Elevated Levels of Oxidized Low Density Lipoprotein Show a Positive Relationship With the Severity of Acute Coronary Syndromes

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Background—There is accumulating data that acute coronary syndromes relate to recent onset activation of inflammation affecting atherosclerotic plaques. Increased blood levels of oxidized low density lipoprotein (ox-LDL) could play a role in these circumstances.

Methods and Results—Ox-LDL levels were measured in 135 patients with acute myocardial infarction (AMI; n=45), unstable angina pectoris (UAP; n=45), and stable angina pectoris (SAP; n=45) and in 46 control subjects using a sandwich ELISA method. In addition, 33 atherectomy specimens obtained from a different cohort of patients with SAP (n=10) and UAP (n=23) were studied immunohistochemically for ox-LDL. In AMI patients, ox-LDL levels were significantly higher than in patients with UAP (P<0.0005) or SAP (P<0.0001) or in controls (P<0.0001) (AMI, 1.95±1.42 ng/5 µg LDL protein; UAP, 1.19±0.74 ng/5 µg LDL protein; SAP, 0.89±0.48 ng/5 µg LDL protein; control, 0.58±0.23 ng/5 µg LDL protein). Serum levels of total, HDL, and LDL cholesterol did not differ among these patient groups. In the atherectomy specimens, the surface area containing ox-LDL–positive macrophages was significantly higher in patients with UAP than in those with SAP (P<0.0001).

Conclusions—This study demonstrates that ox-LDL levels show a significant positive correlation with the severity of acute coronary syndromes and that the more severe lesions also contain a significantly higher percentage of ox-LDL–positive macrophages. These observations suggest that increased levels of ox-LDL relate to plaque instability in human coronary atherosclerotic lesions. (Circulation. 2001;103:1955-1960.)

Key Words: atherosclerosis • coronary disease • myocardial infarction • angina
body (DLH3) and an anti-apolipoprotein B (apoB) polyclonal antibody. DLH3 is specific for ox-LDL and does not bind to native, acetylated, MDA-treated, or glycated LDL.

With the use of this new and highly sensitive method, we measured the levels of ox-LDL in patients with AMI, UAP, and stable angina pectoris (SAP). In addition, we immunohistochemically studied the presence of ox-LDL in coronary atherectomy specimens taken from the culprit lesions responsible for SAP and UAP, although ox-LDL levels in these patients were not available.

Hence, the study is based on a cohort of patients with similar clinical characteristics but in whom different methodologies were applied. The first group contains patients presenting with various degrees of signs and symptoms of a coronary syndrome in whom ox-LDL levels were determined and correlated with the severity of the syndrome. The second group of patients also presented with angina pectoris, but in those patients, only atherectomy specimens obtained from culprit lesions were available and studied immunohistochemically for the presence of ox-LDL.

Methods
The study was approved by the hospital ethical committee, and informed consent was obtained from all patients before the study.

Group I (Plasma ox-LDL)
The cohort consists of 135 patients with either AMI, UAP, or SAP. There were 45 patients with AMI, all of whom were studied within 24 hours after the onset of chest pain. The diagnosis of AMI was based on a history of prolonged ischemic chest pain, characteristic ECG changes, and elevated creatine kinase (>2 times above normal range) within 24 hours after the onset of pain. UAP was diagnosed in 45 patients. UAP was defined as new-onset angina within 2 months after a previous bout; angina with a progressive crescendo pattern, with the anginal episodes increasing in frequency and/or duration; angina that occurred at rest; or angina occurring in the immediate postinfarct period. The patients with UAP were further divided into class I (n=18), class II (n=6), and class III (n=21), according to Braunwald’s criteria. SAP was diagnosed in another 45 patients and defined as chest pain typical of cardiac ischemia on exertion. Of all 135 patients, only 3 patients with SAP were treated with antioxidant drugs (2 patients with probucol and 1 patient with vitamin C); the remaining 132 patients did not receive any antioxidant drugs. A total of 46 age- and sex-matched healthy volunteer blood donors served as controls (29 men and 17 women; aged 58±13 years). Among the control subjects, none had hypercholesterolemia or diabetes mellitus, 5 had a history of hypertension, and 5 were smokers. All 5 hypertensives were in stage I according to the criteria established by the Joint National Committee V, none used antihypertensive medication. Antioxidants were not administered to any controls.

