Circulating oxidized LDL forms complexes with \( \beta_2 \)-glycoprotein I: implication as an atherogenic autoantigen

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Abstract \( \beta_2 \)-glycoprotein I (\( \beta_2 \)-GPI) is a major antigen for antiphospholipid antibodies (Abs, aPL) present in patients with antiphospholipid syndrome (APS). We recently reported (J. Lipid Res., 42: 607, 2001; J. Lipid Res., 43: 1486, 2002) that \( \beta_2 \)-GPI specifically binds to Cu\( ^{2+} \)-oxidized LDL (oxLDL) and that the \( \beta_2 \)-GPI ligands are \( \omega \)-carboxylated 7-ketcholesteryl esters. In the present study, we demonstrate that oxLDL forms stable and nondissociable complexes with \( \beta_2 \)-GPI in serum, and that high serum levels of the complexes are associated with arterial thrombosis in APS. A conjugated ketone function at the 7-position of cholesterol as well as the \( \omega \)-carboxyl function of the \( \beta_2 \)-GPI ligands was necessary for \( \beta_2 \)-GPI binding. The ligand-mediated noncovalent interaction of \( \beta_2 \)-GPI and oxLDL undergoes a temperature- and time-dependent conversion to much more stable but readily dissociable complexes in vitro at neutral pH. In contrast, stable and nondissociable \( \beta_2 \)-GPI-oxLDL complexes were frequently detected in sera from patients with APS and/or systemic lupus erythematos. Both the presence of \( \beta_2 \)-GPI-oxLDL complexes and IgG Abs recognizing these complexes were strongly associated with arterial thrombosis. Further, these same Abs correlated with IgG immune complexes containing \( \beta_2 \)-GPI or LDL. Thus, the \( \beta_2 \)-GPI-oxLDL complexes acting as an autoantigen are closely associated with autoimmune-mediated atherogenesis.—Kobayashi, K., M. Kishi, T. Atsumi, M. L. Bertolaccini, H. Makino, N. Sakairi, I. Yamamoto, T. Yasuda, M. A. Khamashta, G. R. V. Hughes, T. Koike, D. R. Voelker, and E. Matsuura. Circulating oxidized LDL forms complexes with \( \beta_2 \)-glycoprotein I: implication as an atherogenic autoantigen. J. Lipid Res. 2003. 44: 716–726.

Supplementary key words antiphospholipid syndrome • arterial thrombosis • autoantibody

Oxidative modification of LDL is a physiologically relevant mechanism for atherogenesis. Experimental evidence clearly demonstrates that oxidized LDL (oxLDL) exists in vivo in the artery wall and contributes to the initiation and progression of atherosclerotic lesions (1–3). When LDL undergoes oxidation, “biologically active” lipids are generated. The process involves oxidative breakdown of either free polyunsaturated fatty acids or those esterified at the sn-2 position of phospholipids (PLs) to form fatty-acid hydroperoxides. The resulting fatty-acid hydroperoxides decompose to form highly reactive products containing an aldehyde (or ketone) function (4–10). Such active functions can form Schiff-base adducts with lysine residues of the apolipoprotein B (apoB) moiety of LDL (11) and primary amine-containing PLs, such as phosphatidylserine and phosphatidylethanolamine.

Several reports indicate that auto-antibodies (Abs) against oxidatively generated neoepitopes of LDL are present in patients or animals with atherosclerosis. Anti-oxLDL Abs are elevated in patients with early-onset peripheral vascular disease, severe carotid atherosclerosis, and angiographically verified coronary artery disease (12–17). In addition, a monoclonal auto-Ab (EO6) from an apoE-deficient mouse recognizes an adduct formed with ox-
dized phosphatidylcholine, i.e., 1-palmitoyl-2-(5’-oxo) valeryl-$\omega$-glycerol-3-phosphorylcholine and lysine, and its $\beta$-hydroxysteroyl (aldol) condensates (18–20).

The autoimmune disorder antiphospholipid syndrome (APS) is characterized by the presence of a group of heterogeneous antiphospholipid antibodies (Abs, aPL), such as anticardiolipin Abs (aCL) and lupus anticoagulants (LAs), and by the occurrence of thromboembolic complications in the arterial and/or venous vasculature (21, 22). In 1990, it was first reported that a plasma cofactor [22] was subsequently recognized by a mouse monoclonal anti-$\beta_2$-glycoprotein I (23). It was further demonstrated that oxLDL recognition by $\beta_2$-GPI and an anti-$\beta_2$-GPI Abs remains uncertain (30–37).

$\beta_2$-GPI is a member of the short consensus repeats of the complement control protein superfamily, and its fifth domain contains a binding region for negatively charged PLs. X-ray crystal analysis (38) showed that the PL binding is provided by a patch consisting of 14 residues of positively charged amino acids and by a flexible loop between $\beta$11-K117 in domain V. Recent analysis with domain V mutant proteins confirmed interactions of the flexible loop with hydrophobic ligands (39, 40).

