Table 3. cTnT and BNP concentrations simultaneously measured in 137 patients at a mean interval of 31.8 days, subgrouped according to underlying heart diseases

Underlying heart disease	DCM (n = 36)	Ischemic heart disease (n = 33)	Valvular or congenital disease (n = 28)	Hypertensive cardiomyopathy (n = 13)	HCM (n = 16)	Others (n = 11)
Baseline						
BNP, pg/ml (mean ± SE)	662.0 ± 141.5	691.3 ± 143.8	456.5 ± 111.7	402.9 ± 76.4	417.9 ± 90.0	353.2 ± 87.0
Creatine kinase concentration, IU/l (mean ± SE)	89.9 ± 5.7	88.3 ± 7.1	79.0 ± 6.2	89.5 ± 12.8	97.3 ± 11.7	74.1 ± 15.7
cTnT ≥ 0.01 ng/ml						
n (%) of patients	8/36 (22)	16/33 (48)	8/28 (29)	6/13 (46)	2/16 (12)	0/11 (0)
Concentration, ng/ml (mean ± SE)	0.039 ± 0.008	0.072 ± 0.018	0.045 ± 0.013	0.048 ± 0.005	0.035 ± 0.005	–
Follow-up, days (mean ± SE)	38.7 ± 4.1	28.5 ± 3.1	30.7 ± 4.6	24.3 ± 4.0	27.9 ± 6.3	36.8 ± 8.3
BNP, pg/ml (mean ± SE)	257.1 ± 59.0*	310.6 ± 82.7**	274.5 ± 68.9***	186.6 ± 69.1**	265.5 ± 45.5***	151.4 ± 39.4***
cTnT ≥ 0.01 ng/ml						
n (%) of patients	7/36 (19)	13/33 (39)	7/28 (25)	5/13 (38)	3/16 (19)	1/11 (9)
Concentration, ng/ml (mean ± SE)	0.044 ± 0.006	0.044 ± 0.010	0.036 ± 0.008	0.036 ± 0.009	0.033 ± 0.007	0.020

* $P < 0.0001$, ** $P < 0.005$, *** $P < 0.05$ vs baseline

Table 4. Univariate and multivariate analysis of cardiac events

Variable	Univariate			Multivariate		
	P value	Chi-square	Hazard ratio (95% CI)	P value	Chi-square	Hazard ratio (95% CI)
Age	0.1751	1.83	1.023 (0.990–1.058)	0.8416	0.04	1.003 (0.972–1.035)
LVEF	0.0009	11.1	0.961 (0.938–0.984)	0.0846	2.97	0.975 (0.947–1.004)
NYHA III or IV	0.0009	11.0	5.242 (1.976–13.910)	0.6099	0.26	1.339 (0.436–4.116)
cTnT ≥ 0.01 ng/ml	<0.0001	19.7	6.269 (2.792–14.075)	0.0038	8.38	3.481 (1.496–8.100)
Log BNP	<0.0001	20.5	8.041 (3.263–19.818)	0.0306	4.67	3.102 (1.111–8.660)
Creatinine	0.0006	11.8	3.784 (1.771–8.085)	0.0203	5.38	2.764 (1.171–6.524)

CI, confidence interval

significantly higher among patients with high than patients with normal baseline cTnT in the subgroups with dilated cardiomyopathy [3/8 (38%) vs 5 in 33 (15%), $P < 0.05$], ischemic heart disease [7 in 17 (41%) vs 1 in 23 (4%), $P < 0.05$], and valvular or congenital disease [5 in 11 (45%) vs 1 in 42 (2%), $P < 0.01$]. (Seventeen patients had an operation during the observation period. However, none of these patients showed a cardiac event prior to the operation.) Since only four patients had adverse cardiac events among patients with hypertensive disease (n = 1), hypertrophic cardiomyopathy (n = 1), and other diseases (n = 2), statistically valid comparisons could not be made in these subgroups.

Combined cTnT and BNP measurements and long-term outcomes

In the entire population of 190 patients, a low versus high cTnT concentration at baseline, and baseline BNP concentration were both significant independent predictors of long-term outcomes by multivariate Cox proportional hazards regression analysis (Table 4). When groups were created according to cTnT concentrations, low cTnT versus ≥0.01 ng/ml (high cTnT), and BNP concentrations ≥baseline median value of 290.0 pg/ml (high BNP), the adverse cardiac event rate was significantly higher among patients with high concentrations of both cTnT and BNP than among patients who had low cTnT or low BNP concentrations at baseline ($P < 0.0001$, Fig. 2).

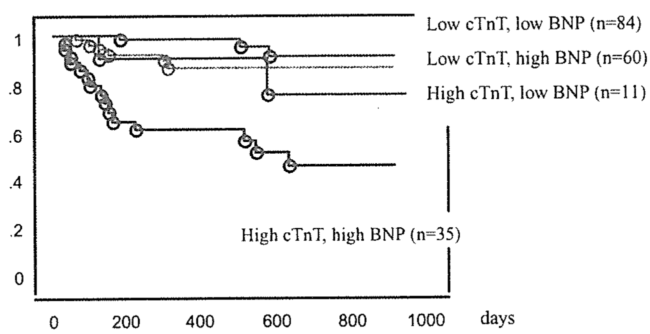

Fig. 2. Adverse cardiac event-free rate in patients with combined measurements of cardiac troponin-T (*cTnT*) and brain natriuretic peptide (*BNP*) concentrations. Patients with high cTnT and high BNP concentrations had the worst prognosis ($P < 0.0001$)

Discussion

Cardiac troponin-T in heart failure from various etiologies

Congestive heart failure is a syndrome caused by a variety of diseases. This is the first study that examined the significance of cTnT and BNP comparing various disease categories at both baseline and follow-up. While cTnT was initially identified as a marker of acute coronary syndrome, we were first to examine its significance in patients with CHF. In an earlier study, we observed persistently high serum cTnT concentrations over several years of follow-up in approximately 30% of our patients with DCM.[5] These patients showed cardiac remodeling and had a poor prognosis. We have also observed high cTnT concentrations in some patients with hypertrophic cardiomyopathy, in its nondilated phase, with preserved systolic function and in the absence of ischemia. Fractional shortening and interventricular septum thickness decreased significantly during follow-up in these patients, indicating that cTnT is a marker of myocyte injury in patients with hypertrophic cardiomyopathy.[15] The results of the current study support our previous reports.

