Relationship between Triglyceride Concentrations and LDL Size Evaluated by Malondialdehyde-modified LDL

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Background: Hypertriglyceridemia is associated with decreased HDL-cholesterol (HDL-C) and increased small dense LDL. In addition, small dense LDL is known to be susceptible to oxidation.

Methods: We measured LDL particle size, using gradient gel electrophoresis, and malondialdehyde-modified LDL (MDA-LDL), using an ELISA, and investigated the association between triglyceride (TG) concentrations, LDL size, and MDA-LDL.

Results: TG concentrations correlated negatively with the predominant LDL size ($r = -0.650$) and HDL-C concentration ($r = -0.556$). The relationship between TG concentration and LDL size, evaluated by measuring MDA-LDL, distinguished subgroups derived from four subfractions of TG concentrations and four distribution ranges of LDL size. These experiments indicated that there is a threshold for oxidation susceptibility at an LDL size of 25.5 nm and a TG concentration of 1500 mg/L. To investigate the relationship between LDL size, MDA-LDL concentration, and other lipids (TGs, HDL-C, apolipoprotein B, and total cholesterol), we evaluated them in control subjects and patients with diabetes mellitus or hypertriglyceridemia. When the size range for normal LDL was postulated to be $25.5 \leq \phi$ (LDL diameter) $< 26.5$ nm, the MDA-LDL concentration was significantly higher in the subgroups of patients with LDL in the size range $24.5 \leq \phi < 25.5$ nm compared with patients with normal LDL. This result also suggests that the threshold is at a LDL size of 23.5 nm.

Conclusion: The threshold for oxidation susceptibility coincided with the point of LDL size separation between the LDL subclass patterns A and B as an atherosclerotic risk.

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Whether hypertriglyceridemia is a risk factor for atherosclerosis remains unclear. Hypertriglyceridemia reflects an increased concentration of any of several lipoproteins, including chylomicrons, VLDL, and intermediate-density lipoprotein (IDL).5 IDL is a known risk factor for atherosclerosis (1, 2), but the roles of chylomicrons and VLDL in atherosclerosis have been controversial for many years.

Austin et al. (3) have shown that LDL subclass pattern B, which is characterized by a preponderance of small dense LDL particles, is associated with an increased risk of myocardial infarction and that it is correlated with increased concentrations of IDL-cholesterol, VLDL-cholesterol, and triglycerides (TGs), and low concentrations of HDL-cholesterol (HDL-C). Because hypertriglyceridemia is concomitant with increasing small dense LDL, hypertriglyceridemic matter is replaced by small dense LDLs. Indeed, it is known that the TG concentration is inversely associated with LDL size. Researchers have reported that when different subpopulations of small dense LDLs are isolated from the same donor, the smallest LDL fraction shows the poorest LDL-receptor binding (4). Furthermore, small LDL is considerably more susceptible

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5 Nonstandard abbreviations: IDL, intermediate-density lipoprotein; TG, triglyceride; HDL-C, HDL-cholesterol; MDA-LDL, malondialdehyde-modified LDL; apo A-I and B, apolipoprotein A-I and B; DM, diabetes mellitus; HbA1c, glycosylated hemoglobin; TC, total cholesterol; TBARS, 2-thiobarbituric acid-reactive substances; and CHD, coronary heart disease.
than normal-sized LDL to oxidation in the presence of copper ions (5, 6). We previously reported that malondialdehyde-modified LDL (MDA-LDL) is distributed to small dense LDL fractions by density gradient ultracentrifugation (7). These findings evoked interest in the relationship between small dense LDL and oxidation. In this study, we measured LDL size by gradient gel electrophoresis and MDA-LDL concentrations by ELISA and thereby evaluated the association between LDL size and MDA-LDL.

Materials and Methods

Reagents

Colloidal gold particles (10 and 15 nm in diameter) were purchased from EY Laboratories, Inc. Carbonic anhydrase derived from bovine erythrocytes was obtained from Roche Diagnostics Co. Monoclonal antibodies against MDA-LDL (ML25) and apolipoprotein B (apo B; monoclonal antibody AB16) were obtained from Daiichi Pure Chemicals Co. Nondenaturing 2–15% polyacrylamide gels were purchased from Daiichi.

