

# 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: 697, 2001; *J. Lipid Res.*, 43: 1486, 2002) that  $\beta_2$ -GPI specifically binds to  $\text{Cu}^{2+}$ -oxidized LDL (oxLDL) and that the  $\beta_2$ -GPI ligands are  $\omega$ -carboxylated 7-ketocholesteryl 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 erythematoses. 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 oxi-

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Abbreviations: Ab, antibody; APS, antiphospholipid syndrome;  $\beta_2$ -GPI,  $\beta_2$ -glycoprotein I; oxLDL, oxidized LDL; PL, phospholipid.

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dized phosphatidylcholine, i.e., 1-palmitoyl-2-(5'-oxo)valeroyl-*sn*-glycero-3-phosphorylcholine and lysine, and its  $\beta$ -hydroxyaldehyde (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 vasculatures (21, 22). In 1990, it was first reported that a plasma cofactor [ $\beta_2$ -glycoprotein I ( $\beta_2$ -GPI)] complexed with negatively charged PLs such as cardiolipin (CL) was an antigenic target for aCL (23–25).  $\beta_2$ -GPI is a 50 kDa protein present in plasma at a concentration of  $\sim 200$   $\mu\text{g}/\text{ml}$ . It binds to negatively charged molecules, including PLs (26) and heparin (27), and to plasma membranes of activated platelets and apoptotic cells on which phosphatidylserine is exposed (28, 29). However, the exact mechanism of the interaction between  $\beta_2$ -GPI and 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 S<sup>311</sup>-K<sup>317</sup> 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 malondialdehyde-modified 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<sup>2+</sup>-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  $\times$  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-ketocholesteryl-9-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<sup>2+</sup>-oxidation of an unsaturated acyl chain moiety in cholesteryl esters (46). All these observations imply that auto-Abs 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.

## Subjects

This study utilized materials from British Caucasian patients with APS and/or SLE [ $n = 127$ ,  $41.0 \pm 11.9$  years (mean  $\pm$  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 [ $40.7 \pm 14.0$  years (mean  $\pm$  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 apoB-100 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

	n	%
Patients		
SLE only	44	
APS	83	
Primary	46	55.4
Secondary	37	44.6
Clinical profile		
Thrombosis	71	55.9
Arterial only	26	20.5
Venous only	27	21.3
Arterial + venous	18	14.2
Pregnancy morbidity	31/116	26.7
Thrombocytopenia	23/123	18.7
Auto-Abs		
$\beta_2$ -GPI-dependent IgG aCL (Anti- $\beta_2$ -GPI-CL IgG Abs)	73/127	57.5
Anti- $\beta_2$ -GPI IgG Abs	46/127	36.2
Anti- $\beta_2$ -GPI-oxLig-1 IgG Abs	60/127	47.2
Lupus anticoagulants	59/108	54.6
$\beta_2$ -GPI-oxLDL complexes	72/127	56.7

Ab, antibody; aCL, anticardiolipin Abs; APS, antiphospholipid syndrome;  $\beta_2$ -GPI,  $\beta_2$ -glycoprotein I; CL, cardiolipin; oxLDL, oxidized LDL; oxLig-1, 7-ketocholesteryl-9-carboxynonanoate; SLE, systemic lupus erythematoses.

## Preparation of human $\beta_2$ -GPI

$\beta_2$ -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- $\beta_2$ -GPI affinity column. To remove any contamination by IgGs, the  $\beta_2$ -GPI-rich fraction was further passed through a protein A Sepharose column. The final  $\beta_2$ -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  $100 \mu\text{g/ml}$  of apoB equivalent and oxidized with  $5 \mu\text{M}$   $\text{CuSO}_4$  in  $10 \text{ mM}$  Hepes and  $150 \text{ mM}$  NaCl (pH 7.4) (Hepes buffer) for various periods at  $37^\circ\text{C}$ . To terminate the oxidation, EDTA (final concentration of  $1 \text{ mM}$ ) was added and the LDL was dialyzed against Hepes buffer containing  $1 \text{ mM}$  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  $0.05 \text{ M}$  barbital 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 ( $10 \text{ mg}$ ,  $0.025 \text{ mmol}$ ) and azelaic acid ( $14.1 \text{ mg}$ ,  $0.075 \text{ mmol}$ ) in acetone ( $1 \text{ ml}$ ) were added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride ( $19.2 \text{ mg}$ ,  $0.10 \text{ mmol}$ ) and 4-(dimethylamino) pyridine ( $6.1 \text{ mg}$ ,  $0.80 \text{ mmol}$ ). The mixture was stirred at room temperature for 2 days, concentrated, and extracted with chloroform. The extract was successively washed with  $2 \text{ M}$  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 ( $8.5 \text{ mg}$ ,  $61\%$  yield).  $^1\text{H-NMR}$  ( $300 \text{ MHz}$ ,  $\text{CDCl}_3$ ):  $\delta = 5.35$  (d,  $^1\text{H}$ , J 5.1 Hz, H-6), 4.59 (m,  $^1\text{H}$ , H-3).

