Autoantibody against, Malondialdehyde-Modified Low Density Lipoprotein in Patients with Non-Diabetic Unstable Angina: A Potential Role in Immunologic Reaction of Plaque Instability

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The role of autoantibody against oxidized low-density lipoprotein (LDL) in the pathogenesis of atherosclerosis is still unknown. The purpose of this study was to determine whether autoantibodies against malondialdehyde (MDA)-modified LDL are associated with coronary artery disease (CAD) and clinical presentations of CAD in non-diabetic patients without acute myocardial infarction (AMI). We determined the serum levels of autoantibody against MDA-modified LDL by ELISA in 71 patients with angiographically significant CAD (≥ 50% diameter stenosis in at least 1 vessel) and 80 controls without angiographically significant CAD. Among the total 151 subjects, 30 subjects did not have any clinical ischemic event, 90 subjects had stable angina symptoms, and 31 subjects had unstable angina symptoms. The autoantibody titer, expressed mean optical density units, was significantly higher in patients with CAD than in controls (0.177 ± 0.014 versus 0.127 ± 0.011, respectively; p=0.006) and higher in unstable angina group than in stable angina group (0.240 ± 0.025 versus 0.145 ± 0.007, respectively; p < 0.001). By logistic regression analysis, the high autoantibody titer was associated significantly with CAD (P=0.008), independent of age, gender, body mass index, triglyceride concentration, and the ratio of total cholesterol-high density lipoprotein (HDL) cholesterol. In multiple regression analysis, presence of CAD, smoking history and low HDL-cholesterol level were independent factors associated with a increased anti-oxLDL Ab titer. The autoantibody titer was significantly lower in nonsmoker than smoker (p=0.019) and higher in low HDL- cholesterol (< 35 mg/dL) group than in HDL-cholesterol group (p=0.012). Elevated autoantibody titer was associated with CAD and the unstable clinical presentation of CAD. Our results suggest that immune response to oxidized LDL may play a role in the pathogenesis of atherosclerosis and plaque instability.

Key Words: Oxidized LDL, autoantibodies, atherosclerosis, coronary artery disease

INTRODUCTION

The oxidative modification of LDL is an important event in the development and progression of atherosclerosis.¹ Oxidative modification of LDL is a prerequisite for rapid accumulation of LDL in macrophages and for the formation of foam cells and fatty plaque development.²³ The oxidation of polyunsaturated fatty acids generates reactive breakdown products, such as MDA; these products subsequently interact with lysine residues of associated proteins to form Schiff base adducts, such as MDA-lysine.⁶⁷ It has been demonstrated that such "oxidation-specific" neoepitopes occur in vivo and are immunogenic.⁶⁷ For example, autoantibody titers to MDA-modified LDL have been found in the plasma of apoE-deficient (apoE⁻/⁻) and LDL receptor-negative (LDLR⁻/⁻) mice and
have been correlated with the progression of atherosclerosis.\textsuperscript{3,9} Furthermore, immunization of LDLR\textsuperscript{-/-} rabbits and mice with MDA-modified LDL led to an amelioration of the progression of atherogenesis.\textsuperscript{30,31}

Several studies have been conducted to investigate the role of autoantibodies against oxLDL. However, the results are conflicting,\textsuperscript{5,12-16} especially in patients with acute AMI\textsuperscript{13,16} and non-insulin dependent diabetes mellitus (NIDDM).\textsuperscript{15} Diabetics may have increased immune complexes for many reasons including immune reactions to nonenzymatically glycated proteins, which are also immunogenic, and contain early forms of oxLDL.

Previously, plasma levels of oxLDL were significantly higher in patients with acute coronary syndromes than in individuals with stable CAD.\textsuperscript{17} However, the study of the relationship between anti-oxLDL Ab titer and clinical presentation of CAD has not been examined in detail. Therefore, the present study was undertaken to determine whether antibodies against MDA-modified LDL are associated with angiographically verified CAD or with clinical presentations in non-diabetic patients without AMI.