Subjects

Study subjects included 91 patients and 42 healthy controls. Among the patients, 49 had diabetes mellitus (DM) and 42 had hypertriglyceridemia without DM. Control subjects and patients with DM or hypertriglyceridemia were matched for age and sex. All DM patients (21 women and 29 men; age range, 30–72 years) were randomly selected from diabetic patients who had glycated hemoglobin (HbA1c) values ≥6% at the onset of study participation. DM was diagnosed according to the criteria of the World Health Organization (8). All patients with hypertriglyceridemia (17 women and 25 men; age range, 29–71 years) were selected from patients who had a serum TG concentration >1500 mg/L and a serum HDL-C <600 mg/L. Among the 49 diabetic subjects, 12 were receiving lipid-lowering drugs and 1 was receiving vitamin E supplementation. Of the 42 hypertriglyceridemic patients, 3 were receiving lipid-lowering medication, 1 was receiving vitamin C supplementation, and 1 was receiving vitamin E supplementation. Control subjects (17 women and 25 men; age range, 31–72 years) were selected from healthy volunteers who had serum total cholesterol (TC) concentrations ≤2200 mg/L and serum TG concentrations ≤1500 mg/L.

Analytical methods for lipids and other analytes

Serum TC, TG, and HDL-C concentrations were determined enzymatically (Kyowa Medex Co., Ltd.). Serum concentrations of apo B and apo A-I were measured with a commercial immunoturbidimetric assay (Daichii). These assays as well as the following electrophoresis were carried out within 1 week after blood was drawn, and serum samples were stored at 4°C. HbA1c in whole blood was measured by HPLC. The reference interval for HbA1c was 4.0–5.8%.

Quantification of MDA-LDL by ELISA

The ELISA method used was based on the same principles as the method used in the previous report by Kotani et al. (7). Briefly, serum samples were diluted 2400-fold in a dilution buffer containing 25 mmol/L HEPES, 3.5 mmol/L sodium dodecyl sulfate, 2 g/L bovine serum albumin (cat. no. A-7030; Sigma), and 1 g/L NaN3 (pH 7.8). The diluted sample was preincubated for 1 h at 37°C and returned to room temperature. Duplicate 100-μL portions of the diluted sample were then added to the wells of plates that were coated with monoclonal antibody against MDA-LDL (ML25; 0.8 μg/well). The reaction was allowed to stand for 1 h at room temperature, and the plates were then washed. β-Galactosidase-conjugated monoclonal antibody against apo B (AB16; 100 μL) was then added, and the mixture was incubated for 30 min at room temperature. Excess enzyme-labeled antibody was removed by washing, and 100 μL of 10 mmol/L o-nitrophenyl-β-galactopyranoside as a substrate was pipetted into the wells. After 2 h, the reaction was stopped by adding 100 μL of 0.2 mol/L sodium carbonate (pH 12). Absorbance in the individual wells was determined at 415 nm with an MPR-4A microplate reader. The intra- and interassay CVs were 6.5% and 9.0%, respectively. Serum samples were used within 4 days after serum was separated by centrifugation and stored at 4°C.

Primary standard was used with preparative MDA-LDL, in which 15% of the total amino groups were modified. We tentatively defined 1 unit/L MDA-LDL as the absorbance obtained with the primary standard at a concentration of 1 mg/L. A calibration curve was prepared by diluting a reference serum as a secondary standard from 300- to 9600-fold with a dilution buffer and calculating the amount of MDA-LDL in the samples. Reference sera were prepared from pooled sera from healthy volunteers, and 100 g/L succrose, 37.5 mmol/L NaCl, and 0.25 mmol/L disodium EDTA were added to each serum pool. The sera were then divided into aliquots and stored at −80°C. For each assay, aliquots of reference sera were thawed and used.

