Clinical Significance of Small Dense Low-Density Lipoprotein Cholesterol Levels Determined by the Simple Precipitation Method

Tsutomu Hirano, Yasuki Ito, Shinji Koba, Miwako Toyoda, Ayako Ikejiri, Haruhisa Saegusa, Jun-ichi Yamazaki, Gen Yoshino

Objective—Recently, we established a simple method for the quantification of small dense LDL cholesterol (C) using heparin–magnesium precipitation. The small dense LDL-C level was identical to cholesterol in the denser LDL fraction with a density of 1.044 to 1.063 g/mL. The aim of this study was to examine clinical significance of this precipitation method for small dense LDL-C.

Methods and Results—Small dense LDL-C was measured by a direct homogenous LDL-C assay in the supernatant that remained after heparin–magnesium precipitation with density <1.044 lipoproteins. In 313 normolipidemic subjects, the mean value of small dense LDL-C was 31±13 mg/dL. In 462 healthy subjects, small dense LDL-C levels were positively correlated with serum triglyceride and LDL-C and were inversely correlated with high-density lipoprotein cholesterol (HDL-C). Combined hyperlipidemia showed the highest small dense LDL-C level among the various types of hyperlipidemia. Patients with type 2 diabetes had an increased small dense LDL-C level (55±17). Patients with coronary heart disease also had increased small dense LDL-C levels (53±30) irrespective of the presence of diabetes, whereas their LDL-C levels were comparable to those of normolipidemic controls (111±31 versus 104±22).

Conclusion—These results suggest that measurement of small dense LDL-C by the present precipitation method is useful to evaluate atherogenic risk and may be applicable to routine clinical examination. (Arterioscler Thromb Vasc Biol. 2004;24:558-563.)

Key Words: small dense LDL cholesterol ■ precipitation ■ hyperlipidemia ■ coronary heart disease ■ diabetes

Recently, small dense low-density lipoprotein (small dense LDL) has been highlighted as a new risk factor for coronary heart disease (CHD) in Westerners and also in the Japanese population, which has relatively lower LDL cholesterol (C) levels. The LDL particle size is usually measured by gradient gel electrophoresis using non-denaturing polyacrylamide according to the method of Krauss et al. However, this procedure requires a long assay time, ie, overnight electrophoresis, staining, and destaining. Of course, this assay does not provide a quantitative determination of small dense LDL. Analytical ultracentrifugation is the standard technique for quantification of small dense LDL; however, this method is also too laborious for general clinical use because it requires special equipment and a long running time.

It is well known that the combination of divalent cations and a polyanion causes the precipitation of apoB-containing lipoproteins, which allows us to measure high-density lipoprotein cholesterol (HDL-C). However, we discovered that the combination of heparin and magnesium (Mg) did not precipitate all of the apo B-containing lipoproteins, but part of LDL remained in the supernatant, which was identical to the small dense LDL fraction (density = 1.044 to 1.063 g/mL) isolated by ultracentrifugation. We subsequently established a simple assay for small dense LDL-C using heparin–Mg precipitation followed by direct measurement of LDL-C.

The aim of this study was to determine the clinical significance of our small dense LDL-C assay. First, we measured small dense LDL-C levels in a large number of healthy subjects with normolipidemia to determine the normal range. Second, we examined subjects with various types of hyperlipidemia. Third, we examined patients with type 2 diabetes and CHD, which are representative diseases associated with an increase of small dense LDL particles.