Plasma levels of total cholesterol, high density lipoprotein (HDL) cholesterol, and LDL cholesterol were measured in the 3 groups of patients and in the control subjects. The following data were obtained: age, sex, and the presence of risk factors (cigarette smoking, hypertension as defined by the Joint National Committee V, diabetes mellitus as defined by the WHO Study Group, and hypercholesterolemia defined as a cholesterol level >220 mg/dL).

Measurement of ox-LDL Levels
Venous blood samples from all patients were obtained on admission to the hospital. The measurement of ox-LDL was performed using a sandwich ELISA method that was previously described. 8 The LDL fraction was separated from blood plasma before the ELISA procedure to minimize potential interferences with other plasma constituents, such as ox-VLDL, anti-ox-LDL autoantibodies, and anti-phospholipid antibodies. The LDL fractions were obtained from the samples by sequential ultracentrifugation. Diluted LDL fractions (5 μg/well) were added to the microtiter wells that were precoated with 0.5 μg of the anti–ox-LDL monoclonal antibody DLH3. After extensive washing, the remaining ox-LDL was detected with a sheep anti-human apoB antibody and an alkaline phosphatase–conjugated anti-sheep IgG antibody. In each ELISA plate, various concentrations of standard ox-LDL, which was prepared by incubating LDL with 5 μmol/L CuSO4 at 37°C for 3 hours, were run simultaneously to determine a standard curve.

Group II (Atherectomy and ox-LDL)
Coronary atherectomy specimens were obtained by directional coronary atherectomy from the culprit lesion in 33 patients who presented with either SAP (n=10) or UAP (n=23); the latter category contained 11 patients in Braunwald’s class I, 9 patients in class II, and 3 in class III. The culprit lesion was identified on the basis of clinical, ECG, and angiographic data. The patients in whom the culprit lesion was not identified were excluded from this study. In all patients, the procedure was performed on a native atherosclerotic lesion. The atherectomy specimens were fixed in methanol-Carnoy’s fixative. From each sample, serial sections were cut at a thickness of 5 μm. Every first and second section was stained with hematoxylin-eosin and an elastic van Gieson stain, respectively; the other sections were used for immunohistochemical staining.

Immunohistochemistry
To identify ox-LDL, a mouse monoclonal antibody (DLH3) was used. The methods of antibody production and specificity testing have been reported previously. Moreover, the presence of apoB was also studied using a polyclonal anti-apoB-100 antibody (Fitzgerald). Immunohistochemical identification of cells was achieved using antibodies directed against smooth muscle cells (SMCs; 1A4, DAKO), endothelial cells (anti-von Willebrand factor antibody, DAKO), macrophages (PGM-1, DAKO), and T cells (CD3, Becton Dickinson).

Single Staining
The sections were subjected to a 3-step staining procedure, with the use of streptavidin-biotin complex, with horseradish peroxidase. Horseradish peroxidase activity was visualized with 3-amino-9-ethylcarbazole, and the sections were faintly counterstained with hematoxylin.

The specificity and results obtained with anti–ox-LDL monoclonal antibody DLH3 were checked by omission of the primary antibodies and use of a nonimmune mouse IgG antibody (DAKO) as a negative control.

**TABLE 1. Characteristics of Patients in Whom ox-LDL Was Quantified**

<table>
<thead>
<tr>
<th></th>
<th>AMI (n=45)</th>
<th>UAP (n=45)</th>
<th>SAP (n=45)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, y</strong></td>
<td>60±10</td>
<td>66±10</td>
<td>63±12</td>
</tr>
<tr>
<td><strong>Men, %</strong></td>
<td>76</td>
<td>62</td>
<td>69</td>
</tr>
<tr>
<td><em><em>Hypertension,</em> %</em>*</td>
<td>40</td>
<td>64</td>
<td>73</td>
</tr>
<tr>
<td><strong>Hypercholesterolemia, %</strong></td>
<td>16</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td><strong>Diabetes mellitus, %</strong></td>
<td>27</td>
<td>36</td>
<td>33</td>
</tr>
<tr>
<td><strong>Smoking, %</strong></td>
<td>67</td>
<td>62</td>
<td>58</td>
</tr>
<tr>
<td><strong>Total cholesterol, mg/dL</strong></td>
<td>191±32</td>
<td>191±42</td>
<td>195±38</td>
</tr>
<tr>
<td><strong>LDL cholesterol, mg/dL</strong></td>
<td>46±10</td>
<td>48±12</td>
<td>44±9</td>
</tr>
<tr>
<td><strong>LDL cholesterol, mg/dL</strong></td>
<td>128±24</td>
<td>119±36</td>
<td>120±39</td>
</tr>
</tbody>
</table>

