ω-Carboxyl variants of 7-ketocholesteryl esters are ligands for β_2-glycoprotein I and mediate antibody-dependent uptake of oxidized LDL by macrophages

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Abstract **β_2-Glycoprotein I (β_2-GPI) is a major antigen for anticardiolipin antibodies (aCL, Abs) present in patients with antiphospholipid syndrome. We recently reported that β_2-GPI specifically binds to oxidized LDL (oxLDL) and that the β_2-GPI's major ligand, oxLig-1 is 7-ketocholesteryl-9-carboxynonanoate (Kobayashi, K., E. Matsuura, Q. P. Liu, J. Furukawa, K. Kaihara, J. Inagaki, T. Atsumi, N. Sakairi, T. Yasuda, D. R. Voelker, and T. Koike. 2001. A specific ligand for β_2-glycoprotein I mediates autoantibody-dependent uptake of oxidized low density lipoprotein by macrophages. *J. Lipid Res.* 42: 697–709). In the present study, we demonstrate that ω-carboxylated 7-ketocholesteryl esters are critical for β_2-GPI binding. A positive ion mass spectrum of a novel ligand, designated oxLig-2, showed fragmented ions at *m/z* 383 and 441 in the presence of acetone, which share features of oxLig-1 and 7-ketocholesterol. In the negative ion mode, ions at *m/z* 627, 625, and 243 were observed. oxLig-2 was most likely 7-ketocholesteryl-12-carboxy (keto) dodecanoate. These ligands were recognized by β_2-GPI. Liposome binding to macrophages was significantly increased depending on the ligand's concentration, in the presence of β_2-GPI and an anti-β_2-GPI Ab. Synthesized variant, 7-ketocholesteryl-13-carboxytridecanoate (13-COOH-7KC), also showed a significant interaction with β_2-GPI and a similar binding profile with macrophages. Methylation of the carboxyl function diminished all of the specific ligand interactions with β_2-GPI. Thus, ω-carboxyl variants of 7-ketocholesteryl esters can mediate anti-β_2-GPI Ab-dependent uptake of oxLDL by macrophages, and autoimmune atherogenesis linked to β_2-GPI interaction with oxLDL.**—Liu, Q., K. Kobayashi, J. Furukawa, J. Inagaki, N. Sakairi, A. Iwado, T. Yasuda, T. Koike, D. R. Voelker, and E. Matsuura. **ω-Carboxyl variants of 7-ketocholesteryl esters are ligands for β_2-glycoprotein I and mediate antibody-dependent uptake of oxidized LDL by macrophages.** *J. Lipid Res.* 43: **1486–1495.**

Supplementary key words antiphospholipid syndrome • atherosclerosis • autoantibody • β_2-glycoprotein I • oxidized LDL • ω-oxidation

The autoimmune disorder, antiphospholipid syndrome (APS), is characterized by the presence of a group of heterogeneous antiphospholipid antibodies (aPL Abs), such as anticardiolipin Abs (aCL) and lupus anticoagulants (LA), in blood, and by occurrence of thromboembolic complications in the arterial and/or venous vasculatures of the patients (1, 2). In 1990, three groups of investigators independently reported that a plasma/serum cofactor complexed with negatively charged phospholipids (PLs), such as cardiolipin, is an antigenic target for aCL (3–5). It is now widely accepted that β_2-glycoprotein I (β_2-GPI) is the major antigen for aCL. However, the mechanisms for interaction between β_2-GPI and anti-β_2-GPI Abs are still uncertain. Two currently proposed mechanisms are: *i*) Binding of β_2-GPI to PL induces a conformational change in the β_2-GPI molecule, thus exposing a cryptic epitope on the protein for the auto-Ab binding, and/or, *ii*) β_2-GPI binding to anionic PL increases the local concentration of β_2-GPI, thus promoting an increase in intrinsic affinity and Ab binding to the protein (6–14).

β_2-GPI is a 50 kDa protein present in plasma at approximately 200 μg/ml. It binds to negatively charged molecules, including PLs (15), heparin (16), and plasma membranes of activated platelets, and apoptotic cells on which phosphatidylserine (PS) is exposed (17, 18). β_2-GPI is a

Abbreviations: Ab, antibody; APS, antiphospholipid syndrome; β_2-GPI, β_2-glycoprotein I; oxLDL, oxidized LDL; PL, phospholipid; oxLig-1, 7-ketocholesteryl-9-carboxynonanoate; 13-COOH-7KC, 7-ketocholesteryl-13-carboxytridecanoate.

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member of the short consensus repeats of the complement control protein superfamily, and its fifth domain has a PL binding region. The X-ray crystal analyses (19) showed that the PL binding is provided by a patch consisting of 14 residues of positively charged amino acids and by a flexible loop of S^{311}-K^{317} in domain V. Recent analysis with domain V's mutant proteins demonstrated interactions of the flexible loop with hydrophobic ligands (20, 21). However, the full details of the structure of in vivo lipid ligands participating in the β_2-GPI binding remains unclear.

β_2-GPI affects not only multiple PL-dependent coagulation pathways but also lipoprotein metabolism (22–24). The oxidation of LDL has been proposed to play a central role in the early phase of atherosclerotic plaque formation, such as the transformation of monocyte-derived macrophages to foam cells (25–30). Accumulating evidence has suggested that the interaction between aPL and malonedialdehyde-modified LDL (MDA-LDL) may be important in relation to the pathogenesis of atherosclerosis and/or atherothrombosis in APS patients (31–33). In 1997, we first reported that β_2-GPI bound directly to oxidized LDL (oxLDL), and that the complex of oxLDL and β_2-GPI was subsequently recognized by anti-β_2-GPI auto-Abs and taken up by macrophages (34). It was further reported that lipid ligands derived from oxLDL were specific for β_2-GPI. The major ligand for β_2-GPI, oxLig-1, was originally reported as 7-ketocholesteryl-9-carboxynonanoate (35). The formal IUPAC name for this compound is 9-oxo-9-(7-ketocholest-5-en-3β-yloxy) nonanoic acid.

In the present report, we now demonstrate that oxLDL recognition by β_2-GPI and an anti-β_2-GPI Ab is provided by an ω-carboxyl function introduced by oxidation of an unsaturated acyl chain of cholesteryl esters.