Procedure for Nondenaturing Gradient Gel Electrophoresis

The diameter of the LDL in the major LDL peak was estimated by nondenaturing polyacrylamide gradient gel electrophoresis using a modified version of the technique described by Krauss and Burke (9). Briefly, 5 μL of serum from each subject was diluted twofold with 400 g/L sucrose and electrophoresed for 24 h at 10°C on 2–15% polyacrylamide gradient gels with a buffer containing 90 mmol/L Tris, 80 mmol/L boric acid, and 3 mmol/L disodium EDTA (pH 8.3). The gels were stained with oil red O. The lane containing the calibrators was stained with Coomassie Blue R250, and a calibration curve was
constructed based on the migration distances of five markers with known diameters: ferritin (12.2 nm), thyroglobulin (17.0 nm), thyroglobulin dimer (23.6 nm; Pharmacia), and protein-coated gold particles (21.1 and 29.2 nm; our preparations). A control serum was run as a reference on each gel. The locations of individual bands were compared with the control serum when each was scanned. The predominant LDL size in each sample lane was calculated from the equation given below after the migration distance of the major LDL peak was measured.

Each gel lane was scanned with a densitometer interfaced with a PC computer. Control serum was drawn from one healthy volunteer and combined with 100 g/L sucrose, 37.5 mmol/L NaCl, and 0.25 mmol/L disodium EDTA. This was divided into aliquots and stored at −80 °C.

PREPARATION FOR PROTEIN-COATED GOLD PARTICLES
When used as a size marker in electrophoresis, a latex particle is not clearly visible. We therefore prepared novel markers using protein-coated gold particles. Briefly, one vial of carbonic anhydrase (50 mg) was diluted to 5 mL in buffer containing 89 mmol/L Tris, 89 mmol/L boric acid, and 2.5 mmol/L disodium EDTA (pH 8.3). We added 200 μL of this carbonic anhydrase solution and 400 μL each of both colloidal gold particles (diameters, 10 and 15 nm, respectively) to a microcentrifuge tube. For all solutions, 1 mL was then vortex-mixed thoroughly and incubated for 1 h at room temperature. The reaction mixture was centrifuged at 12 000 × g for 1 h at 10 °C. After the supernatant was removed, the bottom fraction was adjusted to a final solution by mixing with an equal volume of 500 g/L sucrose containing 2.7 mmol/L disodium EDTA. The final solution was stored at 10 °C for up to 1 month.

DETERMINATION OF DIAMETER OF PROTEIN-COATED GOLD PARTICLES BY SHADOWING
The final solution of protein-coated gold particles was diluted 10-fold in 25 mmol/L phosphate buffer containing 150 mmol/L NaCl (pH 7.2). The diluted sample was placed on a Formvar/carbon-coated grid and immobilized for 0.5 min. Excess fluid was removed with filter paper. The specimen was exposed to platinum shadowing at a 30° angle and irradiated for several minutes using a JEOL JEE-5E vacuum evaporator. The specimen was then immediately placed in a microscope specimen chamber and observed with a JEM-1220 electron microscope at 80 kV.

On the electron micrograph, we measured particle sizes from the shadow cast by platinum shadowing (10). The shadow width was always constant and equal to the particle diameter (Fig. 1). Mean particle sizes were calculated from a sample of 30–100 particles; the mean diameter of the small particles was 21.1 nm, and the mean of the large particles was 29.2 nm.

APPLICATION OF BOTH PROTEIN-COATED GOLD PARTICLES TO ELECTROPHORESIS
The two protein-coated gold particles were used as size markers in the gradient gel electrophoresis. Fig. 2-1 shows that electrophoresis produced clear bands of both particles (lane 2). Using the method of Williams et al. (11) with some modifications, we could calculate the equation for converting molecular diameter (nm) to migration distance (Rf). The equation was derived from the migration dis-
stances of both protein-coated gold particles (diameters, 21.1 and 29.2 nm, respectively) and from the migration distances of the thyroglobulin dimer (23.6 nm), thyroglobulin (17.0 nm), and apoferritin (12.2 nm). The \( R_e \) of each particle measured relative to apoferritin was plotted against particle diameter (nm; Fig 2-2). As a result, LDL diameters were calculated from the following equation:

\[
LDL \text{ migration distance } (R_e) = 2.247e^{-0.0656x}
\]

where \( e \) is the natural logarithm, and \( x \) is the particle diameter (nm).