Values are mean±SD or percent.  
*The difference between AMI and both UAP and SAP is statistically significant (P<0.005).
Immunodouble Staining

For the simultaneous identification of SMCs and macrophages, immunodouble staining was performed based on 2 primary antibodies of a different IgG subclass (1A4/PGM-1), as reported previously. In this immunodouble staining, we visualized the enzymatic activity of β-galactosidase for 1A4 in turquoise (BioGenex Kit, BioGenex) and the activity of alkaline phosphatase for PGM-1 in red (New Fuchsin Kit, DAKO).

To identify cell types that show staining positivity for ox-LDL, we also performed immunodouble staining with PGM-1 (macrophage) and DLH3 (ox-LDL). In this staining, alkaline phosphatase was visualized with fast blue BB (blue, PGM-1) and peroxidase was visualized with 3-amino-9-ethylcarbazole development (red, DLH3).

Quantitative Methods

The surface area occupied by ox-LDL–positive cells was quantified using computer-aided planimetry and expressed as a percentage of the total tissue area of the atherectomy specimen. The area occupied by macrophages was quantified in a similar fashion and likewise expressed as a percentage of the total tissue area. On the basis of these quantifications, an “ox-LDL–positive macrophage score” was calculated as follows:

\[
\text{ox-LDL–positive macrophage score} = \frac{\text{ox-LDL–positive area}}{\text{macrophage-positive area}}
\]

The morphometric analysis was performed by a single investigator who was blinded to the patient’s clinical diagnosis. The results are expressed as mean±SD. The 2 groups were compared with an unpaired Student’s t test or with a Welch’s t test when the variance was heterogeneous. Statistical comparisons between ≥3 groups were performed by 1-way ANOVA and post-hoc multiple comparison using Scheffe’s test. Values of \( P<0.05 \) were considered significant.

Results

Ox-LDL Levels Are Related to Severity of the Coronary Syndrome

Patient characteristics are shown in Table 1. There were no differences in age, sex, or serum levels of total cholesterol, HDL cholesterol, or LDL cholesterol among patients with AMI, UAP, and SAP; only hypertension was less frequent in patients with AMI than in patients with UAP or SAP (\( P<0.005 \)).

As shown in Figure 1, ox-LDL levels in patients with AMI were significantly higher than in patients with UAP (\( P<0.0005 \)) or SAP (\( P<0.0001 \)) or in control subjects (\( P<0.0001 \)) (AMI, 1.95±1.42 ng/5 μg LDL protein; UAP, 1.42±1.14 ng/5 μg LDL protein; SAP, 1.14±0.89 ng/5 μg LDL protein; control group, 0.89±0.69 ng/5 μg LDL protein).

Table 2 shows the baseline characteristics of patients from whom atherectomy specimens were obtained. There were no significant differences in age, sex, or risk factors among the 3 groups.

Table 2. Baseline Characteristics of Patients From Whom Atherectomy Specimens Were Obtained

<table>
<thead>
<tr>
<th></th>
<th>SAP (n=10)</th>
<th>UAP (n=23)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>61±8</td>
<td>57±7</td>
<td>NS</td>
</tr>
<tr>
<td>Men, %</td>
<td>80</td>
<td>91</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>50</td>
<td>35</td>
<td>NS</td>
</tr>
<tr>
<td>Hypercholesterolemia, %</td>
<td>50</td>
<td>43</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>30</td>
<td>35</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>50</td>
<td>57</td>
<td>NS</td>
</tr>
</tbody>
</table>
1.19±0.74 ng/5 μg LDL protein; SAP, 0.89±0.48 ng/5 μg LDL protein; and control, 0.58±0.23 ng/5 μg LDL protein). The levels of ox-LDL in patients with UAP were significantly higher than those in control subjects (P<0.01). Among the 45 patients with UAP, no significant difference existed in ox-LDL levels among the 3 categories of Braunwald’s classification9 (class I, 1.09±0.64 ng/5 μg LDL protein; class II, 1.03±0.84 ng/5 μg LDL protein; class III, 1.31±0.81 ng/5 μg LDL protein).