MATERIALS AND METHODS

Chemicals

L-α-Dipalmitoylphosphatidylserine (DPPS), 7-ketocholesterol (5-cholesten-3β-ol-7-one), cholesteryl linoleate (5-cholesten-3β-ol 3-linoleate), polyinosinic acid [poly(I)], polycytidylic acid [poly(C)], and fucoidan were obtained from Sigma Chemical Co. (St. Louis, MO); dioleoylphosphatidylcholine (DOPC) from Avanti Polar Lipids Inc. (Alabaster, AL); L-3-phosphatidyl-[*N*-methyl-^{3}H]choline, 1, 2-dipalmitoyl ([^{3}H]-DPPC) (80 Ci/mmol) from Amersham-Pharmacia Biotech (Uppsala, Sweden). All other chemicals were from commercial sources and of reagent-grade quality.

Preparation of human β_2-GPI

β_2-GPI was purified from normal human plasma as described (36) with slight modification. Pooled plasma from healthy subjects was subsequently chromatographed on a heparin-Sepharose column, on a DEAE-cellulose column, and on an anti-β_2-GPI affinity column. To remove any contamination by IgGs, the β_2-GPI-rich fraction was further passed through a protein A-Sepharose column. The final β_2-GPI fraction was delipidated by extensive washing with *n*-butanol.

Monoclonal antibodies

The monoclonal anti-human β_2-GPI Ab, Cof-22 (IgG1,κ), was established from BALB/c mice immunized with human β_2-GPI (8). A mouse monoclonal anti-β_2-GPI auto-Ab, WB-CAL-1 (IgG2a, κ), was derived from an (NZW × BXSB) F1 mouse (37). A human monoclonal anti-β_2-GPI auto-Ab, EY2C9 (IgM), was established from peripheral blood lymphocytes from an APS patient (38).

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 (39). The LDL was adjusted to 100 μg/ml and oxidized with 5 μM CuSO4 in PBS for 8 h at 37°C. To terminate the oxidation, 1 mM EDTA was added and dialyzed extensively against PBS containing 1 mM EDTA. The degree of oxidation was estimated as thiobarbituric acid reactive substance (TBARS) value (40) and as migration in agarose electrophoresis.

Lipid extraction and preparative TLC

The lipids from native and oxLDL were isolated, according to the method of Folch, et al. (41). Briefly, lipids were extracted with chloroform-methanol (2:1, v/v) and dried by evaporation. The extracted lipids were spotted on a TLC silica gel-60 plate (2 mm thickness, Merck, Darmstadt, Germany) and developed in chloroform-methanol-30% ammonia-water (120:80:10:5, v/v/v/v, solvent A). Two individual lipid bands, Band-1 and Band-2, containing ligands reactive with β_2-GPI (detected by the ligand blot analysis, as described below), were identified and scraped.

Ligand blot analysis on a TLC plate

For analytical TLC ligand blot, lipids were spotted on a Polygram silica gel G plate (Machery-Nagel, Duren, Germany) and developed in solvent A or in chloroform-methanol (8:1, v/v, solvent B). Ligand blot analysis was performed, as described previously (35). Briefly, after drying and blocking with PBS containing 1% BSA, the plate was simultaneously incubated with β_2-GPI and an anti-β_2-GPI Ab (WB-CAL-1 or EY2C9) for 1 h. In case of Cof-22 Ab, β_2-GPI and the Ab were subsequently incubated for 1 h each. Horseradish peroxidase (HRP)-labeled anti-mouse IgG or HRP-labeled anti-human IgM was then incubated for 1 h. In each step, a plate was extensively washed with PBS. The color was developed with H_2O_2 and 4-methoxy-1-naphtol (Aldrich, Milwaukee, WI). On control TLC plates, ligands were separated and stained with I_2 vapor or with a spray of molybdenum blue.

HPLC

The β_2-GPI-specific ligand, oxLig-2, was purified from the ligand-enriched fraction by a reversed-phase HPLC on a Sephasil Peptide C18 column (4.6 mm × 250 mm; Amersham-Pharmacia Biotech). The scraped band, Band-2, was eluted using a linear gradient of 50–100% solvent C (acetonitrile-isopropanol, 30:70, v/v) against solvent D (water containing 0.2% acetic acid), over 15 min, then 100% solvent C for the following 15 min, at a flow rate of 0.5 ml/min, and absorbance was monitored at 210 nm or 234 nm. The eluate was fractionated every 2 min (1ml/tube). Each fraction was spotted on a TLC plate and subjected to ligand blot analysis with β_2-GPI and EY2C9 Ab.

Synthesis of oxLig-1

To a solution of 7-ketocholesterol (5-cholesten-3β-ol-7-one, 50.1 mg, 0.13 mmol) and azelaic acid (70.6 mg, 0.38 mmol) in acetone (4 ml) was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (WSC; 95.8 mg, 0.50 mmol) and 4-(dimethylamino)pyridine (DMAP; 30.5 mg, 0.25 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 residue was subjected to column chromatography on silica gel using toluene-

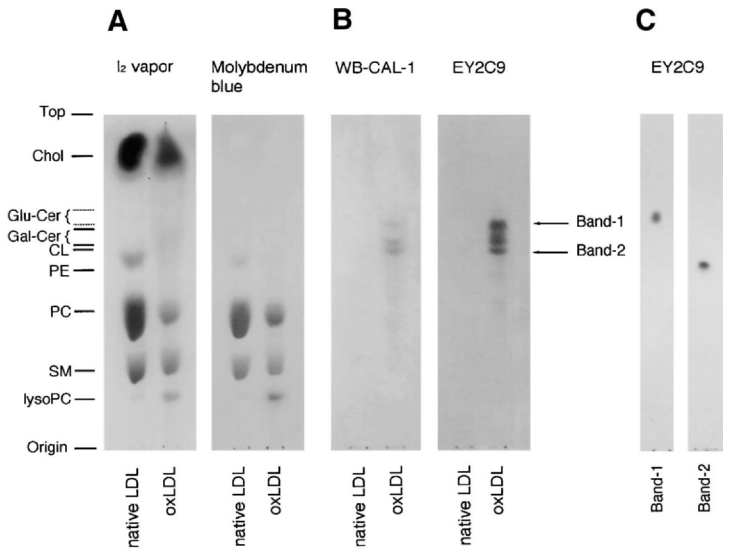

Fig. 1. TLC and ligand blot of lipids extracted from LDLs. Lipids extracted from LDLs were spotted on a silica gel plate and developed in solvent A (A and B) and solvent B (C), respectively. The plate was stained with I_2 vapor and molybdenum blue (A). Ligand blot of lipids extracted from LDLs and incubated with β_2-GPI and an anti-β_2-GPI Ab (WB-CAL-1 or EY2C9) are shown in B. TLC of the isolated Band-1 and Band-2, in solvent B was followed by ligand blot with β_2-GPI and EY2C9 Ab (C).

ethyl acetate (3:1, v/v) to give synthesized oxLig-1 (36.0 mg, 50.4% yield). ^{1}H-NMR and ^{13}C-NMR spectra were obtained at 300 MHz and 75 MHz, respectively, by an ASX-300 spectrometer (Bruker, Billerica, MA). The field desorption (FD) mass spectrum of synthesized oxLig-1 was recorded on a JMS-SX102A spectrometer (JEOL, Tokyo, Japan). ^{1}H-NMR (300.1 MHz, $CDCl_3$): δ = 5.71 (s, ^{1}H, H-6), 4.78–4.69 (m, ^{1}H, H-3); ^{13}C-NMR (75.5 MHz, $CDCl_3$): δ = 202.5, 179.7, 173.4, 164.5, 127.1, 72.4, 55.2, 50.4, 50.2, 45.8, 43.5, 39.9, 38.7, 36.6, 36.1, 29.2, 28.9, 28.4, 25.3, 25.0, 24.2, 23.2, 23.0, 19.3, 17.7, 12.4; *m/z* (FD-MS): 571 [$(M+H)^+$, $C_{36}H_{59}O_5$ requires 571].