Several lines of evidence suggest that the interaction between aPL and mononuclear cell- and platelet-bound LDL (MDA-LDL) may be important in relation to the pathogenesis of atherosclerosis and/or atherothrombosis in APS (41–43). We previously reported that $\beta_2$-GPI bound directly to Cu$^{2+}$-oxLDL, and that the complex of oxLDL and $\beta_2$-GPI was subsequently recognized by a mouse monoclonal anti-$\beta_2$-GPI IgG auto-Ab (WB-CAL-1) established from NZW × BXSB F1 (WB F1) male mouse as a model of APS (44). Uptake of oxLDL by mouse macrophages is significantly increased by phagocytosis of an immune complex consisting of $\beta_2$-GPI, oxLDL, and WB-CAL-1 Ab (44). The major ligand responsible for the $\beta_2$-GPI binding to oxLDL is 7-ketocholesterole9-carboxynonanoate (oxLig-1) (45). It was further demonstrated that oxLDL recognition by $\beta_2$-GPI and an anti-$\beta_2$-GPI Ab, such as WB-CAL-1 Ab and a human monoclonal IgM auto-Ab (EY2C9) derived from an APS patient, requires an $\omega$-carboxyl function introduced by Cu$^{2+}$-oxidation of an unsaturated acyl chain moiety in cholesteryl esters (46). All these observations imply that autoAbs against $\beta_2$-GPI induced in APS patients may be “atherogenic.”

In the present study, we demonstrate that oxLDL forms stable but readily dissociable complexes with $\beta_2$-GPI after an initial noncovalent interaction in vitro. In contrast, stable and nondissociable $\beta_2$-GPI-oxLDL complexes are detected in sera of patients with APS and/or systemic lupus erythematoses (SLE) and are etiologically important. Further, the $\beta_2$-GPI-oxLDL complexes exist as an IgG immune complex in those patients. Clinical analysis indicates that the serum $\beta_2$-GPI-oxLDL complexes are associated with arterial thrombosis.

MATERIALS AND METHODS

Subjects
This study utilized materials from British Caucasian patients with APS and/or SLE [n = 127, 41.0 ± 11.9 years (mean ± SD); range: 16–67 years] who were examined at Lupus Clinic of St. Thomas’ Hospital, London, UK (Table 1). All APS patients were positive for $\beta_2$-GPI-dependent aCL (IgG) and/or LA on two or more occasions at least 6 weeks apart. Clinical records were carefully reviewed retrospectively. One hundred sixteen APS and/or SLE patients were female. Of them, 82 patients fulfilled the new preliminary criteria for APS (47) and seven patients had a history of thrombocytopenia alone. Arterial events comprised stroke, myocardial infarction, and peripheral artery occlusion, confirmed by computed tomography scan, magnetic resonance imaging, or angiography. Deep-vein thrombosis and pulmonary thrombosis were defined as venous thrombosis, confirmed by Doppler ultrasound, venography, or ventilation-perfusion scanning. Pregnancy morbidity was defined according to the preliminary criteria for APS (47). Any patients who had acute thrombosis within 2 months were excluded. Fifty age-matched British Caucasian healthy controls [40.7 ± 14.0 years (mean ± SD); range: 18–66 years] with no history of autoimmune, infectious, or thrombotic diseases were recruited. Informed consent was given for all subjects and the study was approved by both ethics committees of Okayama University Hospital and of St. Thomas’ Hospital.

Monoclonal Abs
Anti-human $\beta_2$-GPI Abs, Cof-22 (IgG1,$\kappa$) and Cof-23 (IgG1,$\kappa$), were established from BALB/c mice immunized with human $\beta_2$-GPI (31). They bind to monomeric $\beta_2$-GPI in solution. Anti-$\beta_2$-GPI auto-Ab, WB-CAL-1 (IgG2a,$\kappa$), was derived from a WB F1 mouse (48). Anti-$\beta_2$-GPI auto-Ab, EY2C9 (IgM), was established from peripheral blood lymphocytes from an APS patient (49). Both WB-CAL-1 and EY2C9 Abs bind only to $\beta_2$-GPI complexed with negatively charged PLs or with oxLDL, but not to monomeric $\beta_2$-GPI in solution. A mouse monoclonal anti-human apoB100 Ab, 1D2 (IgG), was established from BALB/c mouse immunized with human apoB100. The 1D2 Ab reacts with both oxidized and native LDL.

Table 1. Patients’ characteristics

<table>
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<tr>
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<th>n</th>
<th>%</th>
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<td>Auto-Abs</td>
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<tr>
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<tr>
<td>(Anti-$\beta_2$-GPI-CL IgG Abs)</td>
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</tr>
<tr>
<td>Anti-$\beta_2$-GPI IgG Abs</td>
<td>46/127</td>
<td>36.2</td>
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<td>Anti-$\beta_2$-GPI-oxLig-1 IgG Abs</td>
<td>60/127</td>
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<td>Lupus anticoagulants</td>
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<td>$\beta_2$-GPI-oxLDL complexes</td>
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<td>56.7</td>
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</table>

Ab, antibody; aCL, anticardiolipin Abs; APS, antiphospholipid syndrome; $\beta_2$-GPI, $\beta$-glycoprotein I; CL, cardiolipin; oxLDL, oxidized LDL; oxLig-1, 7-ketocholesterole9-carboxynonanoate; SLE, systemic lupus erythematoses.
Preparation of human β2-GPI

β2-GPI was purified from normal human plasma as described (50), with slight modification. Pooled plasma from healthy subjects was sequentially chromatographed on a heparin-Sepharose column, a DEAE-cellulose column, and an anti-β2-GPI affinity column. To remove any contamination by IgGs, the β2-GPI-rich fraction was further passed through a protein A Sepharose column. The final β2-GPI fraction was delipidated by extensive washing with n-butanol.

