

# The association of C-reactive protein with an oxidative metabolite of LDL and its implication in atherosclerosis

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**Abstract** C-reactive protein (CRP) is one of the strongest independent predictors of cardiovascular disease. We have previously reported that oxidized LDL (oxLDL) interacts with  $\beta$ 2-glycoprotein I ( $\beta$ 2GPI), implicating oxLDL/ $\beta$ 2GPI complexes as putative autoantigens in autoimmune-mediated atherosclerotic vascular disease. In this study, we investigated the interaction of CRP with oxLDL/ $\beta$ 2GPI complexes and its association with atherosclerosis in patients with diabetes mellitus (DM). CRP/oxLDL/ $\beta$ 2GPI complexes were predominantly found in sera of DM patients with atherosclerosis. In contrast, noncomplexed CRP isoforms were present in sera of patients with acute/chronic inflammation, i.e., various pyrogenic diseases, rheumatoid arthritis (RA), and DM. Immunohistochemistry staining colocalized CRP and  $\beta$ 2GPI together with oxLDL in carotid artery plaques but not in synovial tissue from RA patients, strongly suggesting that complex formation occurs during the development of atherosclerosis. Serum levels of CRP correlated with soluble forms of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, and oxLDL/ $\beta$ 2GPI complexes correlated with total cholesterol and hemoglobin A1c. **■** Thus, the generation of CRP/oxLDL/ $\beta$ 2GPI complexes seems to be associated with arterial inflammation, hyperglycemia, and hypercholesterolemia. CRP/oxLDL/ $\beta$ 2GPI complexes can be distinguished from pyrogenic noncomplexed CRP isoforms and may represent a more specific and predictive marker for atherosclerosis.—Tabuchi, M., K. Inoue, H. Usui-Kataoka, K. Kobayashi, M. Teramoto, K. Takasugi, K. Shikata, M. Yamamura, K. Ando, K. Nishida, J. Kasahara, N. Kume, L. R. Lopez, K. Mitsudo, M. Nobuyoshi, T. Yasuda, T. Kita, H. Makino, and E. Matsuura. **The association of C-reactive protein with an oxidative metabolite of LDL and its implication in atherosclerosis.** *J. Lipid Res.* 2007. 48: 768–781.

**Supplementary key words**  $\beta$ 2-glycoprotein I • oxidized LDL/ $\beta$ 2-glycoprotein I complexes • diabetes mellitus • oxidized LDL

Atherosclerosis is a pathological condition in which arteries undergo gradual intima thickening, causing decreased elasticity, narrowing, and reduced blood supply. The atherosclerotic involvement of these blood vessels brings about the distinct clinical manifestations of cardiovascular disease (CVD). A characteristic histopathologic finding of atherosclerosis is the focal appearance of macrophage-derived lipid-laden foam cells. The cholesterol that accumulates in foam cells is derived from circulating lipids, mainly from oxidized LDL (oxLDL) (1, 2) generated by vascular inflammation and oxidative stress from different types of pathologic injury. OxLDL is also a proinflammatory chemoattractant agent for macrophages and T lymphocytes, cytotoxic for endothelial cells, and stimulates the release of soluble inflammatory molecules. All of these events not only help to perpetuate a cycle of vascular inflammation and lipid dysregulation within the arterial walls but also create an endothelial prothrombotic state that complicates late stages of atherosclerosis (i.e., arterial thrombosis).

Abbreviations: APS, antiphospholipid syndrome; AT, atherosclerosis; CRP, C-reactive protein; CVD, cardiovascular disease; DM, diabetes mellitus;  $\beta$ 2GPI,  $\beta$ 2-glycoprotein I; HbA1c, hemoglobin A1c; hsCRP, high-sensitivity CRP; ICAM-1, intercellular adhesion molecule-1; IMT, intima media thickness; MAb, monoclonal antibody; oxLDL, oxidized LDL; pI, isoelectric point; RA, rheumatoid arthritis; ROC, receiver operating characteristic; T-chol, total cholesterol; VCAM-1, vascular cell adhesion molecule-1.

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Antiphospholipid syndrome (APS) is an autoimmune disease characterized by the presence of a heterogeneous group of antiphospholipid antibodies (Abs) with arterial and/or venous thromboembolic complications, and/or pregnancy morbidity (3, 4). Antiphospholipid Abs are directed against epitopes that include proteins such as  $\beta$ 2-glycoprotein I ( $\beta$ 2GPI) (4–6), lipids, and protein-lipid complexes. We have demonstrated that anti- $\beta$ 2GPI auto-Abs also recognize oxLDL/ $\beta$ 2GPI complexes (4, 7–10). Further, the *in vitro* macrophage uptake of oxLDL/ $\beta$ 2GPI complexes was significantly increased by IgG anti- $\beta$ 2GPI Abs. (4, 7–10) Thus, findings suggesting that IgG anti- $\beta$ 2GPI auto-Abs may be proatherogenic renewed interest in the immunologic mechanisms participating in atherogenesis (4, 11, 12).

$\beta$ 2GPI interacts with oxLDL via oxidatively induced  $\beta$ 2GPI-specific ligands, e.g., 7-ketocholesterol having an  $\omega$ -carboxyl acyl chain. This interaction may take place in the arterial intima of atherosclerotic lesions, producing stable and nondissociable oxLDL/ $\beta$ 2GPI complexes (4, 8–10). These complexes would then be released into the circulation. In addition to systemic autoimmune diseases (15, 16), oxLDL/ $\beta$ 2GPI complexes have been found in non-autoimmune chronic inflammatory diseases that develop atherosclerosis, such as diabetes mellitus (DM) (13) and chronic nephritis (14). OxLDL/ $\beta$ 2GPI complexes may represent a serologically relevant form of circulating oxLDL, because oxLDL injected intravenously into experimental animals was immediately removed from circulation by the liver (17).

C-reactive protein (CRP) is an acute-phase reactant that belongs to the highly conserved “pentraxin” family and plays a major role in innate immunity (18–20). CRP binds to a range of autologous and exogenous ligands, including phosphorylcholine originating from either the capsular polysaccharide component of microorganisms, native LDL, oxLDL, or apoptotic cells. This interaction moderates the clearance of opsonized CRP by macrophages (21–24). Further, CRP binds to multiple ligands, activating the classical complement pathway, which in turn enhances phagocytosis via complement receptors. Epidemiologic studies have shown an association between CRP and the risk for CVD (25, 26). Multiple clinical studies have demonstrated a predictive relationship between increased CRP levels and atherothrombotic events. It has also been suggested that CRP is not only a serologic marker for atherosclerosis but also a causal risk factor, stimulating adhesion molecule expression and chemokine production by endothelial cells (27, 28). Thus, CRP aids in the first line of host defense against infection and is also involved in inflammatory processes that promote atherosclerosis (atherothrombosis) (29).

In the present study, circulating CRP/oxLDL/ $\beta$ 2GPI complexes demonstrated by ELISA and immunoblot analysis were predominantly detected in DM patients with atherosclerosis. Because CRP/oxLDL/ $\beta$ 2GPI complexes were not detected in patients with pyrogenic diseases and rheumatoid arthritis (RA), they may be a part of the CVD predictor, i.e., high-sensitivity CRP (hsCRP). Immunohistochemistry also revealed the possibility that the com-

plexes are formed in atherosclerotic plaques and released into the circulation. Thus, CRP/oxLDL/ $\beta$ 2GPI complexes, which can be distinguished from pyrogenic noncomplexed CRP isoforms, may be a more specific and predictive marker for atherosclerosis (atherothrombosis).

## MATERIALS AND METHODS

### Subjects and specimens

The present cross-sectional study was performed at Okayama University Hospital (Okayama, Japan). The protocols were approved by the Institutional Ethical Review Board of Okayama University Hospital. Informed consent was given by all participants.

Type 2 DM patients ( $n = 125$ ) fulfilling the diagnostic criteria of the World Health Organization (WHO) (30) were enrolled in the present study. The diagnosis of DM was based on the presence of chronic hyperglycemia and metabolic disturbances of lipid, carbohydrate, and protein metabolism due to defects in insulin production or activity. Impaired fasting glycemia and/or glucose tolerance test results were used according to WHO established criteria. DM patients with significant CVD events, pregnancy, inflammatory disease, and/or steroid use were excluded. DM patients had a male to female ratio of 73:52 and a mean age of  $61.4 \pm 13.3$  years (mean  $\pm$  SD; range 34–67 years). Age-matched RA patients ( $n = 48$ ) and healthy subjects ( $n = 48$ ) were enrolled. All RA patients met the American College of Rheumatology revised criteria (31). RA patients with DM and/or significant CVD events were excluded. RA patients had a male to female ratio of 7:41 and a mean age of  $64.0 \pm 10.0$  years (range 33–80 years). Thirty-five patients with acute pyrogenic diseases (i.e., acute enteritis, acute pancreatitis, acute prostatitis, acute pyelonephritis, acute tonsillitis, appendicitis, bronchitis, diverticulitis of colon, cystitis, epididymitis, femoral necrotizing fasciitis, gonitis, fever of unknown cause, infectious enteritis, mesenteric panniculitis, pharyngitis, or pneumonia) without DM, CVD, or collagen diseases were enrolled. All serum samples were stored at  $-80^\circ\text{C}$  until use.