Synthesis of 7-ketocholesteryl-13-carboxytridecanoate

To a solution of 7-ketocholesterol (50.1 mg, 0.13 mmol) and tridacanedioic acid (brassylic acid; 61.8 mg, 0.25 mmol) in acetone (4 ml) was added WSC (95.8 mg, 0.50 mmol) and DMAP (30.5 mg, 0.25 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, aqueous saturated sodium hydrogencarbonate, and brine, dried over anhydrous magnesium sulfate, and evaporated. The residue was subjected to column chromatography on silica gel using toluene-ethyl acetate (3:1,

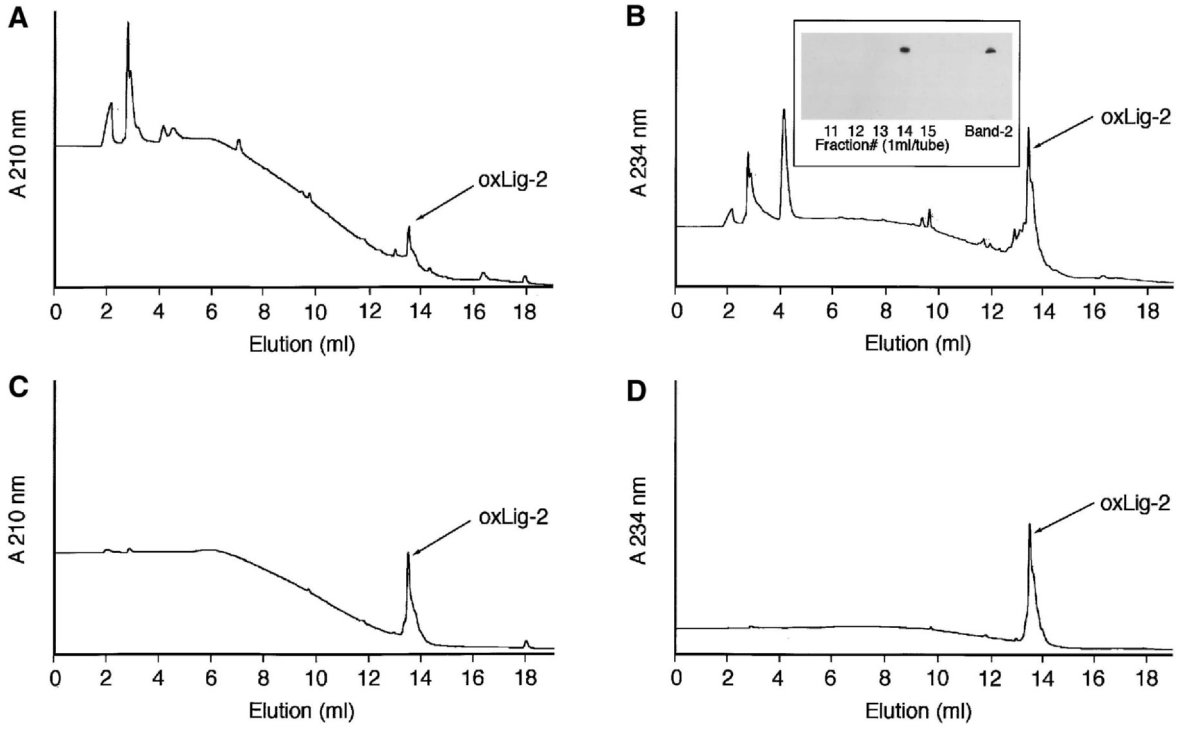

Fig. 2. Elution profiles of Band-2 in reversed-phase HPLC. Isolated Band-2 was applied to a Sephasil-Peptide column with a linear gradient of 50–100% solvent C (acetonitrile-isopropanol 30/70, v/v) against solvent D (water containing 0.2% acetic acid). Absorbance was detected at 210 nm (A) and 234 nm (B) using a flow rate of 0.5 ml/min. Fractions (1 ml/tube) were collected and subjected to ligand blot with β_2-GPI and EY2C9 Ab (B, insert). Fraction # 14 from the first HPLC was re-chromatographed on the same HPLC to confirm its purity (C and D).

