Measurement of Small Dense Low-density Lipoprotein Particles

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Low density lipoprotein (LDL) particles are heterogenous with respect to their size, density, and lipid composition. Among LDL particles, the smaller and denser LDL particles [small dense (sd) LDL] are more atherogenic and the sd LDL phenotype is strongly associated with development of coronary heart disease. Here we will review various methods for measurement of sd LDL. Although ultracentrifugation, nuclear magnetic resonance (NMR) spectroscopy and gradient-gel electrophoresis (GGE) are usually employed for the measurement of sd LDL, such methods are either too laborious or expensive for general clinical use. We recently established a simple precipitation method for the quantification of sd LDL. This method is applicable to routine clinical use and allows the rapid measurement of a large number of samples. J Atheroscler Thromb, 2005; 12: 67–72.

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Introduction

Low density lipoprotein (LDL) particles are heterogenous with respect to their size, density, and lipid composition. Compared with large buoyant LDL, small dense (sd) LDL are thought to be more atherogenic as a result of their better penetration of the arterial wall, lower binding affinity for the LDL receptor, longer plasma half-life, and weaker resistance to oxidative stress (1, 2). Several studies have reported a 2- to 3-fold increase in the risk of coronary heart disease (CHD) in patients with sd LDL (3–5). We have also reported that sd LDL is closely associated with coronary events in the Japanese population, an ethnic group that has lower LDL cholesterol levels compared with Western populations (6, 7). Sd LDL is generated from large triglyceride-rich very-low density lipoprotein (VLDL1) (8). Since hepatic production of VLDL1 is stimulated by insulin resistance, the prevalence of sd LDL is associated with insulin resistance (9). Therefore, sd LDL has been highlighted as a useful new marker for the risk of CHD or type 2 diabetes (10). There are several methods for the assessment of lipoprotein size. Gradient gel electrophoresis (GGE) is used most widely (11), but this method is not a quantitative assay for sd LDL. Atherogenic potency should increase with an increase in the number of atherogenic lipoprotein particles, while the atherogenic potential should be reduced if few particles are present. Therefore, quantification of small dense LDL would be preferable to measurement of LDL size for evaluating the overall atherogenic risk, since it should more clearly indicate the atherogenic potential. Nuclear magnetic resonance (NMR) spectroscopy and ultracentrifugation are standard procedures for quantification of sd LDL, but they require special equipment, and are too difficult to use in daily clinical practice. We recently established a simple method for the quantification of sd LDL using heparin-magnesium precipitation. Here we review various methods for measurement of sd LDL, and also introduce our new method.

Gradient Gel Electrophoresis (GGE)

LDL particle size is most often measured by gradient
gel electrophoresis (GGE) using nondenaturing 2–16% polyacrylamide gel according to the procedure described by Nichols, Krauss, and Musliner (12). Gels containing acrylamide and bis-acrylamide were obtained from BIO CRAFT (Tokyo, Japan). A 10-µl plasma sample is applied to each lane with 20% sucrose and 0.25% bromphenol blue (final concentrations). Electrophoresis is performed in a refrigerated cell (10–15°C) with a prerun for 15 min at 125 V and entry of samples into the stacking gel at 200 V for 4 h and finally at 400 V for 1 h. Then, the gels are stained for lipids overnight with oil-red O in 5% ethanol. Next, the gels are destained in a 45% ethanol solution, and the original gel size is restored in a 5% acetic acid overnight. LDL peak particle size is determined from the migration of standards of known diameters, such as ferritin (12.2 nm), thyroglobulin (17.0 nm), and 39.0 nm latex beads. Pooled plasma is always used as an internal standard. GGE separates LDL particles based on the principle that the particles migrate through the gradient gel until further penetration is restricted by their size. Two distinct LDL size phenotypes have been proposed, these are pattern A with a higher proportion of large buoyant LDL particles (mean diameter > 25.5 nm) and pattern B with a predominance of small dense LDL particles (mean diameter < 25.5 nm) (3–5). Figure 1 shows the representative appearance of LDL bands obtained with GGE. Lanes 1, 6 and 12 show standard plasma with an average LDL diameter of 26.5 nm, lanes 2–5 show plasma samples with pattern A, and lanes 7–8 and 10–11 show plasma samples with pattern B.

Disk Polyacrylamide Gel Electrophoresis

The gel is commercially available (Lipophor Gel, Joko, Tokyo, Japan) (Lipoprint system LDL, Quantimetrix, Redondo Beach, CA, USA) and the run time is relatively short. Fresh serum is placed in glass gel tubes and then a loading gel containing Sudan Black B is added to each tube and mixed with the samples. The loading gel is polymerized using fluorescent light for 40 min and then electrophoresis is done at a constant current of 3 mA/tube for 60 min. Finally, the gel in each glass tube is scanned directly at a wave length of 610 nm. Different LDL subfractions can be identified by their migration distance. Each LDL subfraction has a specific electrophoretic mobility (Rf) relative to HDL when VLDL is at the origin of the separating gel and HDL is at the end of the gel (Fig. 2). Although several LDL subfractions can be detected by this method, we defined the Rf for the highest peak of LDL bands as the peak size of LDL particles. We found an excellent correlation between Rf values obtained by this method and LDL size as determined by the method of Nichols et al. (r = 0.85, p < 0.001, n = 41) (13).