MATERIALS AND METHODS

Subjects

The subjects were selected by random sampling from patients referred from 1997 to 2000 for coronary angiography because of chest pain or that were otherwise clinically suspected of having CAD. The study groups consisted of 71 patients (1-vessel disease: 37, 2-vessel disease: 19, 3-vessel disease: 15) with angiographically significant CAD (≥ 50% diameter stenosis in at least 1 vessel) and a control group that consisted of 80 patients without angiographically significant CAD (< 50% diameter stenosis in all three major vessels). Among the total of 151 subjects, 30 subjects had experienced no clinical ischemic events, 90 subjects had stable angina symptom, and 31 subjects had unstable angina symptom. The following three patient groups may be said to have unstable angina pectoris: 1) patients with new onset (< 2 months) angina that is severe and/or frequent (≥ 3 episodes per day); (2) patients with accelerating angina, i.e., those with chronic stable angina who develop angina that is distinctly more frequent, severe, prolonged, or precipitated by less exertion than previously; (3) those with angina at rest. MB fraction of creatinine kinase and Troponin-T levels were within normal range. Patients with AMI or diabetes mellitus were excluded. The average age, body mass index (BMI), and levels of major CAD risk factors of the patient and control groups are detailed in Table 1 and 2.

Coronary angiography and blood samples

Coronary angiography was performed using the standard Judkin’s technique.\textsuperscript{18} A transluminal narrowing of ≥ 50% was defined as significant.

Table 1. Clinical and Laboratory Characteristics of Control Subjects and Patients with CAD (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects (N=80)</th>
<th>Patients with CAD (N=71)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>26/54</td>
<td>41/30*</td>
</tr>
<tr>
<td>Age, y</td>
<td>55.7 ± 9.8</td>
<td>57.0 ± 9.1</td>
</tr>
<tr>
<td>BMI, kg/m\textsuperscript{2}</td>
<td>24.4 ± 3.4</td>
<td>25.5 ± 3.3</td>
</tr>
<tr>
<td>Smoker, % (n)</td>
<td>11.3 (9)</td>
<td>38.0 (27)\textsuperscript{1}</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>128 ± 18</td>
<td>137 ± 19</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>81 ± 14</td>
<td>85 ± 13</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>182.1 ± 36.5</td>
<td>192.2 ± 32.9</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dl</td>
<td>40.9 ± 12.7</td>
<td>39.7 ± 11.3</td>
</tr>
<tr>
<td>LDL-cholesterol, mg/dl</td>
<td>115.7 ± 31.6</td>
<td>119.4 ± 32.3</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>132.3 ± 92.2</td>
<td>165.5 ± 97.7</td>
</tr>
</tbody>
</table>

\textsuperscript{1}t-test for means and \textsuperscript{2}χ\textsuperscript{2} tests for frequencies.\textsuperscript{p<0.01, 2p<0.001.}

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Table 2. Clinical and Laboratory Characteristics of Control Subjects, Patients with Stable and Unstable Angina Pectoris (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Control (N=30)</th>
<th>Stable Angina (N=90)</th>
<th>Unstable Angina (N=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>10/20</td>
<td>39/51</td>
<td>18/13*</td>
</tr>
<tr>
<td>Age, y</td>
<td>57.9 ± 8.6</td>
<td>55.9 ± 9.9</td>
<td>56.4 ± 9.0</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.7 ± 2.7</td>
<td>25.1 ± 3.7</td>
<td>25.3 ± 3.1</td>
</tr>
<tr>
<td>Smoker, % (n)</td>
<td>16 (4)</td>
<td>23.3 (20)</td>
<td>42.9 (12)*</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>126 ± 19</td>
<td>136 ± 18</td>
<td>133 ± 20</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>89 ± 10</td>
<td>85 ± 12</td>
<td>84 ± 11</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>180.3 ± 36.8</td>
<td>189.3 ± 35.1</td>
<td>187.9 ± 33.1</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dl</td>
<td>37.6 ± 12.3</td>
<td>41.4 ± 11.8</td>
<td>39.2 ± 11.9</td>
</tr>
<tr>
<td>LDL-cholesterol, mg/dl</td>
<td>120.7 ± 35.7</td>
<td>117.9 ± 30.9</td>
<td>114.7 ± 32.3</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>115.3 ± 77.0</td>
<td>152.2 ± 99.9</td>
<td>169.9 ± 95.2</td>
</tr>
</tbody>
</table>

ANOVA for means and χ² test for frequencies.