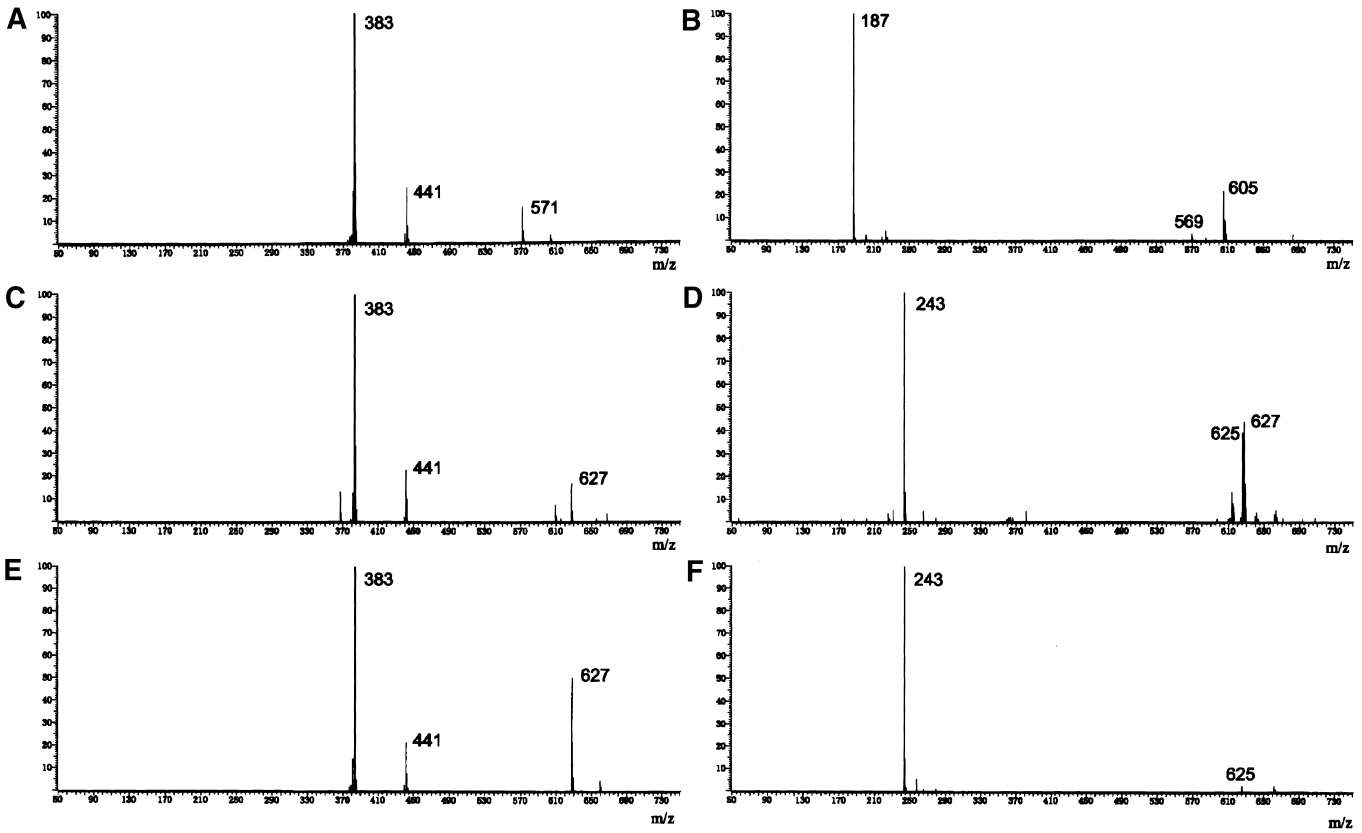

Fig. 3. Liquid chromatography equipped mass spectrometry of purified or synthesized β_2-GPI ligands. Left panels indicate positive ionization mass spectra of 7-ketocholesteryl-9-carboxynonanoate (oxLig-1) (A), oxLig-2 (C), and 7-ketocholesteryl-13-carboxytridecanoate (13-COOH-7KC) (E); right panels indicate negative ionization mass spectrum of oxLig-1 (B), oxLig-2 (D), and 13-COOH-7KC (F).

v/v) to give the product (44 mg, 56.0% yield). NMR spectra and FD mass spectra were measured as described above. ^{1}H-NMR (300.1 MHz, $CDCl_3$): δ = 5.69 (s, ^{1}H, H-6), 4.80-4.67 (m, ^{1}H, H-3); ^{13}C-NMR (75.5 MHz, $CDCl_3$): δ = 202.2, 179.6, 173.1, 164.7, 126.9, 72.3, 55.3, 50.5, 50.1, 45.3, 43.5, 40.6, 39.2, 38.6, 36.5, 36.0, 29.1, 28.8, 28.3, 25.2, 24.9, 24.1, 23.1, 22.9, 19.2, 17.6, 12.3; *m/z* (FD-MS): 627 [$(M+H)^+$, $C_{40}H_{67}O_5$ requires 627].

Liquid chromatography equipped mass spectrometry

Mass spectra of β_2-GPI-specific ligands, synthesized oxLig-1, oxLig-2 purified from Band-2, and synthesized 7-ketocholesteryl-13-carboxytridecanoate 13-COOH-7KC), were obtained by a liquid chromatography equipped mass spectrometry (LC/MS)-2010 spectrometer (Shimadzu Corp., Kyoto, Japan), equipped with a Shimpack FC-ODS column (4.6 mm × 30 mm). The column was developed with a linear gradient of 50–100% solvent D (30% acetone in methanol) against water. Positive and negative ionization mass signals were detected in the mass range of 50–750, as ions generated during atomospheric pressure chemical ionization (APCI).

Methylation of lipid ligands

1-Methyl-3-nitro-1-nitrosomethylguanidine (0.20 g) was added to a mixture of 2 M sodium hydroxide (10 ml) and diethyl ether (10 ml) in an ice bath. The mixture was shaken several minutes and the pale yellow ethereal solution separated was used for methylation. The diazomethane solution (2 ml) was added dropwisely to a solution of lipid ligand (1.0 mg) in diethyl ether (1 ml) at 0°C. Each mixture was stored in refrigerator overnight. TLC of the mixture showed complete disappearance of the starting materials. The solvent was removed by blowing air to give the methyl ester as a white amorphous powder.

ELISA for anti-β_2-GPI Ab binding

Anti-β_2-GPI Ab binding was performed as described (35). Briefly, the lipid ligand (50 μg/ ml, 50 μl/well) was adsorbed by evaporation on a plain polystyrene plate (Immulon 1B, Dynex Technologies Inc., Chantilly, VA) and the plate was then blocked with 1% BSA. A monoclonal anti-β_2-GPI Ab (WB-CAL-1, or EY2C9, 1.0 μg/ml, 100 μl/well) was incubated in PBS containing 0.3% BSA with β_2-GPI (15 μg/ml) for 1 h. In case of Cof-22 Ab, β_2-GPI and the Ab were subsequently incubated for each 1 h. Ab binding was probed using HRP-labeled anti-mouse IgG or anti-human IgM. The color was developed with H_2O_2 and *o*-phenylenediamine and absorbance was measured at 490 nm. Between each step, extensive washing was performed using PBS containing 0.05% Tween 20.

Preparation of liposomes

Liposomes were prepared as described (42), with the following lipid compositions. Lipid molar ratios of 0, 10, 25, 30, and 50% ligand-containing liposomes were made with DOPC-ligand-[^{3}H]DPPC (80 Ci/mmol). The amount of [^{3}H]DPPC was 0.225%. The ligand component was varied to be either cholesteryl linoleate, DPPS, oxLig-1, oxLig-2, methylated oxLig-2 (Me-oxLig-2), 13-COOH-7KC, or methylated 13-COOH-7KC (Me-13-COOH-7KC).