Tissue samples were prepared from human carotid endarterectomy specimens from 17 patients with transient ischemic attack or minor completed strokes before surgery. Synovial tissue samples were prepared from five RA and five osteoarthritis patients. All tissues were fixed with neutral buffered formalin, embedded in paraffin, and sectioned by standard procedures.

### Abs and reagents

Monoclonal antibody (MAb) WB-CAL-1 (IgG<sub>2a</sub>,  $\kappa$ ) was derived from nonimmunized NZW  $\times$  BXSb F1 male mice, an animal model of APS (32). WB-CAL-1 MAb binds to  $\beta$ 2GPI complexed with oxLDL or lipid vesicles containing anionic phospholipids or oxidized cholesteryl esters derived from oxLDL, but not to the noncomplexed form of the protein (5, 7–10). In contrast, another anti- $\beta$ 2GPI MAb, Cof-23 (IgG<sub>1</sub>,  $\kappa$ ), obtained from BALB/c mice immunized with human  $\beta$ 2GPI, recognizes both the noncomplexed and lipid-complex  $\beta$ 2GPI structures (33). Two anti-apolipoprotein B-100 (apoB-100) MAbs were established from BALB/c mice immunized with human native LDL and Cu<sup>2+</sup>-oxLDL, respectively: N2E10 (IgG<sub>2a</sub>,  $\kappa$ ), which recognizes apoB-100 present in either native LDL or oxLDL; and O1F9 (IgG<sub>2a</sub>,  $\lambda$ ), which recognizes apoB-100 present in oxLDL but not in native LDL (i.e., anti-oxLDL Ab). A MAb against scavenger receptor SR-PSOX (34) was used for immunohistochemistry staining. Commercially available goat anti-CRP Abs were obtained from Bethyl Laboratories, Inc. (Montgomery, TX); mouse anti-

CD68 MAb and mouse anti- $\alpha$  actin of smooth muscle cells (SMC $\alpha$ ) MAb from Dako (Kyoto, Japan); horseradish peroxidase (HRP)-labeled rabbit anti-CRP Abs from Medical and Biological Laboratories (Nagoya, Japan); and human CRP, purified from pleural fluid (Chemicon International, Temecula, CA) and from serum (Calbiochem, EMD Biosciences, Inc., La Jolla, CA). Purity of these CRP preparations was checked by SDS-PAGE, yielding a 24 kDa single band under reducing or nonreducing conditions. Both CRP preparations were extensively dialyzed against 10 mM Tris, 150 mM NaCl, 1.25 mM CaCl<sub>2</sub>, pH 7.4 (Tris buffer) to remove NaN<sub>3</sub> before use.  $\beta$ 2GPI was purified from healthy human plasmas, as described (10). Other chemicals were obtained from commercial sources and were of reagent-grade quality.

### Isolation and oxidation of LDL

LDL ( $1.019 < d < 1.063$ ) was isolated by ultracentrifugation from fresh healthy human plasma, as described (10). LDL (100  $\mu$ g/ml of apoB equivalent) in 10 mM Hepes and 150 mM NaCl, pH 7.4, (Hepes buffer) was oxidized by incubation with 5  $\mu$ M CuSO<sub>4</sub> for 12 h at 37°C. Aliquots were taken to determine thiobarbituric acid-reactive substances and electrophoretic migration in agarose gels.

### Reduction of endogenous and/or exogenous endotoxin (lipopolysaccharide) from reagents

Affinity chromatography using a Detoxi-Gel column (Pierce; Rockford, IL) was performed to remove exogenous endotoxin that might have contaminated the CRP and  $\beta$ 2GPI preparations during isolation. In experiments to assess endotoxin involvement in complex formation, additional purification steps were performed. For example, endotoxin-free water and sterile materials, including glassware, which were either commercially apyrogenic or depyrogenated by steam autoclaving and/or dry heat, were used during LDL isolation. Endotoxin levels were determined by the Limulus amoebocyte lysate assay.

### ELISA for oxLDL complexes

*ELISA for oxLDL/ $\beta$ 2GPI complexes.* This procedure was performed as previously described (10). Briefly, anti- $\beta$ 2GPI MAb, WB-CAL-1, was adsorbed onto microtiter plates (Immulon 2HB; Thermo Labsystems, Franklin, MA) by incubating at 8  $\mu$ g/ml (dissolved in Hepes buffer, 50  $\mu$ l/well) at 4°C overnight. After blocking with Hepes buffer containing 1% skim milk, samples diluted 1:100 with Hepes buffer containing 0.5% skim milk were added to the wells (100  $\mu$ l/well) to be incubated for 2 h. The wells were then incubated with HRP-labeled anti-human apoB-100 MAb (N2E10). Extensive washing between steps was performed with Hepes buffer containing 0.05% Tween 20. Color was developed with tetramethylbenzidine and H<sub>2</sub>O<sub>2</sub>. The reaction was terminated, and optical density at 450 nm was measured.

*ELISA for CRP/oxLDL complexes.* Captured anti-apoB-100 MAb, N2E10, was adsorbed onto microtiter plates (Immulon 2HB; Thermo Labsystems) by incubating at 8  $\mu$ g/ml (dissolved in Hepes buffer, 50  $\mu$ l/well) at 4°C overnight. After blocking with 10 mM Tris, 150 mM NaCl, 1.25 mM CaCl<sub>2</sub>, pH 7.4, (Tris buffer) containing 0.5% BSA, samples diluted 1:100 with Tris buffer containing 0.2% BSA were added to each well and incubated for 2 h. The wells were subsequently incubated with HRP-labeled anti-CRP Abs for 1 h. Extensive washing between steps was performed with Tris buffer containing 0.05% Tween 20. Further steps were performed as described above for oxLDL/ $\beta$ 2GPI complexes.

*ELISA for CRP/oxLDL/ $\beta$ 2GPI complexes.* The assay was similar to that for the ELISA for CRP/oxLDL complexes, with the exception that captured N2E10 MAb was replaced by anti- $\beta$ 2GPI MAb WB-CAL-1 on the plate.

### Nondenaturing PAGE (native PAGE) and immunoblot analysis

Nondenaturing PAGE (native PAGE without SDS) was carried out on 2–15% polyacrylamide gradient gels according to the method of Krauss and Burke (35), with slight modifications. A pH of 8.3 was used for running the gels to facilitate the characterization of LDL subclasses. To avoid the influence of protein charge, protein markers having similar isoelectric points (pIs) are generally used in nondenaturing PAGE. A set of commercially available molecular mass markers (Daiichi Pure Chemicals; Tokyo, Japan) for nondenaturing PAGE composed of thyrolobulin (669 kDa, pI 4.6), ferritin (443 kDa, pI 4.5), lactate dehydrogenase (140 kDa, pI 4.0), BSA (66 kDa, pI 4.7), and soybean trypsin inhibitor (20.1 kDa, pI 4.50) was used for the study. Then the proteins were transferred to a polyvinylidene difluoride membrane, and immunoblot was performed with anti-CRP Abs anti- $\beta$ 2GPI MAb (Cof-23) or anti-apo-B100 MAb (N2E10).

### Immunohistochemistry

Details of the immunohistochemical staining procedure were previously described (36). Pretreatment of human carotid endarterectomy and synovial tissue sections in an oil bath (97°C, 20 min in 0.1 M Tris-HCl, pH 6.0) was performed after deparaffinization according to the modified method of Shi, Key, and Kalra (37), except for the CRP staining run. Endogenous peroxidase activity was blocked by treatment with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min, followed by blocking with 2% normal goat serum. Immunohistochemical staining of adjacent sections was carried out using Abs against CD68, SMC $\alpha$ , CRP, SR-PSOX, and apoB in either native LDL or oxLDL (N2E10), apoB in oxLDL (O1F9), noncomplexed and complexed forms of  $\beta$ 2GPI (Cof-23), and complexed form of  $\beta$ 2GPI (WB-CAL-1).

### Other clinical and biological parameters

Intima media thickness (IMT) was measured by B-mode ultrasonography. Three IMT measurements of diastolic images on each side at 10 mm before or after the carotid bifurcation were obtained. Mean IMT was calculated for each point, and the highest value (maximum IMT) was recorded for each subject and was defined as the distance from the lumen-intima interface to the intima-adventitia interface. An IMT cutoff value of 0.75 mm was used to diagnose atherosclerosis. Serum hsCRP was measured by nephelometry, a latex particle-enhanced immunoassay (N-Latex CRPII; Dade Behring, Tokyo, Japan). Total cholesterol (T-chol), hemoglobin A1c (HbA1c), and CRP were measured by routine laboratory methods. Soluble forms of intracellular adhesion molecule-1 (sICAM-1) and vascular cell adhesion molecule-1 (sVCAM-1) were measured by commercially available ELISAs (R and D Systems, Inc.; Minneapolis, MN).

### Statistical analyses

Statistical analysis was performed by StatView software (Abacus Concepts; Berkeley, CA). The Student *t*-test was used to assess possible correlation between levels of CRP or three types of oxLDL complexes and the occurrence of disease. The correlation between two variables was evaluated by the Pearson correlation test. Fisher's exact test was used to compare the appearance of biological markers, CRP and oxLDL complexes. Ninety-five percent confidence interval (95% CI) was calculated



by Woolf's method. The level of  $P < 0.05$  was considered statistically significant.