Plasma Makers for Sd LDL

Each LDL particle contains one large molecule of apo B100 as its constituent protein and cholesterol as the

Fig. 1. Representative appearance of LDL bands in GGE.
The lanes 1, 6 and 12 show standard plasma with an average LDL diameter of 26.5 nm. Lane 2–5 show plasma samples with pattern A, while lanes 7–8 and lanes 10–11 (allows) show plasma samples with pattern B.

Fig. 2. Typical migration pattern of large buoyant LDL (no. 96) and small dense LDL (no. 49) in Lipophor gel.
The electrophoretic mobility (Rf) is calculated from the migration distance between the LDL band and VLDL bands when the distance from VLDL to HDL is 1.0. We assumed that an Rf of 0.4 corresponded to an LDL diameter of 25.5 nm determined by GGE, which is the cut-off value to distinguish LDL size phenotype pattern A (large buoyant LDL) from pattern B (small dense LDL) (see Ref. 13).
major lipid component. The size of LDL particles is largely determined by their cholesterol content (14, 15), so sd LDL is a cholesterol-poor particle. Since over 90% of plasma apo B is recovered in the LDL fraction, the plasma apo B concentration roughly corresponds to the number of LDL particles. Therefore, the LDL-cholesterol concentration divided by plasma apo B concentration approximately indicates the cholesterol content of a LDL particle. We found that the LDL-C/apo B ratio was significantly correlated with LDL size as determined by GGE (Fig. 3). Using this correlation curve, we determined the cut-off value of the LDL-C/apo B ratio corresponding to a LDL diameter of 25.5 nm, and found that the appropriate LDL-C/apo B ratio was 1.2. Thus, the preponderance of sd LDL can be estimated by the extent to which the LDL-C/apo B ratio is less than 1.2. It is well recognized that the plasma TG level is the chief determinant of LDL size, and that 50% of LDL size can be explained by the plasma TG level. According to Austin et al. (4), it is very rare to find subjects with sd LDL who have a TG level below 120 mg/dl. Conversely, few people with hypertriglyceridemia (TG > 200 mg/dl) have large buoyant LDL. Maruyama et al. (16) recommended the TG/HDL-C ratio as a superior marker of sd LDL relative to TG or HDL-C alone. They found that 75% of subjects with sd LDL had a TG/HDL-C ratio greater than 0.9 using mmol/l or 2.0 using mg/dl, while only 25% of the normal LDL group had a ratio above that level.

Proton NMR spectroscopy

Otvos et al. (17) first reported a method of lipoprotein analysis using proton NMR spectroscopy, which measures the signal emitted by plasma lipid methyl groups during magnetic resonance scanning. This method distinguishes the component peaks associated with major lipoproteins and their subclasses, including sd LDL. The major advantage of the NMR method is that it enables measurement of both the size and mass of LDL subclasses simultaneously. A total of 16 subclasses can be measured, including six VLDL subclasses, 4 LDL subclasses, and 5 HDL subclasses. The average lipoprotein particle size is determined by weighting the mass percentage of each subclass relative to its diameter. LDL sizes measured by NMR method are closely correlated with those measured by the GGE method, but are 5 nm smaller (18). Accordingly, 20.5 nm becomes a good cut-off value to distinguish individuals with pattern A or pattern B. Although NMR spectroscopy allows the measurement of both size and mass for all lipoproteins, it requires specialized laboratory equipment and hence has not become popular.

Sequential ultracentrifugation

Large buoyant LDL (d = 1.019–1.044 g/ml) and small dense LDL (d = 1.044–1.063 g/ml) can be separated from plasma by sequential ultracentrifugation with a fixed angle rotor. Serum (2 ml) is mixed with d = 1.045 solution (1 ml) and ultracentrifugation (80,000 rpm (290,000 × g; R avg)) is done for 7 h at 16°C. After discarding the supernatant containing d < 1.019 lipoproteins, 1 ml of d = 1.094 solution is added to the infranatant, and then it is spun at 100,000 rpm (453,000 × g) for 23 h. Large buoyant LDL (d = 1.019–1.044 g/ml) is recovered in the supernatant. One milliliter of d = 1.101 solution is added to the infranatant after removing the supernatant and it is spun at 80,000 rpm (290,000 × g) for 22 h. Finally, 1 ml of the supernatant is taken as sd LDL (d = 1.044–1.063 g/ml). The apo B concentration can be measured in the separated LDL fractions by an immunoturbidimetric assay using a commercially available test kit (Apo B Auto N “DAIICHI”, Daiichi Chemicals, Tokyo, Japan). Menys et al. (19) reported the simplified ultracentrifugation method, in which plasma in two tubes was adjusted to a density of 1.044 and 1.060 g/ml, respectively. Measurement of sd LDL-apo B was done by subtracting the apo B concentration in d > 1.060 lipoproteins from that in d > 1.044 lipoproteins.