Isolation and oxidation of LDL

LDL (d = 1.019–1.063 g/ml) was isolated by preparative ultracentrifugation from fresh normal human plasma, as described (51). The LDL was adjusted to 100 mg/dl NaCl (pH 7.4) (Hepes buffer) for various periods at 37°C. To terminate the oxidation, EDTA (final concentration of 1 mM) was added and the LDL was dialyzed against Hepes buffer containing 1 mM EDTA. Protein concentration was determined using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL), and the degree of oxidation was estimated as thiobarbituric acid-reactive substances (TBARS) value (52) and by electrophoretic migration in agarose gels.

Agarose gel electrophoresis

Native or modified LDLs were spotted on an agarose gel film and subjected to electrophoresis in 0.05 M barbitrate buffer (pH 8.6) using the Pol-E-Film System kit (Herena Laboratories, Urawa, Japan).

Synthesis of oxysterol derivatives of 9-carboxynonanoate

oxLig-1 was synthesized, as previously reported (45). 22-Ketocholesteryl-9-carboxynonanoate (9:COOH-22KC) was synthesized in a similar way. Briefly, to a solution of 22-ketocholesterol (10 mg, 0.025 mmol) and azelaic acid (14.1 mg, 0.075 mmol) in acetonitrile (1 ml) were added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (19.2 mg, 0.10 mmol) and 4-(dimethylamino) pyridine (6.1 mg, 0.080 mmol). The mixture was stirred at room temperature for 2 days, concentrated, and extracted with chloroform. The extract was successively washed with 2 M hydrochloric acid and brine, dried over anhydrous magnesium sulfate, and evaporated. The residues were subjected to column chromatography on silica gel using toluene-ethyl acetate (3:1, v/v) to give 9:COOH-22KC (8.3 mg, 61% yield). 1H-NMR (500 MHz, CDCl3): δ = 5.35 (d, 1H, J 5.1 Hz, H6), 4.59 (m, 1H, H-3). Similar to oxLig-1, the 1H-NMR spectrum of 9:COOH-22KC showed a signal assignable to H-3 at δ = 4.59 ppm as a multiplet, suggesting that the hydroxyl group at this position was esterified. Although the spectrum also revealed a signal of olefinic proton at H-6 in lower magnetic field, spin-spin coupling was observed between the neighboring methylene group. The molecular mass of 9:COOH-22KC was identical to that of oxLig-1. The 9:COOH-22KC was positive in the Lieberman-Burchard reaction, indicating a conjugated ketone at position 7 is not present.

Ligand blot analysis on a TLC plate

For TLC ligand blotting, lipids were spotted on a Polygram silica gel G plate (Machery-Nagel, Duren, Germany) and developed in chloroform-methanol (8:1, v/v). Ligand blot analysis was performed, as described previously (45, 46). Briefly, after drying and blocking with PBS containing 1% BSA, the plate was subsequently and simultaneously incubated with β2-GPI and anti-β2-GPI Ab (Cof-22 and EY2C9, respectively) for 1 h. Subsequently, the plate was incubated with horseradish peroxidase (HRP)-labeled anti-mouse IgG or anti-human IgM for 1 h. In between each step, the plates were extensively washed with PBS. The color was developed with H2O2 and 4-methoxy-1-naphthol. On a control TLC plate, separated ligands were stained with I2 vapor.

ELISA for β2-GPI-oxLDL complexes

Anti-β2-GPI Ab (WB-CAL-1) was adsorbed on a microtiter plate (Immunon 2HB, Dynex Technologies, Inc., Chantilly, VA) by incubating at 8 μg/ml (dissolved in Hepes buffer, 50 μl/well) at 4°C overnight. The plate was blocked with 1% skim milk for 1 h. Serum samples (100-fold diluted) or solutions containing β2-GPI-oxLDL complexes or oxLDL were added to the wells (100 μl/well) and incubated for 2 h. For some experiments, exogenous β2-GPI (25 μg/ml) was present in this step. The wells were subsequently incubated with biotinyl-anti-apoB100 Ab (1D2) for 1 h and HRP-labeled avidin for 30 min. Color was developed with o-phenylenediamine and H2O2. The reaction was terminated by adding 2 N sulfuric acid, and the OD at 490 nm was measured. Between each step, extensive washing was performed using Hepes buffer containing 0.05% Tween 20. Raw OD of samples in individual assays was corrected by mean OD of the blank wells. When 1.0 U/ml was adjusted to 3 SD above the mean of serum samples from 50 normal subjects, 1.0 U/ml of the oxLDL12 h-β2-GPI16 h complex was equilibrated to ~4.5 μg/ml of apoB equivalent. A sample was considered positive when its reactivity was higher than 1.0 U/ml.