*p < 0.05.

Fasting venous blood samples were collected into EDTA tubes. Standardized enzymatic methods were used for the analysis of serum total cholesterol, HDL cholesterol, and triglycerides. Blood samples, which were transferred to tubes without EDTA, were centrifuged at 2000 rpm for 20 minutes. Serum was kept frozen at -70°C until assayed for anti-oxLDL Ab titer.

Analysis of lipid profiles

Total cholesterol was determined by the cholesterol-oxidase method (Daichii, Tokyo, Japan), triglyceride by glycerophosphate oxidase with glycerol blanking method (Asan, Seoul, Korea), HDL cholesterol by direct method using polyethylene glycol-modified enzymes and α-cyclodextrin sulfate (Daichii, Tokyo, Japan) using Hitachi 747 automatic chemistry analyzer (Hitachi, Tokyo, Japan). LDL cholesterol was calculated using the Friedewald equation.

Measurement of autoantibody against MDA-modified LDL

Titers of antibody against MDA-modified LDL were determined by ELISA with a commercially available kit (ImmunoLisa, IMMCO Diagnostics, NY, USA). Prediluted test sera had been incubated at 2-8°C for 2 hours in 96-well microtiter wells precoated with MDA-modified LDL and native LDL. After washing, the wells were incubated with anti-human IgG antibody conjugated with a specific peroxidase at 2-8°C for 2 hours. The wells were washed, pNPP was added, and the wells incubated at room temperature for 30 minutes. Color development was stopped by adding stop solution. Absorbance value was measured at 405 nm using an ELISA reader, LP400 (Sanofi Diagnostics Pasteur, Paris, France).

All measurements were performed blind on coded serum samples. Results were expressed as the mean optical density (MOD) values of the determinations, and level of autoantibody reactivity against oxidized LDL calculated by subtracting the binding of antibodies to native LDL from those to MDA-modified LDL. This approach reduced the possibility of getting false-positive values due to cross-reactivity with both LDL epitopes. The intra-assay and inter-assay coefficients of variation for the oxLDL Ab determinations were 7.9% and 10.8%, respectively.

Statistics

Results are expressed as mean ± SD, as shown in Table 1, the Mann-Whitney U test or t-test were used in group mean comparisons, and risk factor frequencies between the study groups and the controls were compared using χ² test. The effect of different groups on anti-oxLDL Ab reactivity was analyzed by one way ANOVA. The least significance test then was used as a post hoc test to analyze differences among the subgroups. To
find the set of variables that would classify the patients into subjects with CAD or controls, we used logistic regression analysis. To evaluate independent associations among more than three variables, we performed multiple regression analysis. The significance was accepted at \( p < 0.05 \).

**RESULTS**

The levels and differences of major coronary risk factors in patients and control subjects are shown in Table 1 and 2. There was no significant difference in risk factors between control and patient group, except gender and smoking history. Antibodies to MDA-modified LDL were found in both groups, but autoantibody titers were significantly higher in patients with significant CAD than control subjects (0.177 \( \pm \) 0.014 versus 0.127 \( \pm \) 0.011, respectively; \( p = 0.006 \); Fig. 1A). However, there was no significant correlation was found between the autoantibody titer and the extent of CAD (\( p > 0.05 \)). The autoantibody titer was significantly higher in patients with unstable angina pectoris than in those with stable angina pectoris (0.240 \( \pm \) 0.025 versus 0.145 \( \pm \) 0.007, respectively; \( p < 0.001 \); Fig. 1B). However, no significant difference was evident between control subjects and patients with stable angina pectoris (0.134 \( \pm \) 0.024 versus 0.145 \( \pm \) 0.007, \( p > 0.05 \); Fig. 1B). In logistic regression analysis, high anti-oxLDL Ab titer (\( p = 0.008 \)) was significantly associated with CAD (dependent variable), independent of age (\( p = 0.889 \)), gender (\( p = 0.655 \)), BMI (\( p = 0.647 \)), triglyceride concentration (\( p = 0.389 \)), and total cholesterol/HDL cholesterol ratio (\( p = 0.424 \)).