**Isolation of LDL and Its Subspecies**

LDL and its subspecies were isolated by sequential ultracentrifugation from human serum. Sequential ultracentrifugation was performed in a Beckman Model Optima TL ultracentrifuge with a TLA 100.2 fixed-angle rotor and Beckman polycarbonate 1.5-mL centrifuge tubes. Fresh serum was mixed with one-half volume of KBr solution \((d = 1.045 \text{ kg/L})\), which adjusted the density of the mixture to \( d = 1.019 \text{ kg/L} \). The preparative serum (600 \( \mu \text{L} \)) was placed in a tube, and 500 \( \mu \text{L} \) of KBr solution \((d = 1.019 \text{ kg/L})\) was layered on top. After centrifugation at 436 000 \( g \) for 2 h at 15 \( ^\circ \text{C} \), the top layer (500 \( \mu \text{L} \); \( d < 1.019 \text{ kg/L} \)) was collected by aspiration. The bottom fraction was adjusted to \( d = 1.063 \text{ kg/L} \) by mixing with one-half volume of KBr solution \((d = 1.151 \text{ kg/L})\). Subsequently, 500 \( \mu \text{L} \) of KBr solution \((d = 1.063 \text{ kg/L})\) was layered onto this preparative fraction \((600 \mu \text{L})\). Centrifugation was performed again at 436 000 \( g \) for 4 h at 15 \( ^\circ \text{C} \). LDL \((500 \mu \text{L} \) of the 0.1019 < \( d < 1.063 \text{ kg/L} \) fraction) was recovered from the top of the tube. If the bottom fraction \((d > 1.019 \text{ kg/L})\) was divided according to LDL subspecies, that fraction was adjusted to \( d = 1.040 \text{ kg/L} \) by adding one-half volume of KBr solution \((d = 1.082 \text{ kg/L})\). Thereafter, the preparative fraction \((600 \mu \text{L})\) was placed in a tube, 500 \( \mu \text{L} \) of KBr solution \((d = 1.040 \text{ kg/L})\) was layered on top, and the material was then recentrifuged at the conditions described above. The supernatant \((500 \mu \text{L} ; 1.019 < d < 1.063 \text{ kg/L} \) fraction) was recovered as the “normal LDL” fraction. The infranate was mixed with one-half volume of KBr solution \((d = 1.109 \text{ kg/L})\), which adjusted the density of the mixture to \( d = 1.063 \text{ kg/L} \). Finally, small dense LDL was separated by centrifugation at 436 000 \( g \) for 4 h at 15 \( ^\circ \text{C} \). The top layer (500 \( \mu \text{L} ; 1.040 < d < 1.063 \text{ kg/L} \) ) was recovered as the small dense LDL fraction (12). All salt solutions contained 1 mmol/L EDTA.

When the cholesterol concentration in the \( d < 1.019 \text{ kg/L} \) and 1.019 < \( d < 1.063 \text{ kg/L} \) fractions was defined as the amount of HDL-C subtracted from the TC amount in serum, recovery of cholesterol content in the \( d < 1.063 \text{ kg/L} \) fraction was 93.1–97.1%. The interassay CVs were <2.5% as determined by measurement of cholesterol in the LDL fraction. Electrophoresis of the LDL fraction in sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie blue R250 revealed contamination of apo A-I in that fraction that was estimated densitometrically as <1.0% of the total protein moiety. Other lipoproteins in the LDL fraction could not be detected by agarose gel electrophoresis.

**Determination of 2-Thiobarbituric Acid-Reactive Substances in Isolated LDL**

The MDA concentrations in isolated LDL fractions were determined by measuring 2-thiobarbituric acid-reactive substances (TBARS) with a commercially available method (Wako Pure Chemical Industries, Ltd.). Each LDL fraction \((100 \mu \text{L})\) isolated by ultracentrifugation was mixed with 4 mL of distilled water and 1 mL of TBA reagent containing 2-thiobarbituric acid. After vortex-mixing, the samples were incubated in boiling water for 1 h. The complex formed with 2-thiobarbituric acid was extracted with 5 mL of butanol and quantified fluorometrically (excitation, 515 nm; emission, 553 nm). TBARS were expressed as MDA equivalents. The MDA calibrator was prepared from 1,1,3,3-tetraethoxypropane. Addition of the KBr and EDTA solutions used in the LDL purification procedure produced no interference in the assay.