Cell culture and liposome binding assay

A monolayer culture of mouse macrophage-like cell line, J774A.1 (Riken Cell Bank, Tsukuba, Japan), was used for liposome binding experiments. The cells were maintained in RPMI-1640 medium supplemented with 10% FBS. The cells were plated (1 ml/well) into a 12-well culture plate with RPMI 1640 at

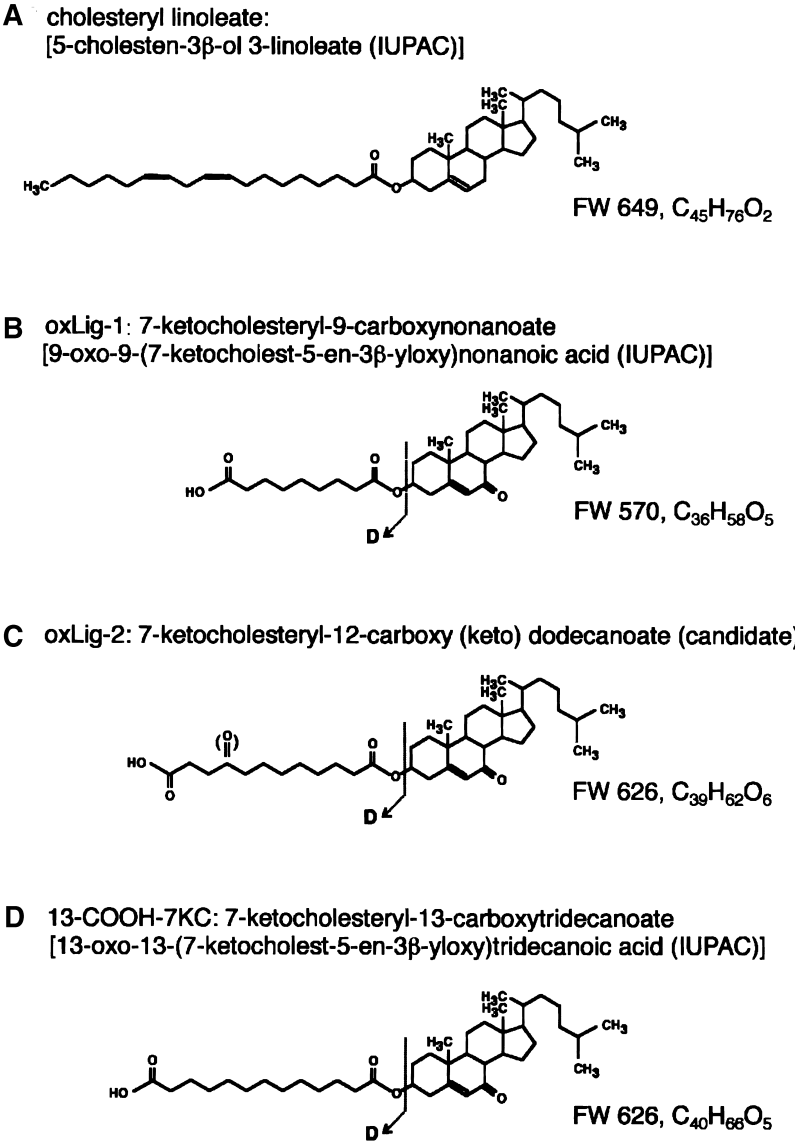

Fig. 4. Structures of cholesteryl esters that serve as precursor and ligands for β_2-GPI. Structures of cholesteryl linoleate (A), oxLig-1 (B) (35), oxLig-2 (C), and 13-COOH-7KC are indicated. A proposed fragmentation schemes of these ligands during APCI-MS are also shown and cleavage at the site (indicated by a dotted allow) yields a daughter fragment D.

8×10^5 cells/ml and incubated for 24 h at 37°C. The medium was replaced with Celgrosser-P medium (Sumitomo Pharmaceutical Co., Tokyo, Japan). After 2 h preincubation at 37°C, 50 μl of liposomes (50 nmol lipid/well) with/or without β_2-GPI (200 μg/ml) and WB-CAL-1 (100 μg/ml) were added to each well, and the cells were then incubated at 4°C for 2 h. The wells were washed with chilled PBS, and the cells were lysed with 1ml of 0.1 N NaOH. An aliquot was taken to determine cellular proteins and radioactivity associated with the cells. Protein concentration was determined using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL).

RESULTS

Detection of β_2-GPI-specific ligands

We first detected extracted lipids from native and Cu^{2+}-oxdized LDL preparations, by different staining procedures applied to TLC plates, developed in solvent A (**Fig. 1A**, **B**). With the I_2 vapor and the molybdenum blue spray, the major change observed due to the Cu^{2+}-oxidation was a small increase in lysophosphatidylcholine (lysoPC). To define the ligands targeted by β_2-GPI and an anti-β_2-GPI auto-Ab (i.e., WB-CAL-1 or EY2C9), ligand blot analysis was performed on the TLC plate. Two major bands and a diffuse lipid band were stained with β_2-GPI and either anti-β_2-GPI auto-Abs. The reactive lipids migrated at similar R_f positions to those of cardiolipin and glycolipids, such as galactosylceramide (Gal-Cer) and glucosylceramide (Glu-Cer). The bands detected by ligand blot were not stained with molybdenum blue spray, indicating that they are not phospholipid (Fig. 1A, B). β_2-GPI ligand-enriched lipids (i.e., Band-1 and -2, indicated by arrows in Fig. 1B) were scraped from the TLC plate (in solvent A) and were subjected to another TLC development in solvent B and subsequent ligand blot with β_2-GPI and EY2C9 (Fig. 1C). The ligand corresponding to the upper band (Band-1) has already been reported to contain oxLig-1 (35). The lower band (Band-2) was further purified by reversed-phase HPLC.

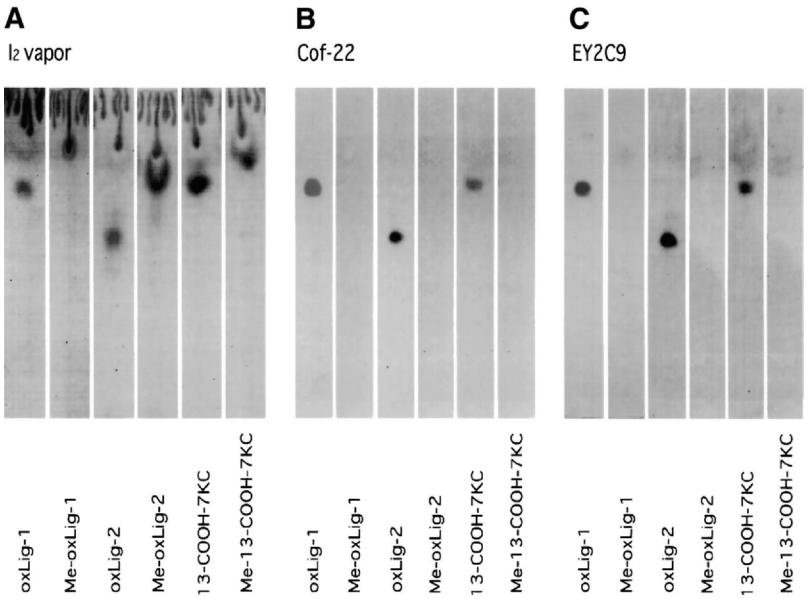

Fig. 5. TLC and ligand blot of methylated ligands. Non-treated oxLig-1, oxLig-2, and 13-COOH-7KC, and methylated oxLig-1, oxLig-2, and 13-COOH-7KC (Me-oxLig-1, Me-oxLig-2, and Me-13-COOH-7KC) were spotted on a silica gel plate and developed in solvent B. The plate was stained with I_2 vapor (A). Ligand blot was performed with β_2-GPI and an anti-β_2-GPI Ab [Cof-22 (B) or EY2C9 Ab (C)].