Similar to oxLig-1, the  $^1\text{H-NMR}$  spectrum of 9-COOH-22KC showed a signal assignable to H-3 at  $\delta = 4.59 \text{ ppm}$  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  $\beta_2$ -GPI and anti- $\beta_2$ -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  $\text{H}_2\text{O}_2$  and 4-methoxy-1-naphthol. On a control TLC plate, separated ligands were stained with I2 vapor.

## ELISA for $\beta_2$ -GPI-oxLDL complexes

Anti- $\beta_2$ -GPI Ab (WB-CAL-1) was adsorbed on a microtiter plate (Immulon 2HB, Dynex Technologies, INC., Chantilly, VA) by incubating at  $8 \mu\text{g/ml}$  (dissolved in Hepes buffer,  $50 \mu\text{l/well}$ ) at  $4^\circ\text{C}$  overnight. The plate was blocked with 1% skim milk for 1 h. Serum samples (100-fold diluted) or solutions containing  $\beta_2$ -GPI-oxLDL complexes or oxLDL were added to the wells ( $100 \mu\text{l/well}$ ) and incubated for 2 h. For some experiments, exogenous  $\beta_2$ -GPI ( $25 \mu\text{g/ml}$ ) was present in this step. The wells were subsequently incubated with biotinyl-anti-apoB-100 Ab (1D2) for 1 h and HRP-labeled avidin for 30 min. Color was developed with *o*-phenylenediamine and  $\text{H}_2\text{O}_2$ . The reaction was terminated by adding 2 N sulfuric acid, and the OD at  $490 \text{ nm}$  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  $1.0 \text{ U/ml}$  was adjusted to 3 SD above the mean of serum samples from 50 normal subjects,  $1.0 \text{ U/ml}$  of the oxLDL<sub>12 h</sub>- $\beta_2$ -GPI<sub>16 h</sub> complex was equilibrated to  $\sim 4.5 \mu\text{g/ml}$  of apoB equivalent. A sample was considered positive when its reactivity was higher than  $1.0 \text{ U/ml}$ .

## ELISA for anti- $\beta_2$ -GPI-lipid IgG Abs

CL (from bovine heart, Sigma Chemical Co.), oxLig-1, or 9-COOH-22KC ( $50 \mu\text{g/ml}$  in ethanol,  $50 \mu\text{l/well}$ ) was adsorbed by evaporation on a plain polystyrene plate (Immulon 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  $\beta_2$ -GPI ( $25 \mu\text{g/ml}$ ) 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  $\beta_2$ -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- $\beta_2$ -GPI IgG Abs

ELISA for anti- $\beta_2$ -GPI IgG Abs was performed as described (30). Briefly,  $\beta_2$ -GPI was adsorbed on polyoxygenated polystyrene plates (carboxylated, Sumilon C, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) by incubating at  $10 \mu\text{g/ml}$  ( $50 \mu\text{l/well}$ ) at  $4^\circ\text{C}$ , 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  $\beta_2$ -GPI-oxLDL complexes."

## ELISA for IgG immune complexes

To determine ELISA for IgG immune complexes (IgG IC) formed with  $\beta_2$ -GPI or LDL, anti- $\beta_2$ -GPI Ab (Cof-23) or anti-apoB100 Ab (1D2) was adsorbed on plain polystyrene plates (Immulon 1B) by incubating overnight at  $5 \mu\text{g/ml}$  ( $50 \mu\text{l/well}$ ) at  $4^\circ\text{C}$ . 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  $\beta_2$ -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.