Methods

Subjects
Four hundred sixty-two persons aged 20 to 72 years old with a wide range of serum lipid levels were enrolled as normolipidemic and
hyperlipidemic subjects. The subjects with normolipidemia (n=313) and some with hyperlipidemia (n=112) were participants in annual medical check-ups, and the other hyperlipidemic subjects (n=37) were outpatients of Showa University Hospital. They were divided into the following 4 groups based on fasting LDL-C and triglyceride (TG) levels: normolipidemia was defined as LDL-C <140 mg/dL and TG <150 mg/dL; hyper LDL-C was LDL-C [metq]140 mg/dL and TG <150 mg/dL; hyper TG was LDL-C <140 mg/dL and TG [metq]150 mg/dL; and hyperchylomicronemia was TG >700 mg/dL and combined the presence of chylomicrons in fasting serum. All of the chylomicronemic subjects had very-low- apologized lipoprotein particle (VLDL) concentration in postheparin plasma (data not shown). The upper limits of normal levels for LDL-C (40 mg/dL) and TG (150 mg/dL) were defined according to the criteria of the Japanese Atherosclerosis Association (2002). All of these subjects were essentially healthy and had no evidence of diseases related to atherosclerosis. They did not have diabetes and fasting serum glucose levels were below 125 mg/dL. None of them had been treated with lipid-lowering drugs such as statins or fibrates, except for the hyperchylomicronemic subjects (6 were being treated with fenofibrate).

One hundred thirteen patients with angiographically documented CHD who had a history of angiina pectoris or myocardial infarction were also enrolled. Clinically significant CHD was defined as ≥50% stenosis of 1 or more branches of the coronary arteries. Furthermore, we enrolled 68 patients with type 2 diabetes, which was defined as a fasting blood glucose [metq]126 mg/dL or use of oral hypoglycemic agents. Patients receiving insulin users were excluded from the study. Forty-eight of the CHD patients had diabetes. There were 44 CHD patients and 21 diabetic patients using treatment with statins for hypercholesterolemia. Patients who were using fibrates were excluded from the study. Informed consent was obtained from each subject, and the study was approved by the local ethics committees.

Small Dense LDL-C Assay
The details and validation of this method have been described elsewhere. In brief, the precipitation reagent (0.1 mL) containing 150 U/mL of heparin sodium salt and 90 mmol/L MgCl2 was added to a serum sample (0.1 mL), and the sample was incubated for 10 minutes at 37°C after mixing. Then each sample was placed in an ice bath and let stand for 15 minutes, after which the precipitate was removed by centrifuging at 15 000 rpm for 15 minutes at 4°C. The precipitate was always packed tightly at the bottom of the tube and the supernatant was clear. An aliquot of the supernatant was removed and the small dense LDL-C was measured by the precipitation method (see Methods).

Biochemical Analysis
Triglyceride, total cholesterol, glucose, and hemoglobin A1c were measured by standard laboratory procedures. Serum apoB and AI were determined by an immunoturbidimetric assay (Diachi Chemicals, Tokyo, Japan). HDL-C was measured by direct homogenous assay of the serum using detergents (HDL-EX; Denka Seiken, Tokyo, Japan). We have previously reported LDL-EX assay selectively measures the narrow-cut LDL with density >1.019 to 1.063 g/mL and shows only a weak cross-react with IDL. Therefore, we could selectively measure small dense LDL-C in the heparin–Mg2+ supernatant without the influence of other lipoproteins. This direct LDL-C assay was performed with an autoanalyzer (type 7170; Hitachi Ltd., Tokyo, Japan). Using autoanalyzer, the assay time was only 10 minutes. The inter-assay and intra-assay of CVs for the precipitation method were 2% to 16% non-denaturing gradient gel electrophoresis according to the method of Nicolai, Krauss, and Musliner.

Statistical analyses was performed using Statview 5.0 software (SAS Japan Ltd, Tokyo, Japan). One-way analysis of variance (ANOVA) and the Bonferroni/Dunn post hoc test were used to compare mean values among groups, and significance was accepted at P<0.05. Pearson linear regression analysis was used to assess correlation between two parameters. Independent associations were assessed by multiple regression analysis.