Purification and characterization of a novel ligand, oxLig-2

The HPLC yielded a novel ligand, we named oxLig-2, from the scraped Band-2 (**Fig. 2A**, **B**). A peak that revealed binding specific for β_2-GPI and EY2C9 was eluted at approximately 26.7 min (equivalent to 13.4 ml of elution volume). To confirm the purity of oxLig-2 (fraction #14), the fraction was re-chromatographed under the same HPLC conditions (Fig. 2C, D) and subjected to the analysis by LC/MS.

A positive ionization mass spectrum of oxLig-2 showed three signals at *m/z* 383, 441, and 627 (**Fig. 3C**). These two smaller peaks, at *m/z* 383 (corresponding to 7-ketocholesterol) and 441 [corresponding to 7-ketocholesterol (+acetone)], were identical to those from oxLig-1 and 13-COOH-7KC (Fig. 3A, C, E). The signals at *m/z* 571, 627, and 627 were detected as a mother ion, $[M+H]^+$, in the positive mass spectra of oxLig-1, oxLig-2, and 13-COOH-7KC, respectively (Fig. 3A, C, E). In contrast, the signals at *m/z* 569, 625, and 625 were detected as a mother ion, $[M-H]^-$, in the negative mass spectra of oxLig-1, oxLig-2, and 13-COOH-7KC, respectively (Fig. 3B, D, F). In analysis of oxLig-2, another signal at *m/z* 627 was also detected as a mother ion of dihyro-oxLig-2 (Fig. 3D). In the negative mode, the signals at *m/z* 187, 243, and 243 were further observed as a daughter ion, $[D-H]^-$, of oxLig-1, oxLig-2, and 13-COOH-7KC, respectively (Fig. 3B, D, F, and **Fig. 4**).

The R_f position of oxLig-2 was lower than those of the related ω-carboxyl derivatives, i.e., oxLig-1 and 13-COOH-7KC, in TLC-ligand blot in either solvent A or B (Fig. 1 and **Fig. 5**), consistent with the deduced difference in polarity. After methylation with diazomethane, the bands corresponding to oxLi-g1, oxLig-2, and 13-COOH-7KC (i.e., Me-oxLig-1, Me-oxLig-2, and Me-13-COOH-7KC, respectively) shifted to higher R_f positions than those of the untreated ligands in TLC analysis in solvent B (Fig. 5A). The peak of oxLig-2 appeared earlier (26.7 min) than those of oxLig-1 (27.3 min) and 13-COOH-7KC (28.9 min) when analyzed by the reversed phase HPLC. Further, peaks of Me-oxLig-1 (29.6 min), Me-oxLig-2 (27.1 min), and 13-COOH-7KC (30.0 min) appeared later than those of untreated ligands, respectively. Interestingly, methylation completely diminished both ligand-interactions with β_2-GPI and an anti-β_2-GPI Ab (either Cof-22 and EY2C9) in the TLC-ligand blot (Fig. 5B, C).

In ELISA for anti-β_2-GPI Abs using a ligand-coated plate, significant binding of anti-β_2-GPI auto-Abs (WB-CAL-1 and EY2C9) was observed to solid phase oxLig-1, oxLig-2, and 13-COOH-7KC, but not solid phase Me-oxLig-1, Me-oxLig-2, and Me-13-COOH-7KC. Identical re-

TABLE 1. Binding of β_2-GPI and anti-β_2-GPI Abs to non-treated or methylated ligands in ELISA

	β_2-GPI Binding (Cof-22 Binding)		WB-CAL-1 Binding		EY2C9 Binding	
Solid Phase Ligand	Non-Treated	Methylated	Non-Treated	Methylated	Non-Treated	Methylated
Chol-linoleate	0.029 ± 0.004	N.T.	0.016 ± 0.007	N.T.	0.058 ± 0.015	N.T.
oxLig-1	1.936 ± 0.033	0.056 ± 0.004	0.958 ± 0.054	0.080 ± 0.020	1.947 ± 0.042	0.066 ± 0.020
oxLig-2	1.607 ± 0.057	0.082 ± 0.008	0.862 ± 0.179	0.107 ± 0.025	1.738 ± 0.008	0.057 ± 0.011
13-COOH	1.645 ± 0.064	0.122 ± 0.037	0.370 ± 0.066	0.067 ± 0.004	0.742 ± 0.021	0.105 ± 0.009

Anti-β_2-GPI Abs were incubated in a non-treated or methylated ligand-coated well in the presence (15 μg/ml) of β_2-GPI. Numbers indicate Ab binding (absorbance at 490 nm), the mean ± SD of triplicate samples. No Ab binding was detected to solid phase cholesterol or 7-ketocholesterol in the ELISA. Chol-linoleate, cholesteryl linoleate; N.T., not tested.

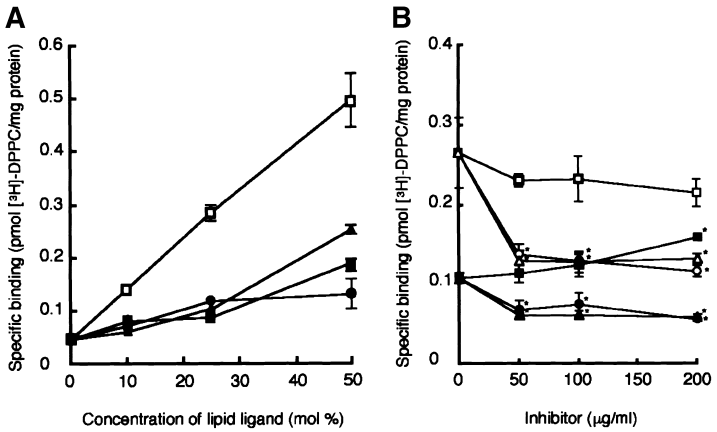

Fig. 6. Direct binding of ligand-containing liposomes to macrophages. A: A monolayer of J774A.1 cells was incubated for 2 h at 4°C with Celgrosser-P medium containing ^{3}H-labeled liposomes with the indicated concentration of ligand (50 nmol lipid/well). DPPS-containing liposomes (open square); oxLig-1-containing liposomes (closed square); oxLig-2-containing liposomes (closed circle); 13-COOH-7KC-containing liposomes (closed triangle). Data shown are the mean ± SD of triplicate samples. Panel B: In the culture system, indicated concentration of poly(I), poly(C), or fucoidan was added. DPPS (30 mol%)-liposomes with poly(I) (open circle), poly(C) (open square), or fucoidan (open triangle); oxLig-1 (30 mol%)-liposomes with poly(I) (closed circle), poly (C) (closed square), or fucoidan (closed triangle). * Student *t*-test, $P < 0.05$.