Although we first reported the significance of cTnT as a marker of myocyte injury in patients with cardiomyopathy, ischemic heart disease, valvular heart disease, congenital disease, and hypertensive heart disease remain predominant causes of chronic heart failure. In this study, we reported the elevation of cTnT in patients with ischemic heart disease, valvular or congenital disease, and hypertensive heart disease without ongoing ischemic events. In patients with ischemic heart disease, it is difficult to completely exclude the possibility that increased cTnT might be related to silent ischemia.[16] However, compared to DCM, left ventricular ejection fraction (LVEF) and left ventricular end-diastolic dimension were identical in patients with ischemic heart disease at baseline (Table 1) and the concentrations of CK, BNP, and cTnT at baseline and follow-up were identical (particularly at follow-up) in both groups (Table 3). We believe that these observations may support the hypothesis that, in patients with ischemic heart diseases (without acute coronary events), persistently elevated cTnT may be a marker of subclinical myocyte injury induced by heart failure in the process of cardiac remodeling,[17,18] rather than induced by silent ischemia.

Recently, Horwich et al. reported the prognostic significance of measuring serum concentrations of cardiac troponin I (cTnI) in patients with CHF, and showed that patients with ischemic and nonischemic causes of heart failure were similar in terms of cTnI level and other laboratory values, NYHA class, LVEF, medications, and hemodynamics.[19] Perna et al. measured cTnT at baseline, and at 3, 6, and 12 months of follow-up in 115 ambulatory patients, two thirds of whom had coronary heart disease, and showed the persistence of myocyte injury in patients with poor prognosis, during optimal therapy of CHF.[20] These two reports also might support our results.

Risk stratification by combined measurements of biochemical markers of myocyte injury and myocardial load

Elevated cTnT was a prognostic factor in patients with DCM, ischemic heart disease, and valvular or congenital disease in this study. However, as heart failure is a complex clinical syndrome, a multimarker approach to risk stratification might be better. Patients with both elevated cTnT and BNP showed the poorest prognosis. Similarly, when patients were subdivided according to the results of cTnT and NT-proBNP, the high molecular weight precursor of BNP, patients with elevations of both cTnT and NT-proBNP concentrations had the worst prognosis in our previous study.[14] Ishii et al. reported that both cTnT and BNP were strong, independent predictors of adverse cardiac events by multivariate analysis on admission[21] and after treatment[22] of CHF. They also showed that patients with elevations of both cTnT and BNP had the worst prognosis.[22]

Therapeutic implications of serial measurements of markers of myocyte injury

Few reports have been published describing the value of serial measurements of troponin,[5,7,20,22] and no troponin-guided therapy has been described thus far. However, after successful treatment of cardiac decompensation, or after a decrease in plasma BNP concentrations, cTnT remains elevated in patients who are at risk of adverse cardiac events. In a preliminary study of 35 patients presenting with decompensated heart failure and pulmonary congestion, we observed a significant decrease in BNP concentrations after treatment. However, cTnT concentrations were elevated in 63% of patients before treatment and remained high in 57%, despite the roentgenographic resolution of pulmonary congestion, after a mean treatment period of 14.3 days.[5] In this study, after a mean treatment period of 31.8 days, BNP significantly decreased and cTnT remained high in 26% of patients. Ishii et al. reported that cTnT remained increased in 35% of patients who had been stabilized, 2 months after initiation of treatment.[22]

These observations suggest that troponin might also be a useful marker in the treatment of patients with CHF, and support the apparent importance of its serial measurement. We have hypothesized that a first therapeutic goal is alleviation of circulatory congestion and lowering of BNP, and a second goal is mitigation of myocyte injury and lowering of cTnT.[8] Persistently increased cTnT concentrations may be a signal to proceed to the next treatment. However, there was no significant difference in treatment protocol during follow-up between patients with elevated cTnT and nonelevated cTnT in this small study (data not shown). Further large studies on treatment protocol with monitoring of cTnT levels might be necessary to determine an effective therapy to reduce persistently elevated cTnT in patients with CHF.

