Oxidized Low-Density Lipoprotein and Autoimmune Antibodies in Patients With Antiphospholipid Syndrome With a History of Thrombosis

Dandan Zhao, MD, PhD,1* Hirokazu Ogawa, MD,2 Xiang Wang, MD,1 Gregory S. Cameron, PhD,1 Darric E. Baty, MD,1 Jeffrey S. Dlott, MD,1 and Douglas A. Triplett, MD, FACP1

Key Words: Oxidized low-density lipoprotein; oxLDL; Anti-oxLDL; Thrombosis; Antiphospholipid syndrome; enzyme-linked immunosorbent assay; ELISA

Abstract

The prevalence and clinical significance of plasma oxidized low-density lipoprotein (oxLDL) and antibodies against oxLDL (anti-oxLDL) were evaluated in patients with antiphospholipid syndrome (APS). OxLDL and IgG anti-oxLDL were determined by enzyme-linked immunosorbent assay in plasma samples from 80 patients with APS. Positive values (mean + 3 SD) for oxLDL and anti-oxLDL were found in 21 (26%) and 19 (24%) of 80 patients with APS, respectively. These values were significantly higher than those in healthy subjects. Levels of oxLDL and anti-oxLDL antibodies in subgroupings of patients with APS who had experienced thrombotic events were compared. There were significant differences among the groups for the levels of both oxLDL and anti-oxLDL antibodies. Pairwise comparisons between the groups yielded similar but not identical results. There was a significant, positive correlation between levels of plasma oxLDL and anti-oxLDL. These results suggest that elevated levels of plasma oxLDL and anti-oxLDL may be risk factors and potential markers for thrombosis, especially for arterial thrombotic events, in patients with APS.

Antiphospholipid syndrome (APS) has been defined as the occurrence of thrombosis (arterial or venous), recurrent fetal loss, and/or thrombocytopenia, in the presence of antiphospholipid antibodies (APAs).1-3 In this autoimmune disorder, atherosclerosis and arterial thrombosis contribute to morbidity and mortality.4-6 A wide spectrum of mechanisms has been proposed to account for arterial thrombosis in patients with APS. Oxidation makes low-density lipoprotein (LDL) more atherogenic,7 as suggested by the presence of oxidized LDL (oxLDL) particles in the early phase of atherosclerotic plaque formation.8,9 Epidemiologic studies have established that an elevated plasma level of LDL represents one of the most important risk factors for the development of atherosclerosis.10 On the other hand, oxLDL is immunogenic, and the generation of antibodies against oxLDL (anti-oxLDL) has been taken as a biologic signature of in vivo LDL oxidation.11,12 Oxidation of LDL can occur in vivo, and oxLDL may enhance LDL uptake by macrophage scavenger receptors, promoting the transformation of macrophages into foam cells.8 Anti-oxLDL has been demonstrated in chronic periaortitis,13 progressive carotid atherosclerosis,14 and APS,15 as well as in atherosclerotic lesions of rabbits and humans.8,16 Such antibodies recognize epitopes expressed in atherosclerotic lesions of rabbits and humans but not in normal arteries.11,17,18 Anti-oxLDL belongs to the family of APAs.19 APAs are associated with both arterial and venous thrombosis, although the risk factors for these two conditions are not the same.20 The question of the direct involvement of plasma oxLDL and anti-oxLDL in the occurrence of thrombosis remains unanswered. We studied the association of oxLDL, anti-oxLDL, and a thrombotic history in patients with APS.
Materials and Methods

Patient Characteristics

A total of 80 patients with APS (Table 1) and 72 healthy control subjects were included in the study. All patients fulfilled the criteria for APS proposed by Harris.21 The study patients were 17 men and 63 women, mean ± SD age, 40.0 ± 14.7 years (range, 19-77 years), and included 61 patients with primary APS, and 19 with systemic lupus erythematosus (SLE). Nine patients with cerebral transient ischemic attack were diagnosed by history alone. All other cases were objectively verified thrombotic events. Fetal loss was defined as recurrent (2 or more) abortions or more than 1 unexpected intrauterine fetal death after the second trimester. Thrombocytopenia was defined as a platelet count less than 100 × 10^3/µL (100 × 10^9/L). Control subjects were healthy volunteers (19 men and 53 women; mean ± SD age, 43.2 ± 12.4 years) without autoimmune diseases, bleeding disorders, unusual thrombotic events, or fetal losses.