**Statistical Analysis**

Data are shown as the mean ± SD. Comparisons of mean values between control subjects and patients with DM or hypertriglyceridemia and between subgroups divided on the basis of LDL size or further subgroups divided by TG concentration were performed with one-way ANOVA followed by the Scheffé test. However, comparison of variables between the subgroups except for subgroups containing only one subject was assessed by the Student \( t \)-test. Spearman rank correlation coefficients were computed to identify correlations that were significantly associated with variations between lipid values. Significance was set at \( P < 0.05 \).

**Results**

**Lipid Values in Control Subjects and Patients**

The mean lipid values in control subjects and patients with DM or hypertriglyceridemia are shown in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Control (( n = 42 ))</th>
<th>DM (( n = 49 ))</th>
<th>Hypertriglyceridemia (( n = 42 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>50.0 ± 11.1</td>
<td>50.6 ± 8.6</td>
<td>50.6 ± 10.3</td>
</tr>
<tr>
<td>TC, mg/L</td>
<td>1810 ± 220</td>
<td>2060 ± 430</td>
<td>2000 ± 390</td>
</tr>
<tr>
<td>HDL-C, mg/L</td>
<td>610 ± 160</td>
<td>490 ± 150</td>
<td>420 ± 90</td>
</tr>
<tr>
<td>TGs, mg/L</td>
<td>840 ± 310</td>
<td>1570 ± 1310</td>
<td>2440 ± 1110</td>
</tr>
<tr>
<td>LDL size, nm</td>
<td>26.1 ± 0.9</td>
<td>25.7 ± 1.2</td>
<td>24.6 ± 1.1</td>
</tr>
<tr>
<td>MDA-LDL, units/L</td>
<td>52 ± 14</td>
<td>87 ± 50</td>
<td>96 ± 35</td>
</tr>
<tr>
<td>apo A-I, mg/L</td>
<td>1430 ± 170</td>
<td>1240 ± 220</td>
<td>1250 ± 180</td>
</tr>
<tr>
<td>apo B, mg/L</td>
<td>800 ± 140</td>
<td>1010 ± 270</td>
<td>1040 ± 230</td>
</tr>
</tbody>
</table>

*Values are the mean ± SD.

\( a, b, c \) Statistical difference between control subjects and those with either DM or hypertriglyceridemia: \( a P < 0.01 \); \( b P < 0.001 \).
For subjects with DM or hypertriglyceridemia, HDL-C and apo A-I concentrations were lower \((P < 0.001)\) than those in control subjects. The predominant LDL size in hypertriglyceridemic patients was significantly lower \((P < 0.001)\) than that in control subjects. No significant difference in LDL size existed between control and diabetic subjects. TG, MDA-LDL, and apo B concentrations were significantly higher in subjects with DM or hypertriglyceridemia than in control subjects \([P < 0.001]\) except for the difference in TG concentrations between diabetic patients and control subjects \((P < 0.01)\).

**Correlations between Lipids**

The correlation coefficients for comparisons between lipid values for three distinct groups (controls, DM patients, and hypertriglyceridemic patients) combined are shown in Table 2. LDL particle size was inversely correlated with TG and MDA-LDL concentrations and positively correlated with the HDL-C concentration. HDL-C concentration was weakly correlated with other lipid values. MDA-LDL was correlated with TG concentration and LDL size. Overall, TGs, LDL size, and MDA-LDL were closely correlated.