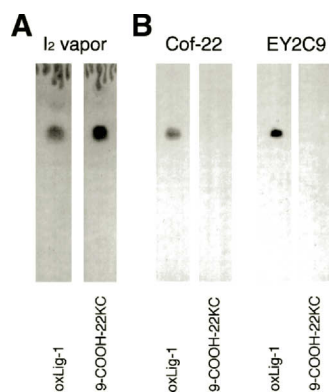
## RESULTS

### 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  $\text{Cu}^{2+}$ -mediated oxLDL.

### $\beta_2$ -GPI interaction with LDL undergoing $\text{Cu}^{2+}$ -mediated oxidation

LDL (100  $\mu\text{g}/\text{ml}$  of apoB equivalent) was oxidized by incubating with 5  $\mu\text{M}$   $\text{CuSO}_4$  for 12 h at 37°C (oxLDL<sub>12h</sub>), 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<sub>12h</sub> 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<sub>12h</sub> and not with native LDL. Complex formation at pH 7.4 was almost completely inhibited in the presence of heparin or  $\text{MgCl}_2$  (Fig. 2C). The inhibition was also observed with  $\text{CaCl}_2$  in the same manner (data not shown). These data indicate that  $\beta_2$ -GPI can initially form dissociable noncovalent complexes with oxLDL<sub>12h</sub>. In contrast, relatively stable complexes between oxLDL and  $\beta_2$ -GPI were consistently observed when oxLDL<sub>12h</sub> was incubated at pH 7.4 with  $\beta_2$ -GPI for 16 h at 37°C (oxLDL<sub>12h</sub>- $\beta_2$ -GPI<sub>16h</sub>). The subsequent addition of heparin or  $\text{MgCl}_2$  at pH 7.4 failed to disrupt oxLDL<sub>12h</sub>- $\beta_2$ -



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

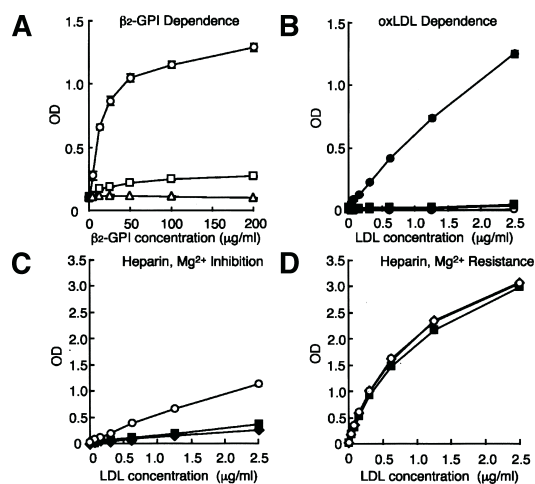
**TABLE 2.**  $\beta_2$ -GPI binding to solid phase  $\omega$ -carboxyl variants of oxysterol ester, detecting in ELISA with anti- $\beta_2$ -GPI Abs

Solid Phase Lipid	$\beta_2$ -GPI Binding (OD)		
	With Cof-22	With WB-CAL-1	With EY2C9
oxLig-1	1.194 $\pm$ 0.099 (0.041 $\pm$ 0.001)	0.441 $\pm$ 0.007 (0.013 $\pm$ 0.004)	0.878 $\pm$ 0.031 (0.013 $\pm$ 0.001)
9-COOH-22KC	0.217 $\pm$ 0.016 (0.067 $\pm$ 0.013)	0.063 $\pm$ 0.004 (0.031 $\pm$ 0.011)	0.130 $\pm$ 0.024 (0.046 $\pm$ 0.008)

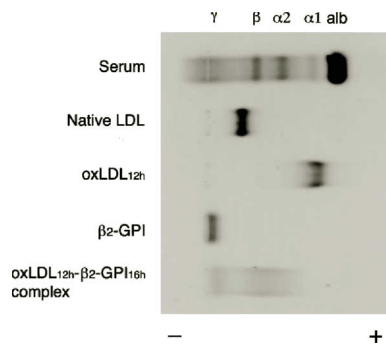
9-COOH-22KC, 22-ketocholesteryl-9-carboxynonanoate. Lipid coated plates were subsequently incubated with  $\beta_2$ -GPI and Cof-22 Ab or were simultaneously incubated with  $\beta_2$ -GPI and WB-CAL-1 or EY2C9 Ab. Binding to the solid phase lipid in the absence of  $\beta_2$ -GPI is indicated in parentheses. Data are indicated as the OD (mean  $\pm$  SD of triplicate samples).

GPI<sub>16h</sub> complex formation (Fig. 2D). LDL,  $\beta_2$ -GPI, and their complexes were applied to agarose gels for electrophoresis. As shown in Fig. 3, the increased negative charge in LDL that was gained by the incubation with  $\text{CuSO}_4$  for 12 h at 37°C (oxLDL<sub>12h</sub>) was neutralized by the interaction with  $\beta_2$ -GPI (oxLDL<sub>12h</sub>- $\beta_2$ -GPI<sub>16h</sub>).