Blood Samples

Blood was obtained by venipuncture from the antecubital vein after informed consent and collected into plastic tubes containing a 0.105 mol/L concentration of sodium citrate in a ratio of 9 parts blood to 1 part anticoagulant. The samples were centrifuged twice at 2500 g for 15 minutes at 6°C to obtain platelet-poor plasma (<10 × 10^9/µL [<10 × 10^3/L]). Lupus anticoagulant (LA) activity was identified according to the recommendations of the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis.22 All plasma samples were divided into aliquots and kept frozen at −70°C until assayed. The protocol and informed consent were approved by the Ball Memorial Hospital (Muncie, IN) Institutional Review Board.

Preparation of Human Plasma LDL

Plasma LDL (density, 1.019-1.063 g/L) was isolated from fresh normal plasma as previously described,23 after density adjustment with potassium bromide, by ultracentrifugation at 100,000g for 24 hours (L8-60M ultracentrifuge, rotor 45 titanium, Beckman, Fullerton, CA). The crude LDL preparation was dialyzed against phosphate-buffered saline (PBS) containing a 1-µmol/L concentration of EDTA, passed through a Millex-GV (Millipore, Bedford, MA) filter (0.22 µm pore size) to remove aggregates, and stored at 4°C. Purified LDL showed a single band on 1% agarose gel electrophoresis in borate buffer. LDL was quantified based on protein concentration (Bio-Rad Protein Assay Kit, Hercules, CA). This LDL was used as a standard for subsequent determinations.

Modification of LDL

LDL oxidation was carried out by incubation of 100 µg/mL of LDL (in EDTA-free PBS) with a 5-µmol/L concentration of cupric sulfate at 37°C for 8 hours. The reaction was terminated by adding a 1-mmol/L concentration of EDTA and then dialyzed extensively against PBS at 4°C for 24 hours to remove cupric ions. Quantification of oxLDL was based on protein concentration. This oxLDL was used as a standard for subsequent determinations.

oxLDL Enzyme-Linked Immunosorbent Assay

An enzyme-linked immunosorbent assay (ELISA) for human plasma oxLDL was developed in our laboratory (D. Zhao, MD, PhD, et al, unpublished data). Briefly, microtiter plates (Immulon 2HB, Dynex Technologies, Chantilly, VA) were coated with 50 µL of WB-CAL-1, a murine monoclonal anticardiolipin antibody (aCL),24 at 10 µg/mL in PBS. The plates were incubated at 4°C overnight. After washing 3 times with PBS containing 0.05% polysorbate 20, plates were blocked with 1% nonfat dry milk (Bio-Rad) in PBS and placed at room temperature for 1 hour, after which the solution was removed. After washing, 0- to 10-µg/mL oxLDL solutions were diluted with 1% nonfat dry milk as a standard. Plasma samples from healthy subjects and patients with APS diluted 2:100 in 1% nonfat dry milk containing 0.1 mg/mL of beta-glycoprotein I (GPI), in the total volume of 100 µL, were added to the wells. After a 1-hour incubation at room temperature, the plates were washed, and 100 µL of biotinylated 1D2, a mouse monoclonal anti-apolipoprotein B 100 antibody (Yamasa, Choshi, Japan), diluted 1:10,000 was added to the wells and incubated at room temperature for 1 hour. After washing, plates were incubated with HRP-Avidin (Zymed, South San Francisco, CA), diluted 1:3,000, at room temperature for 15 minutes. Then, a 1.85-µmol/L concentration of o-phenylenediamine (Sigma, St Louis, MO) in a 0.1-µmol/L concentration of citric acid buffer (pH 5.0) containing 0.05% hydrogen peroxide was added as a