Density gradient ultracentrifugation

Density gradient ultracentrifugation is performed on plasma samples to calculate the relative LDL flotation rate and density distribution of lipoprotein cholesterol (20, 21). A discontinuous salt density gradient is created in an ultracentrifuge tube. Plasma samples are centrifuged at 65,000 rpm for 70 min at 10°C in a vertical rotor. Then, the fractions are collected from the bottom of the centrifuge tube, and the cholesterol level in each fraction is
measured. The relative flotation rate (Rf), which characterizes LDL peak buoyancy as a continuous variable, is calculated as the fraction number containing the LDL cholesterol peak divided by the total number of fractions collected. Davies, Graham, and Griffin recently reported a simple method for the detection of sd LDL by gradient ultracentrifugation using a self-forming density gradient of iodixanol (22). The total run time was shortened to 3 h.

Precipitation method for the quantification of sd LDL

It is well known that a combination of divalent cations and polyanion precipitates apo B-containing lipoproteins, which allows HDL-cholesterol to be measured. However, we (23) found that the combination of heparin and magnesium (Mg) did not precipitate all apo B-containing lipoproteins, since the denser LDL remained in the supernatant. Figure 4 shows the changes of lipid, apo B, and apo AI concentrations in the supernatant after heparin-Mg precipitation or heparin-manganase (Mn) precipitation with variation of heparin concentration. A higher concentration of heparin completely precipitated apo B and LDL-C in the presence of Mn, whereas apo B and LDL-C remained in the supernatant after heparin-Mg precipitation.

This precipitation method is performed as follows. The precipitation reagent (0.1 ml) containing 15 U/ml heparin sodium salt and 90 mmol/l MgCl₂ is added to each serum sample (0.1 ml), followed by mixing and incubation for 10 min at 37°C. Then the samples are placed in an ice bath and left to stand for 15 min, after which the precipitate is collected by centrifuging at 15,000 rpm for 15 min at 4°C. This procedure yields a precipitate packed tightly at the bottom of the tube and a clear supernatant. An aliquot of the supernatant is removed for analysis of LDL-cholesterol and apo B. The LDL-cholesterol level in the heparin-Mg supernatant (containing sd LDL (d = 1.044–1.063 g/ml) and HDL) is directly and selectively measured by the homogenous method (LDL-EX, Denka Seiken, Tokyo, Japan). This direct LDL cholesterol assay can be done by an autoanalyzer, reducing the assay time to 10 min. Sd LDL-apo B in the heparin-Mg²⁺ supernatant can also be measured by an immunoturbidometric assay. However, because there is a strong correlation between sd LDL-C and sd LDL-apo B (r = 0.94, p < 0.0001), we decided to only measure sd LDL-C. Comparison with the ultracentrifugation method gave a correlation coefficient of r = 0.91 and an equation of y = 1.090 x – 1.8 (n = 146) (Fig. 5). There was a positive correlation with the total LDL-C level and also with the triglyceride level, while there was a negative correlation with LDL particle size and the HDL-C level. Ito et al. (24) refined this method by using a Millipore filter to eliminate the debris that are often found in the supernatant when severely hypertriglyceridemic

![Fig. 4. Effect of altering the concentrations of heparin on the LDL-cholesterol, HDL-cholesterol, triglycerides (TG), apo AI, and apo B levels in the supernatant after the heparin-Mg or the heparin-Mn precipitation.](image)

A constant concentration of MnCl₂ (30 mmol/l) or MgCl₂ (90 mmol/l) was used and only the heparin concentration was varied.

![Fig. 5. Correlation between ultracentrifugation and the precipitation method.](image)

Lipoprotein with density of 1.044–1.063 g/ml was separated by the ultracentrifugation method.
Small Dense LDL Assay

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Serum 0.2 ml Heparin-Mg 0.2 ml

Precipitation 37°C 5000 x g

10 min 1 min

Infranatant

Precipitate (VLDL, LDL, Large LDL)

Filter

Step 2

Autoanalyser

Step 1

Direct LDL-cholesterol (C) assay by the homogenous method

Infranatant

Sd LDL-C

Fig. 6. The procedure of the improved precipitation method.
The precipitation reagent (0.1 ml) containing 150 U/ml heparin sodium salt and 90 mmol/l MgCl₂ is added to each serum sample (0.1 ml), and incubated for 10 min at 37°C. Then sd LDL (d = 1.044–1.063 g/ml) and HDL are collected by filtering off the more buoyant lipoproteins, after which LDL-cholesterol is measured by the homogenous method.

serum is tested (Fig. 6). This improved method filters out large buoyant LDL and other apo B-containing lipoproteins, which form aggregates with the heparin-Mg reagent, and can completely eliminate non-specific debris. The assay time for this precipitation procedure is shortened to 11 min.

In summary, although the importance of measurement of sd LDL is well recognized, there is no standard assay procedure in general clinical use. We established a simple method for the quantification of sd LDL-C in serum using heparin-Mg precipitation. This precipitation method is applicable to routine clinical examination and allows the rapid measurement of a large number of samples.

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References
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