Increased Circulating Malondialdehyde-Modified LDL Levels in Patients With Coronary Artery Diseases and Their Association With Peak Sizes of LDL Particles

Kosei Tanaga, Hideaki Bujo, Masahiro Inoue, Keiji Mikami, Kazuo Kotani, Kazuo Takahashi, Takashi Kanno, Yasushi Saito

Abstract—Recent establishment of a sensitive ELISA system using antibodies against malondialdehyde-modified low density lipoprotein (MDA-LDL) made it possible to determine the circulating oxidized lipoprotein levels. Here, we investigated the serum levels of MDA-LDL in 62 patients with coronary artery disease (CAD) compared with the levels in 42 patients without CAD [groups CAD(+) and CAD(−), respectively], which are adjusted for age, serum total cholesterol, LDL and high density lipoprotein cholesterol, and triglyceride levels. Serum MDA-LDL levels were 113.4±49.1 IU/L in CAD(+), which were significantly higher than the levels in CAD(−) (85.2±22.5 IU/L, P<0.005). The ratio of MDA-LDL/LDL cholesterol was 0.95±0.32 in CAD(+), indicating a significant increase compared with the ratio in CAD(−) (0.68±0.19, P<0.0005). The positive correlation of MDA-LDL level and the ratio of MDA-LDL/LDL cholesterol with intima-media thickness in carotid arteries was observed. Age was not clearly associated with the MDA-LDL level (P=0.865). The serum MDA level was positively correlated with LDL cholesterol (P<0.0001) and with triglycerides (P<0.001) and negatively correlated with high density lipoprotein cholesterol (P<0.05). Furthermore, the MDA-LDL level was negatively correlated with the peak size of the LDL particle (P<0.01). The LDL subclasses that were identified by using the sera collected from the subjects by sequential ultracentrifugation showed that the ratios of MDA-LDL/apolioprotein B in LDL3 and LDL4 were nearly 3-fold higher than those in LDL1 and LDL2 for CAD(+) and CAD(−). These results indicate that the circulating MDA-LDL level is increased in CAD(+), independent of the serum LDL cholesterol level but in association with the peak size of LDL particles. The measurement of serum MDA-LDL level may be useful for the identification of patients with advanced atherosclerosis.

Key Words: malondialdehyde-modified LDL ■ coronary artery disease ■ ELISA ■ atherosclerosis

Oxidized LDLs, as modified LDLs, are thought to play key roles in the progression of atherosclerosis (see reviews1–3). The modification of LDL by oxidation alters its native properties: oxidized LDL becomes incorporated into macrophages by scavenger receptors4 and modulates the gene expression involved in the cellular function of endothelial cells and smooth muscle cells in the vessel walls.5–8 Increasing evidence of atherosclerosis as an inflammatory disease raised the possibility of detection of circulating markers in the serum: increased levels of C-reactive protein and of cytokines, such as interleukins,9,10 In this context, the detection and quantification of circulating oxidized LDL might reflect the severity and phases of atherosclerosis.

Until now, some new methods to measure the levels of circulating oxidized LDL have been established, and their clinical relevance has been suggested by use of the detection system for some parts of oxidized LDL.11–14 The ELISA method for measurements of serum oxidized and malondialdehyde (MDA)-modified LDL (MDA-LDL) has been suggested as being useful for the identification of patients with acute coronary syndrome.15–17 Toshima et al18 have suggested that the sensitivity of oxidized LDL, which does not include MDA-LDL, is a better biochemical risk marker for coronary artery disease (CAD).15 Furthermore, the circulating oxidized LDL has been proven to exist in macrophages in human atheroma.15 We have recently reported a sensitive method to measure circulating MDA-LDL,11 and we have revealed that its level is increased in patients with diabetes or hypertriglyceridemia.18 The MDA-LDL level was highly correlated with the particle size of LDLs in their sera.18 In the present study, therefore, we measured the serum levels of MDA-LDL in the patients with CAD [CAD(+)] and compared these levels with

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the levels in the patients without CAD [CAD(-)]; adjustments were made for age and for serum total cholesterol (TC), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), and triglyceride (TG) levels. We clearly found an increased serum level of MDA-LDL in CAD(+), and the level was positively correlated with the intima-media thickness (IMT) of the carotid arteries. Furthermore, the circulating level was negatively correlated with the peak size of LDL particles, and increased levels were observed in the LDL subfractions with increased densities, which are known to be a risk for coronary atherosclerosis. Thus, the circulating MDA-LDL level is likely associated with advanced atherosclerosis, possibly related to the peak size of LDL particles.