Table 1

| Characteristics of 80 Patients With Antiphospholipid Syndrome (APS) |
|------------------|-----------------|
| Number (%) of Patients |
| Primary antiphospholipid syndrome | 61 (76) |
| Antiphospholipid syndrome secondary to systemic lupus erythematosus | 19 (24) |
| Anticardiolipin antibody positive | 57 (71) |
| Lupus anticoagulant positive | 69 (86) |
| Clinical manifestations |
| Thrombosis | 46 (58) |
| Arterial | 19 (24) |
| Venous | 14 (18) |
| Arterial and venous | 13 (16) |
| Recurrent miscarriage | 28 (35) |
| Thrombocytopenia | 27 (34) |
substrate at room temperature for exactly 10 minutes. The reaction was terminated by adding 2N sulfuric acid following the 10-minute incubation at room temperature. The optical density was read at a wavelength of 492 nm. The concentration of plasma oxLDL was calculated from the standard curve produced using the purified oxLDL as described.

Anti-oxLDL ELISA

Half of a microtiter plate (Immulon 2 HB) was coated with native LDL and the other half with Cu\(^{+2}\)-LDL (oxLDL), both at 50 \(\mu\)L of a 10 \(\mu\)g/mL solution in PBS, and incubated at 37\(^\circ\)C for 2 hours and then overnight at 4\(^\circ\)C. Plates were washed 3 times with PBS containing 0.05% polysorbate 20, and wells were blocked with 200 \(\mu\)L of PBS containing 1% bovine serum albumin for 2 hours at room temperature. After washing, 50 \(\mu\)L of plasma diluted with 1% bovine serum albumin at 1:200 containing 0.2mg/mL of beta\(_2\)-GPI was added in duplicate. After 2 hours of incubation at room temperature, the plates were washed 3 times and then incubated with 100 \(\mu\)L of alkaline phosphatase–conjugated monoclonal murine antihuman IgG (gamma chain specific) diluted 1:1,000 in dilution buffer for 1 hour at room temperature. Four washings followed, and the color reaction was developed by the addition of 100 \(\mu\)L of substrate (5 mg/mL p-nitrophenyl phosphate in 10% diethanolamine containing a 0.5-mmol/L concentration of magnesium chloride), incubated for 1 hour at room temperature. The reaction was stopped by adding 100 \(\mu\)L of a 100-mmol/L concentration of EDTA solution. Absorbance values at 405 nm were obtained with an ELISA plate reader and SOFTmax analysis software (Molecular Device, Menlo Park, CA). All reagents were purchased from Sigma. Each sample was measured in duplicate, and binding of antibody to oxLDL was calculated by subtracting the binding to native LDL from that to Cu\(^{+2}\)-LDL. Consistency was monitored in the ELISAs by including a plasma sample previously identified as having a high anti-oxLDL titer in each plate. Samples with values greater than mean + 3 SDs for 72 healthy control plasma samples were identified as positive.

Statistical Analysis

The data are expressed as optical density or concentration (\(\mu\)g/mL), and group averages are given on the appropriate figures. Tests for significance of differences between groups were conducted using the Mann-Whitney \(U\) test for 2-group comparisons and the Kruskal-Wallis test for comparisons of more than 2 groups. When statistical significance was indicated by the Kruskal-Wallis test, the Dunn multiple comparison test was used for pairwise comparisons. Determination of correlation between factors was calculated using the Spearman rank correlation procedure. A probability value of .05 or less was considered statistically significant.

Results

Effects of beta\(_2\)-GPI on Anti-oxLDL ELISA

In our preliminary experiments, we examined anti-oxLDL binding in different beta\(_2\)-GPI concentrations by randomly selecting plasma samples from 5 patients with APS. The results showed a similar binding trend toward beta\(_2\)-GPI concentrations, although individuals demonstrated different levels of anti-oxLDL binding to oxLDL. It is likely that beta\(_2\)-GPI enhances the binding of anti-oxLDL to oxLDL; however, it is apparent that beta\(_2\)-GPI-independent binding of anti-oxLDL also occurs (data not shown). Based on these data, we added purified exogenous beta\(_2\)-GPI (0.2 mg/mL) in our anti-oxLDL assay.