Figure 2 shows the relationship between ox-LDL levels and the risk factors studied; none of the risk factors showed a statistically significant correlation.

**Immunohistochemical Quantification in Atherectomy Specimens**

Patient characteristics are shown in Table 2. Age, sex, and presence of risk factors did not differ among patients with either UAP or SAP. In the lesions of patients with UAP, abundant ox-LDL positivity was found in macrophage-derived foam cells; immunodouble staining for macrophages and ox-LDL revealed distinct ox-LDL positivity in macrophage-derived foam cells (Figure 3). Moreover, in these lesions, ox-LDL and apoB colocalized in macrophage-derived foam cells (Figure 4). In contrast, in the atherectomy specimens of patients with SAP, ox-LDL positivity was sparse and, when present, was localized to macrophages (Figure 5); in these macrophage-derived foam cells, colocalization of ox-LDL and apoB was occasionally found. In these experiments, sections treated with a nonimmune IgG antibody gave a negative result (Figure 4B). Figure 6 shows the ox-LDL-positive macrophage score for each individual lesion in the 2 groups. The ox-LDL-positive macrophage score was significantly higher (P<0.0001) in patients with UAP (0.49±0.26) than in patients with SAP (0.07±0.07).

**Discussion**

To the best of our knowledge, this is the first study to demonstrate that levels of ox-LDL have a positive correlation with the severity of the underlying acute coronary syndrome using an anti–ox-LDL monoclonal antibody (DLH3) and an apoB polyclonal antibody to measure ox-LDL in the circulating LDL fractions of blood plasma.8 DLH3 is specific for ox-LDL because it does not bind to native, acetylated, MDA-treated, or glycated LDL.8 The molecules recognized by DLH3 are oxidized phosphatidylcholine (ox-PC), including 1-palmitoyl-2-(9-oxononanoyl) PC and proteins modified by ox-PC. Among the many anti–ox-LDL monoclonal antibodies, DLH3 is one of the few whose epitopes on ox-LDL are well characterized. Strictly speaking, one may argue that we have not actually measured the plasma ox-LDL levels, because LDL was separated from blood plasma before the ELISA procedure. However, this method avoids interference with other plasma substances and, in fact, is highly sensitive in detecting minute amounts of ox-LDL particles.8
Watson et al. demonstrated that ox-PC separated from minimally modified LDL (MM-LDL), prepared by mild oxidation of LDL, was capable of inducing monocyte adhesion to endothelial cells and neutrophil migration. These studies suggest that ox-PC is one of the key molecules in ox-LDL and is directly involved in the early development of atherosclerosis. Measuring ox-LDL in circulating plasma using the DLH3 antibody could provide a means to monitor the behavior of ox-PC particles as part of ox-LDL in plasma LDL fraction.

A number of previous studies have been devoted to detecting ox-LDL in circulating plasma. Holvoet et al. developed a competition ELISA method to detect MDA-LDL and ox-LDL using monoclonal antibodies. They used the anti–ox-LDL antibody 4E6, which reacts not only to ox-LDL but also to MDA-LDL when >120 lysine residues per apoB molecule are modified with MDA. The antibody 1H11, which they used to detect MDA-LDL, binds 100 times more effectively to MDA-LDL than to ox-LDL. They reported that the ox-LDL concentration is ~3 times higher than that of MDA-LDL, probably because the 4E6 antibody that they used for ox-LDL detection binds to a rather broad array of differently modified types of LDL. A number of lipid peroxidation products formed during the oxidation of LDL can react with apoB; therefore, different types of modifications occur simultaneously on ox-LDL particles. Because MDA is one of those lipid peroxidation products that is highly reactive to lysine residues, MDA-LDL has been widely used as a way to detect and quantify ox-LDL. However, despite the fact that ox-LDL contains MDA-induced modifications of the apoB protein, MDA-LDL cannot be considered identical to ox-LDL.

