Development of a Homogeneous Assay for Measurement of Small Dense LDL Cholesterol

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BACKGROUND: Plasma concentrations of small dense (sd)-LDL are associated with the prevalence of cardiovascular events. However, the special equipment and long assay times required for sd-LDL measurement have hindered its clinical application. Herein, we report development of a simple homogeneous assay for sd-LDL-cholesterol (C) adaptable to autoanalyzers.

MATERIALS AND METHODS: We identified suitable surfactants and phospholipases by screening for those selective for the sd-LDL fraction (d 1.044–1.063 kg/L) and for the dissociation of other lipoproteins, including large buoyant LDL (lb-LDL). Principal characteristics of this assay were compared with ultracentrifugal isolation of LDL subfractions and with our previous heparin-magnesium precipitation assay for sd-LDL. We measured sd-LDL-C concentrations in 460 healthy, normolipidemic individuals.

RESULTS: We used a polyoxethylene benzylphenyl ether derivative to dissociate triglyceride-rich lipoproteins and HDLs, whereas sphingomyelinase proved most effective for dissociation of lb-LDL from LDL owing to the higher sphingomyelin content in the lb-LDL subfractions. A polyoxethylene styrenephenyl ether derivative protected sd-LDL against the dissociative actions of sphingomyelinase and cholesterol oxidase/esterase during an initial incubation step. Next, polyoxethylene alkyl ether dissociated sd-LDL-C and the cholesterol released from sd-LDL were subsequently measured by using cholesterol oxidase/esterase. The homogeneous method correlated excellently with ultracentrifugation for sd-LDL-C (y = 0.99x – 0.09, R² = 0.91, n = 60) and exhibited within-run precision CVs <1.1%. The distribution of sd-LDL-C was skewed, and the central 95% of sd-LDL-C concentrations ranged from 0.24 to 0.88 mmol/L (9.4–34.0 mg/dL).

CONCLUSIONS: The homogeneous assay allows reproducible measurement of sd-LDL-C within 10 min and appears promising in further investigations of the clinical significance of sd-LDL-C.

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LDL, an established atherogenic lipoprotein, can be fractionated into large buoyant (lb) and small dense (sd) particles based on size and density (1). An abundance of clinical evidence has shown that sd-LDL particles are more atherogenic than lb-LDL particles (2–5) and that a high sd-LDL cholesterol (C) concentration is closely associated with a high prevalence of cardiovascular disease (6). The selective measurement of the sd-LDL-C concentration is thus important for evaluating the actual atherogenic risk of individuals.

The sd-LDL is traditionally measured by ultracentrifugation (7) or gradient gel electrophoresis (GGE) (8). Yet these methods are both unsuitable for routine analysis, as each requires expensive equipment, complicated techniques, and long assay times. A recently developed method of nuclear magnetic resonance (NMR) imaging is capable of simultaneously determining the sizes and numbers of LDL particles (9), whereas HPLC for lipoprotein analysis enables the determination of lipid concentrations in various lipoprotein subfractions (10). However, both methods have limitations. HPLC is too laborious, time-consuming, and expensive for routine clinical use. The instrumentation and devices required for NMR are too costly for general clinical laboratories to feasibly acquire.

We previously developed a simple precipitation method for sd-LDL-C quantification consisting of 2 steps: removal of apolipoprotein B (apoB)-containing sd-LDL–free lipoproteins by precipitation with heparin and magnesium (Mg), followed by LDL-C measurement by the homogeneous method (11). Later, the

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3 Nonstandard abbreviations: lb, large buoyant; sd, small dense; C, cholesterol; GGE, gradient gel electrophoresis; NMR, nuclear magnetic resonance; apoB, apolipoprotein B; PL, phospholipid; PLase, phospholipase; SMase, sphingomyelinase; SM, sphingomyelin; TOOS, N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline; 4-AA, 4-aminoantipyrine; PC, phosphatidylcholine; TG, triglyceride; TRL, TG-rich lipoprotein.
procedure was further simplified by using a filter to trap the aggregated lipoproteins with heparin-Mg (12). Yet even with this simplification, the technique still required an offline sample pretreatment that hindered the smooth integration of this method into general clinical examination. Therefore, there has been need for the development of a fully automated assay for sd-LDL-C.