Results
Small Dense LDL-C in Normolipidemic Subjects
Figure 1 upper panels shows the distribution of small dense LDL-C levels in 313 normolipidemic control subjects. The mean small dense LDL-C value was 31±13 mg/dL, and the median (50% percentile) was 28 mg/dL. Small dense LDL-C levels were significantly higher in males than in females (34±13 versus 28±11 mg/dL, P<0.0001). However, TG was a significant determinant of small dense LDL-C (Figure 2) and males had higher TG levels than females (74±28 versus 56±21 mg/dL, P<0.0001). Therefore, the gender difference of small dense LDL-C disappeared after adjustment for TG.

Figure 1. Histogram and percentiles of small dense LDL-C in healthy subjects with normolipidemia (TG <150 mg/dL and LDL-C <140 mg/dL) (upper panel). Histogram and percentiles of small dense LDL-C in patients with CHD (lower panel). Small dense LDL-C was measured by the precipitation method (see Methods).

Figure 2. Correlation between small dense LDL-C and serum lipids in healthy subjects with various lipid levels. Subjects with severe hypertriglyceridemia (TG >400 mg/dL) were excluded (D).
The percentage of small dense LDL-C, calculated as small dense LDL-C/total LDL-C, was ≈30% (Table 1).

**Small Dense LDL-C Levels in Subjects With Various Type of Hyperlipidemia**
Table 1 shows serum lipid and small dense LDL-C levels in subjects with various types of hyperlipidemia. Subjects with hyper LDL-C had increased small dense LDL-C levels, but the percent small dense LDL-C was not higher than in normolipidemic subjects. Large buoyant (LB) LDL-C can be calculated by subtracting small dense LDL-C from total LDL-C. It was found that hyper LDL-C subjects had the highest levels of LB LDL-C among all of groups. Subjects with hyper TG also had a significant increase of small dense LDL-C and percent small dense LDL-C, whereas LB LDL-C was significantly lower than in normolipidemic controls. Subjects with combined hyperlipidemia had the highest small dense LDL-C level among the groups. They also had higher levels of LB LDL-C and percent small dense LDL-C compared with normolipidemic controls. Among subjects with hyper LDL-C, we found higher small dense LDL-C in males than in females (59±13 mg/dL, P<0.05). However, the TG level was also significantly higher in males than in females (113±20 versus 64±20 mg/dL, P<0.0001), so the gender difference of small dense LDL disappeared after adjustment for TG. In the subjects with isolated hypertriglyceridemia, small dense LDL levels were comparable between males and females (64±20 versus 55±19 mg/dL). In the subjects with combined lipemia, small dense LDL levels were also comparable between males and females (84±26 versus 90±23 mg/dL). Subjects with hyperchylomicronemia had low LDL-C levels, but small dense LDL-C was higher than in normolipidemic controls. Thus, percent small dense LDL-C was high and LB LDL-C was low.

Figure 2 depicts the relationship between small dense LDL-C and serum lipids in the normolipidemic and hyperlipidemic subjects. Small dense LDL-C showed significant positively correlation with LDL-C (Figure 2A) and an inverse correlation with HDL-C (Figure 2B). Small dense LDL-C exhibited a positive correlation with serum TG, but subjects with severe hyper TG (including chylomicronemia) had disproportionately low levels of small dense LDL-C, which resulted in loss of the association between these parameters (Figure 2C). When subjects with TG >400 mg/dL were excluded, correlation between small dense LDL-C and TG became substantially stronger (Figure 2D). TG was inversely correlated with HDL-C (r=0.381, P<0.0001). Multiple linear regression analysis revealed that either LDL-C or TG was independently associated with small dense LDL-C in the subjects without severe hyper TG.