To further examine the processes of complex formation, a series of time-course studies was performed. Figure 4A reveals the time-dependent generation of TBARS in



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



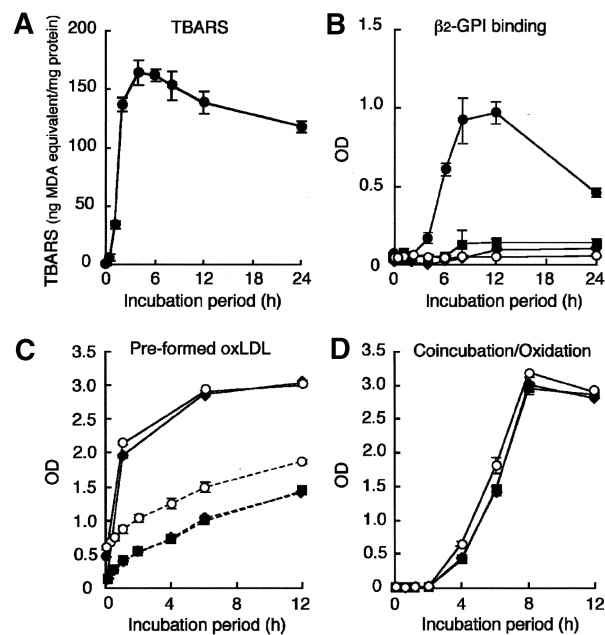
**Fig. 3.** The negative charge in oxLDL is partially neutralized by interaction with  $\beta_2$ -GPI. Native LDL, oxLDL,  $\beta_2$ -GPI, and oxLDL<sub>12h</sub>- $\beta_2$ -GPI<sub>16h</sub> complexes were analyzed by electrophoresis on an agarose gel and visualized by staining with amido black.

CuSO<sub>4</sub>-treated LDL. The TBARS were rapidly generated in LDL preparations exposed to the Cu<sup>2+</sup> ion at 37°C, with a peak at 4 h. In contrast, oxidation of LDL that generated the  $\beta_2$ -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 MgCl<sub>2</sub>. These data are consistent with our previous observations demonstrating that  $\beta_2$ -GPI binds to Cu<sup>2+</sup>-oxLDL but not to MDA-modified LDL (44).

The preformed oxLDL<sub>12h</sub> (final concentration at 100  $\mu$ g/ml of apoB equivalent) was also incubated with  $\beta_2$ -GPI (100  $\mu$ g/ml) for different periods at 4°C or 37°C (Fig. 4C). The formation of  $\beta_2$ -GPI-oxLDL complexes was temperature- and time-dependent. The complexes were not dissociated by the addition of heparin or MgCl<sub>2</sub> after the incubation at pH 7.4. Figure 4D indicates that the stable interaction between  $\beta_2$ -GPI and oxLDL was generated during the Cu<sup>2+</sup>-oxidation process even in the presence of  $\beta_2$ -GPI.

### Stability of in vitro $\beta_2$ -GPI-oxLDL complexes at different pHs

The stable complexes appeared at neutral pH and are possibly Schiff-base adducts formed between  $\epsilon$ -amines of lysine residues of  $\beta_2$ -GPI and oxidatively generated aldehydes on the Cu<sup>2+</sup>-mediated oxLDL vesicles. To test this, we analyzed the stability of nonreduced and NaCNBH<sub>3</sub>-reduced complexes at basic pH conditions. As shown in Fig. 5, no dissociation was observed in the reduced oxLDL<sub>12h</sub>- $\beta_2$ -GPI<sub>16h</sub> complexes at any pH conditions tested in the absence of MgCl<sub>2</sub>. In the presence of MgCl<sub>2</sub>, 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 Cu<sup>2+</sup>-oxLDL and the PL binding patch on the  $\beta_2$ -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 NaCNBH<sub>3</sub> (e.g., a hydrophobic pocket).

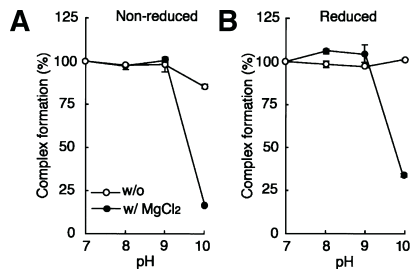


**Fig. 4.** Time-course study of complex formation between Cu<sup>2+</sup>-mediated oxLDL and  $\beta_2$ -GPI. A: Thiobarbituric acid reactive substances in LDL (treated with 5  $\mu$ M CuSO<sub>4</sub> for indicated period) were measured. B:  $\beta_2$ -GPI-oxLDL complexes formed by incubating Cu<sup>2+</sup>-oxLDL (2.5  $\mu$ g/ml of apoB equivalent) with  $\beta_2$ -GPI (0  $\mu$ g/ml, open circles; 25  $\mu$ g/ml, closed circles) in the assay wells, and ELISA was performed. The ELISA with 25  $\mu$ g/ml of  $\beta_2$ -GPI was also performed in the presence of heparin (100 U/ml, closed squares) or MgCl<sub>2</sub> (10 mM; closed diamonds). C:  $\beta_2$ -GPI-oxLDL complexes generated by incubation of preformed oxLDL<sub>12h</sub> (100  $\mu$ g/ml) with  $\beta_2$ -GPI (100  $\mu$ g/ml) during indicated periods at 4°C (dotted lines) or at 37°C (solid lines) were detected in the ELISA. The ELISA was also performed in the absence (open circles) or presence of heparin (100 U/ml; closed squares) or MgCl<sub>2</sub> (10 mM; closed diamonds). D:  $\beta_2$ -GPI-oxLDL complexes were formed by the simultaneous incubation of LDL (100  $\mu$ g/ml) and  $\beta_2$ -GPI (100  $\mu$ g/ml) during the Cu<sup>2+</sup> (5  $\mu$ M) oxidation at 37°C and ELISA of  $\beta_2$ -GPI-oxLDL complexes (2.5  $\mu$ g/ml equivalent of apoB) were detected in the ELISA. The ELISA was also performed in the absence (open circles) or presence of heparin (100 U/ml; closed squares) or MgCl<sub>2</sub> (10 mM; closed diamonds). Results are expressed as the mean  $\pm$  SD of triplicate samples.