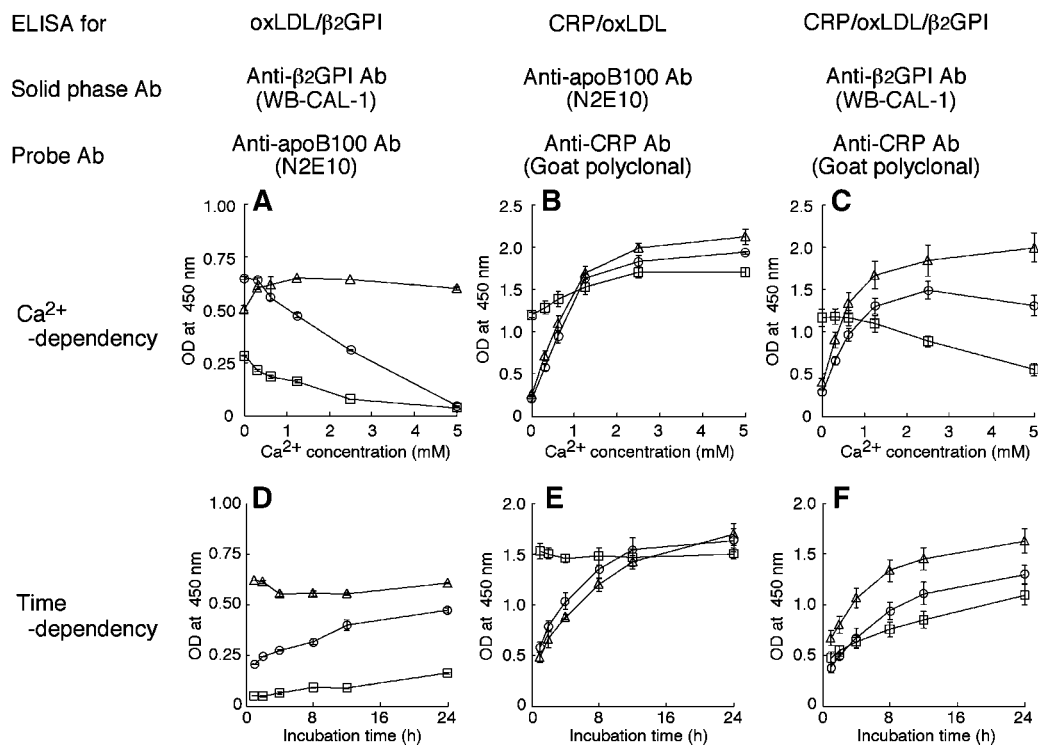
## RESULTS

### Formation of CRP/oxLDL/ $\beta$ 2GPI complexes

The nature of the interaction between oxLDL and  $\beta$ 2GPI has been previously characterized and described (8–10). We first investigated the interaction among  $\text{Cu}^{2+}$ -oxLDL,  $\beta$ 2GPI, and CRP. **Figure 1A–C** depicts the calcium dependency of the interaction of oxLDL with CRP and oxLDL with  $\beta$ 2GPI. Figure 1A shows that the interaction between oxLDL and  $\beta$ 2GPI was calcium independent from 0 to 5 mM of  $\text{Ca}^{2+}$  ion. In contrast, the interaction between oxLDL and CRP was calcium dependent (Fig. 1B). The complexes were not dissociated even if excess EDTA or  $\text{Ca}^{2+}$  ion was added after the completion of the reaction (data not shown). At physiological calcium concentrations

(approximately 1.25 mM), both the calcium-independent and -dependent interactions took place (Fig. 1A–C). CRP/oxLDL/ $\beta$ 2GPI complexes were formed proportionally when these three components were coincubated (Fig. 1C). No direct interaction between CRP and  $\beta$ 2GPI was observed under any experimental conditions. Figure 1D–F reveals the time-dependent formation of oxLDL complexes with CRP and/or  $\beta$ 2GPI in the presence of 1.25 mM of  $\text{Ca}^{2+}$  ion at 37°C. The interaction of oxLDL with CRP and oxLDL with  $\beta$ 2GPI occurred in a time-dependent as well as dose-dependent manner. These two interactions progressed gradually and reached a plateau after ~12 h.

While the interaction between  $\beta$ 2GPI and oxLDL was calcium independent, the interaction between CRP and oxLDL was calcium dependent. At physiological concentrations of calcium, long incubations (of several hours) were required for the in vitro formation of nondissociable CRP/oxLDL/ $\beta$ 2GPI complexes or oxLDL/ $\beta$ 2GPI complexes. The oxLDL-negative charges acquired during



**Fig. 1.** Profiles of complex formation among  $\text{Cu}^{2+}$ -oxidized LDL (oxLDL),  $\beta$ 2-glycoprotein I ( $\beta$ 2GPI), and C-reactive protein (CRP). For the experiment, oxLDL/ $\beta$ 2GPI and CRP/oxLDL complexes were preformed by incubating  $\text{Cu}^{2+}$ -oxLDL [1 mg apolipoprotein B (apoB) equivalent/ml] and  $\beta$ 2GPI (1 mg/ml) in the absence of  $\text{CaCl}_2$ , and  $\text{Cu}^{2+}$ -oxLDL (1 mg apoB equivalent/ml) and CRP (1 mg/ml) in the presence of 2 mM  $\text{CaCl}_2$ , respectively, at 37°C for 16 h. Subsequently, the complexes were purified by size exclusion column chromatography, as shown in Fig. 2B. A–C: CRP, oxLDL,  $\beta$ 2GPI, preformed oxLDL/ $\beta$ 2GPI complexes, and/or preformed CRP/oxLDL complexes were incubated at 37°C for 16 h in the presence of different concentrations of  $\text{CaCl}_2$ . Concentration of each material was 50  $\mu\text{g}/\text{ml}$  protein or 50  $\mu\text{g}/\text{ml}$  apoB-100 equivalent. Reaction was terminated by immediate freeze, and an aliquot of each specimen was diluted up to 0.5  $\mu\text{g}/\text{ml}$  of apoB equivalent (1:100 dilution) to apply to the ELISAs. Generation of oxLDL/ $\beta$ 2GPI complexes (A), CRP/oxLDL complexes (B), or CRP/oxLDL/ $\beta$ 2GPI complexes (C) was detected in ELISAs, as described in Materials and Methods. D–F: CRP, oxLDL,  $\beta$ 2GPI, preformed oxLDL/ $\beta$ 2GPI complexes, and/or preformed CRP/oxLDL complexes were incubated at 37°C for different periods in the presence of 1.25 mM  $\text{CaCl}_2$ , and generated complexes (D–F) were measured by ELISA, as described above. All values in the in vitro experiments are expressed as the mean  $\pm$  SD. Circles, CRP + oxLDL +  $\beta$ 2GPI; triangles, preformed oxLDL/ $\beta$ 2GPI complexes + CRP; squares, preformed CRP/oxLDL complexes +  $\beta$ 2GPI. Error bars represent mean  $\pm$  SD.

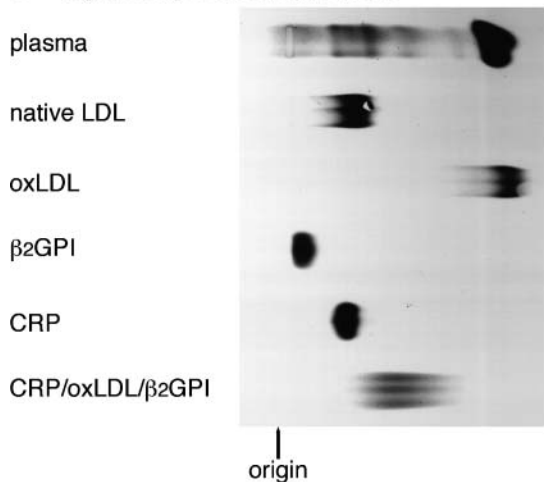
CuSO<sub>4</sub> incubation were neutralized by the interaction with these proteins (Fig. 2A).

We also confirmed that significant in vitro production of CRP/oxLDL/β<sub>2</sub>GPI complexes occurs at reduced endotoxin concentrations (28 pg/ml). However, the potential influence of such small amounts of endotoxin contaminating the reaction mixture on complex formation should be investigated in further studies.