**Relationships among TG Concentration, LDL Size, and MDA-LDL**

On the basis of distribution of the total LDL particles, we divided particles into four LDL size groups with diameters \((f) < 24.5\) nm, \(24.5 \leq f < 25.5\) nm, \(25.5 \leq f < 26.5\) nm, and \(f \geq 26.5\) nm. The distribution of TGs was separated into four intervals: \(0 < \text{TGs} \leq 1500\) mg/L, \(1500 < \text{TGs} \leq 3000\) mg/L, \(3000 < \text{TGs} \leq 4500\) mg/L, and \(\text{TGs} > 4500\) mg/L. Fig. 3 shows a diagram of the subgroup combinations for ranges of LDL size and TG concentration. Mean values of MDA-LDL in each subgroup were calculated. The bottom panel in Fig. 3 shows the number of subjects in each subgroup and the statistical analysis for mean comparisons of MDA-LDL between the normal group (LDL size, \(25.5 \leq f < 26.5\) nm; \(0 \leq \text{TGs} \leq 1500\) mg/L) and each of the other subgroups. \(+++, P < 0.001; +++, P < 0.01.\)

TG concentration within the reference interval possess small dense LDLs, with MDA-LDL concentrations inversely related to LDL size. At TG concentrations \(\geq 3000\) mg/L, there was virtually no large LDL \((f \geq 25.5\) nm).

**Distribution of MDA-LDL in Control Subjects and Patients**

We compared MDA-LDL concentrations in each distribution range of LDL size between control subjects and patients with DM and with hypertriglyceridemia (Fig. 4).

**Table 2. Spearman correlation\(^a\) between lipid values.**

<table>
<thead>
<tr>
<th></th>
<th>TGs</th>
<th>HDL-C</th>
<th>LDL size</th>
<th>MDA-LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGs</td>
<td>-0.556</td>
<td>-0.650</td>
<td>0.644</td>
<td></td>
</tr>
<tr>
<td>HDL-C</td>
<td>0.448</td>
<td>-0.482</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL size</td>
<td></td>
<td></td>
<td>-0.567</td>
<td></td>
</tr>
<tr>
<td>MDA-LDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) All P values of correlation coefficients are \(< 0.001.\)
There were statistically more \((P < 0.05)\) diabetic patients than control subjects with LDL size ranges of \(\phi < 24.5 \text{ nm} \) and \(25.5 \leq \phi < 26.5 \text{ nm}\). In the LDL size distribution of \(25.5 \leq \phi < 26.5 \text{ nm}\), patients with hypertriglyceridemia had significantly higher \((P < 0.01)\) MDA-LDL concentrations than control subjects; in the \(\phi < 24.5 \text{ nm} \) size range, patients with hypertriglyceridemia had significantly lower MDA-LDL concentrations \((P < 0.05)\) than diabetic patients. In the other LDL size ranges, MDA-LDL concentrations in both patient groups were somewhat higher than those in control subjects, but the differences did not reach statistical significance.

### Comparisons between lipids in each LDL size range

Assuming a LDL size range of \(25.5 \leq \phi < 26.5 \text{ nm} \) in control subjects as a normal LDL, we compared TC, HDL-C, TG, MDA-LDL, and apo B concentrations in individual subjects on the basis of normal LDL and other LDL size ranges. Table 3 shows the comparisons between lipid values and LDL size in control subjects. None of the lipid values for control subjects in the LDL size ranges \(\phi < 25.5 \text{ nm} \) or \(\phi \geq 26.5 \text{ nm} \) differed significantly from those in the subgroup with normal LDL. For DM patients, the concentrations of MDA-LDL and apo B for patients in the LDL size ranges \(< 25.5 \text{ nm}\) were significantly higher than those in control subjects with normal LDL. TC and TG concentrations were significantly higher only in DM patients with LDL size range \(\phi < 24.5 \text{ nm}\). For patients with hypertriglyceridemia, the TG concentrations in patients in the three LDL size ranges \(< 26.5 \text{ nm}\) were significantly higher than those in control subjects with normal LDL. Conversely, the concentration of HDL-C in patients in the same LDL size-range subgroups was significantly lower than that in control subjects with normal LDL. MDA-LDL and apo B concentrations were significantly higher in patients in the LDL size ranges \(24.5 \leq \phi < 25.5 \text{ nm} \) and \(\phi < 24.5 \text{ nm}\), and \(25.5 \leq \phi < 26.5 \text{ nm}\) and \(24.5 \leq \phi < 25.5 \text{ nm}\), respectively.