ELISA for anti-β2-GPI-lipid IgG Abs

CL (from bovine heart, Sigma Chemical Co.), oxLig-1, or 9:COOH-22KC (50 μg/ml in ethanol, 50 μl/well) was adsorbed by evaporation on a plain polystyrene plate (Immunon 1B), and the plate was then blocked with 1% BSA. Purified monoclonal auto-Abs or serum samples (100-fold diluted) were incubated in the wells with or without β2-GPI (25 μg/ml) for 1 h, and HRP-labeled anti-mouse IgG or anti-human IgG or IgM was then added. Further steps were performed as described in “ELISA for β2-GPI-oxLDL complexes.” Raw OD of individual samples was corrected by mean OD of the blank wells. OD variation among plates was normalized by using a positive control. A sample was considered to be positive when its Ab titer was higher than 3 SD above the mean OD of plasma samples of 50 normal subjects.

ELISA for anti-β2-GPI IgG Abs

ELISA for anti-β2-GPI IgG Abs was performed as described (30). Briefly, β2-GPI was adsorbed on polyoxygenated polystyrene plates (carboxylated, Sumilon C, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) by incubating at 10 μg/ml (50 μl/well) at 4°C, overnight, and the plates were blocked with 3% gelatin. Serum samples were diluted 100-fold and incubated in the wells for 1 h. HRP-labeled anti-human IgG was then added to the plates. Further steps were performed as described in “ELISA for β2-GPI-oxLDL complexes.”

ELISA for IgG immune complexes

To determine ELISA for IgG immune complexes (IgG IC) formed with β2-GPI or LDL, anti-β2-GPI Ab (Cof-23) or anti-apoB100 Ab (1D2) was adsorbed on plain polystyrene plates (Immunon 1B) by incubating overnight at 5 μg/ml (50 μl/well) at 4°C. The plates were then blocked with 1% BSA. Serum samples (100-fold diluted) were incubated in the wells for 1 h and HRP-labeled anti-human IgG was added. Further steps were performed as described in “ELISA for β2-GPI-oxLDL complexes.”

Statistical analysis

Statistical analysis was performed by StatView software (Abacus Concepts, Berkeley, CA). Fisher’s exact test was used to compare the occurrence of auto-Abs and clinical histories. Ninety-five percent confidence interval (95% CI) was calculated by Woolf’s method.
RESULTS

Role of 7-ketone function as a ligand for \( \beta_2 \)-GPI binding

We compared the binding of \( \beta_2 \)-GPI to two positional ketone variants of \( \omega \)-carboxyl oxysterol esters (i.e., oxLig-1 and 9-COOH-22KC) in ligand blot and ELISA using anti-\( \beta_2 \)-GPI Abs as a probe. \( \beta_2 \)-GPI preferentially bound to the 7-keto-variant (oxLig-1) but not to 9-COOH-22KC in the ligand blot as detected by Cof-22 or EY2C9 Ab (Fig. 1). In the ELISA using a ligand-coated plate, \( \beta_2 \)-GPI binding to solid-phase oxLig-1 rather than 9-COOH-22KC was detected with anti-\( \beta_2 \)-GPI Abs (Cof-22, WB-CAL-1, or EY2C9) (Table 2). These data demonstrate that the ketone function at position 7 of the cholesterol backbone is a critical determinant for high-affinity interaction between \( \beta_2 \)-GPI and its ligands, e.g., oxLig-1, derived from Cu\(^{2+} \)-mediated oxLDL.

\( \beta_2 \)-GPI interaction with LDL undergoing Cu\(^{2+} \)-mediated oxidation

LDL (100 \( \mu \)g/ml of apoB equivalent) was oxidized by incubating with 5 \( \mu \)M CuSO\(_4\) for 12 h at 37\( ^\circ \)C (oxLDL\( _{12 \text{ h}} \)), and the oxidation was terminated by addition of EDTA. In ELISA for detecting \( \beta_2 \)-GPI-oxLDL complexes, the OD was increased only when oxLDL\( _{12 \text{ h}} \) was incubated with exogenous \( \beta_2 \)-GPI in the assay wells. The formation of complexes was dependent upon the concentration of both \( \beta_2 \)-GPI and oxLDL (Fig. 2A, B). Significant complex formation occurred only with oxLDL\( _{12 \text{ h}} \) and not with native LDL. Complex formation at pH 7.4 was almost completely inhibited in the presence of heparin or MgCl\(_2\) (Fig. 2C). The inhibition was also observed with CaCl\(_2\) in the same manner (data not shown). These data indicate that \( \beta_2 \)-GPI can initially form dissociable noncovalent complexes with oxLDL\( _{12 \text{ h}} \). In contrast, relatively stable complexes between oxLDL and \( \beta_2 \)-GPI were consistently observed when oxLDL\( _{12 \text{ h}} \) was incubated at pH 7.4 with \( \beta_2 \)-GPI for 16 h at 37\( ^\circ \)C (oxLDL\( _{12 \text{ h}} \)-\( \beta_2 \)-GPI\( _{16 \text{ h}} \)). The subsequent addition of heparin or MgCl\(_2\) at pH 7.4 failed to disrupt oxLDL\( _{12 \text{ h}} \)-\( \beta_2 \)-GPI