Patients With APS and Healthy Subjects

A positive status (mean + 3 SD) for oxLDL and anti-oxLDL was found in 26% (21/80) and 24% (19/80), respectively, of patients with APS but in only 3% (2/72) and 6% (4/72), respectively, of control subjects. Likewise, mean levels of oxLDL and anti-oxLDL were significantly higher in patients than in control subjects (both \(P < .0001\) ) [Figure II]. Positive levels of oxLDL and anti-oxLDL were detected in 18% (11/61) of patients with primary APS. In patients whose APS was secondary to SLE, 47% (9/19) were positive for oxLDL and 42% (8/19) were positive for anti-oxLDL.

Levels of oxLDL and anti-oxLDL antibodies for patients with APS with a history of thrombosis, patients with APS without a history of thrombosis, and healthy control subjects were measured by the described ELISA techniques and compared for differences [Figure II]. There were significant differences among the groups for both the oxLDL levels and the anti-oxLDL levels (\(P = .001\) or less, Kruskal-Wallis).

Pairwise comparison of the groups indicated that each of the 3 groups was significantly different from the other 2 groups [Table II].

Patients With APS

Levels of oxLDL and anti-oxLDL in subgroupings of patients with APS who had experienced thrombotic events were compared using the ELISA technique described in the “Materials and Methods” section. The patients were classified as having had an arterial thrombotic event (ATH), an arterial/venous thrombotic event (A/VTH), a venous thrombotic event (VTH), or no thrombotic event (NoTh). The
Table 2
Comparison of oxLDL and Anti-oxLDL Antibody Levels in Patients With APS and Healthy Control Subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>oxLDL</th>
<th>Anti-oxLDL</th>
<th>Dunn Grouping †</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS (Th)</td>
<td>46</td>
<td>40.03</td>
<td>0.841</td>
<td>A</td>
</tr>
<tr>
<td>APS (NoTh)</td>
<td>34</td>
<td>26.90</td>
<td>0.593</td>
<td>—</td>
</tr>
<tr>
<td>Control subjects</td>
<td>72</td>
<td>20.22</td>
<td>0.424</td>
<td>—</td>
</tr>
</tbody>
</table>

Anti-oxLDL, antibodies against oxLDL; APS, antiphospholipid syndrome; NoTh, no thrombotic event; oxLDL, oxidized low-density lipoprotein; Th, thrombotic event.

† Mean concentration and optical density value as measured by the enzyme-linked immunosorbent assay methods described in the “Materials and Methods” section.

Pairwise comparisons made using the Dunn multiple comparison test. Statistical results were the same for the oxLDL and the anti-oxLDL antibody levels. Groups with the same letter designations were not significantly different, alpha = .05.
average values for the oxLDL levels and the anti-oxLDL levels are shown in **Figure 3**. There were significant differences among the groups for the levels of both oxLDL and anti-oxLDL antibodies \((P = .002\) or less, Kruskal-Wallis).

Pairwise comparisons between the groups yielded similar, but not identical results. For the oxLDL levels, 2 groupings were significantly different. The ATh, A/VTh, and the VTh groups formed a subset with the groups not significantly different. The VTh and the NoTh groups formed a second such subset. The NoTh group had significantly lower levels of oxLDL than did the ATh group or the VTh group.

For the anti-oxLDL levels, there were 3 subsets formed of pairs of groups that were not significantly different from each other. These subsets consisted of the following pairs: (1) the ATh and A/VTh groups, (2) the A/VTh and VTh groups, and (3) the VTh and NoTh groups. However, the VTh and the NoTh groups had significantly lower levels of the antibodies than did the ATh group, and the NoTh group had significantly lower levels than the A/VTh group **Table 3**.