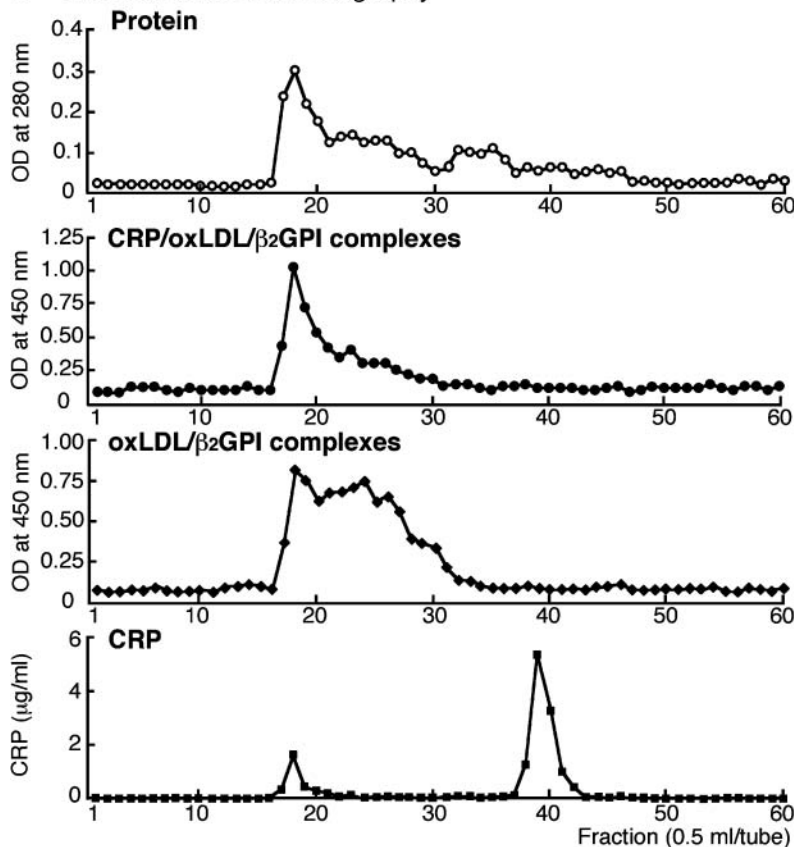
Figure 2B shows the elution profile of size exclusion chromatography of the reaction mixture containing CRP and oxLDL/β<sub>2</sub>GPI complexes. CRP/oxLDL/β<sub>2</sub>GPI and

oxLDL/β<sub>2</sub>GPI complexes were mainly eluted in fractions 17–30 (mainly 17–22) and 17–30 (mainly 20–30), respectively. CRP was detected in the first peak (corresponding to the size of CRP/oxLDL/β<sub>2</sub>GPI particles) and in a later fraction (38–42), where the commercially available pentameric CRP (120 kDa; derived from pleural fluid or serum) was eluted. The contamination of the modified CRP isoform having a molecular mass of 75 kDa (38) in the CRP protein(s) eluted in fractions larger than 50 kDa cannot be excluded. Thus, these results indicate that CRP/oxLDL/β<sub>2</sub>GPI complexes can cross-react in the hsCRP assay. The

### A Agarose gel electrophoresis



### B Size exclusion chromatography



**Fig. 2.** In vitro formation of CRP/oxLDL/β<sub>2</sub>GPI complexes. Cu<sup>2+</sup>-OxLDL (1 mg/ml of apoB equivalent) was incubated with β<sub>2</sub>GPI (1 mg/ml) for 16 h at 37°C to form oxLDL/β<sub>2</sub>GPI complexes. The purified oxLDL/β<sub>2</sub>GPI (1 mg/ml of apoB equivalent) was further incubated with CRP (purified from human pleural fluid; 0.1 mg/ml) in the presence of 2 mM CaCl<sub>2</sub> for 16 h at 37°C, to form nondissociable CRP/oxLDL/β<sub>2</sub>GPI complexes. The complexes were purified by size exclusion chromatography. A: Electrophoresis on an agarose gel was performed and proteins were visualized by amido black staining. The negative charge in oxLDL was significantly neutralized by the complex formation with CRP and β<sub>2</sub>GPI. B: Elution profile of the size exclusion column chromatography of CRP/oxLDL/β<sub>2</sub>GPI complexes is shown. The reaction mixture (100 μl aliquot) containing CRP and oxLDL/β<sub>2</sub>GPI complexes was applied on a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech) equipped with fast-protein liquid chromatography and eluted with Tris buffer containing 1.25 mM CaCl<sub>2</sub>. CRP/oxLDL/β<sub>2</sub>GPI and oxLDL/β<sub>2</sub>GPI complexes in 100-fold diluted fractions were detected by ELISAs, and CRP in nondiluted fractions was detected by high-sensitivity CRP nephelometry.

total recovery of CRP from all eluates (including noncomplexed CRP) detected by hsCRP nephelometry varied in individual experiments, with ranges between 38% and 70%.

### Diagnostic accuracy of CRP/oxLDL/ $\beta$ 2GPI complex assay

The principle of the ELISA for CRP/oxLDL/ $\beta$ 2GPI complexes is schematically represented in Fig. 3A. The assay was specific for CRP/oxLDL/ $\beta$ 2GPI complexes without any cross-reactivity to CRP/oxLDL complexes or oxLDL/ $\beta$ 2GPI complexes (Fig. 3B). In addition, the CRP/oxLDL/ $\beta$ 2GPI complex value (30 ng/ml of apoB equivalent) was not affected by addition of excess amounts of oxLDL/ $\beta$ 2GPI complexes (300 ng/ml of apoB equivalent, expected as pathophysiological concentration) in the CRP/oxLDL/ $\beta$ 2GPI ELISA. The ELISA for oxLDL/ $\beta$ 2GPI complexes was previously described elsewhere (10).

In the present study, the clinical significance of circulating CRP/oxLDL/ $\beta$ 2GPI, oxLDL/ $\beta$ 2GPI, and CRP/oxLDL complexes was assessed by ELISA. Cutoff values for these ELISAs were analyzed by two methods: a receiver operating characteristic (ROC) curve using DM patients,

and by calculating one to five standard deviations (SDs) above mean values using 48 healthy subjects. For the ROC analysis, atherosclerosis was diagnosed according to IMT measurements. A mean + 3 SD of the healthy subjects was used as cutoff value. One U/ml was defined as the mean + 3 SD of controls. The assay was reproducible, with intra- and inter-assay coefficients of variation not exceeding 7.0%. The same procedure was used to calculate the CRP cutoff value (0.16 mg/dl).

Diagnostic accuracy of the CRP/oxLDL/ $\beta$ 2GPI complex ELISA for atherosclerosis, as compared with CRP nephelometry and the other two complex ELISAs, was assessed by determining sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), odds ratio (OR), and 95% confidence interval (CI) (Table 1). ROC curves for these three markers were similar (data not shown). In the present study, a subgroup of 69 DM patients (49 patients without atherosclerosis and 20 patients with atherosclerosis) was analyzed. Both CRP/oxLDL/ $\beta$ 2GPI and CRP/oxLDL ELISAs showed lower sensitivity (25%) but 100% specificity and PPV, and similar NPV (76.6%) compared with the CRP assay. The measurement of circulating CRP/oxLDL/ $\beta$ 2GPI complexes may reliably help to predict the development of atherosclerosis. Thus, a major benefit of these complex ELISAs is that they allow the exclusion of cross-reactive noncomplexed CRP isoforms frequently found in sera of patients with various unrelated acute/chronic infections.

### Serum levels of oxLDL/ $\beta$ 2GPI and CRP/oxLDL/ $\beta$ 2GPI complexes

Figure 4 shows the serum levels of CRP, oxLDL/ $\beta$ 2GPI, CRP/oxLDL, and CRP/oxLDL/ $\beta$ 2GPI complexes in patients with various diseases and in healthy subjects. Elevated levels of CRP were observed in patients with DM, RA, and pyrogenic diseases [25.6% (32/125), 75.0% (36/48), and 100% (35/35), respectively] (Fig. 4A). Statistical significance was observed between DM and healthy subjects, RA and healthy subjects, and DM and RA patients. OxLDL/ $\beta$ 2GPI complexes were found in 44.0% of DM patients (55/125) but not in healthy subjects, RA patients, or patients with pyrogenic diseases (Fig. 4B). Seven DM patients had high levels of CRP/oxLDL (5.6%) and nine had high levels of CRP oxLDL/ $\beta$ 2GPI complexes (7.2%). Five DM patients had both (Fig. 4C, D). Although none of the patients in the other groups showed high levels of CRP/oxLDL/ $\beta$ 2GPI