![Fig. 1. Ligand blot analysis on two \( \omega \)-carboxyl variants of oxysterol ester 7-ketocholesterol-9-carboxynonoate (oxLig-1) and 22-ketocholesterol-9-carboxynonoate. The developed ligands on a TLC plate were stained with I\(_2\) vapor (A) and ligand blot was performed with anti-\( \beta_2 \)-glycoprotein 1 (\( \beta_2 \)-GPI) antibodies (Abs), i.e., Cof-22 (B) and EY2C9 (C).](image)

![Fig. 2. Profiles of complex formation between Cu\(^{2+} \)-mediated oxidized LDL (oxLDL) and \( \beta_2 \)-GPI. A: oxLDL\( _{12 \text{ h}} \) [0 (open triangles), 0.16 (open squares), or 2.5 \( \mu \)g/ml of apolipoprotein B (apoB) equivalent (open circles)] was incubated with various concentrations of \( \beta_2 \)-GPI in the assay wells, and ELISA for \( \beta_2 \)-GPI-oxLDL complexes was performed. B: Indicated concentrations of oxLDL\( _{12 \text{ h}} \) (LDL treated with 5 \( \mu \)M CuSO\(_4\) for 12 h at 37\( ^\circ \)C, circles) or native LDL (squares) were incubated in the absence (open symbols) or presence (25 \( \mu \)g/ml, closed symbols) of \( \beta_2 \)-GPI, and the ELISA was performed in the absence (open circles), or presence of heparin (100 U/ml; closed squares) or MgCl\(_2\) (10 mM; closed diamonds). D: oxLDL\( _{12 \text{ h}} \)-\( \beta_2 \)-GPI\( _{16 \text{ h}} \) complexes were prepared by incubating oxLDL\( _{12 \text{ h}} \) (100 \( \mu \)g/ml) with \( \beta_2 \)-GPI (100 \( \mu \)g/ml) at 37\( ^\circ \)C for 16 h. The ELISA was performed with the complexes (2.5 \( \mu \)g/ml of apoB equivalent) in the absence (open circles) or presence of heparin (100 U/ml; closed squares) or MgCl\(_2\) (10 mM; closed diamonds). Results are expressed as the mean \( \pm \) SD of triplicate samples.](image)

TABLE 2. \( \beta_2 \)-GPI binding to solid phase \( \omega \)-carboxyl variants of oxysterol ester, detecting in ELISA with anti-\( \beta_2 \)-GPI Abs

<table>
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<tr>
<th>Solid Phase Lipid</th>
<th>With Cof-22</th>
<th>With WB-CAL-1</th>
<th>With EY2C9</th>
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<tr>
<td>oxLig-1</td>
<td>1.194 ± 0.099</td>
<td>0.441 ± 0.007</td>
<td>0.878 ± 0.031</td>
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<td>(0.041 ± 0.001)</td>
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<tr>
<td>9-COOH-22KC</td>
<td>0.217 ± 0.016</td>
<td>0.065 ± 0.004</td>
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<td>(0.067 ± 0.013)</td>
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| 9-COOH-22KC, 22-ketocholesterol-9-carboxynonoate: Lipid coated plates were subsequently incubated with \( \beta_2 \)-GPI and Cof-22 Ab or were simultaneously incubated with \( \beta_2 \)-GPI and WB-CAL-1 or EY2C9 Ab. Binding to the solid phase lipid in the absence of \( \beta_2 \)-GPI is indicated in parentheses. Data are indicated as the OD (mean \( \pm \) SD of triplicate samples).
CuSO₄-treated LDL. The TBARS were rapidly generated in LDL preparations exposed to the Cu²⁺ ion at 37°C, with a peak at 4 h. In contrast, oxidation of LDL that generated the β₂-GPI binding proceeded with a lag and reached its maximum after ~12 h (Fig. 4B). The complex formation was almost completely inhibited by the addition of heparin or MgCl₂. These data are consistent with our previous observations demonstrating that β₂-GPI binds to Cu²⁺-oxLDL but not to MDA-modified LDL (44).

The preformed oxLDL₁₂ h (final concentration at 100 μg/ml of apoB equivalent) was also incubated with β₂-GPI (100 μg/ml) for different periods at 4°C or 37°C (Fig. 4C). The formation of β₂-GPI-oxLDL complexes was temperature- and time-dependent. The complexes were not dissociated by the addition of heparin or MgCl₂ after the incubation at pH 7.4. Figure 4D indicates that the stable interaction between β₂-GPI and oxLDL was generated during the Cu²⁺-oxidation process even in the presence of β₂-GPI.

Stability of in vitro β₂-GPI-oxLDL complexes at different pHs

The stable complexes appeared at neutral pH and are possibly Schiff-base adducts formed between ε-amines of lysine residues of β₂-GPI and oxidatively generated aldehydes on the Cu²⁺-mediated oxLDL vesicles. To test this, we analyzed the stability of nonreduced and NaCNBH₃-reduced complexes at basic pH conditions. As shown in Fig. 5, no dissociation was observed in the reduced oxLDL₁₂ h β₂-GPI₁₆ h complexes at any pH conditions tested in the absence of MgCl₂. In the presence of MgCl₂, 82% of nonreduced complexes dissociated at pH 10, whereas 69% of reduced complexes dissociated. The stable complexes may be formed by both electrostatic interaction and Schiff-base formation between an oxidized moiety on Cu²⁺-oxLDL and the PL binding patch on the β₂-GPI molecule that is composed of 14 positively charged amino acid residues and a hydrophobic loop. These findings also indicate that the adduct is either not a Schiff base, or if it is a Schiff base, it resides in an environment that is not accessible to NaCNBH₃ (e.g., a hydrophobic pocket).