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References

1. Hunt SA, Baker DW, Chin MH, Cinquegrani MP, Feldman AM, Francis GS, Ganiats TG, Goldstein S, Gregoratos G, Jessup ML,

Noble RJ, Packer M, Silver MA, Stevenson LW, Gibbons RJ, Antman EM, Alpert JS, Faxon DP, Fuster V, Gregoratos G, Jacobs AK, Hiratzka LF, Russell RO, Smith SC; American College of Cardiology; American Heart Association (2002) ACC/AHA guidelines for the evaluation and management of chronic heart failure in the adult: executive summary. J Heart Lung Transplant 21:189–203
2. Jessup M, Brozena S (2003) Heart failure (review). N Engl J Med 348:2007–2018
3. Alpert JS, Thygesen K, Antman E, Bassand JP (2000) Myocardial infarction redefined – a consensus document of the Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction. J Am Coll Cardiol 36:959–969
4. Jaffe AS, Ravkilde J, Roberts R, Naslund U, Apple FS, Galvani M, Katus H (2000) It's time for a change to a troponin standard. Circulation 102:1216–1220
5. Sato Y, Yamada T, Taniguchi R, Nagai K, Makiyama T, Okada H, Kataoka K, Ito H, Matsumori A, Sasayama S, Takatsu Y (2001) Persistently increased serum concentrations of cardiac troponin T in patients with idiopathic dilated cardiomyopathy are predictive of adverse outcomes. Circulation 103:369–374
6. Yazici M, Demircan S, Durna K, Sahin M (2005) The relation between endothelin-1 levels and myocardial injury in chronic ischemic heart failure. Heart Vessels 20:95–99
7. Sato Y, Taniguchi R, Makiyama T, Nagai K, Okada H, Yamada T, Matsumori A, Takatsu Y (2002) Serum cardiac troponin T and plasma brain natriuretic peptide in patients with severe cardiac decompensation. Heart 88:647–648
8. Sato Y, Kita T, Takatsu Y, Kimura T (2004) Biochemical markers of myocyte injury in heart failure. Review Heart 90:1110–1113
9. Setsuta K, Seino Y, Takahashi N, Ogawa T, Sasaki K, Harada A, Takano T, Kishida H, Hayakawa H (1999) Clinical significance of elevated levels of cardiac troponin T in patients with chronic heart failure. Am J Cardiol 84:608–611
10. Koh E, Nakamura T, Takahashi H (2004) Troponin-T and brain natriuretic peptide as predictors for adriamycin-induced cardiomyopathy in rats. Circ J 68:163–7
11. Tsutamoto T, Wada A, Maeda K, Hisanaga T, Maeda Y, Fukai D, Ohnishi M, Sugimoto Y, Kinoshita M (1997) Attenuation of compensation of endogenous cardiac natriuretic peptide system in chronic heart failure: prognostic role of plasma brain natriuretic peptide concentration in patients with chronic symptomatic left ventricular dysfunction. Circulation 96:509–516
12. Rodeheffer RJ (2004) Measuring plasma B-type natriuretic peptide in heart failure. J Am Coll Cardiol 44:740–49
13. Richardson P, McKenna W, Bristow M, Maisch B, Mauntner B, O'Connell J, Olsen E, Thiene G, Goodwin J, Gyarfas I, Martin I, Nordet P (1996) Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the definition and classification of cardiomyopathies. Circulation 93:841–842
14. Taniguchi R, Sato Y, Yamada T, Ooba M, Higuchi H, Matsumori A, Kimura T, Kita T (2004) Combined measurements of cardiac troponin T and N-terminal pro-brain natriuretic peptide in patients with heart failure. Circ J 68:1160–1164
15. Sato Y, Taniguchi R, Nagai K, Makiyama T, Okada H, Yamada T, Matsumori A, Takatsu Y (2003) Measurements of cardiac troponin T in patients with hypertrophic cardiomyopathy (scientific letter). Heart 89:659–660
16. Sato Y, Taniguchi R, Yamada T, Matsumori A (2003) Measurements of serum cardiac troponin T in patients with heart failure (letter). Am Heart J 145:e18
17. Cohn JN, Ferrari R, Sharp N (2000) Cardiac remodeling-concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. J Am Coll Cardiol 35:569–582.
18. St John Sutton MG, Sharpe N (2000) Left ventricular remodeling after myocardial infarction: pathophysiology and therapy. Circulation 101:2981–2988
19. Horwich TB, Patel J, MacLellan WR, Fanarow GC (2003) Cardiac troponin I is associated with impaired hemodynamics, progressive left ventricular dysfunction, and increased mortality rates in advanced heart failure. Circulation 108:833–838
20. Perna ER, Macin SM, Canella JP, Augier N, Stival JL, Cialzeta JR, Pitzus AE, Garcia EH, Obregon R, Brizuela M, Barbagelata A (2004) Ongoing myocardial injury in stable severe heart failure: value of cardiac troponin T monitoring for high-risk patient identification. Circulation 110:2376–2382
21. Ishii J, Nomura M, Nakamura Y, Naruse H, Mori Y, Ishikawa T, Ando T, Kurokawa H, Kondo T, Nagamura Y, Ezaki K, Hishida H (2002) Risk stratification using a combination of cardiac troponin T and brain natriuretic peptide in patients hospitalized for worsening chronic heart failure. Am J Cardiol 89:691–695
22. Ishii J, Cui W, Kitagawa F, Kuno T, Nakamura Y, Naruse H, Mori Y, Ishikawa T, Nagamura Y, Kondo T, Oshima H, Nomura M, Ezaki K, Hishida H (2003) Prognostic value of combination of cardiac troponin T and B-type natriuretic peptide after initiation of treatment in patients with chronic heart failure. Clin Chem 49:2020–2026

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 β2-glycoprotein I (β2GPI), implicating oxLDL/β2GPI complexes as putative autoantigens in autoimmune-mediated atherosclerotic vascular disease. In this study, we investigated the interaction of CRP with oxLDL/β2GPI complexes and its association with atherosclerosis in patients with diabetes mellitus (DM). CRP/oxLDL/β2GPI 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 β2GPI 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/β2GPI complexes correlated with total cholesterol and hemoglobin A1c. Thus, the generation of CRP/oxLDL/β2GPI complexes seems to be associated with arterial inflammation, hyperglycemia, and hypercholesterolemia. CRP/oxLDL/β2GPI 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 β2-glycoprotein I • oxidized LDL/β2-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).

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Abbreviations: APS, antiphospholipid syndrome; AT, atherosclerosis; CRP, C-reactive protein; CVD, cardiovascular disease; DM, diabetes mellitus; β2GPI, β2-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 heterogenous 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 β2-glycoprotein I (β2GPI) (4–6), lipids, and protein-lipid complexes. We have demonstrated that anti-β2GPI auto-Abs also recognize oxLDL/β2GPI complexes (4, 7–10). Further, the in vitro macrophage uptake of oxLDL/β2GPI complexes was significantly increased by IgG anti-β2GPI Abs. (4, 7–10) Thus, findings suggesting that IgG anti-β2GPI auto-Abs may be proatherogenic renewed interest in the immunologic mechanisms participating in atherogenesis (4, 11, 12).

β2GPI interacts with oxLDL via oxidatively induced β2GPI-specific ligands, e.g., 7-ketocholesterol having an ω-carboxyl acyl chain. This interaction may take place in the arterial intima of atherosclerotic lesions, producing stable and nondissociable oxLDL/β2GPI complexes (4, 8–10). These complexes would then be released into the circulation. In addition to systemic autoimmune diseases (15, 16), oxLDL/β2GPI complexes have been found in nonautoimmune chronic inflammatory diseases that develop atherosclerosis, such as diabetes mellitus (DM) (13) and chronic nephritis (14). OxLDL/β2GPI 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/β2GPI complexes demonstrated by ELISA and immunoblot analysis were predominantly detected in DM patients with atherosclerosis. Because CRP/oxLDL/β2GPI 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 complexes are formed in atherosclerotic plaques and released into the circulation. Thus, CRP/oxLDL/β2GPI 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 ± 13.3 years (mean ± 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 ± 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°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_{2a}, κ) was derived from nonimmunized NZW × BXSB F1 male mice, an animal model of APS (32). WB-CAL-1 MAb binds to β2GPI 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-β2GPI MAb, Cof-23 (IgG_1, κ), obtained from BALB/c mice immunized with human β2GPI, recognizes both the noncomplexed and lipid-complex β2GPI structures (33). Two anti-apolipoprotein B-100 (apoB-100) MAbs were established from BALB/c mice immunized with human native LDL and Cu^{2+}-oxLDL, respectively: N2E10 (IgG_{2a}, κ), which recognizes apoB-100 present in either native LDL or oxLDL; and O1F9 (IgG_{2a}, λ), 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-α actin of smooth muscle cells (SMCα) 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 $CaCl_2$, pH 7.4 (Tris buffer) to remove NaN_3 before use. β2GPI 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 μg/ml of apoB equivalent) in 10 mM Hepes and 150 mM NaCl, pH 7.4, (Hepes buffer) was oxidized by incubation with 5 μM $CuSO_4$ 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 β2GPI 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/β2GPI complexes. This procedure was performed as previously described (10). Briefly, anti-β2GPI MAb, WB-CAL-1, was adsorbed onto microtiter plates (Immulon 2HB; Thermo Labsystems, Franklin, MA) by incubating at 8 μg/ml (dissolved in Hepes buffer, 50 μl/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 μl/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 tetramethylbendizine and H_2O_2. 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 μg/ml (dissolved in Hepes buffer, 50 μl/well) at 4°C overnight. After blocking with 10 mM Tris, 150 mM NaCl, 1.25 mM $CaCl_2$, pH7.4, (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/β2GPI complexes.