**Small Dense LDL-C in Patients With CHD**
The distribution of small dense LDL-C in 113 patients with CHD is depicted in Figure 1 (lower panels). The CHD patients had significantly higher small dense LDL-C levels than normolipidemic healthy controls, and the range of values was skewed rightward. The mean small dense LDL-C level was 53 mg/dL, and the median was 48 mg/dL in the CHD patients. Thus, the median of small dense LDL-C in patients with CHD corresponded to the 90 percentile in normolipidemic control subjects. However, LDL-C levels were not increased in the patients with CHD and were comparable to those in normolipidemic controls (111±31 versus 104±22 mg/dL). We compared 44 statin users with 69 non-statin users, but did not find any significant differences of LDL-C (123±39 versus 117±31 mg/dL), TG (120±82 versus 122±61 mg/dL), and small dense LDL-C (53±32 versus 52±25 mg/dL). Small dense LDL levels were significantly associated with the levels of apo B (r=0.726, P<0.0001), LDL-C (r=0.626, P<0.0001), TG (r=0.346, P<0.0002), and HDL-C (r=−0.240, P<0.02), but not with LDL size (r=−0.186) or apo AI (r=−0.107). Multiple regression analysis revealed that only apo B was independently associated with small dense LDL-C (standard regression coeffi-


Discussion

LDL size is not correlated with the LDL-C level,\textsuperscript{1–6} but the small dense LDL level showed a strong correlation with LDL-C, which is the most powerful risk factor for CHD. This suggests that quantification of small dense LDL can more clearly indicate the overall atherogenic potential than assessment of LDL size. Griffin et al\textsuperscript{9} reported that LDL-III (equivalent to small dense LDL with density \( \langle 1.044 \) mg/dL) was significantly increased in CHD patients and that the relative risk of CHD was increased 4.5-fold in individuals with LDL-III levels (protein plus lipid) higher than 100 mg/dL when compared with those with lower levels. Their study strongly suggested that quantification of small dense LDL might be useful for the assessment of CHD risk. However, their analytical ultracentrifugation technique was too laborious for general clinical use. Therefore, we developed a simple and rapid assay for the quantification of small dense LDL using heparin–Mg precipitation.\textsuperscript{10}

We observed that small dense LDL-C values obtained using our precipitation method were similar to those obtained by the ultracentrifugation method, and there was an excellent correlation between the two methods (\( y = 0.049x + 1.049 \times 10^{-1}, r = 0.884, P < 0.0001 \)).\textsuperscript{10} The small dense LDL level determined using the precipitation method was inversely associated with the mean LDL particle diameter measured by gradient gel electrophoresis, but it was not related to LB LDL.\textsuperscript{10} Because small dense LDL particles are cholesterol-depleted,\textsuperscript{14} we initially thought that small dense LDL–apoB

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### Table 2. Small Dense LDL-C and Various Parameters in Patients With CHD and Type 2 Diabetes (DM)

<table>
<thead>
<tr>
<th></th>
<th>Normolipidemic</th>
<th>DM Alone</th>
<th>CHD Alone</th>
<th>CHD With DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (male/female)</td>
<td>313 (140/168)</td>
<td>68 (30/38)</td>
<td>65 (49/16)</td>
<td>48 (33/15)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>38±12</td>
<td>38±12*</td>
<td>63±13*</td>
<td>63±11*</td>
</tr>
<tr>
<td>BMI</td>
<td>21±3</td>
<td>25±4*</td>
<td>24±3*</td>
<td>24±3*</td>
</tr>
<tr>
<td>Hemoglobin A1c (%)</td>
<td>NA</td>
<td>7.0±1.4†</td>
<td>5.2±0.5</td>
<td>7.1±1.6‡</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>82±10</td>
<td>153±68‡</td>
<td>106±22*</td>
<td>146±51†</td>
</tr>
<tr>
<td>LDL size (nm)</td>
<td>26.0±0.4‡</td>
<td>25.6±0.5*</td>
<td>25.6±0.5*</td>
<td>25.4±0.5*</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>64±26</td>
<td>163±88†§</td>
<td>110±80*</td>
<td>132±66*</td>
</tr>
<tr>
<td>Total-C (mg/dL)</td>
<td>188±27</td>
<td>217±36†§</td>
<td>190±37</td>
<td>191±42</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>104±22</td>
<td>162±20†</td>
<td>108±19</td>
<td>119±41</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>66±16</td>
<td>61±14</td>
<td>49±8†</td>
<td>53±9†</td>
</tr>
<tr>
<td>apoAI (mg/dL)</td>
<td>NA</td>
<td>134±27</td>
<td>122±23†</td>
<td>114±23†</td>
</tr>
<tr>
<td>apoB (mg/dL)</td>
<td>NA</td>
<td>106±21‡§</td>
<td>91±25</td>
<td>93±28</td>
</tr>
<tr>
<td>Small dense LDL-C (mg/dL)</td>
<td>31±13</td>
<td>55±17*</td>
<td>51±17*</td>
<td>54±29*</td>
</tr>
<tr>
<td>% Small dense LDL-C</td>
<td>29±9</td>
<td>36±11</td>
<td>42±20†</td>
<td>45±18†</td>
</tr>
<tr>
<td>Estimated LB LDL-C (mg/dL)</td>
<td>73±17</td>
<td>90±24‡§</td>
<td>70±31</td>
<td>65±27*</td>
</tr>
</tbody>
</table>