Nondissociable β₂-GPI-oxLDL complexes exist in patient sera

We screened serum samples from patients with APS and/or SLE for high levels of β₂-GPI-oxLDL complexes. β₂-GPI-oxLDL complexes were previously characterized in 20 sera. This group showed high concentrations of serum complexes with a range of 2.1–13.7 U/ml, and a mean concentration of 4.48 U/ml (cutoff value: 1.0 U/ml). As shown in Fig. 6, native LDL did not form complexes upon incubation with β₂-GPI at 37°C for 16 h. In contrast, oxLDL₁₂ h β₂-GPI₁₆ h complexes were stable at pH 7.4, even in the presence of heparin or MgCl₂. The typical binding pattern was also shown for preexisting oxLDL-β₂-GPI complexes detected in five serum samples at pH 7.4. In all 20 tested samples, the complexes that were preformed in vivo were stable at neutral pH, even in the presence of heparin and MgCl₂ (The ODs in the cases with heparin and MgCl₂ were 121 ± 25.1% and 128 ± 13.6%
of control condition, respectively). The preformed complexes present in serum samples were also consistently observed after the 16 h-incubation with MgCl2 at pH 10 at 37°C (104 ± 10.9%), that can dissociate the complexes formed in vitro (Fig. 5). We interpret these findings to indicate that nondissociable and covalent adducts between β2-GPI and in vivo oxLDL are formed. We propose that our in vitro adducts are intermediates in the formation of the nondissociable complexes.

**Clinical significance of β2-GPI-oxLDL complex and its auto-Ab**

In the ELISA, we obtained an apparent calibration curve for oxLDL12h-β2-GPI16h complexes within a range of 10 ng/ml to 1.25 μg/ml of apoB equivalent. The ELISA was not affected by the high concentration of endogenous and monomeric β2-GPI in serum samples, because WB-CAL-1 Ab used in the ELISA is highly specific for β2-GPI complexed with oxLDL. In the present study, the β2-GPI-oxLDL complexes were positive in 58.7% (27/46), 54.1% (20/37), and 56.8% (25/44) of patients with the primary APS, APS with SLE (secondary APS), and SLE without APS, respectively (Fig. 7).

Anti-β2-GPI-oxLig-1 IgG Abs were found in 71.7% (33/46), 59.5% (22/37), and 11.4% (5/44) of patients with the primary APS, APS with SLE (secondary APS), and SLE without APS, respectively. The individual anti-β2-GPI-oxLig-1 IgG Ab titers from this group of 127 patients are strongly correlated with both β2-GPI-dependent IgG aCL and anti-β2-GPI IgG Abs (correlation coefficient; r² = 0.69 and 0.81, respectively) (Fig. 8). As shown in Fig. 9, there also was a good correlation between IgG IC with β2-GPI and anti-β2-GPI IgG Abs (r² = 0.50) (Fig. 9A), IgG IC with β2-GPI and anti-β2-GPI-oxLig-1 IgG Abs (r² = 0.50) (Fig. 9B), and IgG IC with LDL and IgG IC with β2-GPI (r² = 0.40) (Fig. 9C). However, a good correlation between levels of β2-GPI-oxLDL complex and titers of any of these Abs was not observed (data not shown).

In Table 3, the correlation between anti-β2-GPI-oxLig-1 IgG Abs (not β2-GPI-oxLig-1 complex antigen) and thrombosis was calculated and the relative risk of having thrombosis was approximated by odds ratio. The first line showed the correlation between Abs and all thrombosis in all 127 patients; therefore the referent was patients without any thrombosis. Abs were correlated with thrombosis among β2-GPI-oxLDL the complex antigen-positive patients’ group (the second line) and among the antigen-negative patients’ group as well (the third line). The correlation between anti-β2-GPI-oxLig-1 IgG Abs and arterial and venous thrombosis was presented in (B) and (C) in the same fashion, respectively. The relative risk in the β2-GPI-oxLDL antigen-positive patients’ group was higher than that in the antigen-negative patients’ group. It is of interest because the presence of β2-GPI-oxLDL antigen may be an additional risk of having arterial thrombosis in patients with anti-β2-GPI-oxLDL Abs.

**DISCUSSION**

We previously reported that the major lipid ligands responsible for β2-GPI binding to Cu²⁺-mediated oxLDL are ω-carboxylated 7-ketocholesterol esters such as oxLig-1, and that the ω-carboxyl moiety is also essential for β2-GPI recognition (45, 46). The in vitro interaction between β2-GPI and Cu²⁺-oxLDL is initially reversible by Mg²⁺ treatment but progresses to a much more stable interaction requiring Mg²⁺ and a high pH to be dissociated. In contrast, stable and nondissociable complexes between oxLDL and β2-GPI are found in serum samples from patients with APS and/or SLE. We further detected the complexes as IgG-immune complexes containing LDL and...
2-GPI in sera from those patients, and statistical analysis indicates that the serum 2-GPI-oxLDL complexes are associated with arterial thrombosis.