Specific interactions of surfactants with lipoproteins have been successfully used to develop direct lipoprotein-C assays in which non-LDL lipoproteins are dissociated with a specific surfactant to allow the cholesterol to be accessed as a nonchromogenic product. A homogeneous assay for LDL-C was established by eliminating non-LDL lipoproteins with a specific surfactant (13). The surface of lipoproteins consists of various phospholipids (PLs), and phospholipase (PLase) by use of a test kit based on the precipitation method with filtration (12) (sd-LDL-C “Seiken”; Denka Seiken) and SM according to the method of Hojati and Jiang (18). Phosphatidylcholine (PC) was determined by subtracting SM from PL.

Materials and Methods

Materials

Cholesterol esterase (EC3.1.1.13 *Pseudomonas* sp.) was obtained from Asahi Kasei Pharma, cholesterol oxidase (EC1.1.3.6 microorganism) and peroxidase (EC1.11.1.7 Horseradish) from Toyobo, and catalase (EC1.11.1.6 *Corynebacterium glutamicum*) from Roche Diagnostics. N-Ethyl-N-(2-hydroxy-3-sulfolpropyl)-3-methylaniline (TOOS) and Good’s buffer (PIPES) were supplied from Dojindo Laboratories, 4-aminoantipyrine (4-AA) from Nittobo, and sodium azide from Nacalai Tesque. Surfactants (a polyoxyethylene benzylphenyl ether derivative, a polyoxyethylene styrenephenyl ether derivative, and polyoxyethylene alkyl ether) were obtained from Kao Corporation. PLase A2, C, D, lysophospholipase, glycerophospholipase, and SMase derived from *Streptomyces* sp. were obtained from Asahi Kasei Pharma.

Measurements

We measured total cholesterol, LDL cholesterol, and PL by use of commercially available test kits [T-CHO, LDL-EX(N), and PL-S, respectively; Denka Seiken]. We determined the LDL size phenotype by 2%–16% polycrylamide GGE according to the method of Krauss and colleagues (2). We measured sd-LDL-C by use of a test kit based on the precipitation method with filtration (12) (sd-LDL-C “Seiken”; Denka Seiken) and SM according to the method of Hojati and Jiang (18). Phosphatidylcholine (PC) was determined by subtracting SM from PL.

Separation of LDL Subfraction by Gel Filtration

We separated serum samples into lipoprotein fractions by gel filtration chromatography using Superose 6HR 10/30 columns (Pharmacia). Each sample (0.2 mL) was loaded, and each lipoprotein fraction was eluted with PBS buffer at a flow rate of 0.5 mL/min. The column effluent was collected in 0.5-mL fractions, and the cholesterol concentration of each was measured by enzymatic assay (13).

Screening of Surfactants and PLase

We used the following 2 reagents to select the specificity of surfactants and PLase and evaluate assay performance. Reagent 1 consisted of 600 U/L cholesterol esterase, 500 U/L cholesterol oxidase, 1200 KU/L catalase, 2 mmol/L *N*-ethyl-*N*-(2-hydroxy-3-sulfolpropyl)-3-methylaniline, sodium salt, and dihydrate in PIPES buffer (50 mmol/L, pH 7.0). Reagent 2 consisted of 5 KU/L peroxidase, 4 mmol/L 4-aminoantipyrine, 0.05% (wt/vol) sodium azide, and 1% (wt/vol) poloxymethylene alkyl ether, in piperazine-N,N’-bis(2-ethanesulfonic acid) buffer. A 1500-μL al-

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iquot of reagent 1 was added to 3 μL sample, and the solution was incubated at 37 °C for 5 min (step 1). Next, 50 μL of reagent 2 was added, and the reaction mixture was incubated at 37 °C for 5 min (step 2).