ELISA for CRP/oxLDL/β2GPI 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-β2GPI 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 thyroblobulin (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-β2GPI 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 staying run. Endogenous peroxidase activity was blocked by treatment with 3% H_2O_2 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, SMCα, CRP, SR-PSOX, and apoB in either native LDL or oxLDL (N2E10), apoB in oxLDL (O1F9), noncomplexed and complexed forms of β2GPI (Cof-23), and complexed form of β2GPI (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. Ninty-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/β2GPI complexes

The nature of the interaction between oxLDL and β2GPI has been previously characterized and described (8–10). We first investigated the interaction among Cu^{2+}-oxLDL, β2GPI, and CRP. **Figure 1A–C** depicts the calcium dependency of the interaction of oxLDL with CRP and oxLDL with β2GPI. Figure 1A shows that the interaction between oxLDL and β2GPI was calcium independent from 0 to 5 mM of 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 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/β2GPI complexes were formed proportionally when these three components were coincubated (Fig. 1C). No direct interaction between CRP and β2GPI was observed under any experimental conditions. Figure 1D–F reveals the time-dependent formation of oxLDL complexes with CRP and/or β2GPI in the presence of 1.25 mM of Ca^{2+} ion at 37°C. The interaction of oxLDL with CRP and oxLDL with β2GPI 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 β2GPI 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/β2GPI complexes or oxLDL/β2GPI complexes. The oxLDL-negative charges acquired during

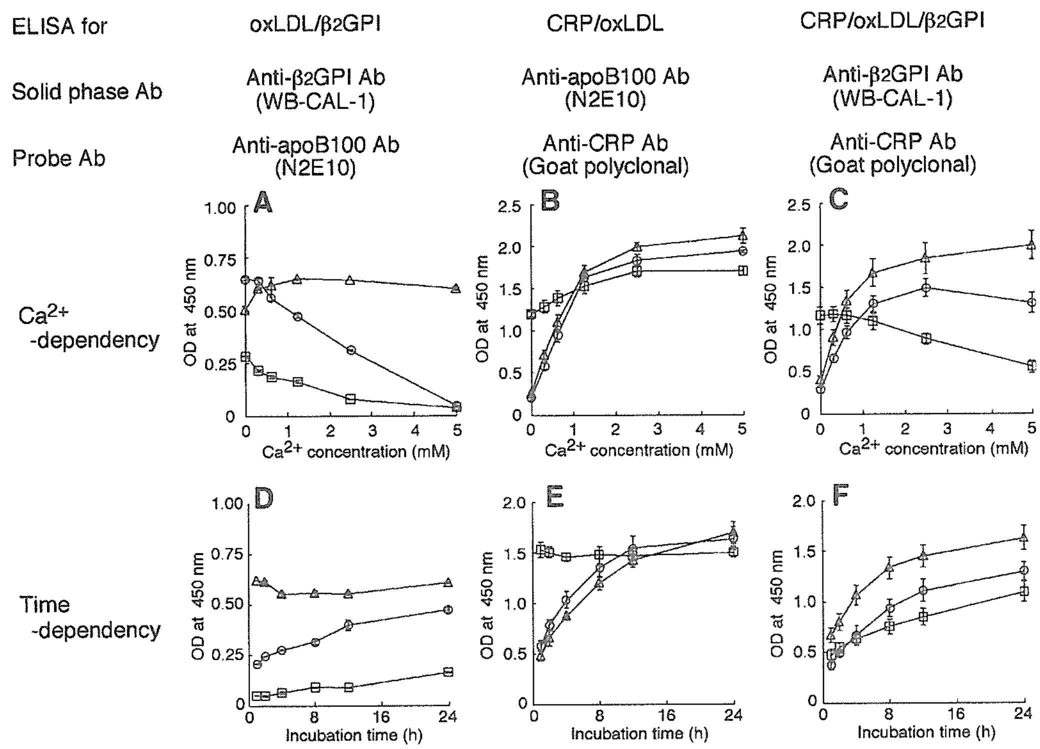

Fig. 1. Profiles of complex formation among Cu^{2+}-oxidized LDL (oxLDL), β2-glycoprotein I (β2GPI), and C-reactive protein (CRP). For the experiment, oxLDL/β2GPI and CRP/oxLDL complexes were preformed by incubating Cu^{2+}-oxLDL [1 mg apolipoprotein B (apoB) equivalent/ml] and β2GPI (1 mg/ml) in the absence of $CaCl_2$, and Cu^{2+}-oxLDL (1 mg apoB equivalent/ml) and CRP (1 mg/ml) in the presence of 2 mM $CaCl_2$, 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, β2GPI, preformed oxLDL/β2GPI complexes, and/or preformed CRP/oxLDL complexes were incubated at 37°C for 16 h in the presence of different concentrations of $CaCl_2$. Concentration of each material was 50 μg/ml protein or 50 μg/ml apoB-100 equivalent. Reaction was terminated by immediate freeze, and an aliquot of each specimen was diluted up to 0.5 μg/ml of apoB equivalent (1:100 dilution) to apply to the ELISAs. Generation of oxLDL/β2GPI complexes (A), CRP/oxLDL complexes (B), or CRP/oxLDL/β2GPI complexes (C) was detected in ELISAs, as described in Materials and Methods. D–F: CRP, oxLDL, β2GPI, preformed oxLDL/β2GPI complexes, and/or preformed CRP/oxLDL complexes were incubated at 37°C for different periods in the presence of 1.25 mM $CaCl_2$, and generated complexes (D–F) were measured by ELISA, as described above. All values in the in vitro experiments are expressed as the mean ± SD. Circles, CRP + oxLDL + β2GPI; triangles, preformed oxLDL/β2GPI complexes + CRP; squares, preformed CRP/oxLDL complexes + β2GPI. Error bars represent mean ± SD.