sults were obtained with Cof-22, a mouse monoclonal anti-β_2-GPI Ab obtained from a human β_2-GPI-immunized mouse (**Table 1**). All three Abs failed to bind to solid phase cholesterol, 7-ketocholesterol, and cholesteryl linoleate. From all of these results and the previously reported observations (43), the most likely structure of oxLig-$_2$ was concluded to be that of 7-ketocholesteryl-12-carboxy (keto)-dodecanoate, one of the oxidized products derived from cholesteryl linoleate (Fig. 4). However, the exact location of the ketone-group was not assigned.

Liposome binding to macrophages

Direct binding of liposomes containing oxLig-1, oxLig-2, or 13-COOH-7KC to mouse macrophages, i.e., J774A.1 cells, was compared with that of liposomes containing DPPS. DPPS-containing liposomes showed binding dependent upon the concentration of DPPS. In contrast, the liposomes containing oxLig-1, oxLig-2, or 13-COOH-7KC displayed relatively weak or negligible binding to the cells (**Fig. 6A**). Further, we have done inhibition experiments to see whether scavenger receptor(s) is involved in the binding of liposomes containing β_2-GPI ligands. As shown in Fig. 6B and **Table 2**, binding of oxLig-1-liposomes to macrophages was inhibited by the addition of poly(I) or fucoidan but not by poly(C). Similar results were obtained with DPPS-liposomes. In contrast, the binding of oxLig-2 or 13-COOH-liposomes was not affected even by the addition of poly(I) or poly(C). These results indicate that the scavenger receptor(s) may primarily be involved in binding of liposomes containing DPPS to macrophages but may only be weakly involved in those with β_2-GPI ligand-containing liposomes. Conversely, the uptake of oxLig-1, oxLig-2, and 13-COOH-7KC-containing liposomes by J774A.1 cells was significantly enhanced in the presence of both β_2-GPI and an anti-β_2-GPI Ab (WB-CAL-1), as compared with control binding of cholesteryl linoleate-liposomes (**Fig. 7A–D**). In contrast, binding of liposomes was almost completely eradicated by methylation of oxLig-1, oxLig-2, or 13-COOH-7KC (Fig. 7C, D). The β_2-GPI and anti-β_2-GPI Ab-mediated binding of ligand-containing liposomes was not affected either by poly(I) or poly(C).

DISCUSSION

We previously reported that the major lipid ligand, oxLig-1, specific for β_2-GPI and anti-β_2-GPI auto-Abs de-

TABLE 2. Effect of scavenger receptor's inhibitors on direct or β_2-GPI/antibody-mediated binding of ligand-containing liposomes to macrophages

Ligand	Control (w/o) Binding	w/poly(I) Binding	w/poly(I) Control	w/poly(I) *P*	w/poly(C) Binding	w/poly(C) Control	w/poly(C) *P*
			%			%	
(A) Direct binding							
oxLig-1	0.107 ± 0.007	0.073 ± 0.015	(68.8)	0.004	0.123 ± 0.014	(115)	N.S.
oxLig-2	0.076 ± 0.012	0.079 ± 0.016	(104)	N.S.	0.078 ± 0.010	(103)	N.S.
C13-COOH-7KC	0.100 ± 0.005	0.085 ± 0.017	(85.0)	N.S.	0.119 ± 0.012	(119)	N.S.
(B) β_2-GPI/antibody-mediated binding							
oxLig-1	4.08 ± 0.57	4.02 ± 0.23	(98.5)	N.S.	4.04 ± 0.30	(99.0)	N.S.
oxLig-2	1.98 ± 0.19	2.34 ± 0.20	(118)	N.S.	2.53 ± 0.45	(128)	N.S.
C13-COOH-7KC	0.761 ± 0.11	0.912 ± 0.30	(120)	N.S.	0.797 ± 0.18	(105)	N.S.

poly(I), polyinosinic acid; poly(C), polycytidylic acid; *P*, Student's *t*-test; N.S., not significant.

J774A.1 cells were incubated with ligand-containing liposomes, β_2-GPI, and WB-CAL-1 in the presence (100 μg/ml) or absence of poly(I)/ or poly(C). Numbers indicate Ab binding (absorbance at 490 nm), the mean ± SD of triplicate samples.