In screening, some lipoproteins were dissociated to water and oxygen by actions of cholesterol esterase/oxidase and catalase to form a nonchromogenic product in step 1. The lipoprotein left over from step 1 conferred a purple-red color with the coupler in the presence of peroxidase in step 2, and cholesterol was measured spectrophotometrically at dual wavelengths of 600 nm (main) and 700 nm (subsidiary). This assay was performed in an autoanalyzer (Hitachi 7180).

Screening of surfactant A. In screening for surfactant A, various surfactants were added to reagent 1 followed by measurement of each lipoprotein isolated by ultracentrifugation. These lipoprotein measurements (made using total cholesterol reagents) were compared for the various surfactants investigated.

Screening of SMase. To determine an optimal PLase, several PLases were added to reagent 1 including surfactant A. Measurements of sd-LDL-C were performed on several sera and compared with sd-LDL-C as measured by the precipitation method.

Screening of surfactant B. Surfactant B was chosen by the following approach: various surfactants were added to reagent 1 along with surfactant A and SMase. Subsequent measurements were made of lb-LDL-C and sd-LDL-C purified by ultracentrifugation using total cholesterol reagents, and these measurements were compared for the various surfactants investigated.

LDL SIZE MEASUREMENT BY ELECTRON MICROSCOPY

Serum lipoprotein particle sizes before and after treatment with the reagents of the sd-LDL-C homogeneous method were observed by electron microscopy according to the method of Nakata et al. (20), with modifications. Grids coated with collodion and carbon were made hydrophilic by 0.1% (wt/vol) poly-l-lysine and floated on a drop of sample suspension for 5 min. After removing excess fluid by dabbing the edge of each grid with a piece of paper, the grids were floated on drops of 2% phosphotungstic acid for 2 min and immediately drained to near dryness on absorbed filter paper. Lipoproteins were stained with osmium.

REFERENCE INTERVAL OF sd-LDL-C DETERMINED BY THE HOMOGENEOUS ASSAY

We determined the reference interval of sd-LDL-C by measuring serum samples from 460 normolipidemic healthy individuals [LDL <3.64 mmol/L (140 mg/dL), TG <1.65 mmol/L (150 mg/dL), and HDL-C >1.04 mmol/L (40 mg/dL)] according to the Japan Atherosclerosis Society guidelines [21]. Written informed consent was obtained from all individuals, and the local ethics committee approved the experimental protocol.

Results

In total, we screened 174 surfactants for their ability to dissociate TRL and HDL. Through this screening, polyoxyethylene benzylphenyl ether derivative (surfactant A) was found to be the most effective agent for selectively dissociating TRL and HDL without affecting LDL. Surfactant A specifically dissociated TRL and HDL, while LDL remained intact, irrespective of the presence of hypertriglyceridemia (see Supplemental Fig. 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue1).

We examined compositional changes in LDL with increased density. Fig. 1 shows the cholesterol concentration (A) and PC and SM concentrations (B) in LDL subspecies fractionated by the ultracentrifugation. The cholesterol, PC, and SM concentrations all increased, reached peak values at fraction 11, and then decreased as the LDL density increased (11–19). The SM/LDL particle ratio, estimated by the apoB concentration, consistently fell as the LDL density rose. The SM/PC ratio plateaued between fractions 1 and 11, then decreased as the LDL density increased (11–19). The densities corresponding to the LDL subfractions are shown in Fig. 1D. These results suggested SM as a target lipid for separating LDL subfractions.