### Measurements of TBARS in fractions of normal and small dense LDL

When MDA-LDL concentrations in 20 randomly selected subjects were compared with the corresponding TBARS concentrations in LDL isolated by ultracentrifugation, we found no correlation between variables \((r = 0.076; \text{ data not shown})\). However, MDA-LDL in isolated LDL correlated with the MDA-LDL in serum \((r = 0.138; P < 0.01)\). Furthermore, we compared TBARS in fractions of normal \((1.019 < d < 1.040 \text{ kg/L})\) and small dense LDL \((1.040 < d < 1.063 \text{ kg/L}; \text{ Table 4})\). In all four randomly selected cases, TBARS were localized in the fractions of small dense LDL. Cholesterol and apo B were also present at higher concentrations in normal LDL than in small dense LDL.

### Discussion

There exists increasing evidence of a positive correlation between plasma TG concentrations and increased risk of coronary heart disease (CHD) \((13)\). HDL-C concentrations and predominant LDL sizes show an inverse relationship with CHD \((3, 14, 15)\). Relatively high TG concentrations generally mean reduced HDL-C concentrations and a predominance of small LDL particles, implying that the existing concentrations are closely related \((16)\). These relationships are difficult to interpret in terms of cause. One hypothesis is that prolonged presence of the TG-rich lipoproteins in the circulation leads to increased exchange of their TG for cholesterol ester in HDL as well as LDL by cholesterol ester transfer protein \((17)\). These neutral lipid exchanges decrease the HDL-C concentration. In addition, TG-enriched LDL may be removed by hepatic lipase,
leading to small dense LDL. We first confirmed associations between concentrations of both TG and HDL-C and the LDL subclasses. As described in other reports (16, 18), the TG concentration correlated more closely with the predominant LDL size (r = −0.650) than with the HDL-C concentration (r = −0.556).

Austin and Krauss (18) categorized LDL particles into two types, the A and B patterns. Pattern A is defined as a normal LDL pattern with a major peak at a LDL size ≥25.5 nm in diameter and is associated with an increased risk of CHD. One hypothesis is that small dense LDLs are responsible for oxidation, increasing atherogenic risk (5–7). When the MDA-LDL concentration was evaluated as an index of LDL oxidation (19), it was higher in subjects with DM or hypertriglyceridemia than in control subjects. This finding supports previous evidence of progressive oxidation in patients with such disorders (20–22).

We assessed the relationship between LDL size and TG concentration by measuring MDA-LDL concentrations in corresponding subgroups (Fig. 3). Using statistical analysis for comparison of the mean MDA-LDL in the normal LDL size group (LDL size, 25.5 ≤ Φ ≤ 26.5 nm; 0 < TG < 1500 mg/L) and each subgroup, we observed that 25.5 nm (for LDL particle size) and 1500 mg/L (for TG concentration) were the cutoff values marking susceptibility of LDL to oxidation. This finding supports a LDL particle diameter of 25.5 nm as the dividing point between LDL subclasses A and B. In addition, even when concentrations of TG were ≤1500 mg/L, it was apparent not only that small dense LDLs were present in some subgroups but also that concentrations of MDA-LDL increased in these subgroups with decreasing LDL size.

Overall, subgroups of subjects with a LDL diameter <25.5 nm and with increased TGs showed more significantly increased concentrations of MDA-LDL.

When we examined the relationship between LDL size and the concentration of MDA-LDL in control subjects and patients with DM or hypertriglyceridemia, we found that LDL particle diameters and MDA-LDL concentrations were inversely related in all groups (Fig. 4). MDA-LDL concentrations differed between controls and patients regardless of LDL size range. Furthermore, in the same groups, we evaluated the relationship between lipid markers and LDL size. When we postulated the particle size range of 25.5 ≤ Φ < 26.5 nm in control subjects to be normal, only MDA-LDL in both patient groups was significantly increased when the LDL size was just below normal compared with normal LDL (Table 3). It is understood that both small dense LDL and large buoyant LDL have reduced clearance from circulation because of their lower affinities for the LDL receptor (23). Therefore, our postulate has merit for defining normal LDL as LDL particles with a size range 25.5 ≤ Φ < 26.5 nm. This also suggests that the separation point for susceptibility to LDL oxidation is at 25.5 nm. TG concentrations in subjects with hypertriglyceridemia increased as LDL size decreased from 26.5 nm. The relationship between TG concentration and LDL size distribution diminishes to a certain extent at higher TG concentrations.