Foam cell formation is regarded as the hallmark of early atherosclerosis, and LDL is the major source of lipids deposited in these cells. The binding of modified LDL to scavenger receptors on macrophages leads to unregulated cholesterol accumulation and the formation of foam cells with development of atherosclerotic lesions. Recently, we identified the structure of two major ligands, which provide 2-GPI binding to Cu^{2+}-oxLDL and anti-2-GPI Ab mediated-phagocytosis by macrophages, to be oxLig-1 and 7-ketocholesteryl-12-carboxy (keto) dodecanoate (oxLig-2) (45, 46). In the present study, we demonstrated that the conjugated ketone function at position 7 of the cholesterol backbone of the ligands is required for high-affinity binding for 2-GPI and cannot be replaced by a ketone at the 22 position (Fig. 1 and Table 2).

A patch consisting of 14 positively charged amino acid residues, and a flexible loop in domain V of 2-GPI were reported to be critical for interaction with amphiphilic compounds such as CL, phosphatidylserine, phosphatidic acid, and phosphatidylycerine (38–40). We previously reported that an interaction with oxLDL was also provided by the same binding site of 2-GPI (53). The conjugated ketone function at position 7 of the cholesterol backbone of the ligands is required for high-affinity binding for 2-GPI and cannot be replaced by a ketone at the 22 position (Fig. 1 and Table 2).

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β₂-GPI in sera from those patients, and statistical analysis indicates that the serum β₂-GPI-oxLDL complexes are associated with arterial thrombosis.

Fig. 7. Serum levels of β₂-GPI-oxLDL complexes detected in ELISA. β₂-GPI-oxLDL complexes were detected in 100-fold diluted sera from normal subjects and patients with the primary antiphospholipid syndrome (PAPS), APS with systemic lupus erythematoses (SLE) (the secondary APS), and SLE without APS. Cutoff value (1 U/ml) was adjusted to 3 SD above the mean levels of 50 normal subjects. A number indicates mean level in each subject group.

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Fig. 8. Correlation among β₂-GPI-related IgG Ab titers detected in three different ELISA systems. A: β₂-GPI-dependent IgG antcardiolipin Abs (anti-β₂-GPI-cardiolipin IgG Abs) versus anti-β₂-GPI-oxLig-1 IgG Abs. B: Anti-β₂-GPI IgG Abs (detected in ELISA using a β₂-GPI-coated polyoxygenated plate) versus anti-β₂-GPI-oxLig-1 IgG Abs.
NaCNBH₃ (i.e., 200 mM) was ineffective for reduction of the imine in Schiff-base adducts. This result raises the possibility that the stable and nondissociable complexes between oxLDL and β₂-GPI may be generated by other mechanisms, such as the Michael reaction or direct oxidation of lysine residues by alkoxyl radicals of polyunsaturated fatty acids.

In the present study, we demonstrate that oxLDL circulates in patients with APS and/or SLE (54.1–58.7%) in stable and nondissociable complexes with β₂-GPI (Fig. 7). Many reports demonstrate that oxLDL is preferentially taken up by macrophages via scavenger receptors and lead to foam cell formation and development of atherosclerotic lesions. However, there is incomplete information about oxLDL circulating in the blood stream of patients with atherosclerosis. Even though we did not measure the free form of oxLDL in patient sera, it is likely that oxLDL generated in vivo is complexed with endogenous β₂-GPI (the plasma concentration of β₂-GPI is ~200 μg/ml). As shown in Fig. 4D, in the presence of β₂-GPI, LDL that underwent in vitro oxidation formed stable adducts with increasing incubation time at neutral pH. Furthermore, the stable interaction between β₂-GPI and oxLDL was observed under several different in vitro conditions, including in buffer alone or in buffer containing 1% BSA or 50% human serum (data not shown). Thus, β₂-GPI ligands related to oxLig-1 and oxLig-2 provide specific interaction between β₂-GPI and oxLDL to form stable complexes in the presence of excess levels of other plasma/serum proteins.

The association of aPL with serious clinical complications such as arterial and/or venous thrombosis, recurrent fetal loss, and thrombocytopenia has been established in patients with APS. aCLs were initially considered to be directed to acidic PLs such as CL, but now it is widely accepted that β₂-GPI is the true antigen for aCL. In 1998, we showed that anti-β₂-GPI IgG Abs could be a serologic marker for arterial thrombosis in SLE patients, while anti-MDA-LDL IgG Abs were not associated with arterial thrombosis (43). In the present study, we demonstrate a good correlation among titers of anti-β₂-GPI-CL IgG Abs, anti-β₂-GPI IgG Abs, and anti-β₂-GPI-oxLig-1 IgG Abs (Fig. 8). The appearance of anti-β₂-GPI-oxLig-1 IgG Abs was better correlated with a history of arterial thrombosis rather than with venous thrombosis (Table 3). These findings suggest that β₂-GPI-oxLig-1 (i.e., β₂-GPI-oxLDL) complexes may be the true target antigen for the previously characterized aCL. The anti-β₂-GPI-oxLig-1 IgG Abs appears to be an excellent candidate for inducing autoimmune-mediated atherothrombosis/atherosclerosis.