Fig. 2 demonstrates the linear regression curves between sd-LDL-C concentrations determined by the precipitation method and cholesterol concentration after treatment with various PLases in presence of surfactant A (the homogeneous method). Serum samples were obtained from healthy volunteers. Cholesterol concentrations treated with PLase A2, PLase C, PLase D, lysophospholipase, and glycerophospholipase were weakly correlated with sd-LDL-C determined by the precipitation method ($R^2 = 0.13–0.67$). On the other hand, the cholesterol concentration treated with SMase was highly correlated with sd-LDL-C determined by the precipitation method ($R^2 = 0.895$). The SMase concentration was set at 600 U/L since a decline and a plateau of the cholesterol reaction during the rise of the SMase concentration to 500 U/L were observed (see online Supplemental Fig. 2). Ultimately, SMase was selected as the PLase most effective in dissociating lb-LDL and preserving sd-LDL. It was also found, however, that homogeneous assay results after treatment with surfactant A and SMase still had a nonzero intercept against the sd-LDL-C concentration as determined by the precipitation method [i.e., the intercept was 0.3 mmol/L (12.3 mg/dL)]. This nonzero intercept
suggested that the specificity of the homogeneous method with surfactant A and SMase was not sufficient to accurately determine sd-LDL-C values.

Fig. 3A shows the reactivity of narrow-cut lb-LDL (fraction 3, d 1.033 kg/L) and narrow-cut sd-LDL (fraction 15, d 1.059 kg/L) with the total-cholesterol assay after treatment by surfactant A (0.07% wt/vol) with and without SMase (600 U/L), where the absorbance without surfactant A was set as 100%. Both lb-LDL-C and sd-LDL-C reacted well with the total-cholesterol assay in the absence of SMase. In contrast, only 3% of the lb-LDL-C and 18% of the sd-LDL-C reacted with the cholesterol assay in the presence of SMase.

After screening for a second surfactant (surfactant B) to protect sd-LDL against the reaction of SMase, we found that a polyoxyethylene styrenephenyl ether derivative (Kao Corp.) can protect sd-LDL against the actions of SMase and cholesterol-oxidase/esterase reaction (R1). Thus, the sd-LDL-C that escapes from the action of these enzymes can be measured by the standard total-cholesterol assay (R2). Figs. 5A and B shows the reaction of the homogeneous method with ultracentrifugally isolated LDL obtained from serum of the lb-LDL (A) and sd-LDL (B) phenotype determined by GGE (19). The sd-LDL homogeneous method selectively elicited a reaction with the sd-LDL (fractions 13–20), whereas the LDL homo-
Geneous elicited a reaction with the total LDL fraction. The homogeneous assay sd-LDL-C results for 60 samples from healthy volunteers were compared with those obtained by ultracentrifugation. Serum samples were obtained from these individuals who had variable serum lipid levels [TG, 0.3–5.2 mmol/L (30–472 mg/dL), LDL-C, 1.6–5.2 mmol/L (63–199 mg/dL), and HDL-C, 0.9–2.5 mmol/L (35–97 mg/dL)]. The sd-LDL-C concentrations determined by the homogeneous method and ultracentrifugation were excellently correlated in all subjects ($y = 1.282x + 0.68, R^2 = 0.6657$). No significant differences were found between the mean (standard deviation) sd-LDL-C concentrations determined by the homogeneous method and by ultracentrifugation in the overall study population [0.81 (0.06) vs 0.91 (0.06) mmol/L, or 31.2 (2.2) vs 35.0 (2.1) mg/dL, respectively; $P = 0.220$] or in the dyslipidemic subjects [TG, 1.65 mmol/L (150 mg/dL); LDL-C, >3.64 mmol/L (140 mg/dL); or HDL-C, <1.04 mmol/L (40 mg/dL); 1.06 (0.08) vs 1.15 (0.08) mmol/L, or 40.6 (3.1) vs 43.9 (3.0) mg/dL, respectively; $P = 0.445$]. Fig. 6 shows the Deming fit for the sd-LDL-C values determined by the homogeneous method and ultracentrifugation. The Deming fit plot analysis revealed no significant proportional difference between the methods.