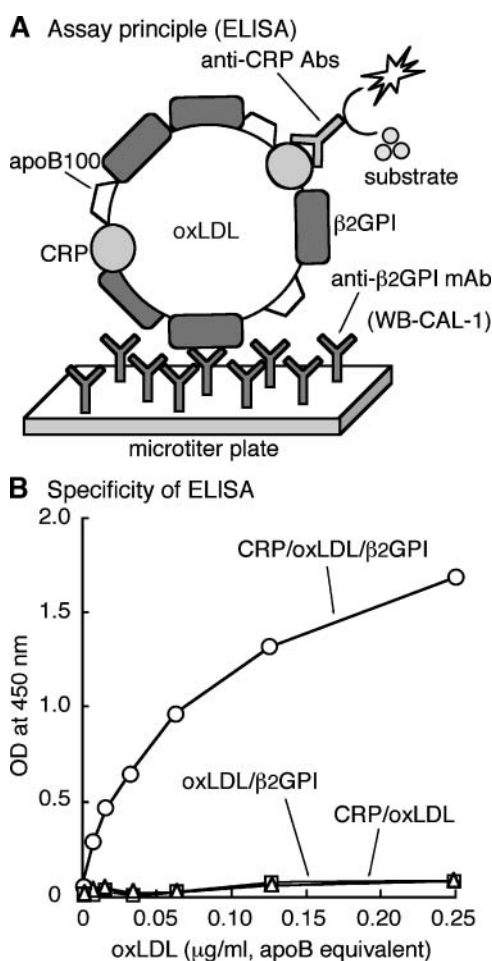
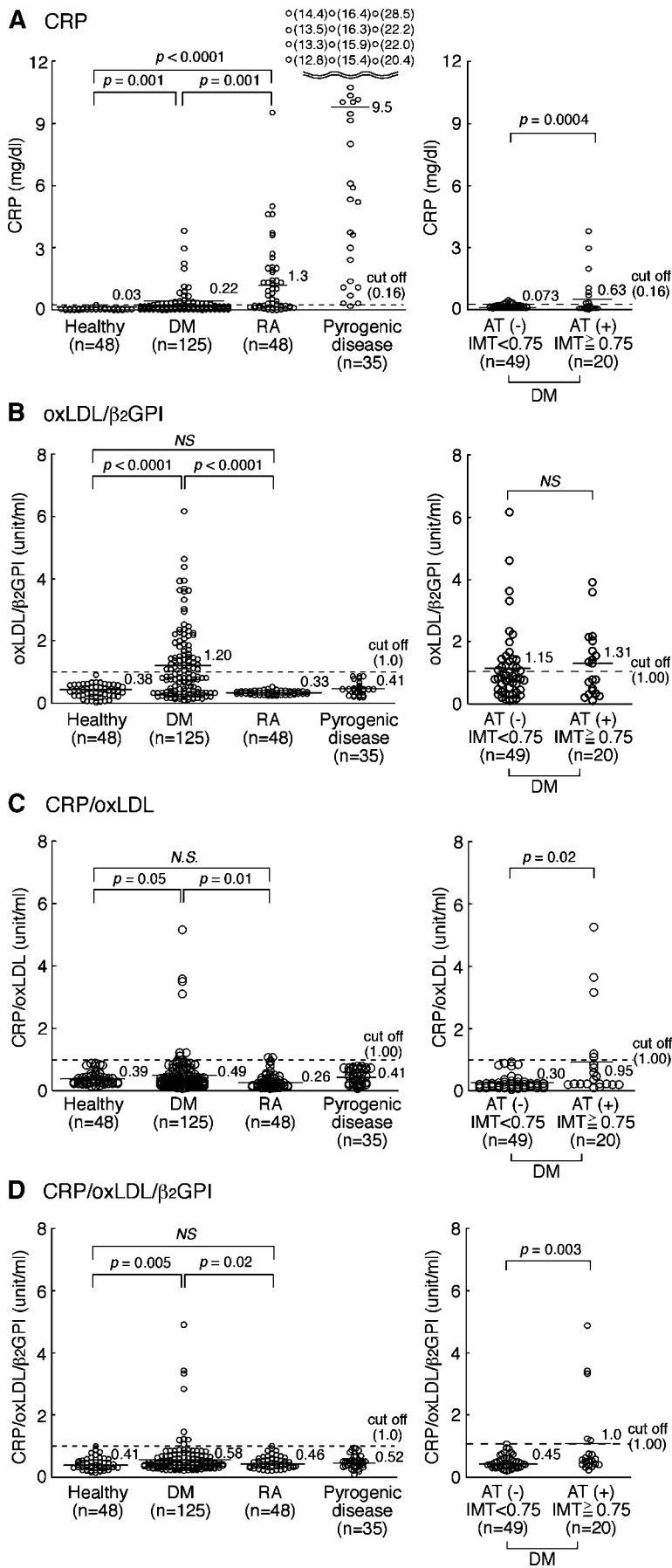


Fig. 3. Sandwich ELISA for oxLDL/ $\beta$ 2GPI complexes. Assay principle of the ELISA (A) and specificity (cross-reactivity) (B) are shown. Circles, CRP/oxLDL/ $\beta$ 2GPI; squares, oxLDL/ $\beta$ 2GPI; triangles, CRP/oxLDL.

TABLE 1. Diagnostic accuracy of CRP and oxLDL complexes

	Sensitivity	Specificity	PPV	NPV	OR	95% CI
	%					
CRP	45	90	64	80	7.2	2.0–26
OxLDL/ $\beta$ 2GPI complexes	50	57	32	74	1.3	0.5–3.8
CRP/oxLDL complexes	25	100	100	77	—	—
CRP/oxLDL/ $\beta$ 2GPI complexes	25	100	100	77	—	—

CRP, C-reactive protein;  $\beta$ 2GPI,  $\beta$ 2-glycoprotein I; PPV, positive predictive value; NPV, negative predictive value; OR, odds ratio; oxLDL, oxidized LDL; 95% CI, 95% confidence interval.



**Fig. 4.** Serum levels of CRP and three kinds of oxLDL complexes in patients with diabetes mellitus (DM), rheumatoid arthritis (RA), or pyrogenic disease. Serum CRP was measured by the routine or high-sensitivity methods (A). Serum oxLDL/β2GPI complexes (B), CRP/oxLDL complexes (C), and CRP/oxLDL/β2GPI complexes (D) were detected by ELISA. Complex levels are expressed in arbitrary units. A sample was considered positive when its complex level was higher than the cutoff value, 1 U/ml. The number of patients and mean values for each group are shown. The Student's *t*-test was performed.  $P < 0.05$  was considered statistically significant. NS, not significant.



complexes, two RA patients had slightly elevated levels of CRP/oxLDL complexes (just above the cutoff value). CRP/oxLDL/ $\beta$ 2GPI and CRP/oxLDL complex levels between DM and healthy subjects, DM and RA, and DM and patients with pyrogenic diseases were statistically significant.

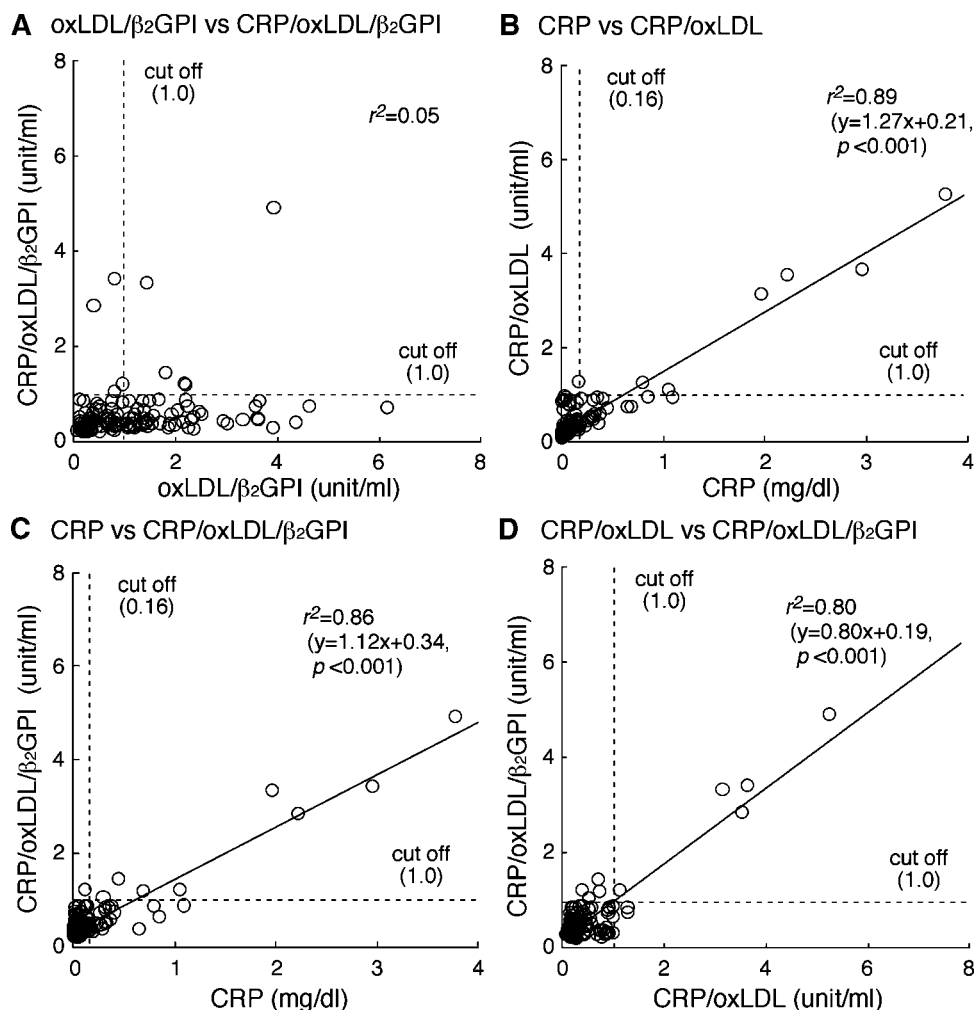
Sixty-nine DM patients, a subpopulation of 125 DM patients described in Materials and Methods, were divided into two groups according to the presence (AT+) or absence (AT-) of atherosclerosis (AT+ with IMT >0.75 mm, n = 20 and AT- with IMT <0.75 mm, n = 49). Serum levels of CRP, CRP/oxLDL and CRP/oxLDL/ $\beta$ 2GPI complexes but not oxLDL/ $\beta$ 2GPI complexes were significantly higher in AT+ than those in AT- (Fig. 4).