$CuSO_4$ incubation were neutralized by the interaction with these proteins (**Fig. 2A**).

We also confirmed that significant in vitro production of CRP/oxLDL/β2GPI 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/β2GPI complexes. CRP/oxLDL/β2GPI and oxLDL/β2GPI 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/β2GPI 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/β2GPI complexes can cross-react in the hsCRP assay. The

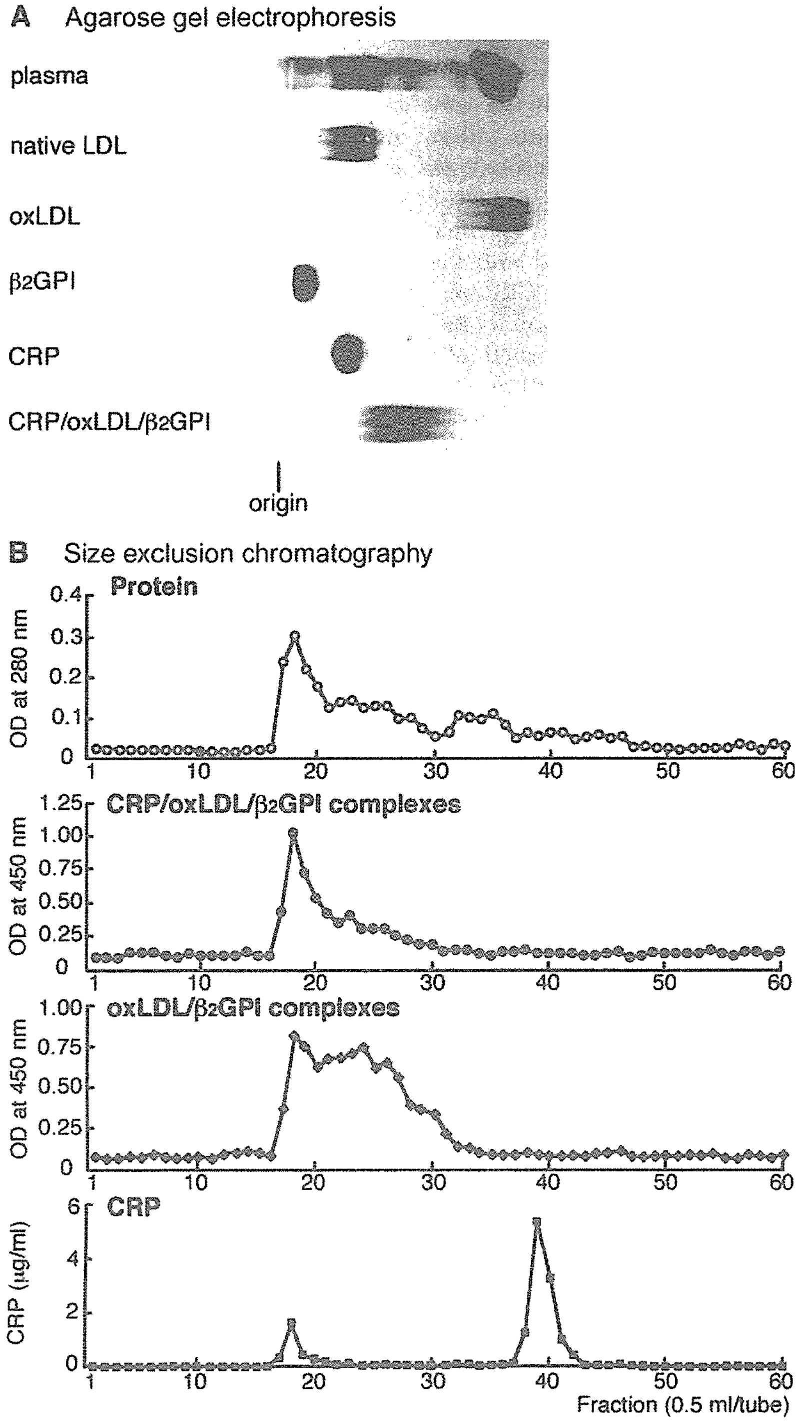

Fig. 2. In vitro formation of CRP/oxLDL/β2GPI complexes. Cu^{2+}-OxLDL (1 mg/ml of apoB equivalent) was incubated with β2GPI (1 mg/ml) for 16 h at 37°C to form oxLDL/β2GPI complexes. The purified oxLDL/β2GPI (1 mg/ml of apoB equivalent) was further incubated with CRP (purified from human pleural fluid; 0.1 mg/ml) in the presence of 2 mM $CaCl_2$ for 16 h at 37°C, to form nondissociable CRP/oxLDL/β2GPI 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 β2GPI. B: Elution profile of the size exclusion column chromatography of CRP/oxLDL/β2GPI complexes is shown. The reaction mixture (100 μl aliquot) containing CRP and oxLDL/β2GPI 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 $CaCl_2$. CRP/oxLDL/β2GPI and oxLDL/β2GPI 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/β2GPI complex assay

The principle of the ELISA for CRP/oxLDL/β2GPI complexes is schematically represented in **Fig. 3A**. The assay was specific for CRP/oxLDL/β2GPI complexes without any cross-reactivity to CRP/oxLDL complexes or oxLDL/β2GPI complexes (Fig. 3B). In addition, the CRP/oxLDL/β2GPI complex value (30 ng/ml of apoB equivalent) was not affected by addition of excess amounts of oxLDL/β2GPI complexes (300 ng/ml of apoB equivalent, expected as pathophysiological concentration) in the CRP/oxLDL/β2GPI ELISA. The ELISA for oxLDL/β2GPI complexes was previously described elsewhere (10).