**Fig. 2.** Linear regression curves between the sd-LDL-C values determined by the precipitation method [Hirano et al. (12)] and sd-LDL-C values determined by the homogeneous method with surfactant A and various phospholipases. Among the homogeneous methods using phospholipases, that using sphingomyelinase exhibited the highest correlation with sd-LDL-C, as determined by the precipitation method.

**Fig. 3.** (A), The effect of SMase on the reactivity of narrow-cut lb-LDL (ultracentrifugation fraction 3, d 1.033 kg/L) and narrow-cut sd-LDL (fraction 15, d 1.059) with the total-cholesterol assay in the presence of surfactant A. The absorbance without surfactant A was set at 100%. (B), Effect of surfactant B on the reactivity (% absorbance) of lb-LDL (fraction 3, d 1.033 kg/L) and sd-LDL (fraction 15, d 1.049) with the total-cholesterol assay system in the presence of surfactant A and SMase. The absorbance without surfactant A and SMase was set at 100%.
between the 2 methods (slope 1.04, 95% CI 0.94–1.13, SE 0.48, \( P = 0.41 \)), but a slight constant negative intercept was found for the homogeneous method in comparison with ultracentrifugation (intercept \( -0.13 \) mmol/l \( = -5.0 \) mg/dL), 95% CI \(-0.22\) to \(-0.05\), SE 0.042, \( P < 0.005 \)).

The homogeneous sd-LDL-C method also exhibited a close association with sd-LDL-C determined by the precipitation method (\( y = 0.99x - 0.04 \), \( R^2 = 0.82 \)) (see online Supplemental Fig. 3).

Electron microscopic observations confirmed that only LDL particles with a diameter of 15–20 nm (average 17 nm) were left after the incubation of plasma with surfactants A and B and SMase (see online Supplemental Fig. 4).

We evaluated the basic characteristics of the homogeneous assay for sd-LDL-C (see online Supplemental Table 1). To investigate for possible interferences, we used Interference Check A Plus (Sysmex) made from human blood. The precision for the measurement of sd-LDL-C was determined by duplicate determinations, in 2 runs/day for 21 days (total \( n = 84 \)). The CVs for within-run precision were \( <1.1\% \), and total CVs were \( <2.2\% \). The assay did not show evidence of interference with 2.52 mmol/L (50 mg/dL) ascorbic acid, 5.0 g/L hemoglobin (hemolysate), 0.51 mmol/L (30 mg/dL) conjugated bilirubin, or 0.51 mmol/L (30 mg/dL) unconjugated bilirubin. When we examined the effect of triglyceride on the assay by adding isolated TRL from severe hypertriglyceridemic subjects, we found that plasma TG concentrations \(<11\) mmol/L \(<1000\) mg/dL) did not affect the sd-LDL-C measurement (see online Supplemental Fig. 5). Mean sd-LDL-C using serum vs plasma for the sample showed a negligible difference \( [1.00 + 0.04 vs 0.93 + 0.04 \) mmol/L (38.6 + 1.8 vs 35.9 + 1.7 mg/dL), respectively, \( n = 53, P = 0.266 \)], and the serum samples were stable until the fourth day of incubation at 4 °C.

The distribution of sd-LDL-C levels was skewed in normolipidemic individuals. The central 95% of sd-LDL-C concentrations ranged from 0.24 to 0.88 mmol/L (9.4–34.0 mg/dL) (see online Supplemental Fig. 6).