There was no correlation between serum levels of oxLDL/ $\beta$ 2GPI and CRP/oxLDL/ $\beta$ 2GPI complexes (Fig. 5A). In contrast, strong correlation between CRP and CRP/oxLDL/ $\beta$ 2GPI complexes (Fig. 5B), CRP and CRP/oxLDL (Fig. 5C), and CRP/oxLDL and CRP/oxLDL/ $\beta$ 2GPI (Fig. 5D) were observed in DM patients. Thus, CRP/

oxLDL and CRP/oxLDL/ $\beta$ 2GPI found in DM patients may be a part of the hsCRP currently used as a predictive marker for CVD. Further, these correlations seem to indicate that CRP/oxLDL complexes in DM patients were mostly complexed with  $\beta$ 2GPI.

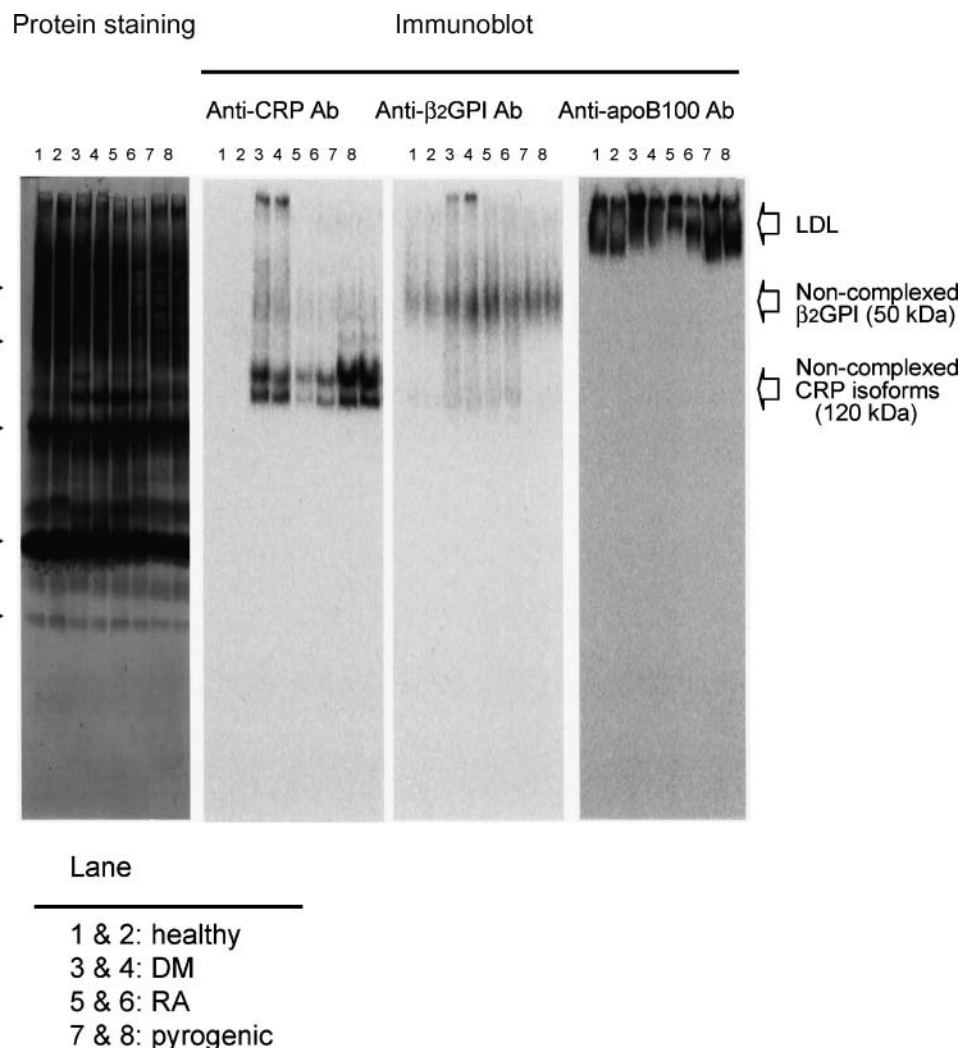
#### Detection of CRP/oxLDL/ $\beta$ 2GPI complexes in serum samples by immunoblot analysis

To confirm the presence of oxLDL complexes containing CRP and  $\beta$ 2GPI, nonreducing PAGE (native PAGE without SDS)/immunoblot analysis was performed in serum samples from healthy subjects, CRP/oxLDL/ $\beta$ 2GPI complex-positive DM patients with atherosclerosis, CRP-positive RA patients, and CRP-positive patients with pyrogenic diseases (seven samples from each group). Typical immunoblot patterns from two samples of each group are shown in Fig. 6. Two noncomplexed CRP isoforms were detected in DM and RA patients and those with pyrogenic diseases. In contrast, noncomplexed  $\beta$ 2GPI was detected in



**Fig. 5.** Correlation among serum levels of CRP/oxLDL/ $\beta$ 2GPI complexes, oxLDL/ $\beta$ 2GPI, CRP/oxLDL complexes, and CRP. Serum levels of these complexes and CRP in individual DM patients are indicated. (A) Correlation between OXLDL/ $\beta$ 2GPI and CRP/OXLDL/ $\beta$ 2GPI complexes; (B) correlation between CRP and CRP/OXLDL complexes; (C) correlation between CRP and CRP/OXLDL/ $\beta$ 2GPI complexes; (D) correlation between CRP/OXLDL and CRP/OXLDL/ $\beta$ 2GPI complexes. The correlation between two variables was evaluated by the Pearson's correlation test.





**Fig. 6.** Immunoblot analysis of oxLDL complexes in serum samples. All proteins were stained in the left panel. Transferred proteins onto a PVDF membrane were reacted with anti-CRP Ab, anti-β<sub>2</sub>GPI Ab, or anti-apoB100 Ab. Serum samples from healthy subjects (lanes 1 and 2), DM patients (lanes 3 and 4), RA patients (lanes 5 and 6), or patients with pyrogenic disease (lanes 7 and 8) were run on the non-denaturing PAGE (native PAGE without SDS). The proteins were transferred onto the polyvinylidene difluoride membrane, and immunoblot analysis was performed with rabbit anti-CRP antiphospholipid antibodies, mouse anti-β<sub>2</sub>GPI monoclonal antibody (MAb), Cof-23, or mouse anti-apoB100 MAb, N2E10. The arrows indicate positions of marker proteins (on the left side) and of LDL, noncomplexed β<sub>2</sub>GPI or noncomplexed CRP isoforms (on the right side).

all serum samples. The complex forms of LDL with CRP and of β<sub>2</sub>GPI were only detected in the DM sera (at the upper position of the LDL band). As described in Materials and Methods, the gels were run at pH 8.3, much closer to the reported pI of CRP (5.3 and 7.4) (39) and β<sub>2</sub>GPI (5.0 to 7.0) (40) than those of molecular markers (4.0 to 4.7) used in this experiment. Actually, mobility of CRP and β<sub>2</sub>GPI in agarose gel electrophoresis (run at pH 8.6) was relatively smaller than that of BSA (pI 4.7) (Fig. 2A). Therefore, molecular sizes of pentameric CRP (120 kDa) and β<sub>2</sub>GPI (50 kDa) determined by nondenaturing PAGE in this study were not consistent with previous reports.