### Nondissociable $\beta_2$ -GPI-oxLDL complexes exist in patient sera

We screened serum samples from patients with APS and/or SLE for high levels of  $\beta_2$ -GPI-oxLDL complexes.  $\beta_2$ -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  $\beta_2$ -GPI at 37°C for 16 h. In contrast, oxLDL<sub>12h</sub>- $\beta_2$ -GPI<sub>16h</sub> complexes were stable at pH 7.4, even in the presence of heparin or MgCl<sub>2</sub>. The typical binding pattern was also shown for preexisting oxLDL- $\beta_2$ -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 MgCl<sub>2</sub> (The ODs in the cases with heparin and MgCl<sub>2</sub> were 121  $\pm$  25.1% and 128  $\pm$  13.6%





**Fig. 5.** Stability of oxLDL<sub>12 h</sub>-β<sub>2</sub>-GPI<sub>16 h</sub> complexes and their NaCNBH<sub>3</sub>-reduced forms at different pH conditions. oxLDL<sub>12 h</sub>-β<sub>2</sub>-GPI<sub>16 h</sub> complexes (100 μg/ml apoB equivalent) were treated with 200 mM NaCNBH<sub>3</sub> in PBS at pH 7.4 for 16 h to be reduced. The nonreduced and reduced complexes were incubated at indicated pH for 16 h at 37°C in the absence or presence of 10 mM MgCl<sub>2</sub>. β<sub>2</sub>-GPI-oxLDL complexes were measured in these preparations containing 300 ng/ml of LDL (apoB equivalent) in the ELISA. Results are expressed as the mean ± SD of triplicate samples.

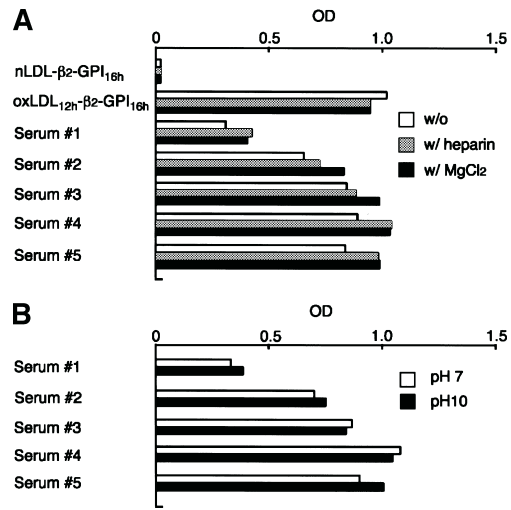
of control condition, respectively). The preformed complexes present in serum samples were also consistently observed after the 16 h-incubation with MgCl<sub>2</sub> at pH 10 at 37°C (104 ± 10.9%), that can dissociate the complexes formed in vitro (Fig. 5). We interpret these findings to indicate that nondissociable and covalent adducts between β<sub>2</sub>-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 β<sub>2</sub>-GPI-oxLDL complex and its auto-Abs

In the ELISA, we obtained an apparent calibration curve for oxLDL<sub>12 h</sub>-β<sub>2</sub>-GPI<sub>16 h</sub> 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 β<sub>2</sub>-GPI in serum samples, because WB-CAL-1 Ab used in the ELISA is highly specific for β<sub>2</sub>-GPI complexed with oxLDL. In the present study, the β<sub>2</sub>-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-β<sub>2</sub>-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-β<sub>2</sub>-GPI-oxLig-1 IgG Ab titers from this group of 127 patients are strongly correlated with both β<sub>2</sub>-GPI-dependent IgG aCL and anti-β<sub>2</sub>-GPI IgG Abs (correlation coefficient;  $r^2 = 0.69$  and  $0.81$ , respectively) (Fig. 8). As shown in Fig. 9, there also was a good correlation between IgG IC with β<sub>2</sub>-GPI and anti-β<sub>2</sub>-GPI IgG Abs ( $r^2 = 0.50$ ) (Fig. 9A), IgG IC with β<sub>2</sub>-GPI and anti-β<sub>2</sub>-GPI-oxLig-1 IgG Abs ( $r^2 = 0.50$ ) (Fig. 9B), and IgG IC with LDL and IgG IC with β<sub>2</sub>-GPI ( $r^2 = 0.40$ ) (Fig. 9C). However, a good correlation between levels of β<sub>2</sub>-GPI-oxLDL complex and titers of any of these Abs was not observed (data not shown).