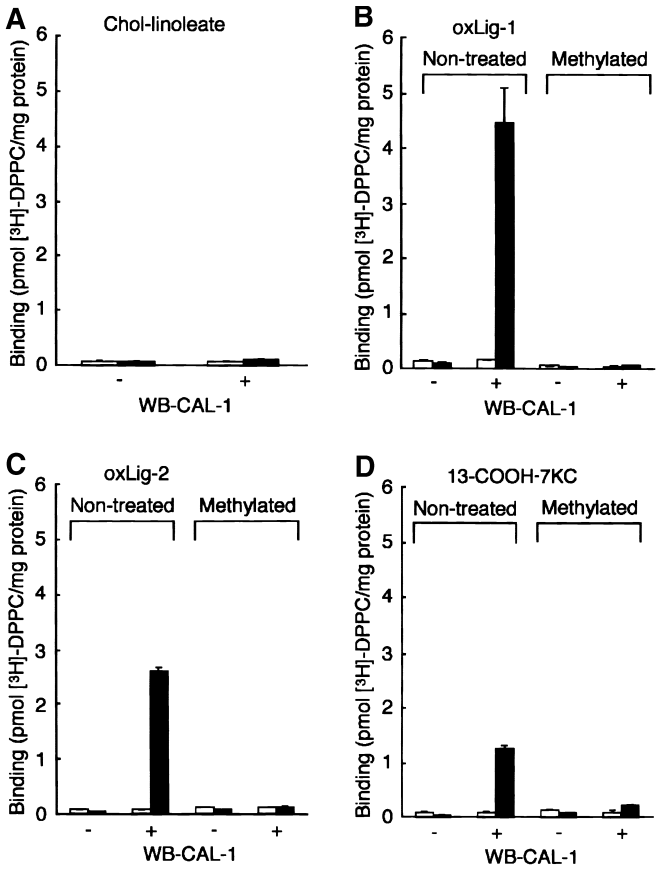

Fig. 7. β_2-GPI and anti-β_2-GPI Ab-dependent binding of ligand-containing liposomes to macrophage. A monolayer of J774A.1 cells was incubated for 2 h at 4°C with Celgrosser-P medium containing ^{3}H-labeled liposomes containing 30 mol% ligand (50 nmol lipid/well) in the presence (closed square)/or absence (open square) of β_2-GPI (200 μg/ml) and WB-CAL-1 (100 μg/ml). A: Cholesteryl linoleate-containing liposome; B: oxLig-1-containing liposomes; C: oxLig-2-containing liposomes; D: 13-COOH-7KC-containing liposomes. In panels B, C, and D, binding of methylated ligand-liposomes was also compared. Data are indicated as the mean ± SD of triplicate samples.

rived from the lipid oxidation of LDL (35), is oxLig-1 (Fig. 4). In the present study, we isolated another ligand (oxLig-2), which we characterized to be keto-dodecanoate. Derivatization of such ligands now demonstrate that an ω-carboxyl function introduced by Cu^{2+}-oxidation is critical for an interaction with β_2-GPI and its ligands.

Foam cell formation is regarded as the hallmark of early atherogenesis, and LDL is the major source of the lipid deposited in foam cells (26). Native LDL, under normal physiological conditions, cannot induce foam cell formation. The binding of modified LDL to scavenger receptors and possibly other cell surface sites on macrophages leads to unregulated cholesterol accumulation and the formation of foam cells with development of atherosclerotic lesions (44, 45).

Although the nature of the agents responsible for LDL oxidation in vivo is unknown, several candidates have been proposed (46–49). LDL oxidized with Cu^{2+} ion in vitro exhibits the physicochemical and immunological properties of oxLDL extracted from atherosclerotic lesions (28).

The Cu^{2+}-dependent oxidative products include cholesterol /or oxysterols esterified with 9- or 13-hydroperoxy (or hydroxy)-octadecadienoate, with 9-oxononanoate, or with 9-caboxynonanoate, some of which have also been shown to be present in atherosclerotic plaques (43, 50, 51). In the present study, we provide evidence that oxLig-2 is keto-dodecanoate (Fig. 4). Methylation of oxLig-2 indicated the presence of a carboxyl function on its acyl chain, but the exact location of the ketone-group cannot be assigned by mass spectrometry. Nevertheless, cholesteryl linoleate, which constitutes one of the major cholesteryl esters of LDL, has four carbons with double bonds susceptible to oxygenation at positions C9, C10, C12, and C13.

7-Ketocholesterol is a major oxysterol present in the Cu^{2+}-treated oxLDL (52–55). We have also synthesized a 22-ketocholesterol analog of oxLig-1 (i.e., 22-ketocholesteryl-9-carboxynonanoate; 9-COOH-22KC) in the same manner for synthesizing oxLig-1. However, 9-COOH-22KC did not have the same properties of β_2-GPI ligand (data not shown). The observation suggests that 7-ketocholesterol may also be responsible for binding to β_2-GPI, as well as an ω-carboxyl function on shortened fatty acid chains generated by the Cu^{2+}-oxidation of LDL.

It is now well established that anti-β_2-GPI Abs, found in APS patients, bind a complex of β_2-GPI and negatively charged PLs, such as cardiolipin, PS, and phosphatidic acid (36). In our recent (35) and present studies, however, negatively charged PLs are very minor components of oxLDL.

The flexible loop in the C-terminus and a particular cluster consisting of 14 residues of positively charged amino acid residues in domain V of β_2-GPI has a critical role for interaction with amphiphilic compounds such as cardiolipin, PS, phosphatidic acid, and phosphatidylglycerol (19–21, 36). Although β_2-GPI did not bind to cholesterol, 7-ketocholesterol, or cholesteryl linoleate, significant binding was observed to oxLig-1, oxLig-2, and 13-COOH-7KC. Thus, these oxysterol esters having an ω-carboxylated acyl chain, constitute a new class of an amphiphilic ligand suitable for β_2-GPI. Further, the observation that methylation of these ligands diminished the β_2-GPI interaction indicates that a free carboxyl residue is required for the recognition.

As previously described, in vivo uptake of oxLDL via scavenger receptor(s) of macrophages may play a central role in atherogenesis. The term, scavenger receptor(s), refers to a number of different cell-surface proteins that can bind chemically or biologically modified lipoproteins. Various scavenger receptors that bind oxLDL have been found on macrophages, including class A scavenger receptors (56), CD36 (57), human homolog CD68 (58), a lectin-like oxLDL receptor-1 (LOX-1) (59), and Fcγ receptor (60).

Although PS-containing liposomes showed significant binding to macrophages dependent upon the PS concentration, those containing oxLig-1, oxLig-2, and 13-COOH-7KC did not (Fig. 6). The simultaneous addition of β_2-GPI and an anti-β_2-GPI Ab significantly increased the binding

of liposomes containing oxLig-1, oxLig-2, and 13-COOH-7KC to the macrophages (Fig. 7). Further, poly(I) did not have any inhibitory effect on the binding of ligand-containing liposomes in the presence of β_2-GPI and an anti-β_2-GPI Ab. These results suggest that β_2-GPI and anti-β_2-GPI Ab mediated uptake of oxLDL occurs through Fcγ receptor on macrophages but not via scavenger receptor(s) (61–64).

In the present study, we demonstrate that oxidized cholesteryl esters, especially those with 7-ketocholesterol and an ω-carboxyl function in the acyl chain are ligands for β_2-GPI and anti-β_2-GPI auto-Abs. Such auto-Abs are found in APS patients and in an animal model, the WB F1 mouse. Furthermore, one major class of biochemically oxidized compounds derived from plasma LDL consists of ω-carboxylated oxysterols such as oxLig-1 and oxLig-2. Although 13-COOH-7KC is an artificially synthesized compound, it also showed significant binding to β_2-GPI as well as oxLig-1 and -2.

Most recently, we observed that high levels of circulating immune complexes containing oxLDL, β_2-GPI, and anti-β_2-GPI auto-Abs in the blood stream, were associated with development of arterial thrombosis in APS patients (unpublished observations). Thus, ω-carboxylation of oxysterol esters to form the autoantigenic complex of β_2-GPI bound to oxLDL may have patho-physiologically (etiologically) important roles, especially in development of APS and atherosclerosis.

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