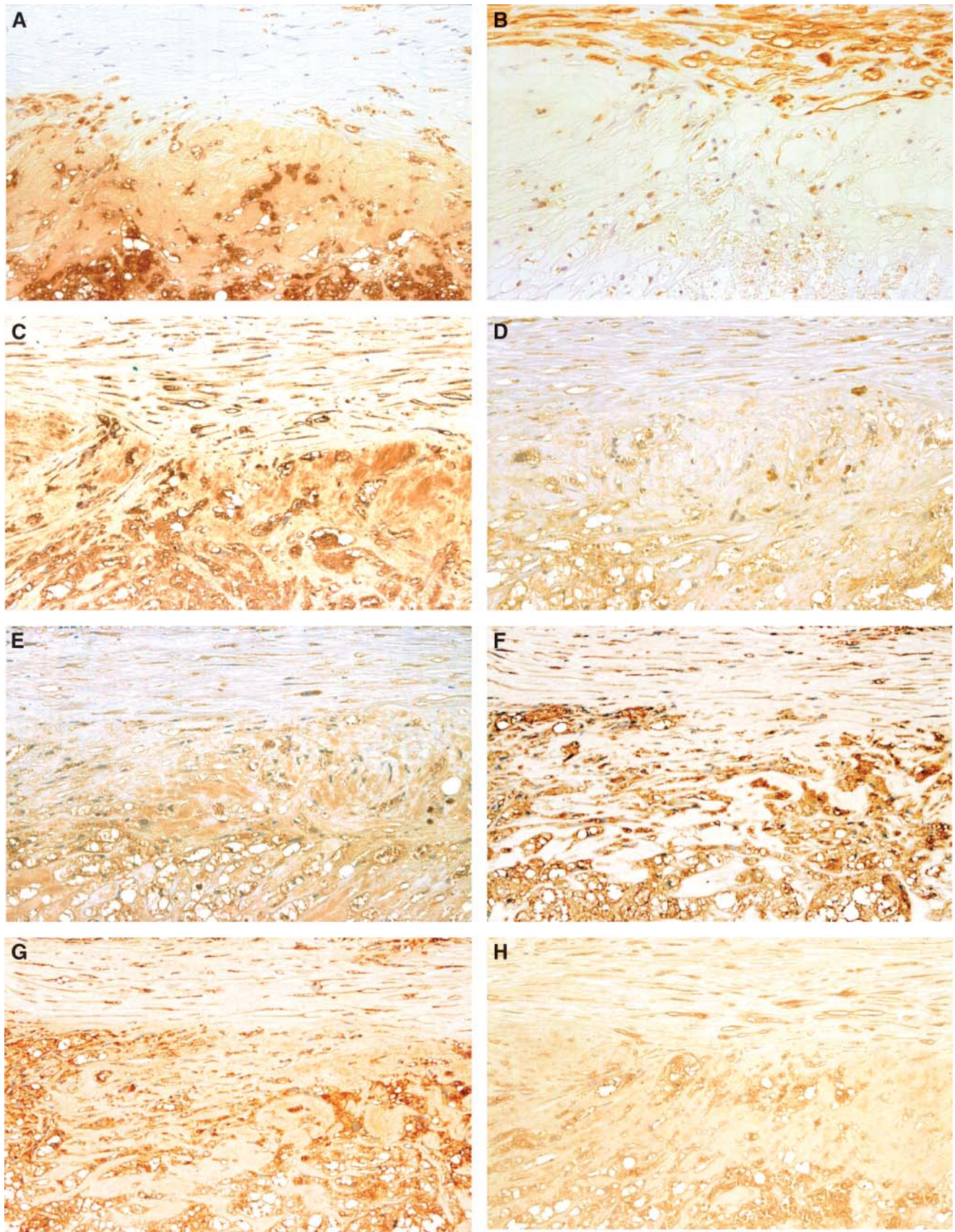
#### Co-localization of β<sub>2</sub>GPI, CRP, and oxLDL in macrophages and smooth muscle cells from atherosclerotic lesions

We investigated the localization of β<sub>2</sub>GPI, CRP, and oxLDL in atherosclerotic carotid arteries and RA synovial

tissues by immunohistochemistry. As shown in **Fig. 7**, clusters of foamy macrophages and layered smooth muscle cells with lipid droplets were commonly observed in carotid endarterectomy specimens (Fig. 7A, B). These foamy macrophages and smooth muscle cells strongly expressed scavenger receptors for oxLDL (SR-PSOX) (Fig. 7C). Furthermore, CRP (Fig. 7D), apoB and oxLDL (Fig. 7E, F, respectively), noncomplexed/complexed β<sub>2</sub>GPI (Fig. 7G), and the complexed form of β<sub>2</sub>GPI (Fig. 7H) were always detected in these macrophages and smooth muscle cells. The presence of β<sub>2</sub>GPI in carotid atherosclerotic plaques is consistent with the previous report by George et al. (41).

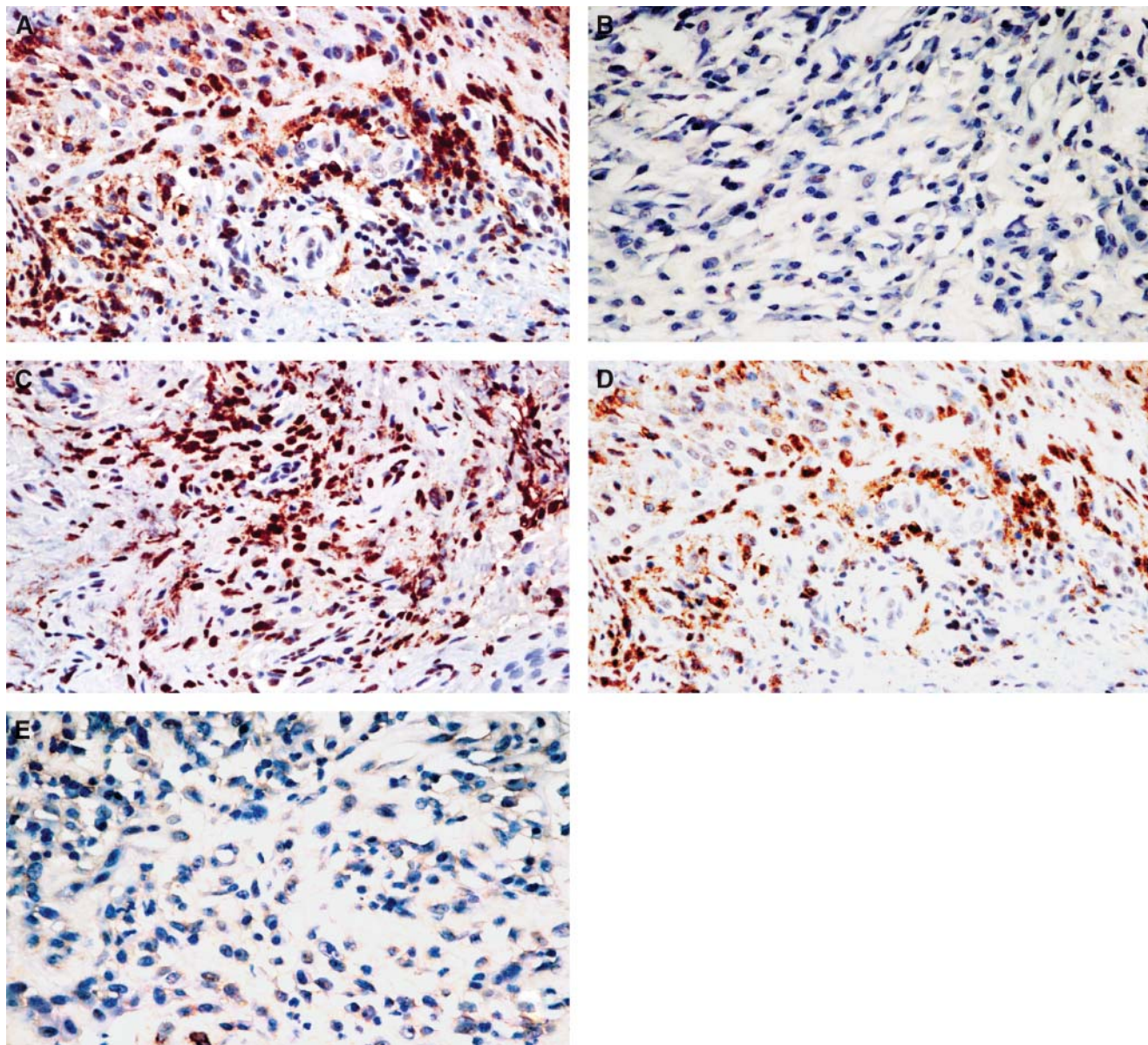
Macrophage infiltration was also commonly observed in RA synovium (**Fig. 8A**) and colocalized with oxLDL (Fig. 8C), and some macrophages obviously showed CRP immunoreactivity (Fig. 8D). Interestingly, no expression





**Fig. 7.** Immunostaining of carotid atherosclerotic lesions (original magnification:  $\times 60$ ). Round foamy macrophages were observed in the lower half of the slides (A) (CD68), whereas spindle-shaped and foamy smooth muscle cells existed in the upper half (B) ( $SMC\alpha$ ). Staining of serial sections indicated that almost all macrophages and smooth muscle cells expressed SR-PSOX (C), CRP (D), apoB (E), oxLDL (F),  $\beta 2$ GPI (G), and the complexed form of  $\beta 2$ GPI (H).





**Fig. 8.** Immunostaining of RA synovium (original magnification:  $\times 100$ ). Severe infiltration of macrophages was observed in RA synovial tissue (A) (CD68). oxLDL (C) and CRP (D) were detected in these cells. Positive staining of SR-PSOX (B) and the complexed form of  $\beta 2$ GPI (E) was not obtained in the synovial macrophages.

of SR-PSOX (Fig. 8B) and no presence of  $\beta 2$ GPI (Fig. 8E) was observed. In contrast, oxLDL, CRP, and  $\beta 2$ GPI were not detected in osteoarthritis synovial tissues (data not shown).

#### Association with biological markers related to atherosclerosis

To further evaluate the implication of CRP and these oxLDL complexes in atherosclerosis, the association with several biological serum markers was analyzed. As shown in **Table 2**, serum CRP levels were positively associated with those of sICAM-1 and sVCAM-1. In contrast, oxLDL/ $\beta 2$ GPI complexes were associated with T-chol and HbA1c. However, there was no association between CRP/oxLDL/ $\beta 2$ GPI complexes and any markers determined in this study. The

results support the previous observations that CRP activates endothelial cells (29) and that oxidative stress (i.e., oxidation of LDL and complex formation) in the intima is related to hypercholesterolemia and hyperglycemia.