However, when all tested APS/SLE patients were divided into two subgroups, i.e., the β₂-GPI-oxLDL complex positive and negative, a stronger association between anti-β₂-GPI-oxLig-1 Ab and episodes of those clinical manifestations was observed in the positive subgroup than in the negative one. In auto-Ab-positive APS patients, IgG immune complexes with β₂-GPI and LDL were also detected. Although the mechanisms of in vivo oxidation of LDL remain unclear, the resultant β₂-GPI-oxLDL complexes may have a pathogenic role as an autoantigen to induce the

**TABLE 3.** Association with anti-β₂-GPI-oxLig-1 IgG Abs and thrombosis in APS

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Fisher’s Exact Test (P)</th>
<th>Odds Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombosis (arterial and/or venous)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients (total, n = 127)</td>
<td>1.7 × 10⁻⁷</td>
<td>7.65</td>
<td>3.41–17.2</td>
</tr>
<tr>
<td>(β₂-GPI-oxLDL positive, n = 72)</td>
<td>5.9 × 10⁻⁵</td>
<td>8.21</td>
<td>2.79–24.2</td>
</tr>
<tr>
<td>(β₂-GPI-oxLDL negative, n = 55)</td>
<td>0.0014</td>
<td>6.87</td>
<td>2.01–23.5</td>
</tr>
<tr>
<td>Arterial thrombosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients (total, n = 127)</td>
<td>6.9 × 10⁻⁷</td>
<td>7.45</td>
<td>3.21–17.3</td>
</tr>
<tr>
<td>(β₂-GPI-oxLDL positive, n = 72)</td>
<td>4.8 × 10⁻⁵</td>
<td>10.2</td>
<td>2.98–34.7</td>
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<tr>
<td>(β₂-GPI-oxLDL negative, n = 55)</td>
<td>0.0043</td>
<td>5.63</td>
<td>1.68–18.9</td>
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<tr>
<td>Venous thrombosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients (total, n = 127)</td>
<td>0.026</td>
<td>2.23</td>
<td>1.06–4.68</td>
</tr>
<tr>
<td>(β₂-GPI-oxLDL positive, n = 72)</td>
<td>0.066</td>
<td>2.37</td>
<td>0.89–6.29</td>
</tr>
<tr>
<td>(β₂-GPI-oxLDL negative, n = 55)</td>
<td>0.20</td>
<td>1.93</td>
<td>0.63–6.14</td>
</tr>
</tbody>
</table>
development of thrombosis, especially arterial thrombosis, in APS. The ELISA methodology using solid-phase native or ox-LDL to measure Abs against oxLDLs and/or to measure IC with LDL is problematic. In previous reports (58–60), competitive ELISA for anti-oxLDL Abs and preparation of samples with polyethylene glycol for their detection have been proposed to minimize nonspecific binding of Abs to LDL solid phases. The system described in this report has relatively low nonspecific binding because the stable ox-LDL-β2-GPI complexes formed in vitro do not have the high negative charge of Cu²⁺-ox-LDL. Furthermore, we applied two types of Ab-capture ELISAs using anti-β2-GPI Ab and anti-apoB100 for detecting IgG IC with β2-GPI and IgG IC with LDL, respectively. These two ELISAs are not affected by high titers of rheumatoid factors and endogenous levels of β2-GPI. Although extremely high levels of lipids (>350 mg/dl of total cholesterol, i.e., in cases of familial hypercholesterolemia) can exert a dose-dependent effect on IC levels, this was not a problem for the current study, since none of the patients were hypercholesterolemic (>300 mg/dl). As shown in Fig. 9, there were statistically significant correlations between anti-β2-GPI IgG and IgG IC with β2-GPI, between anti-β2-GPI-oxLig-1 IgG and IgG IC with β2-GPI, and between IgG IC with β2-GPI and IgG IC with LDL (ox-LDL). All of these correlations indicate that the presence of IgG (anti-β2-GPI) IC with the β2-GPI-oxLDL (ox-LDL) complexes in the APS sera. In addition, the contaminated IgG (anti-oxLDL) IC with LDL could not be excluded.

George et al. reported that LDL-receptor-deficient mice fed a chow diet and immunized with β2-GPI had accelerated atherosclerosis (61). β2-GPI was abundant within subendothelial regions and intimal-medial borders of human atherosclerotic plaques, and colocalized with monocytes and CD4-positive lymphocytes (62). Thus, there is increasing circumstantial evidence of an autoimmune mechanism involving β2-GPI and oxLDL in the atherogenesis of APS. This is the first report that stable and nondissociable β2-GPI-oxLDL complexes are found in patient sera and that the complexes may be a quantifiable risk factor for arterial thrombosis in APS. However, the β2-GPI-oxLDL complexes were found not only in APS but also in the Ab-negative and nonthrombotic SLE and chronic nephritis (data not shown). The observation indicates that the serum complex level alone does not predict clinical manifestation in APS. It is understood that abnormalities in lipid and lipoprotein metabolism are commonly associated with diverse renal diseases and that hyperlipidemia and increased plasma lipoproteins such as LDL contribute to the high incidence of atherosclerotic cardiovascular events and mortality noted in patients with renal disease. These findings also raise important new issues about the clinical significance of circulating β2-GPI-oxLDL complexes in blood stream of patients with coronary artery diseases.

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