In the present study, the clinical significance of circulating CRP/oxLDL/β2GPI, oxLDL/β2GPI, 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,

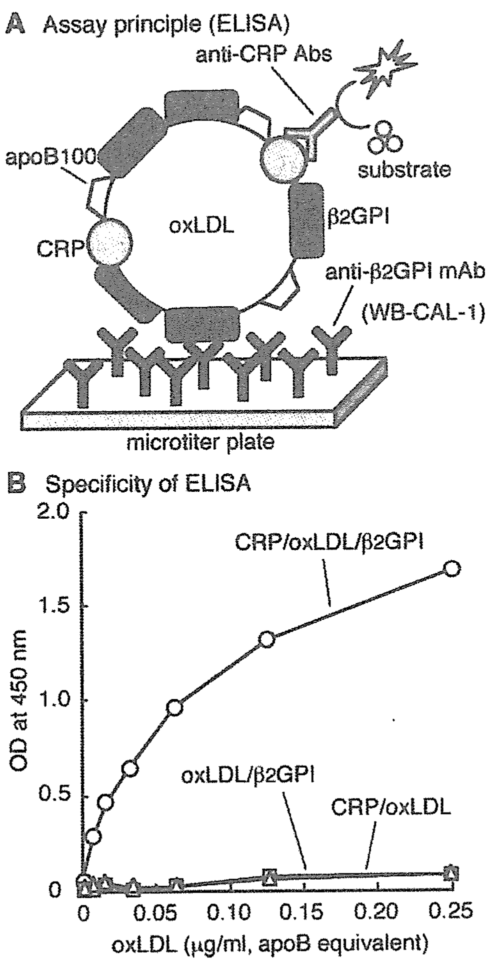

Fig. 3. Sandwich ELISA for oxLDL/β2GPI complexes. Assay principle of the ELISA (A) and specificity (cross-reactivity) (B) are shown. Circles, CRP/oxLDL/β2GPI; squares, oxLDL/β2GPI; triangles, CRP/oxLDL.

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/β2GPI 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/β2GPI 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/β2GPI 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/β2GPI and CRP/oxLDL/β2GPI complexes

Figure 4 shows the serum levels of CRP, oxLDL/β2GPI, CRP/oxLDL, and CRP/oxLDL/β2GPI 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/β2GPI 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/β2GPI 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/β2GPI

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/β2GPI complexes	50	57	32	74	1.3	0.5–3.8
CRP/oxLDL complexes	25	100	100	77	—	—
CRP/oxLDL/β2GPI complexes	25	100	100	77	—	—

CRP, C-reactive protein; β2GPI, β2-glycoprotein I; PPV, positive predictive value; NPV, negative predictive value; OR, odds ratio; oxLDL, oxidized LDL; 95% CI, 95% confidence interval.

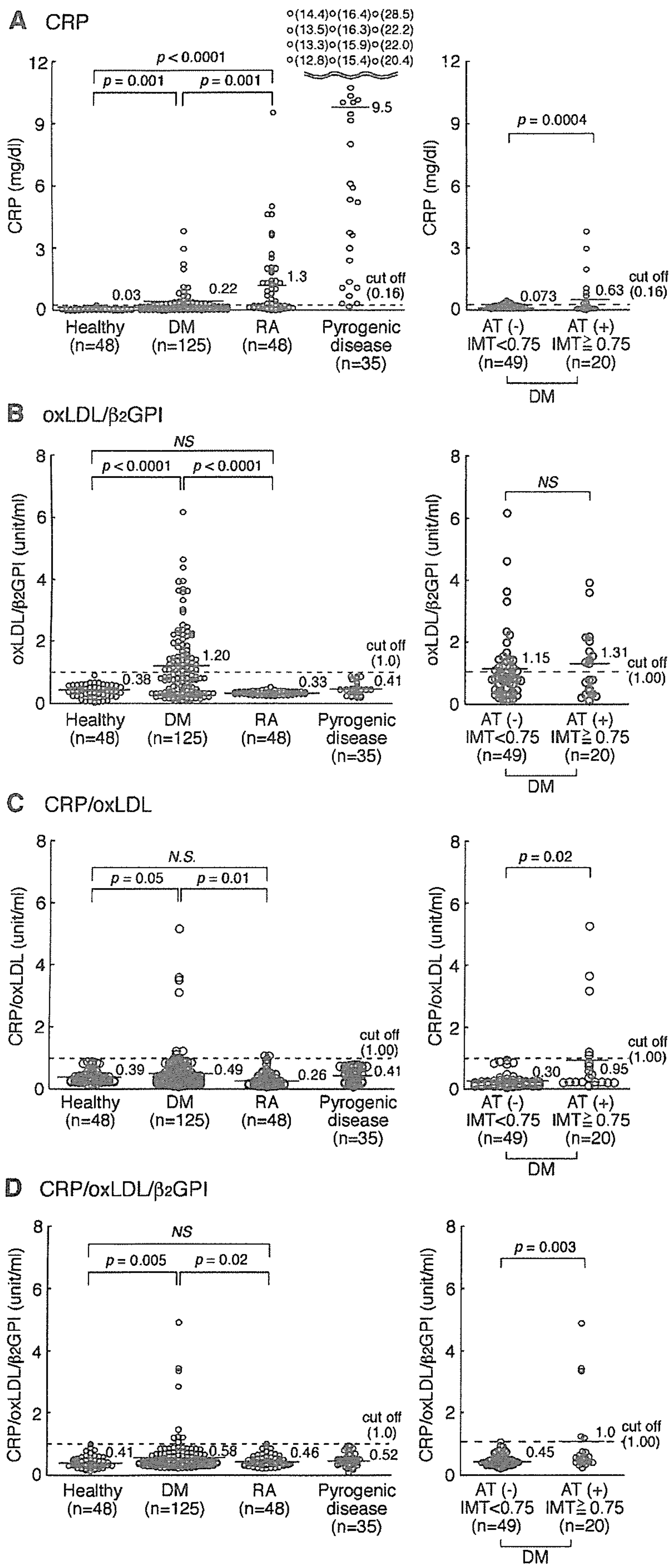

Fig. 4. Serum levels of CRP and three kinds of oxLDL complexes in patients with diabetes mellitus (DM), rheumatoid arthritis (RA), or pyrogenic disease. Serum CRP was measured by the routine or high-sensitivity methods (A). Serum oxLDL/β2GPI complexes (B), CRP/oxLDL complexes (C), and CRP/oxLDL/β2GPI 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/β2GPI 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/β2GPI complexes but not oxLDL/β2GPI complexes were significantly higher in AT+ than those in AT− (Fig. 4).

There was no correlation between serum levels of oxLDL/β2GPI and CRP/oxLDL/β2GPI complexes (**Fig. 5A**). In contrast, strong correlation between CRP and CRP/oxLDL/β2GPI complexes (Fig. 5B), CRP and CRP/oxLDL (Fig. 5C), and CRP/oxLDL and CRP/oxLDL/β2GPI (Fig. 5D) were observed in DM patients. Thus, CRP/oxLDL and CRP/oxLDL/β2GPI 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 β2GPI.