#### DISCUSSION

We have recently demonstrated that oxLDL interacts with  $\beta 2$ GPI and the presence of oxLDL/ $\beta 2$ GPI complexes circulating in patients with atherosclerotic and inflammatory diseases, such as systemic lupus erythematosus, APS, DM, and chronic nephritis (4, 6, 10). High levels of oxLDL/ $\beta 2$ GPI complexes were also present in atherosclerosis-prone

TABLE 2. Association between CRP, three types of oxLDL complexes, and biological markers in DM patients

Biological Marker	CRP			oxLDL/ $\beta$ 2GPI Complexes			CRP/oxLDL Complexes			CRP/oxLDL/ $\beta$ 2GPI Complexes		
	OR	95% CI	<i>P</i>	OR	95% CI	<i>P</i>	OR	95% CI	<i>P</i>	OR	95% CI	<i>P</i>
T-chol	0.52	0.14–2.0	NS	2.9	1.0–8.2	0.04	0.71	0.08–6.3	NS	0.52	0.06–4.4	NS
HbA1c	0.58	0.16–2.1	NS	5.9	1.2–29	0.03	0.74	0.08–6.7	NS	1.0	0.12–9.0	NS
sICAM-1	2.5	1.1–5.8	0.03	0.83	0.41–0.7	NS	1.6	0.40–6.2	NS	1.4	0.35–5.4	NS
sVCAM-1	3.5	1.4–8.9	0.007	0.54	0.26–1.1	NS	0.18	0.04–0.91	NS	1.5	0.36–6.4	NS

DM, diabetes mellitus; T-chol, total cholesterol; HbA1c, hemoglobin A1c; sICAM-1, soluble form of intercellular adhesion molecule-1; sVCAM-1, soluble form of vascular cell adhesion molecule-1; NS, not significant.  $P < 0.05$  was considered statistically significant.

mice with *apoe*<sup>-/-</sup> and *ldlr*<sup>-/-</sup> genotypes, especially those fed a high-cholesterol diet (unpublished observations). These observations strongly suggested that increased serum levels of oxLDL/ $\beta$ 2GPI complexes could represent a novel and clinically useful serologic marker for the assessment of atherosclerosis. In this study, we found CRP/oxLDL/ $\beta$ 2GPI (and CRP/oxLDL) as well as oxLDL/ $\beta$ 2GPI complexes in sera of patients with DM. CRP/oxLDL/ $\beta$ 2GPI (and CRP/oxLDL) complexes were particularly present in patients with IMT-diagnosed atherosclerosis, and their levels strongly correlated with hsCRP but not with oxLDL/ $\beta$ 2GPI complexes. Interestingly, LDL complexes containing  $\beta$ 2GPI were not present in sera of patients with RA or pyrogenic diseases. Routine CRP/hsCRP nephelometry for diagnosing CVD may provide false-positive results. In contrast, in the present study, the CRP/oxLDL/ $\beta$ 2GPI complex ELISA showed 100% specificity and positive predictive value due to the lack of reactivity of samples from pyrogenic diseases and RA.

We postulate that CRP/oxLDL/ $\beta$ 2GPI complexes are mainly, or possibly, only formed in atherosclerotic lesions based on the following observations: Immunohistochemistry of carotid artery plaques showed colocalization of oxLDL,  $\beta$ 2GPI, and CRP with SR-PSOX-positive foamy macrophages and activated/transformed smooth muscle cells. Our previous (10) and present in vitro studies actually demonstrated a stoichiometric interaction between Cu<sup>2+</sup>-oxLDL and  $\beta$ 2GPI and that stable/non-dissociable complexes of oxLDL/ $\beta$ 2GPI and CRP/oxLDL/ $\beta$ 2GPI were gradually generated up to 24 h of incubation at 37°C (Fig. 1D–F). OxLDL injected intravenously into experimental animals was quickly removed from the circulation by the liver (half life  $\sim$ 10 min), due to its negative charge (17). Even though high concentrations of  $\beta$ 2GPI (about 200  $\mu$ g/ml) are present in the circulation, it seems impossible that oxLDL could form complexes with circulating  $\beta$ 2GPI in such a short period of time. In contrast, a 6-month follow-up study of DM patients indicated that serum levels of these complexes were very steady (data not shown). Therefore, CRP/oxLDL/ $\beta$ 2GPI and oxLDL/ $\beta$ 2GPI complexes found in the circulation were probably generated in atherosclerotic lesions, not in the blood stream.

It is now widely accepted that CRP contributes to arterial inflammation and pro-atherosclerotic phenotypes by upregulating adhesion molecules of endothelial cells, such as ICAM-1 and VCAM-1 (42). Dyslipoproteinemia and hyperglycemia contribute to oxidation of LDL, which leads to the progression of atherosclerosis via different mecha-

nisms by activating or damaging endothelial cells with the participation of CRP (43). Two positive associations were observed in the present study: one between serum CRP and sICAM-1/sVCAM-1 levels, and another between oxLDL/ $\beta$ 2GPI complex and T-chol/HbA1c levels, both supporting the mechanisms mentioned above. As shown in Table 2, there were no significant associations of serum CRP/oxLDL/ $\beta$ 2GPI complexes with these pro-atherosclerotic parameters, because CRP/oxLDL/ $\beta$ 2GPI complexes may represent a unique combination and a novel marker for the assessment of atherosclerosis severity. These complexes are formed by two distinct mechanisms: CRP generated from vascular inflammation, and oxLDL (oxLDL/ $\beta$ 2GPI) generated from oxidative stress within the intima.

The interaction of CRP with oxLDL is considered a key event linked to atherosclerosis. It has been recently reported that monomeric or structurally modified (not pentameric) CRP isoform(s) can bind to apoB and lipid ligands either on native LDL or oxLDL in a calcium-dependent manner (24). Interestingly, our native PAGE/immunoblot analysis also revealed the presence of two noncomplexed CRP isoforms in all CRP/oxLDL/ $\beta$ 2GPI complexes-positive DM sera, and one of them may be a spontaneously dissociated monomeric form and/or a structurally modified form (Fig. 6). Another possibility is that the doublet bands come from the pentraxin form of CRP and have different pIs because of their different conformation, as previously reported (39). In contrast, as we previously described elsewhere, the  $\omega$ -carboxyl function at the acyl chain of cholesteryl esters in oxLDL was responsible for the initial  $\beta$ 2GPI binding (4, 8–10). The carboxyl function is a late oxidative product that appears after severe lipid peroxidation of LDL, but not in minimally modified LDL. Taken together, these results indicate that circulating CRP/oxLDL/ $\beta$ 2GPI complexes are most probably generated from highly oxidized LDL in atherosclerotic lesions but not from native or minimally modified LDL.

As shown in the present study, significant amounts of noncomplexed isoforms of CRP were detected in sera of DM patients with atherosclerosis, as well as CRP/oxLDL/ $\beta$ 2GPI complexes (immunoblot analysis in Fig. 6). It is generally thought that circulating CRP comes from the liver and that the hepatic production of CRP is mainly up-regulated by inflammatory cytokines, such as interleukin-6. However, it has been recently reported that CRP can also be produced locally in atherosclerotic lesions by endothelial cells, smooth muscle cells, and monocytic cells (44–46). It



is still under discussion, but CRP as an autocrine and a paracrine factor probably promotes atherosclerosis by interfering with endothelial cell regulation, altering vascular smooth muscle cell and/or monocyte/macrophage functions (29). As described herein,  $\beta$ 2GPI complexes with oxLDL or CRP/oxLDL are most probably generated only in atherosclerotic lesions; however, it is still unclear whether oxLDL is complexed with liver or locally generated CRP.

Finally, atherosclerotic complications have been described in RA patients, but  $\beta$ 2GPI complexes with oxLDL or CRP/oxLDL complexes were not detected in the sera from these patients. We demonstrated that oxLDL,  $\beta$ 2GPI, and CRP were colocalized with SR-PSOX-positive foamy macrophages and activated or transformed smooth muscle cells in carotid artery plaques, but not with  $\beta$ 2GPI in RA synovial tissues. In addition, synovial macrophages did not express scavenger receptor SR-PSOX, nor did they develop into foam cells. This can be interpreted as indicating that synovial macrophages are not sufficiently activated. LDL in RA synovial fluid was slightly more electronegative than LDL from matched plasma samples, possibly representing minimally modified LDL (47, 48). Because changes of minimally modified LDL are much less pronounced than those produced by  $\text{Cu}^{2+}$  treatment, the finding of minimal cholesterol or cholesteryl ester loading in synovial macrophages was expected. The degree of LDL oxidation and macrophage activation in carotid atherosclerotic plaques, unlike that in synovial tissue, is strong enough to cause the binding of  $\beta$ 2GPI. Thus, the development of atherosclerosis in DM patients may occur as a result of a mechanism somewhat different from that in RA patients.

In conclusion, oxLDL complexes containing both  $\beta$ 2GPI and CRP are formed under inflammatory and oxidative stress conditions in atherosclerotic lesions and are released into the circulation as electrostatically neutral and stable complexes. Determination of circulating CRP/oxLDL/ $\beta$ 2GPI complexes may be useful in assessing the development of atherosclerosis and/or in diagnosing atherosclerosis. However, the clinical significance of these novel markers (CRP/oxLDL/ $\beta$ 2GPI and oxLDL/ $\beta$ 2GPI) as well as CRP/oxLDL should be further elucidated in larger clinical studies. ■

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