In Table 3, the correlation between anti-β<sub>2</sub>-GPI-oxLig-1 IgG Abs (not β<sub>2</sub>-GPI-oxLig-1 complex antigen) and throm-

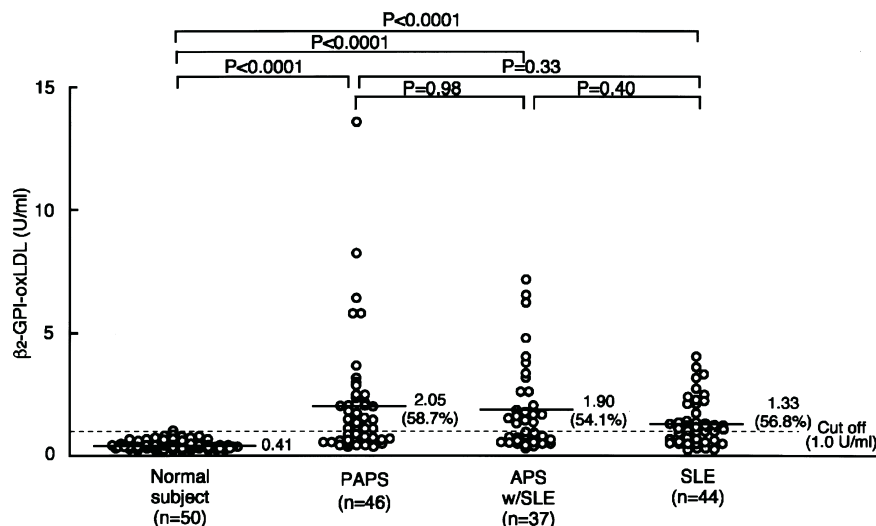


**Fig. 6.** β<sub>2</sub>-GPI-oxLDL complexes present in patient sera. A: Native LDL (nLDL)-β<sub>2</sub>-GPI<sub>16 h</sub> (the reaction mixture of native LDL and β<sub>2</sub>-GPI by the 16 h incubation at 37°C, as a negative control) (300 ng/ml apoB equivalent), oxLDL<sub>12 h</sub>-β<sub>2</sub>-GPI<sub>16 h</sub> (300 ng/ml), or 100-fold diluted β<sub>2</sub>-GPI-oxLDL complex-positive sera were incubated in the absence or presence of heparin (100 U/ml) or MgCl<sub>2</sub> (10 mM) at pH 7.4. B: The β<sub>2</sub>-GPI-oxLDL complex-positive sera were preincubated at pH 7 or pH 10 in the presence of MgCl<sub>2</sub> (10 mM) for 16 h at 37°C, and the ELISA was performed. Results are expressed as the mean of duplicate samples.

bolism 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 β<sub>2</sub>-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-β<sub>2</sub>-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 β<sub>2</sub>-GPI-oxLDL antigen-positive patients' group was higher than that in the antigen-negative patients' group. It is of interest because the presence of β<sub>2</sub>-GPI-oxLDL antigen may be an additional risk of having arterial thrombosis in patients with anti-β<sub>2</sub>-GPI-oxLDL Abs.

## DISCUSSION

We previously reported that the major lipid ligands responsible for β<sub>2</sub>-GPI binding to Cu<sup>2+</sup>-mediated oxLDL are ω-carboxylated 7-ketocholesterol esters such as oxLig-1, and that the ω-carboxyl moiety is also essential for β<sub>2</sub>-GPI recognition (45, 46). The in vitro interaction between β<sub>2</sub>-GPI and Cu<sup>2+</sup>-oxLDL is initially reversible by Mg<sup>2+</sup> treatment but progresses to a much more stable interaction requiring Mg<sup>2+</sup> and a high pH to be dissociated. In contrast, stable and nondissociable complexes between oxLDL and β<sub>2</sub>-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



**Fig. 7.** Serum levels of  $\beta_2$ -GPI-oxLDL complexes detected in ELISA.  $\beta_2$ -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.