Detection of CRP/oxLDL/β2GPI complexes in serum samples by immunoblot analysis

To confirm the presence of oxLDL complexes containing CRP and β2GPI, nondenaturing PAGE (native PAGE without SDS)/immunoblot analysis was performed in serum samples from healthy subjects, CRP/oxLDL/β2GPI 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 β2GPI was detected in

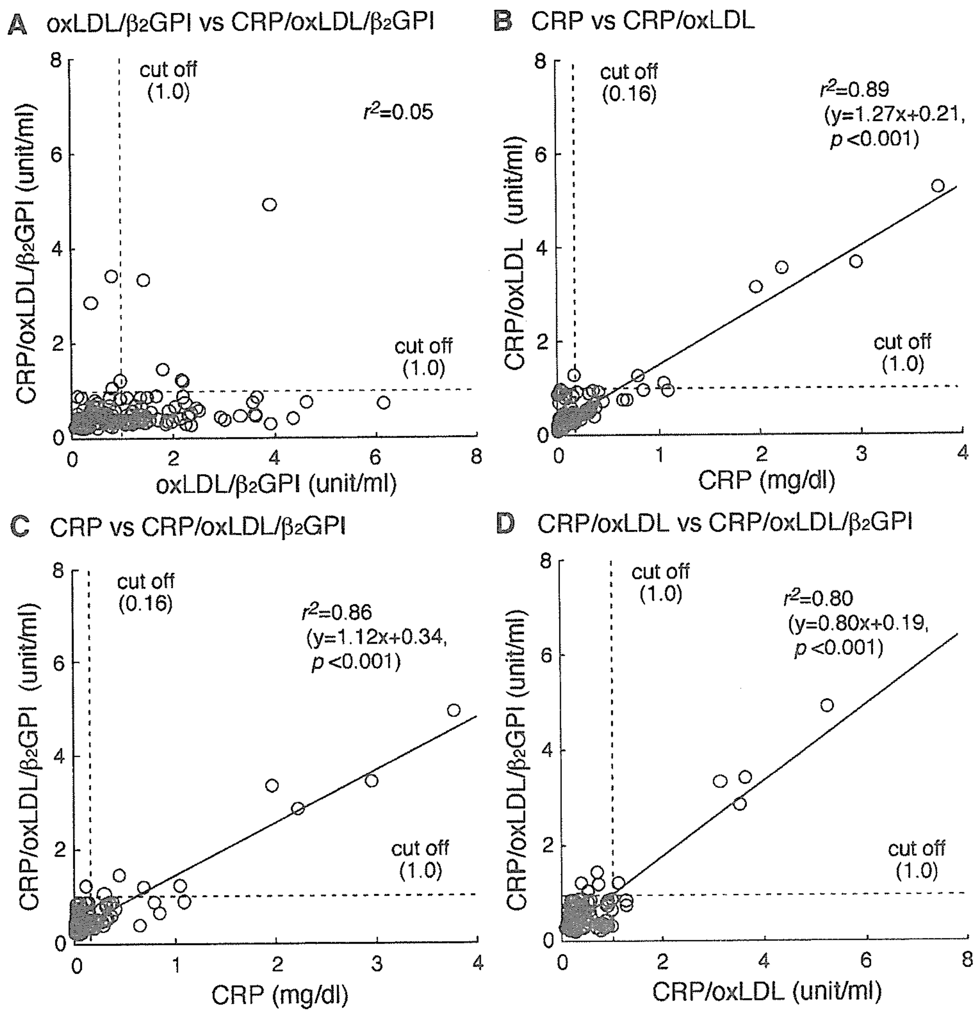

Fig. 5. Correlation among serum levels of CRP/oxLDL/β2GPI complexes, oxLDL/β2GPI, CRP/oxLDL complexes, and CRP. Serum levels of these complexes and CRP in individual DM patients are indicated. (A) Correlation between OXLDL/β2GPI and CRP/OXLDL/β2GPI complexes; (B) correlation between CRP and CRP/OXLDL complexes; (C) correlation between CRP and CRP/OXLDL/β2GPI complexes; (D) correlation between CRP/OXLDL and CRP/OXLDL/β2GPI complexes. The correlation between two variables was evaluated by the Pearsons' correlation test.