Discussion

In previous experiments with a homogeneous method (LDL-EX; Denka Seiken) (13) using the surfactant to dissociate TRL and HDL, selectively measuring narrow-cut LDL in a density range of 1.019–1.063 kg/L was successful. On that basis, we believed it would be possible to establish a homogeneous method for sd-

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**Fig. 4. The simple principle of the homogeneous assay for sd-LDL-C.**

Surfactant A (polyoxyethylene benzylphenyl ether derivative) reacts with TRLs and HDL, and cholesterol in these lipoproteins is eliminated by the action of cholesterol oxidase/esterase and catalase in step 1. Sphingomyelinase specifically reacts with lb-LDL, while surfactant B (polyoxyethylene styrenephenyl ether derivative) protects sd-LDL from the actions of sphingomyelinase and cholesterol oxidase/esterase (R1). The sd-LDL-C that escapes via the action of these enzymes is measured by the standard cholesterol assay in step 2.
LDL by finding a substance effective in dissociating lb-LDL. Liu et al. (14) reported that SM or phosphatidylinositol and phosphatidylserine were more abundant in lb-LDL than in sd-LDL. Kontush et al. (22) found that this abundance of sphingomyelin diminished in parallel with a progressive increase in HDL density. Similarly, we observed that the SM content of LDL particles and the SM/PC ratio decreased as the LDL particle density rose. Several articles have demonstrated that SMase aggregates and fuses LDL particles (15–17). We speculated that SMase might preferentially decompose lb-LDL particles containing abundant SM, while imposing only minimal effects on sd-LDL particles containing smaller amounts of SM. In tests to determine which PLases were most suitable for sd-LDL measurement, we found that the homogeneous assay system using SMase correlated most closely with the established method for sd-LDL-C precipitation.

SMase dissociated lb-LDL almost completely and sd-LDL partially, a finding that could have been predicted by the levels of SM in lb-LDL and sd-LDL. Given that SMase would be expected to partially dissociate the sd-LDL particles, we knew that another reagent would be required to protect the sd-LDL particles against the action of SMase. To find the best candidate, we screened the effectiveness of various surfactants in protecting sd-LDL against the actions of SMase, cholesterol oxidase/esterase, and catalase in step 1. On the basis of these investigations, we selected polyoxyethylene styrenephenyl ether derivative as surfactant B. The reactivity of sd-LDL-C with the cholesterol assay in the presence of surfactant B was strongly protected, rising from 18% to 82%. With this finding, it appeared that surfactant B was critical for the protection of sd-LDL particles against dissociation with SMase and cholesterol oxidase/esterase. The precise mechanisms for surfactant B–induced protection of sd-LDL particles in step 1 remain unknown.

The new homogeneous method gave good agreement with the ultracentrifugation method, a standard procedure for the measurement of sd-LDL (d 1.044–1.063 kg/L). Specifically, a regression equation, $y = 1.04x - 0.13$.
0.99x – 0.09, R² = 0.91, was obtained when comparing the homogeneous method with ultracentrifugation, a better correlation than obtained with our previous precipitation method (y = 1.05x + 1.0, R² = 0.78) (11). In addition, the absolute values of sd-LDL-C were almost identical between the ultracentrifugation and homogeneous methods. In these experiments, the homogeneous method was also confirmed to have specificity for the sd-LDL subfractions obtained from 2 different LDL phenotypes, patterns A and B. Electron microscopic observations also supported the finding that the homogeneous system selectively dissociated other lipoproteins while excluding sd-LDL particles. All of these observations strongly corroborate the validity of the new homogeneous assay for selectively determining sd-LDL-C and dissociating lb-LDL-C. However, precise mechanisms to explain why the homogeneous method selectively decomposes d < 1.044 kg/L lipoproteins have not been identified. Compositional changes of sd-LDL particles, such as a lower SM, PL, or cholesterol (23), might occur around densities of 1.044 kg/L. Further study will be required to elucidate this important issue.

The distribution of sd-LDL-C concentrations determined by the homogeneous method was skewed. The mean sd-LDL-C in normolipidemic individuals was approximately 0.5 mmol/L, or almost identical to the value measured earlier by the precipitation method (24). As a technique now proven to enable the measurement of sd-LDL-C in a fully automated manner with a short assay time, our homogeneous method is expected to be helpful in advancing understanding of the clinical significance of sd-LDL-C in various clinical studies.

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