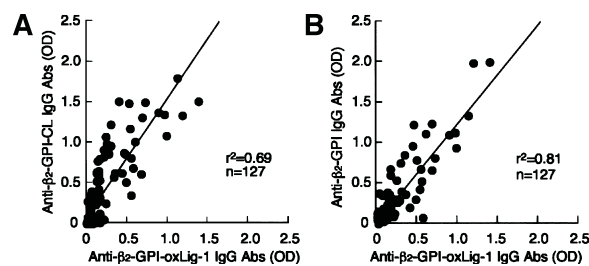
$\beta_2$ -GPI in sera from those patients, and statistical analysis indicates that the serum  $\beta_2$ -GPI-oxLDL complexes are associated with arterial thrombosis.

Foam cell formation is regarded as the hallmark of early atherogenesis, 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  $\beta_2$ -GPI binding to  $\text{Cu}^{2+}$ -oxLDL and anti- $\beta_2$ -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  $\beta_2$ -GPI and cannot be replaced by a ketone at the 22 position (Fig. 1 and Table 2).

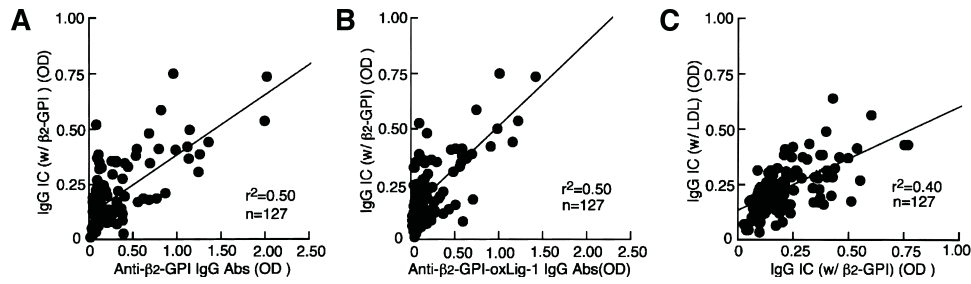
A patch consisting of 14 positively charged amino acid residues, and a flexible loop in domain V of  $\beta_2$ -GPI were reported to be critical for interaction with amphiphilic compounds such as CL, phosphatidylserine, phosphatidic acid, and phosphatidylglycerol (38–40). We previously reported that an interaction with oxLDL was also provided by the same binding site of  $\beta_2$ -GPI (53). The conjugated ketone of the ligands may orient to hydrophilic space together with  $\omega$ -carboxyl function, which results in providing specific binding to  $\beta_2$ -GPI. In general, a conjugated ketone is less likely to actively form Schiff-base adducts than an  $\omega$ -aldehyde. The  $\beta_2$ -GPI ligands may be involved in a noncovalent and electrostatic interaction between oxLDL and  $\beta_2$ -GPI at neutral pH because the interaction is inhibited either by  $\text{MgCl}_2$ ,  $\text{CaCl}_2$ , or heparin.

It is now well established that anti- $\beta_2$ -GPI Abs found in sera from APS patients bind to a complex of  $\beta_2$ -GPI and

negatively charged PLs, such as CL, phosphatidylserine, and phosphatidic acid, in ELISA using a PL-coated microtiter plate (50). Hörkkö et al. (54) recently demonstrated that aCLs present in APS patients react with the Schiff-base adducts formed between oxidized CL and  $\beta_2$ -GPI. However, such negatively charged PLs are very minor components of LDL. The  $\text{Cu}^{2+}$ -mediated oxidative products in LDL include cholesterol or oxysterols esterified with 9- or 13-hydroperoxy (or hydroxy)-octadecadienoate, 9-oxononanoate, or with 9-carboxynonanoate, some of which have also been shown to be present in atherosclerotic plaques (55–57). As we previously reported (45, 46),  $\omega$ -carboxyl-oxysterol esters such as oxLig-1 and oxLig-2, but not oxidized PLs, were detected in  $\text{Cu}^{2+}$ -oxLDL as major ligands for  $\beta_2$ -GPI binding. The nature of these in vitro and in vivo adducts has not yet been chemically defined, but conjugates between  $\beta_2$ -GPI and some oxidatively modified forms of cholesteryl esters, as well as oxidized PLs, are the most likely candidates. In the present study, treatment of oxLDL<sub>12 h</sub>- $\beta_2$ -GPI<sub>16 h</sub> complex with excess



**Fig. 8.** Correlation among  $\beta_2$ -GPI-related IgG Ab titers detected in three different ELISA systems. A:  $\beta_2$ -GPI-dependent IgG anticardiolipin Abs (anti- $\beta_2$ -GPI-cardiolipin IgG Abs) versus anti- $\beta_2$ -GPI-oxLig-1 IgG Abs. B: Anti- $\beta_2$ -GPI IgG Abs (detected in ELISA using a  $\beta_2$ -GPI-coated polyoxygenated plate) versus anti- $\beta_2$ -GPI-oxLig-1 IgG Abs.



