low; SAA ≤ 8 μg/mL, n = 15, middle; 8 < SAA ≤ 100 μg/mL, n = 9, high; SAA > 100 μg/mL, n = 25) were determined by native-PAGE, followed by CBB staining (A) and immunoblotting using an anti-apoA-I antibody (B) and anti-SAA antibody (C). HDL from a healthy subject and purified apoA-I were also included as controls. The figure shows the representative profiles of HDL from four patients with low SAA (patient 1, 3.3 μg/mL, and patient 2, 2.4 μg/mL) and high SAA (patient 3, 2238.4 μg/mL, and patient 4, 832.4 μg/mL).

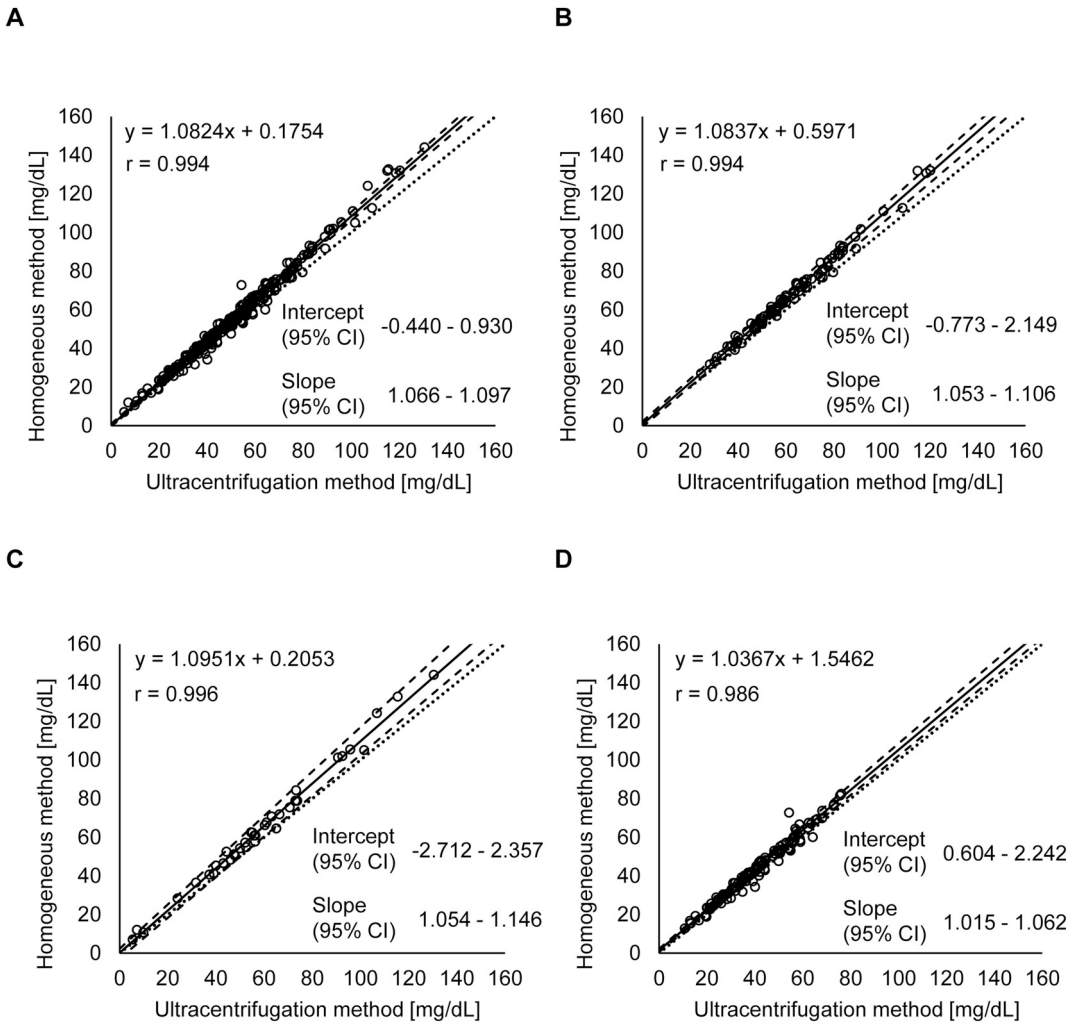

Fig. 2. Comparison of HDL-C between the homogeneous method and the ultracentrifugation method.
Comparison of HDL-C between the two methods were estimated for all of the patients (A) and the patients with low (B), middle (C), and high (D) SAA concentrations by using Passing-Bablok regression. Figure shows the observations with the regression line (solid line), regression line confidence bands (dashed lines) and identity line (x = y, dotted line). Formula of the regression line along with its 95% upper and lower confidence interval limits for the slope (β_1) and intercept (β_0) was included in the respective figures.