Methods

Subjects
The present study included 62 patients with angiographically proven CAD [CAD(+)] and 42 healthy control subjects [CAD(-)]. Angiograms of CAD(+) showed at least 50% stenosis of 1, 2, or 3 coronary arteries. A total of 42 healthy volunteers matched for age and TC, LDL-C, and HDL-C levels served as CAD(-), ie, the control group. Among the CAD(-) group, none had diabetes mellitus or thyroid or endocrinological diseases. The CAD(-) group had no clinical evidence of CAD and an absence of significant atherosclerotic lesions, which was confirmed by ultrasound scanning of carotid arteries, as previously described (IMT <1.0 mm). The frequency of smoking was not significantly different between 2 groups.

Measurement of IMT of the Common Carotid Artery
Examination of IMT was carried out with an ultrasound scanner (SSD-1200CV, Aloka) equipped with a linear 7.5-MHz transducer. IMT was defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface of the far wall. The measurement of IMT in the common carotid artery was made along a 10-mm-long section just proximal to the carotid bulb. Average IMT was calculated from right and left IMTs of the common carotid arteries.

Measurement of MDA-LDL
The ELISA method used was based on the same principles as previously reported by Kotani et al. Serum samples were drawn after an overnight fast, used within 4 days after the serum was separated by centrifugation, and stored at 4°C. Samples were diluted 2000-fold in a dilution buffer containing SDS. Duplicate 100-μL of 0.2 mol/L sodium carbonate (pH 12). L-(0.2 mol/L sodium carbonate (pH 12). L-β-Galactosidase conjugated monoclonal antibody against MDA-LDL (ML25) has previously been shown to recognize MDA residue but has not been shown to be specific for MDA-LDL. The reaction was stopped with AB16 has been shown to recognize MDA-LDL. LDL oxidized with a modified version of the technique described by Krauss and Burke. Briefly, 5 μL of serum from each subject was diluted 2-fold with 400 g/L sucrose and electrophoresed for 24 hours at 10°C on 2% to 15% polyacrylamide gradient gels with buffer containing 90 mmol/L Tris, 80 mmol/L boric acid, and 3 mmol/L 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 on the basis of the migration distance of 5 markers with known diameters as follows: 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). 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 major LDL peaks was measured. Each gel lane was scanned with a densitometer interfaced with a PC computer. Control serum was drawn from 1 healthy volunteer and combined with 100 g/L sucrose, 37.5 mmol/L NaCl, and 0.25 mmol/L EDTA. This was divided into aliquots and stored at −80°C.

Lipid Analysis and Ultracentrifugation of Lipoproteins
TC, HDL-C, and TG levels were measured by enzymatic methods. ApoB concentration was determined by using commercially available immunoturbidimetric assay kits (Daichi Pure Chemicals). LDL-C levels were calculated by the Friedewald formula: LDL-C = TC−HDL-C−TG/5. LDL and its subspecies were isolated by sequential ultracentrifugation from human serum. The isolation techniques have been described previously. The lipoproteins in the 1.022<d<1.032, 1.032<d<1.038, 1.038<d<1.050, and 1.050<d<1.063 kg/L fractions (where d indicates density) were defined as LDL1, LDL2, LDL3, and LDL4, respectively.

Statistical Analysis
Data are presented as mean±SD. The Student t test was used when means were compared. Simple regression was performed to study the association. A value of P<0.05 was considered statistically significant.

Results
Clinical and lipid profiles of CAD(+)) in the present study are shown in the Table. The serum MDA-LDL levels in these 62 subjects were compared with the 42 control CAD(-), whose age, TC, LDL-C, HDL-C, and TG levels were adjusted to those of CAD(+). The lipid levels in males and females were not significantly different between CAD(+) and CAD(-). Serum MDA-LDL levels were 113.4±49.1 IU/L in CAD(+) and 85.2±22.5 IU/L in CAD(-) (Figure 1). There were statistically significant differences between CAD(+) and CAD(-) in circulating MDA-LDL levels (P<0.0005). Then, we compared the ratio of MDA-LDL/LDL-C in both groups. Figure 1D shows that the ratios of MDA-LDL/LDL-C were 0.95±0.32 in CAD(+) and 0.68±0.19 in CAD(-), indicating a significant difference between CAD(+) and CAD(-) (P<0.0005). We next compared the MDA-LDL levels or the ratios of MDA-LDL/LDL-C between CAD(+) and CAD(-) in males and females. As shown in Figure 1B, 1C, 1E, and 1F, the MDA-LDL levels and the ratios of MDA-LDL/LDL-C
Clinical and Lipid Profiles of the Subjects in Study