In multiple regression analysis, presence of CAD, smoking history and low HDL-cholesterol level were independent factors associated with an increased anti-oxLDL Ab titer (Table 3). Autoantibody titers were significantly different between nonsmokers and smokers (0.139 \( \pm \) 0.019 versus 0.173 \( \pm \) 0.018, respectively; \( p = 0.019 \); Fig. 2A) and between subjects with high HDL-cholesterol levels (>35 mg/dl) and low HDL-cholesterol levels (0.129 \( \pm \) 0.009 versus 0.180 \( \pm \) 0.020, respectively; \( p = 0.012 \); Fig. 2B). When these two risk factors, i.e. low HDL cholesterol and smoking, were taken into consideration, no significant difference in the autoantibody titers of patients with or without risk factors (low HDL cholesterol or smoker) were found in either the stable angina group (0.134 \( \pm \) 0.014 versus 0.118 \( \pm \) 0.008, \( p > 0.05 \); Fig. 3A) or in the unstable angina group (0.251 \( \pm \) 0.033 versus 0.225 \( \pm \) 0.026, \( p > 0.05 \); Fig. 3B). However, subjects with unstable angina pectoris showed markedly higher autoantibody titers than subjects with stable angina pectoris, independent of the presence of risk factors. The autoantibody titer was higher in patients with unstable angina, than in those with stable angina with low HDL cholesterol levels or smoking history (0.251 \( \pm \) 0.033 versus 0.134 \( \pm \) 0.014, \( p = 0.032 \); Fig. 4A), and higher than in those without a smoking history and with high HDL cholesterol level (0.225 \( \pm \) 0.026 versus 0.118 \( \pm \) 0.007, \( p < 0.001 \); Fig. 4B).

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**Fig. 1.** (A) Antibody reactivities (mean \( \pm \) SEM; OD units) against MDA-modified LDL in control subjects and patients with CAD. (B) Antibody reactivity (mean \( \pm \) SEM; OD units) against MDA-modified LDL in control subjects and in subjects with stable angina pectoris and unstable angina pectoris. CAD; coronary artery disease, SA; stable angina, UA; unstable angina. *p-test, #ANOVA.
Table 3. Multiple Regression Analysis of Factors Affecting Anti-oxLDL Ab titer

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>$\beta$-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.157</td>
<td>0.092</td>
</tr>
<tr>
<td>Gender (female=1, male=2)</td>
<td>0.046</td>
<td>0.684</td>
</tr>
<tr>
<td>Smoking (nonsmoker=1, smoker=2)</td>
<td>0.196</td>
<td>0.055</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>-0.201</td>
<td>0.045</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>0.148</td>
<td>0.11</td>
</tr>
<tr>
<td>CAD (control=1, patient=2)</td>
<td>0.313</td>
<td>0.001</td>
</tr>
</tbody>
</table>

$R^2=0.160$ (p=0.004)

*Factors associated with anti-oxLDL Ab titer were evaluated by multiple regression analysis. Because of the skewed distribution of Ab titer, this variable was log-transformed before analysis. Dummy variables were used for gender (1 for female, 2 for male), smoking status (1 for nonsmoker, 2 for smoker), coronary artery disease (1 for control subjects, 2 for patients) and unstable angina (1 for control or stable angina, 2 for unstable angina) in the model. Abbreviations: CAD; coronary artery disease; UA, unstable angina; SA, stable angina; $\beta$, standard regression coefficient; p, level of significance; $R^2$, multiple coefficient of determination.

Fig. 2. (A) Antibody reactivities (mean ± SEM; OD units) against MDA-modified LDL in subjects with smoking history and without smoking history. (B) Antibody reactivity (mean ± SEM; OD units) against MDA-modified LDL in subjects with high (>35 mg/dl) and low (≤35 mg/dl) HDL cholesterol level. *t-test.