of a patient with inflammation was correctly measured by the use of common homogeneous assays. Hence, we assessed the homogeneous assay for HDL-C in patients with varied SAA concentrations by comparison with the ultracentrifugation method. Measurements of both methods were correlated for samples obtained from patients without inflammation, indicating that the ultracentrifugation method constructed in our laboratory accurately measured the concentrations; this method included a volume correction using albumin values in both the serum and bottom fraction after ultracentrifugation. Despite the extent to which HDL can be remodeled by associating with SAA, the correlation of both methods was also present in patients with inflammation. However, the ratios of HDL-C obtained by the homogeneous assay to those obtained by the ultracentrifugation method indicated significantly lower values in patients with high SAA concentrations. This could be explained by the presence of lipoprotein (a) (Lp(a)) in the HDL fractions obtained from the patients with high SAA concentrations by the ultracentrifugation method. Lp(a) is known to have the characteristics of an acute phase reactant, indicating that higher concentrations of Lp(a) are expected in the patients with high SAA concentrations, compared to those in the patients with low SAA concentrations [13,14]. In addition, the density of Lp(a) is falls between LDL and HDL [15], indicating that a non-negligible amount of Lp(a) could be present in the HDL fraction obtained by ultracentrifugation. Similarly, the reason that the HDL-C values obtained by the homogeneous assay showed a lower trend than those obtained by the ultracentrifugation method could be explained by the existence of a basal level of Lp(a) in all serum samples. Nevertheless, a high correlation between the homogeneous assay and the ultracentrifugation method was unexpectedly obtained in the patients with high SAA concentrations.

A limitation of this study was use of only one homogeneous assay. Although further investigation with the use of another homogeneous assay would be ideal, we hypothesize that the difference could be explained by the principles of the homogeneous assay, in which TC is measured after specific inhibition of an enzymatic reaction against some lipoproteins (VLDL and LDL but not HDL) by α-cyclodextrin sulfate [16]. This means that the values obtained by the homogeneous assay used here for the HDL-C measurement are not affected by a change in the structure of HDL. It also indicates the possibility that HDL-C values obtained when a homogeneous assay with a different principle that induces a selective and direct reaction for HDL alone is used for comparison with the ultracentrifugation method. It has been reported that the HDL-C concentrations obtained by other homogeneous assays indicated a large bias toward extremely low HDL-C concentrations in diseased subjects but not in normal subjects [2]. It is well known that extremely low HDL-C values are frequently obtained from subjects with inflammation.

5. Conclusion

Structural diversity, such as HDL particle size and SAA distribution, was observed in HDLs obtained from patients with inflammation; however, the HDL-C values measured by homogeneous assay were consistent with those obtained by ultracentrifugation method, regardless of SAA concentrations, an indicator of the presence or absence of inflammation. Although HDL-C measurements obtained with the homogeneous assay reagents correlated with those obtained from ultracentrifugation [2], further investigation is needed to understand the reaction characteristics of other homogeneous assays against SAA-containing HDL.

Declaration of conflicting interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

This study was approved by the Institutional Research Ethics Committee of the Faculty of Medicine, The University of Tokyo (No. 3333-20), and the Ethics Committee of the Faculty of Medicine, Tokyo Medical and Dental University (No. 1182).

Contributorship

MS, RO, and MT designed the study. RO supervised the study. MN and SO collected the serum samples and analyzed HDL-C (homogeneous assay) and SAA. MS carried out the main research, including the HDL-C measurement by ultracentrifugation, and AY and KY assisted with native-PAGE and cholesterol measurement. HL performed statistical analysis. RO, KT, and MT analyzed the data. MS and RO wrote the manuscript, and HL, MT and YY revised the manuscript.

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