**Table 3**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean*</th>
<th>Dunn Grouping†</th>
</tr>
</thead>
<tbody>
<tr>
<td>OxLDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATh ((n = 19))</td>
<td>39.22</td>
<td>A  —</td>
</tr>
<tr>
<td>A/VTh ((n = 13))</td>
<td>45.54</td>
<td>A  —</td>
</tr>
<tr>
<td>VTh ((n = 14))</td>
<td>35.99</td>
<td>A  B</td>
</tr>
<tr>
<td>NoTh ((n = 34))</td>
<td>26.90</td>
<td>—  B</td>
</tr>
<tr>
<td>Anti-oxLDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATh ((n = 19))</td>
<td>0.833</td>
<td>A  —  —</td>
</tr>
<tr>
<td>A/VTh ((n = 13))</td>
<td>1.085</td>
<td>A  B  —</td>
</tr>
<tr>
<td>VTh ((n = 14))</td>
<td>0.557</td>
<td>—  B  C</td>
</tr>
<tr>
<td>NoTh ((n = 34))</td>
<td>0.593</td>
<td>—  —  C</td>
</tr>
</tbody>
</table>

Anti-oxLDL, antibodies against oxLDL; ATh, arterial thrombotic event; A/VTh, arterial/venous thrombotic event; NoTh, no thrombotic event; oxLDL, oxidized low-density lipoprotein; VTh, venous thrombotic event.

* Mean oxLDL concentration and optical density value as measured by the enzyme-linked immunosorbent assay methods described in the „Materials and Methods“ section.

† Pairwise comparisons made using the Dunn multiple comparison test. Groups with the same letter designations were not significantly different, alpha = .05.

**Correlation Between Levels of Plasma oxLDL and Anti-oxLDL**

There was a significant, positive correlation between levels of plasma oxLDL and anti-oxLDL **Figure 4**. However, some plasma was clearly high level in the oxLDL assay and low level in the anti-oxLDL assay, and vice versa.

**Discussion**

Patients with APS have many risk factors for arterial diseases, including hypertension and abnormalities in the lipid profile. Several studies have shown that oxLDL has an important role in vascular events.25,26 We present evidence for the novel idea that oxLDL and anti-oxLDL are associated with thrombosis, especially arterial thrombosis, in patients with APS.

The present study reveals that patients with APS, when compared with healthy control subjects, have significantly higher levels of oxLDL and anti-oxLDL (Figure 1). Levels of oxLDL and anti-oxLDL also are significantly higher in patients with APS with a history of thrombosis than in both healthy subjects and patients with APS without a history of thrombosis (Table 2). oxLDL, at concentrations higher than...
those inducing cell stimulatory responses, may provoke injury and inflammation in the vascular wall by toxic and inhibitory actions. OxLDL stimulates endothelial cells and monocytes to make tissue factor, enhances platelet aggregation, and promotes procoagulant cascades by increasing tissue thromboplastin activity. Other proatherogenic effects that may be related to platelet activating factor—like lipids formed in LDL during oxidation include enhancement of endothelial adhesiveness and smooth muscle cell proliferation. The oxidation of LDL results in many structural modifications and the formation of a large number of neoepitopes. OxLDL is more immunogenic than its native counterpart and induces the production of a heterogeneous population of specific anti-oxLDL autoantibodies. The generation of anti-oxLDL probably reflects the increased oxidation of LDL in atherosclerosis; however, the magnitude of the immune reaction may reflect the individual’s responsiveness to the chronic inflammatory process in the arterial wall rather than the amount of in vivo oxLDL. Extensive data have accumulated suggesting that titers of such antibodies may be of diagnostic and prognostic value. Our data indicate a significant association between oxLDL and anti-oxLDL levels and clot formation and demonstrate that oxLDL and anti-oxLDL may be important in thrombogenesis in patients with APS.