Fig. 3. (A) Antibody reactivities (mean ± SEM; OD units) against MDA-modified LDL in subjects with risk factors (low HDL cholesterol or smoker) and in subjects without risk factors (high HDL cholesterol and non-smoker) in stable angina group. (B) Antibody reactivity (mean ± SEM; OD units) against MDA-modified LDL in subjects with risk factors (low HDL cholesterol or smoker) and in subjects without risk factors (high HDL cholesterol and non-smoker) in unstable angina group. *Mann-Whitney U test.
DISCUSSION

The present study demonstrates that the plasma levels of autoantibody titer against MDA-modified LDL are significantly elevated in patients with CAD than control subjects and that these levels are significantly higher in unstable angina than in those with a stable clinical presentation. However, there was no significant difference of the autoantibody titers between the stable angina group and control group. In addition, there was no significant association between the autoantibody titer and the severity of CAD. These data thus show that the increase of antibody reactivity levels against MDA-modified LDL is dependent on the unstable clinical presentation. Therefore, these results suggest that immune response to oxidized LDL may be associated with plaque instability and elevated autoantibody titer might be a useful marker for plaque vulnerability. These results are consistent with previous studies, which indicated that plasma levels of MDA-modified LDL are associated with acute coronary syndromes.\(^{17,20}\) However, this study is the first clinical study which demonstrates the association of autoantibody titer against MDA-modified LDL and unstable clinical presentation.

In human atherogenesis, anti-oxidized LDL autoantibody blocks the uptake of oxidized LDL by macrophages and also bound to the apoptotic cells and inhibited their phagocytosis by macrophages.\(^{21}\) Apoptosis is a major event occurring during atheromatous plaque development. Apoptotic cells, presented under oxidative stress, express oxidatively modified moieties on their surface that mediate macrophage recognition and phagocytosis. The occurrence of phosphatidylinositol serine (PS) in the exoplasmic leaflet of the plasma membrane is considered as one of the hallmarks of cells undergoing apoptosis and more generally constitutes one of the determinants for the phagocytosis of apoptotic cells to be rapidly cleared.\(^{22}\) Once accessible, PS acquires a procoagulant potential, owing to its ability to promote the surface assembly and the catalytic efficiency of the characteristic enzyme complexes of the blood coagulation cascade,\(^{23}\) including the tissue factor (TF)/factor VIIa complex.\(^{24}\) This parallels PS externalization in activated platelets, which constitutes the basis of the platelet coagulant response.\(^{25}\) Recent in vitro studies suggest that removal of apoptotic cells may be inefficient in atheromatous plaques. Indeed, oxidized phospholipids, as well as antibodies directed against them, which are abundant in advanced plaques, affect recognition of apoptotic or damaged cells by macrophages.\(^{19}\) Therefore, it is likely that the capacities of clearance of apoptotic cells are reduced in foam macrophages that are in an oxidation-rich environment.

Our study showed that the antibody reactivity levels against oxidized LDL were significantly higher in subjects with low HDL-cholesterol levels than in subjects with high HDL cholesterol concentrations. Since HDL-cholesterol is able to pass through the vascular endothelium and reaches to the subendothelial space of the intima, there might be an interaction between HDL and LDL. Previous studies have shown that HDL-cho
lesterol prevents the cytotoxicity and atherogenic properties of LDL and that HDL inhibits the oxidation of LDL-cholesterol. HDL (apolipoprotein E) may inhibit the immunogenic response against oxidized LDL and thus diminish the formation of anti-oxLDL Ab. Our results might reflect the protective effect of HDL against LDL oxidation in vivo.

In this study, the autoantibody titer against MDA-modified LDL was found to be significantly higher in smokers than non-smokers. Exposure to cigarette smoke increases immunoreactivity for TF, vascular cell adhesion molecule-1 (VCAM-1), and macrophages in the atheromatous plaques of apoE-/- mice. In addition, smoking is associated with increased TF immunoreactivity and activity in human carotid plaques. In apoE-/- mice, TF content paralleled VCAM-1 and macrophage immunoreactivity. These findings suggest that smoking may increase plaque macrophage content through increased VCAM-1 expression. A similar mechanism was described in association with increased monocyte adhesion to endothelial cells exposed to cigarette smoke condensate.

In conclusion, the present study has shown that elevated autoantibody titer was associated with CAD and the unstable clinical presentation of CAD. However, the present study was done under the limited numbers of patients and did not involve sequential time analysis according to the clinical course. Moreover, this study has limitation that IVUS study was not performed. A prospective investigation of the immunogenic role of MDA-modified LDL or oxidized LDL in the progression of coronary atherosclerosis and/or plaque instability and associated histopathologic studies are needed.

REFERENCES

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