**Fig. 9.** Correlation among IgG Ab titers and levels of IgG immune complexes. A: IgG immune complex with  $\beta_2$ -GPI (IgG IC w/  $\beta_2$ -GPI) versus anti- $\beta_2$ -GPI IgG Abs. B: IgG IC with  $\beta_2$ -GPI versus anti- $\beta_2$ -GPI-oxLig-1 IgG Abs. C: IgG immune complex with LDL (IgG IC with LDL) versus IgG IC with  $\beta_2$ -GPI.

NaCNBH<sub>3</sub> (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  $\beta_2$ -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  $\beta_2$ -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  $\beta_2$ -GPI (the plasma concentration of  $\beta_2$ -GPI is  $\sim$ 200  $\mu$ g/ml). As shown in Fig. 4D, in the presence of  $\beta_2$ -GPI, LDL that underwent in vitro oxidation formed stable adducts with increasing incubation time at neutral pH. Furthermore, the stable interaction between  $\beta_2$ -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,  $\beta_2$ -GPI ligands related to oxLig-1 and oxLig-2 provide specific interaction between  $\beta_2$ -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  $\beta_2$ -GPI is the true antigen for aCL. In 1998, we showed that anti- $\beta_2$ -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- $\beta_2$ -GPI-CL IgG Abs, anti- $\beta_2$ -GPI IgG Abs, and anti- $\beta_2$ -GPI-oxLig-1 IgG Abs (Fig. 8). The appearance of anti- $\beta_2$ -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  $\beta_2$ -GPI-oxLig-1 (i.e.,  $\beta_2$ -GPI-oxLDL) complexes may be the true target antigen for the previously characterized aCL. The anti- $\beta_2$ -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  $\beta_2$ -GPI-oxLDL complex positive and negative, a stronger association between anti- $\beta_2$ -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  $\beta_2$ -GPI and LDL were also detected. Although the mechanisms of in vivo oxidation of LDL remain unclear, the resultant  $\beta_2$ -GPI-oxLDL complexes may have a pathogenic role as an autoantigen to induce the

TABLE 3. Association with anti- $\beta_2$ -GPI-oxLig-1 IgG Abs and thrombosis in APS


Subjects	Fisher's Exact Test ( <i>P</i> )	Odds Ratio	95% CI
Thrombosis (arterial and/or venous)			
Patients (total, n = 127)	$1.7 \times 10^{-7}$	7.65	3.41–17.2
( $\beta_2$ -GPI-oxLDL positive, n = 72)	$5.9 \times 10^{-5}$	8.21	2.79–24.2
( $\beta_2$ -GPI-oxLDL negative, n = 55)	0.0014	6.87	2.01–23.5
Arterial thrombosis			
Patients (total, n = 127)	$6.9 \times 10^{-7}$	7.45	3.21–17.3
( $\beta_2$ -GPI-oxLDL positive, n = 72)	$4.8 \times 10^{-5}$	10.2	2.98–34.7
( $\beta_2$ -GPI-oxLDL negative, n = 55)	0.0043	5.63	1.68–18.9
Venous thrombosis			
Patients (total, n = 127)	0.026	2.23	1.06–4.68
( $\beta_2$ -GPI-oxLDL positive, n = 72)	0.066	2.37	0.89–6.29
( $\beta_2$ -GPI-oxLDL negative, n = 55)	0.20	1.93	0.61–6.14



development of thrombosis, especially arterial thrombosis, in APS.

The ELISA methodology using solid-phase native or oxLDL 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 prepurification of samples with polyethyleneglycol 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 oxLDL- $\beta_2$ -GPI complexes formed in vitro do not have the high negative charge of  $\text{Cu}^{2+}$ -oxLDL. Furthermore, we applied two types of Ab-capture ELISAs using anti- $\beta_2$ -GPI Ab and anti-apoB100 for detecting IgG IC with  $\beta_2$ -GPI and IgG IC with LDL, respectively. These two ELISAs are not affected by high titers of rheumatoid factors and endogenous levels of  $\beta_2$ -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  $\beta_2$ -GPI IgG and IgG IC with  $\beta_2$ -GPI, between anti- $\beta_2$ -GPI-oxLig-1 IgG and IgG IC with  $\beta_2$ -GPI, and between IgG IC with  $\beta_2$ -GPI and IgG IC with LDL (oxLDL). All of these correlations indicate that the presence of IgG (anti- $\beta_2$ -GPI) IC with the  $\beta_2$ -GPI-LDL (oxLDL) 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  $\beta_2$ -GPI had accelerated atherosclerosis (61).  $\beta_2$ -GPI was abundant within sub-endothelial 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  $\beta_2$ -GPI and oxLDL in the atherogenesis of APS.

This is the first report that stable and nondissociable  $\beta_2$ -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  $\beta_2$ -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  $\beta_2$ -GPI-oxLDL complexes in blood stream of patients with coronary artery diseases. 

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