In the present study, we found that levels of ox-LDL were ~4 times higher in patients with AMI than in control subjects. This observation strongly suggests that ox-LDL in circulating plasma could serve as a marker for cardiovascular events. Recently, similar results were reported by Holvoet et al. The mean plasma ox-LDL level for AMI patients determined in the present study was 1.95 ng of ox-LDL/5 μg of LDL protein, and these amounts correspond to 0.04% of the total LDL. However, in the system reported by Holvoet et al. using the 4E6 antibody, 3.44 mg/dL of ox-LDL was detected in AMI patients; this amounts to ~5% of the total LDL. It is likely that the antigen detected by their system is a large variety of conformationally modified LDL and that the ox-PC–modified ox-LDL detected by our system is a
part of it. Despite differences, however, both methods reveal that a change in the ox-LDL levels has occurred in AMI patients, thus providing good evidence for the involvement of oxidative modification of LDL in acute cardiovascular events.

The present study showed that ox-LDL levels related directly to the severity of acute coronary syndromes. However, the observations provide no insight into the question of whether these increased levels also reflect the atherosclerotic burden within the coronary arteries. Nevertheless, the findings are of interest because the atherosclerotic plaques underlying AMI usually present as lipid-rich plaques with abundant inflammation and plaque complications, such as surface erosion or rupture with adherent thrombus. Moreover, coronary atherectomy specimens have revealed that the culprit lesions from UAP patients contain a significantly higher number of macrophages and T lymphocytes than those from SAP patients. Hence, an increased number of inflammatory cells in coronary atherosclerotic plaques is related to an increase in the severity of the acute coronary syndrome. As mentioned previously, oxidative modification of lipoproteins is widely accepted as a key event in the genesis of atherosclerosis. Moreover, previous studies have suggested that ox-LDL may also play a role in triggering thrombosis by inducing platelet adhesion and by decreasing the fibrinolytic capacities of endothelial cells. Hence, our observation that ox-LDL levels relate directly to the severity of acute coronary syndromes suggests that raised ox-LDL levels may have a destabilizing effect on plaque composition, most likely by enhancing the inflammatory processes and surface thrombosis.

The question arises regarding what causes high levels of ox-LDL in patients with UAP and AMI. Are systemic changes involved that alter the lipid profile or is it the atherosclerotic process itself that could be held responsible? At this stage, it is fair to state that this remains speculative. Previous in vitro studies have documented that macrophages and lymphocytes are capable of oxidizing LDL. The culprit lesions of patients with AMI contain abundant macrophages and T lymphocytes, as previously demonstrated. Under these circumstances, ox-LDL in macrophage-derived foam cells may be enhanced within unstable plaques in association with the progression of plaque inflammation. On this basis, one could hypothesize that the ox-LDL present within unstable plaques may be released into the blood stream in patients with severe endothelial injuries, such as plaque erosion or rupture. Moreover, previous in vitro studies have demonstrated that neutrophils can oxidatively modify LDL into a form that is rapidly incorporated by macrophages. Neutrophils are known to accumulate at sites of plaque rupture or erosion in patients with AMI. Hence, one could also hypothesize that neutrophils, which may accumulate at sites of inflammatory reactions in unstable, eroded, or ruptured plaques, especially at early stages after injuries, could contribute to an increase in the ox-LDL levels in the blood.

Our immunohistochemical study using atherectomy specimens clearly demonstrates that the number of ox-LDL-positive macrophages in the culprit lesions of UAP patients is significantly higher than in those of SAP patients. It is presently well accepted that intraplaque inflammation plays a key role in plaque destabilization and, hence, in the pathophysiology of acute coronary syndromes. Our present findings not only support this concept, but also suggest a pivotal role for ox-LDL in the genesis of coronary plaque instability and the development of acute coronary syndromes.

In conclusion, this study demonstrates for the first time that ox-LDL levels relate directly to the severity of coronary syndromes.

References
15. van der Wal AC, Becker AE, van der Loos CM, et al. Site of initial rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation. 1994;89:36–44.
19. Lamb DJ, Wikins GM, Leake DS. The oxidative modification of low density lipoprotein by human polymorphonuclear leukocytes to a form recognized by the lipoprotein scavenger pathway. Biochim Biophys Acta. 1994;1213:231–237.
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