<table>
<thead>
<tr>
<th></th>
<th>CAD(+)</th>
<th>CAD(−)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>62</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Sex (male/female), n</td>
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<td>21/21</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>67.5±8.9</td>
<td>65.9±6.5</td>
<td>NS</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.1±3.0</td>
<td>23.4±3.0</td>
<td>NS</td>
</tr>
<tr>
<td>TC, mg/dL</td>
<td>202.4±36.5</td>
<td>208.6±23.4</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-C, mg/dL</td>
<td>121.7±33.8</td>
<td>126.4±21.2</td>
<td>NS</td>
</tr>
<tr>
<td>TGs, mg/dL</td>
<td>138.6±63.4</td>
<td>142.1±94.3</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
<td>54.1±11.9</td>
<td>59.8±16.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

Male

|                |        |        |      |
| n              | 41     | 21     |      |
| Age, y         | 65.5±8.9 | 67.3±6.3 | NS   |
| BMI, kg/m²     | 24.7±3.0 | 24.1±2.9 | NS   |
| TC, mg/dL      | 200.4±25.9 | 206.5±22.5 | NS   |
| LDL-C, mg/dL   | 120.0±22.0 | 128.8±21.8 | NS   |
| TGs, mg/dL     | 150.0±68.4 | 150.5±106.5 | NS   |
| HDL-C, mg/dL   | 50.7±9.6  | 55.9±14.5 | NS   |

Female

|                |        |        |      |
| n              | 21     | 21     |      |
| Age, y         | 71.4±7.6  | 68.5±5.3  | NS   |
| BMI, kg/m²     | 23.0±2.7  | 22.7±2.9  | NS   |
| TC, mg/dL      | 206.2±50.7 | 210.7±24.1 | NS   |
| LDL-C, mg/dL   | 124.8±48.7 | 124.0±20.2 | NS   |
| TGs, mg/dL     | 116.4±43.9 | 133.8±80.1 | NS   |
| HDL-C, mg/dL   | 60.9±12.6  | 63.7±17.0  | NS   |

BMI indicates body mass index. Quantitative data represent mean±SD.

were significantly higher in CAD(+) compared with CAD(−) in males and females.

We next analyzed the IMT of carotid arteries in all subjects to determine the clinical significance of the increased circulating MDA-LDL levels in CAD(+). Figure 2A shows that the MDA-LDL levels are significantly positively correlated with IMT (P<0.0001). Furthermore, the ratio of MDA-LDL/LDL-C was significantly positively correlated with IMT (P<0.0001). These results together with the above results in Figure 1 indicate that circulating MDA-LDL levels are increased in the subjects with advanced atherosclerosis.

Figure 3 shows the correlation of serum MDA-LDL levels with age (panel A), LDL-C (panel B), HDL-C (panel C), and TGs (panel D). Age did not show a clear relationship with the MDA-LDL level (P=0.0865, Figure 2A). LDL-C was positively correlated with the MDA-LDL level (P<0.0001). On the other hand, HDL-C was inversely correlated with MDA-LDL (P<0.05). The plasma TG level was positively correlated with MDA-LDL (P<0.0001). These results indicate that the circulating MDA-LDL levels are associated with the TG and HDL-C levels as well as with the LDL-C levels.

We next assessed the relationship between the LDL particle size and serum MDA-LDL level by measuring the peak size of LDL by gradient gel electrophoresis. Figure 4 shows that the MDA-LDL level is negatively correlated with the LDL size (P<0.01). Thus, the MDA-LDL level was associated with the peak size of LDL in the subjects, probably suggesting its metabolic relationship with the smaller particle of LDL.

To further address the possibility of an association of the serum MDA-LDL level and the LDL particle size, we measured the MDA-LDL levels in the LDL subclasses identified by ultracentrifugation with the use of sera from all subjects. Figure 5 shows the serum apoB and MDA-LDL levels and the ratios of MDA-LDL/apoB in the LDL subclasses prepared from CAD(+) or CAD(−). The apoB and MDA-LDL levels showed similar distribution in the subfractions between CAD(+) and CAD(−). ApoB was most abundant in LDL2 fractions, followed by LDL3 fractions in CAD(+) and CAD(−). Nearly 50% of MDA-LDL was localized in LDL3 fractions in both groups. The ratios of MDA-LDL/apoB in LDL3 and LDL4 were increased nearly 3-fold compared with those in LDL1 and LDL2 in both groups. In CAD(+), the ratios of MDA-LDL/apoB in LDL3 and LDL4 were significantly higher than those in CAD(−). These results indicate that the increased circulating MDA-LDL particles are localized mostly in the dense LDL fractions.