NA indicates not available.

\( *P<0.001–0.05 \) vs normolipidemic.

\( †P<0.001–0.05 \) vs DM alone.

\( ‡P<0.05–0.001 \) vs CHD alone.

\( §P<0.05–0.001 \) vs CHD with DM.

\( †‡L DL size was measured in 124 normolipidemic subjects.\)

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The coefficient=0.509, \( t \) value=3.30, \( P<0.0013 \), whereas LDL-C, TG, and HDL-C showed no association.

**Small Dense LDL-C in Patients With Type 2 Diabetes and CHD**

Table 2 shows various serum parameters and small dense LDL-C in levels in the patients with type 2 diabetes and CHD. When our CHD patients were divided into 2 groups based on the presence or absence of diabetes, the diabetic subjects had higher levels of fasting serum glucose and hemoglobin A1c, and these levels were identical to those in diabetic patients without CHD. LDL was significantly smaller in the diabetic patients and in the patients with CHD (irrespective of the presence of diabetes) than in normolipidemic controls. Serum TG was significantly increased in the patients with diabetes. Patients with CHD also had higher TG levels than normolipidemic controls, but the TG level was lower than in those with diabetes. Total-C and LDL-C levels were significantly increased in diabetic subjects but were not increased in CHD patients irrespective of the presence of diabetes and were comparable to the levels in normolipidemic controls. CHD patients had significantly lower HDL-C levels than normolipidemic controls or patients with diabetes alone. Apo B was significantly increased in patients with diabetes, but apo AI was decreased in patients with CHD. Small dense LDL-C levels were similarly increased in patients with diabetes, CHD, or diabetes plus CHD when compared with the levels in normolipidemic controls. However, the ratio of small dense LDL-C to total LDL-C was not increased in patients with diabetes alone, but this was significantly decreased in patients with CHD alone or those with diabetes plus CHD. LB LDL was increased in patients with diabetes, but not in patients with CHD. We did not find any differences of serum parameters between statin users and nonusers among the patients with type 2 diabetes (data not shown).
or protein may precisely reflect the mass of these atherogenic lipoproteins whereas small dense LDL-C does not. However, we found a very strong correlation between small dense LDL-C and small dense LDL-apoB values ($r=0.944$, $P<0.0001$), suggesting that measurement of cholesterol is sufficient to evaluate the small dense LDL mass and that protein measurement is not essential. It is likely that the cholesterol content of each particle is homogenous in the small dense LDL fraction, so the number of particles (apoB) determines the cholesterol level.