We also examined whether MDA-LDL in serum detected by our assay was directly comparable with TBARS in isolated LDL particles. Subsequently, although the former was associated with MDA-LDL in LDL, we found no correlation between MDA-LDL in serum and TBARS in LDL. Whereas the MDA value obtained as a TBARS concentration is provided from the reaction between free MDA and 2-thiobarbituric acid, the ELISA detects LDL modified by MDA but not free MDA. Thus, there is a

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**Table 4. Comparisons of lipids and TBARS in normal and small dense LDL.**

<table>
<thead>
<tr>
<th>Subject 1</th>
<th>Subject 2</th>
<th>Subject 3</th>
<th>Subject 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery, %</td>
<td>89.1</td>
<td>91.2</td>
<td>91.4</td>
</tr>
<tr>
<td>TGs, mg/L</td>
<td>1570</td>
<td>2230</td>
<td>1960</td>
</tr>
<tr>
<td>TC, mg/L</td>
<td>1920</td>
<td>2500</td>
<td>1980</td>
</tr>
<tr>
<td>HDL-C, mg/L</td>
<td>310</td>
<td>370</td>
<td>350</td>
</tr>
<tr>
<td>MDA-LDL, units/L</td>
<td>67</td>
<td>77</td>
<td>54</td>
</tr>
<tr>
<td>Normal LDL&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol, mg/L</td>
<td>450</td>
<td>1220</td>
<td>540</td>
</tr>
<tr>
<td>apo B, mg/L</td>
<td>300</td>
<td>710</td>
<td>380</td>
</tr>
<tr>
<td>MDA, μmol/L</td>
<td>0.94</td>
<td>0.77</td>
<td>0.95</td>
</tr>
<tr>
<td>Small dense LDL&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol, mg/L</td>
<td>650</td>
<td>450</td>
<td>530</td>
</tr>
<tr>
<td>apo B, mg/L</td>
<td>590</td>
<td>370</td>
<td>450</td>
</tr>
<tr>
<td>MDA, μmol/L</td>
<td>1.25</td>
<td>0.90</td>
<td>2.91</td>
</tr>
</tbody>
</table>

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<sup>a</sup> When cholesterol concentration in the d < 1.063 kg/L fractions was defined as the amount of HDL-C subtracted from the TC amount in serum, recovery was estimated.

<sup>b</sup> Normal LDL fraction is 1.019–1.040 kg/L, and small dense LDL is 1.040 < d < 1.063 kg/L.

<sup>c</sup> Measured in serum.

<sup>d</sup> Determined by measuring TBARS.
possibility that the reactants detected by each method differ. This may be the reason that the MDA-LDL concentration in serum was not correlated with the TBARS concentration in isolated LDL. However, TBARS are generally used as a marker of lipid oxidation (24). It was important to compare TBARS concentrations between fractions of normal (1.019 < d < 1.040 kg/L) and small dense LDL (1.040 < d < 1.063 kg/L). TBARS localized in the fraction of small dense LDL, supporting our finding that a LDL particle size of 25.5 nm is the cutoff for susceptibility of LDL to oxidation.

In conclusion, we evaluated the relationship between TG concentration and LDL size, using an ELISA to measure MDA-LDL concentrations, and deduced that decreased LDL particle size with an increased TG concentration leads to an increased concentration of small dense LDLs with oxidative modification, thereby increasing atherogenic risk. The threshold of susceptibility to oxidative modification appears to be at a LDL particle diameter of 25.5 nm. This coincides with the point of distinction between LDL subclass patterns A and B proposed by Austin and Krauss (18).

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References