**Figure 1.** Comparison of plasma MDA-LDL levels between CAD(+) and CAD(−). Bar graph shows the plasma MDA-LDL levels (A, B, and C) or MDA-LDL/LDL-C ratios (D, E, and F) of CAD(+) and CAD(−) in all subjects (A and D), men (B and E), and women (C and F), respectively. Data are presented as mean±SD. ***P<0.0005, **P<0.001, and *P<0.05.

**Figure 2.** Correlation of IMT with serum MDA-LDL level (A) or MDA-LDL/LDL-C (B).
The oxidative modification of LDL is believed to play a key role in the progression of atherosclerosis (see reviews). One of the oxidized forms, MDA-LDL, has been isolated from the sera of patients with CAD. We have recently established a new sensitive method to measure MDA-LDL in the serum by using ELISA. In the present study, we could show the increased serum levels of immunoreactive MDA-LDL in CAD compared with CAD. The ratio of MDA-LDL and LDL was obviously higher in the CAD for males and females. Furthermore, the level and ratio were positively correlated with the progression of IMT in carotid arteries. These results clarified the clinical significance of immunoreactive serum MDA-LDL level as a factor in association with the progression of atherosclerosis. Next, an analysis of the relationship between age and serum lipid levels showed a positive correlation with LDL-C and TGs but no significant correlation with age. These clear relationships of MDA-LDL with other lipids suggest a biochemical and pathological connection with the functional properties of LDL particles. As a consequence, the MDA-LDL level was negatively correlated with the peak sizes of LDL particles and was mostly distributed in the heavier LDL subfractions separated by ultracentrifugation. Taken together, the circulating MDA-LDL levels are probably related to the densities of LDL particles, which are known to be affected by serum TG levels as well as LDL-C levels.

Previous experiments using the measurements of circulating autoantibodies to MDA-LDL have shown the importance of oxidized product, MDA-LDL, in the vessel wall in the process and regression of atherosclerosis. Furthermore, a positive association of autoantibodies against MDA-LDL with carotid atherosclerosis has been suggested in a case-control study. Recently, it has become possible to detect immunoreactive MDA-LDL in the serum. Holvoet et al have shown that plasma levels of MDA-LDL were significantly higher in patients with acute coronary syndromes than in patients with stable CAD. However, the histological distribution in atheromatous plaques in humans revealed that compared with other immunoreactive oxidized LDL, immunoreactive MDA-LDL was not much observed in macrophages in the shoulder areas. Thus, the clinical significance of immunoreactive MDA-LDL in the serum has not been fully established in association with CAD. To approach this topic, we measured the serum levels of MDA-LDL in CAD (+) all of whom were diagnosed by coronary angiography) by using a sensitive ELISA method recently established by us. As a result, we found significantly higher plasma levels of MDA-LDL in CAD (+) compared with CAD (−) whose levels of LDL-C were adjusted.

Another important finding in the present study is the relationship between serum levels of MDA-LDL and TGs. So far, it is known that small dense LDL, in association with an increased serum TG level, is a risk factor for coronary atherosclerosis. It has been also become clear that the dense LDL subpopulation is more susceptible to oxidative modification and, therefore, may contribute more to foam cell formation than the large LDL. Our results using LDL subfractions showed the higher concentrations of MDA-LDL in the dense fractions of molecules. These findings suggest that the atherogenic properties of MDA-LDL identified in the present study are partly caused by the higher production of modified LDL from the dense LDL particles, although the latter mechanism should be studied in detail. In agreement
with our results, the increased atherogenic risk associated with the pattern B phenotype has been suggested to result from increased concentrations of lipoprotein subpopulations that are relatively susceptible to oxidative modification.\textsuperscript{28} LDL subclass pattern B, characterized by a preponderance of small dense LDL particles, is associated with an increased risk of myocardial infarction, independent of age and sex.\textsuperscript{19} The close relationships between the circulating MDA-LDL and TG levels in addition to the LDL-C level seem indicative of common pathways for atherogenic oxidized lipoproteins derived from TG-rich lipoproteins. The obvious difference in the ratio of MDA-LDL/apoB in dense LDL fractions that is shown between CAD(+) and CAD(−) in Figure 5 suggests that the production pathways of MDA-LDL from dense LDL fractions play a key role in the progression of atherosclerosis.

In conclusion, we clarified the clinical significance of increased serum levels of MDA-LDL for the presence of CAD and the progression of carotid atherosclerosis by using the recently established sensitive ELISA method, independent of the serum LDL-C level. This level in serum was closely related to TG level and the LDL particle size, thus suggesting a pathological connection of TG-rich lipoproteins, small dense LDL, and oxidized LDL. The circulating level of MDA-LDL may reflect the local levels of oxidized lipoproteins and the following progression of atherosclerosis. Further histochemical and metabolic analyses are needed to address these important issues.

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References

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