Our results show that in subgroupings of patients with APS, levels not only of oxLDL but also of anti-oxLDL in patients with an arterial thrombotic event are significantly higher than those found in other groups of patients with APS (Table 3); this indicates an association between oxLDL and anti-oxLDL in patients with APS and a clinical history of arterial thrombosis in those patients. The correlation between anti-oxLDL and disease progression in carotid atherosclerosis and the predictive value of anti-oxLDL for myocardial infarction have been reported, supporting the involvement of anti-oxLDL in the development of atherosclerosis. Several studies indicate that oxLDL antibodies serve as markers of the pathogenic determinants of atherothrombosis, such as oxidation of LDL, endothelial dysfunction, and arterial inflammation. A number of mechanisms have been hypothesized to explain the pathophysiology of thrombosis in APS. The atherosclerotic process could be implicated as one of them. Thrombosis seldom occurs without underlying atherosclerosis. Antibodies against beta2-GPI also have been found in patients with APS and SLE and reportedly are associated with thrombosis; however, there is still no general agreement on the specific mechanism or even the relationship between anti-oxLDL and thrombosis. Our results lead us to hypothesize that oxLDL and its antibodies have an important role in atherosclerotic complications in patients with APS; whether the results are clinically significant can be answered only through further research with larger data sets.

Our study showed that 17 (37%) of 46 patients with a thrombotic history had high levels of plasma oxLDL and 16 (35%) of 46 had antibodies to oxLDL. Interestingly, we found a strong correlation between plasma oxLDL and anti-oxLDL, high levels of which were associated significantly with a history of thrombosis, especially arterial thrombosis in patients with APS (Figure 4). Twelve (26%) of 46 patients were double-positive, while only 3 (9%) of the 34 patients without thrombosis were double-positive with regard to these 2 factors. Thus, both oxLDL and anti-oxLDL may be important in evaluating the risk of arterial thrombosis in patients with APS. In fact, it has been hypothesized that oxLDL may combine with anti-oxLDL, leading to the formation of an immune complex, the uptake of which by Fc receptors on macrophages occurs in synergy and faster than by the scavenger pathway. An in vitro study has shown that uptake of radiolabeled oxLDL by a monocyte/macrophage–like cell line was more rapid in the presence of anti-oxLDL than the uptake of oxLDL alone. In theory, these phenomena could lead to quicker formation of foam cells, resulting in induction of atherosclerotic and thrombotic pathways.

In the present study, we used copper-oxidized LDL as a standard or an antigen in our ELISA. Experiments conducted in vitro have shown that LDL may be oxidized by several types of cells, including endothelial, smooth muscle, and macrophages. Oxidation of LDL occurs in vivo, as well. At present, there is general agreement about the use of copper-oxidized LDL as an antigen in an anti-oxLDL assay. Our plasma oxLDL and anti-oxLDL ELISA tests were conducted with a physiologic concentration (0.2 mg/mL) of beta2-GPI. In our anti-oxLDL ELISA, APS plasma was diluted (1:200),
corresponding to a beta_{2}-GPI level of 1 µg/mL, which reduced the chance of detecting cofactor activity of endogenous beta_{2}-GPI. It is well known that the behavior of aCLs in relation to the cofactor beta_{2}-GPI is heterogeneous. It has been shown that 2 types of aCL exist: cofactor dependent and cofactor independent.42 Moreover, among cofactor-dependent aCL it seems there are differences in cofactor requirements.33 Vaarala et al15 reported that aCLs in patients with SLE cross-react with malondialdehyde-modified LDL. Studies in patients with APS, however, failed to show any cross-reaction between aCL and anti-oxLDL antibodies.39 Our study also demonstrated that the levels of anti-oxLDL did not correlate with those of anti–beta_{2}-GPI (data not shown); therefore, we agree that anti-oxLDL may represent a distinct subset of antibodies coexisting with beta_{2}-GPI-dependent aCL.44 Further study is necessary to demonstrate whether anti-oxLDL is really beta_{2}-GPI dependent by using beta_{2}-GPI-depleted plasma.

We have confirmed that oxLDL and its antibodies may be risk factors and potential markers for thrombosis, especially for arterial thrombotic events, in patients with APS. Proposed mechanisms have been reviewed, and areas that need more extensive study have been discussed. Differences in the method used for the measurement of anti-oxLDL and/or in ethnicity of the study group may explain why we obtained results different from those of Aho et al20; however, any explanation remains speculative. Longitudinal prospective studies are needed to investigate the predictive value of plasma oxLDL and anti-oxLDL for atherosclerosis and thrombosis in patients with APS.

References