The mean small dense LDL-C level in healthy subjects without hyperlipidemia was $\approx 30$ mg/dL, which was $\approx 30\%$ of total LDL-C. Because small dense LDL-C was correlated with either TG or LDL-C, it was increased in hypertriglyceridemia and hypercholesterolemia. As expected, small dense LDL-C was markedly increased in combined hyperlipidemia, and the majority (86%) of LDL-C was recovered in the small dense LDL fraction. Familial combined hyperlipidemia is characterized by high levels of LDL particles (hyper apoB) and a preponderance of small dense LDL, and it is a representative disease associated with a high incidence of CHD. Therefore, measurement of small dense LDL-C may be useful when screening for familial combined hyperlipidemia. Our subjects with severe hypertriglyceridemia including chylomicronemic subjects had only slightly elevated small dense LDL-C levels. It is likely that the subjects with a TG level $>400$ mg/dL had a low LPL activity, which impaired the conversion of TG-rich lipoproteins to LDL and resulted in a disproportionately low small dense LDL-C concentration relative to the TG level. Only modest elevation of small dense LDL-C in subjects with severe hypertriglyceridemia might explain why the incidence of CHD is not increased further despite a massive increase in TG levels.

We previously demonstrated that LB LDL-C, estimated as total LDL-C minus small dense LDL-C, significantly correlated with the value actually determined by the ultracentrifugation method. The estimated LB LDL-C level was higher in subjects with hypercholesterolemia than in those with combined hyperlipidemic. The greater atherogenic potential of small dense LDL compared with LB LDL may explain the higher incidence of CHD in combined hyperlipidemia than in isolated hypercholesterolemia. Small dense LDL-C showed a significant positive correlation with LDL-C and a negative correlation with HDL-C, which are well-established risk factors for CHD. Thus, small dense LDL-C could be considered as an integral marker for CHD risk.

Small dense LDL-C was substantially increased, although total LDL was not, in our patients with CHD. We also enrolled CHD patients being treated with statins in this study, which could lead to underestimation of LDL-C level in some patients. However, the same results were obtained in our statin-free CHD patients. The present result is in good agreement with our previous finding that CHD patients have a high prevalence of the small dense LDL phenotype (pattern B) despite normal LDL-C levels. Our patients with CHD had higher TG levels than normolipidemic controls. Because a decrease of LDL size and the small dense LDL-C level are strongly associated with the serum TG level, it is likely that an increase of small dense LDL particles in CHD is caused by a modest increase of serum TG. LDL size is determined not only by the fasting TG level but also by the postprandial increase of TG. Several studies have demonstrated that patients with CHD have postprandial hyperlipidemia. Thus, an increase of fasting and postprandial TG levels might contribute to the high prevalence of small dense LDL particles in patients with CHD. Low HDL-C levels in CHD patients might also imply the presence of abnormal TG metabolism. We found that the serum apoC concentration was significantly associated with small dense LDL-C level, independently of LDL-C, HDL-C, and TG. Therefore, the presence of hyper apoC may identify CHD patients with a high small dense LDL-C concentration.

A number of previous studies have demonstrated a high prevalence of small dense LDL particles in patients with type 2 diabetes. We found that not only the prevalence of small dense LDL but also the concentration of small dense LDL was substantially increased in patients with type 2 diabetes. It is unknown why serum lipid levels were higher in patients with diabetes alone than in those who had diabetes plus CHD in the present study; however, it is likely that patients with both diseases paid more attention to serum lipid levels by restricting their diet. We previously reported that LDL size did not further decrease in CHD patients even when they had diabetes. Similarly, the present study demonstrated that the presence of diabetes did not affect small dense LDL-C in CHD patients. These results suggest that small dense LDL-C is a powerful predictor of CHD for diabetic and nondiabetic populations.

In conclusion, we determined the normal range of small dense LDL-C and found remarkably high small dense LDL-C levels in patients with CHD or type 2 diabetes by a simple assay. We plan to apply this method in a large cohort study to confirm the atherogenicity of small dense LDL particles.

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