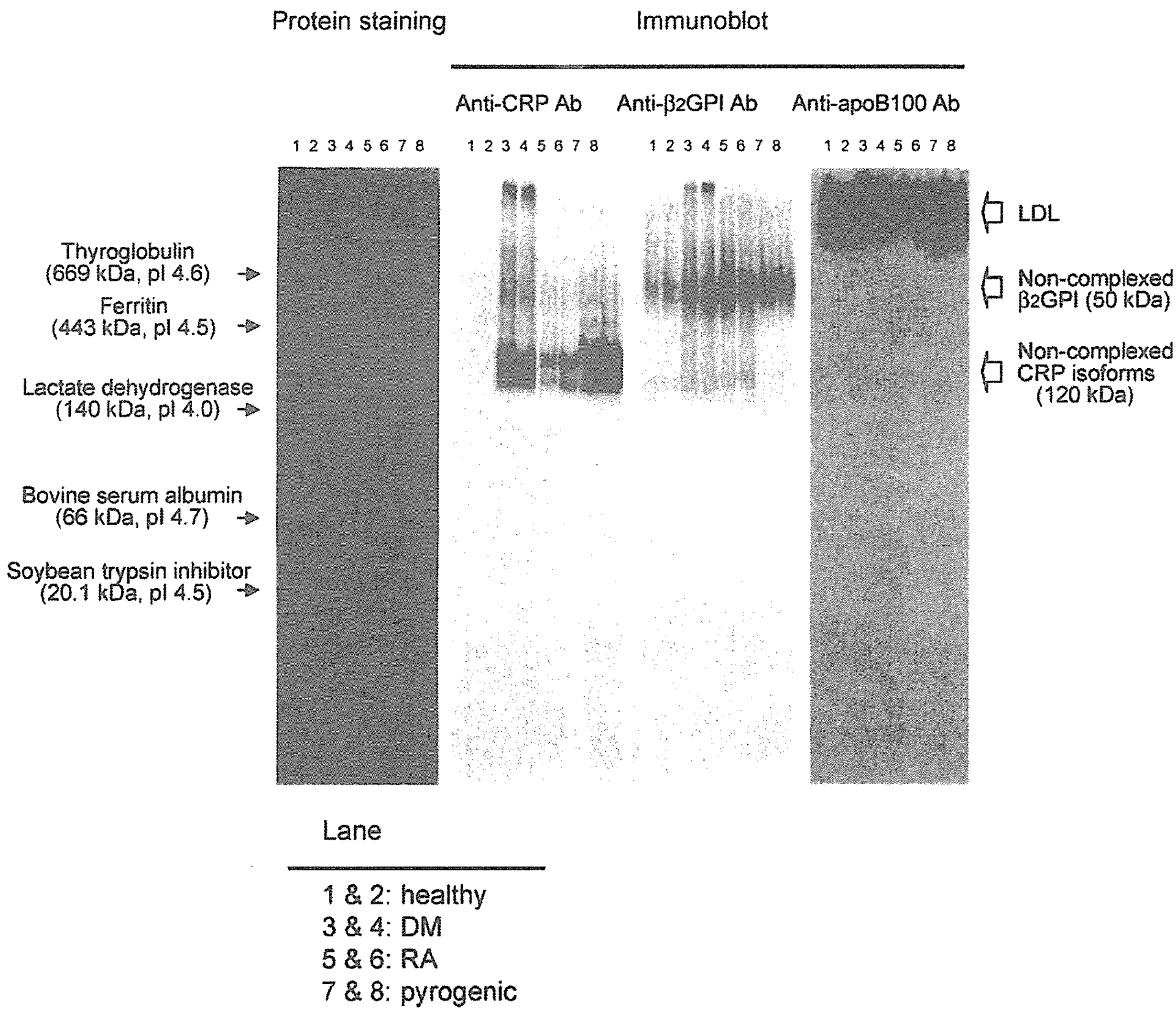

Fig. 6. Immunoblot analysis of oxLDL complexes in serum samples. All proteins were stained in the left panel. Transferred proteins onto a PVDF membrane were reacted with anti-CRP Ab, anti-b2GPI Ab, or anti-apoB100 Ab. Serum samples from healthy subjects (lanes 1 and 2), DM patients (lanes 3 and 4), RA 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-β2GPI 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 β2GPI or noncomplexed CRP isoforms (on the right side).

all serum samples. The complex forms of LDL with CRP and of β2GPI 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 β2GPI (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 β2GPI 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 β2GPI (50 kDa) determined by nondenaturing PAGE in this study were not consistent with previous reports.

Co-localization of β2GPI, CRP, and oxLDL in macrophages and smooth muscle cells from atherosclerotic lesions

We investigated the localization of β2GPI, 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 cartoid 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 β2GPI (Fig. 7G), and the complexed form of β2GPI (Fig. 7H) were always detected in these macrophages and smooth muscle cells. The presence of β2GPI 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

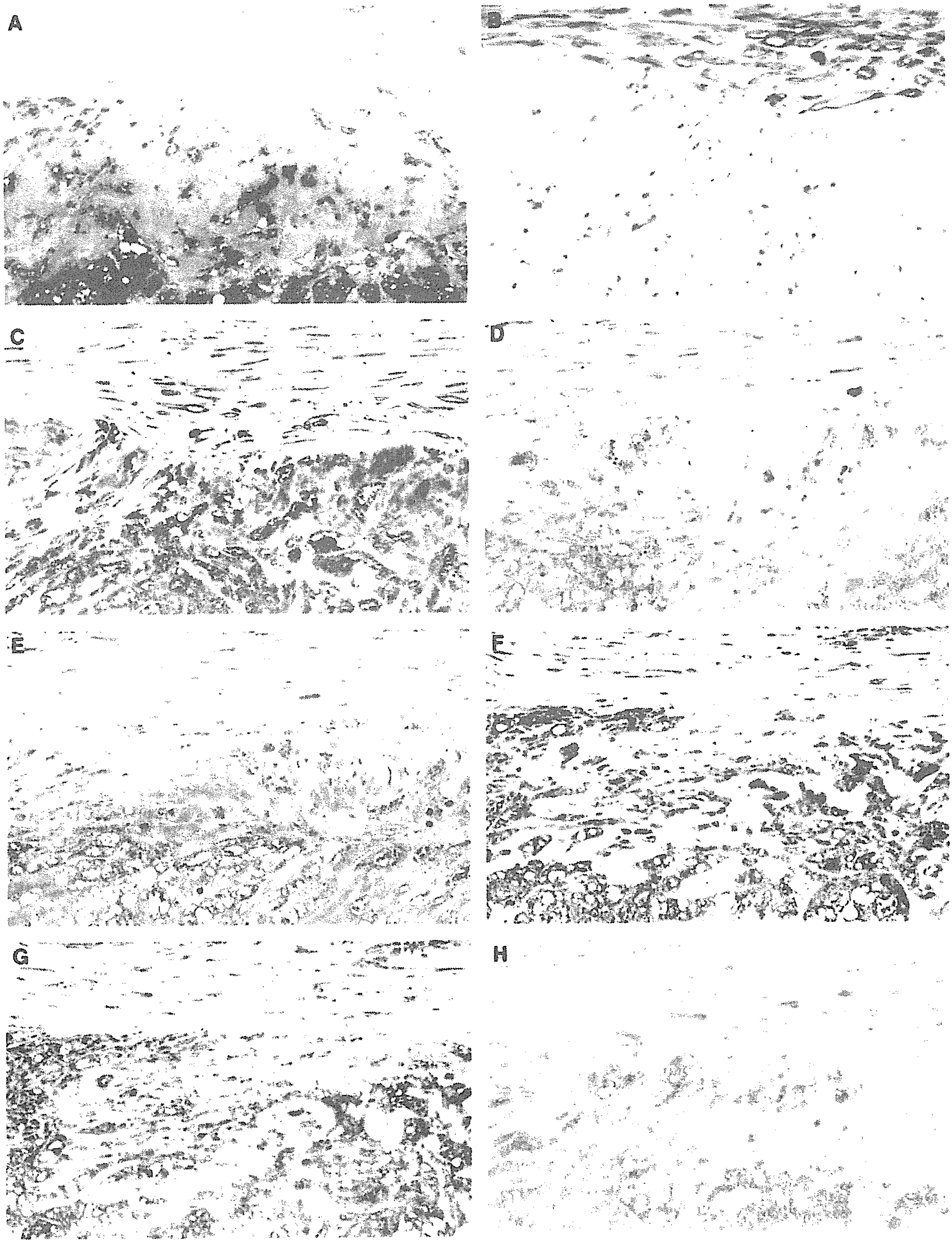

Fig. 7. Immunostaining of carotid atherosclerotic lesions (original magnification: ×60). Round foamy macrophages were observed in the lower half of the slides (A) (CD68), whereas spindle-shaped and foamy smooth muscle cells existed in the upper half (B) (SMCα). Staining of serial sections indicated that almost all macrophages and smooth muscle cells expressed SR-PSOX (C), CRP (D), apoB (E), oxLDL (F), β2GPI (G), and the complexed form of β2GPI (H).

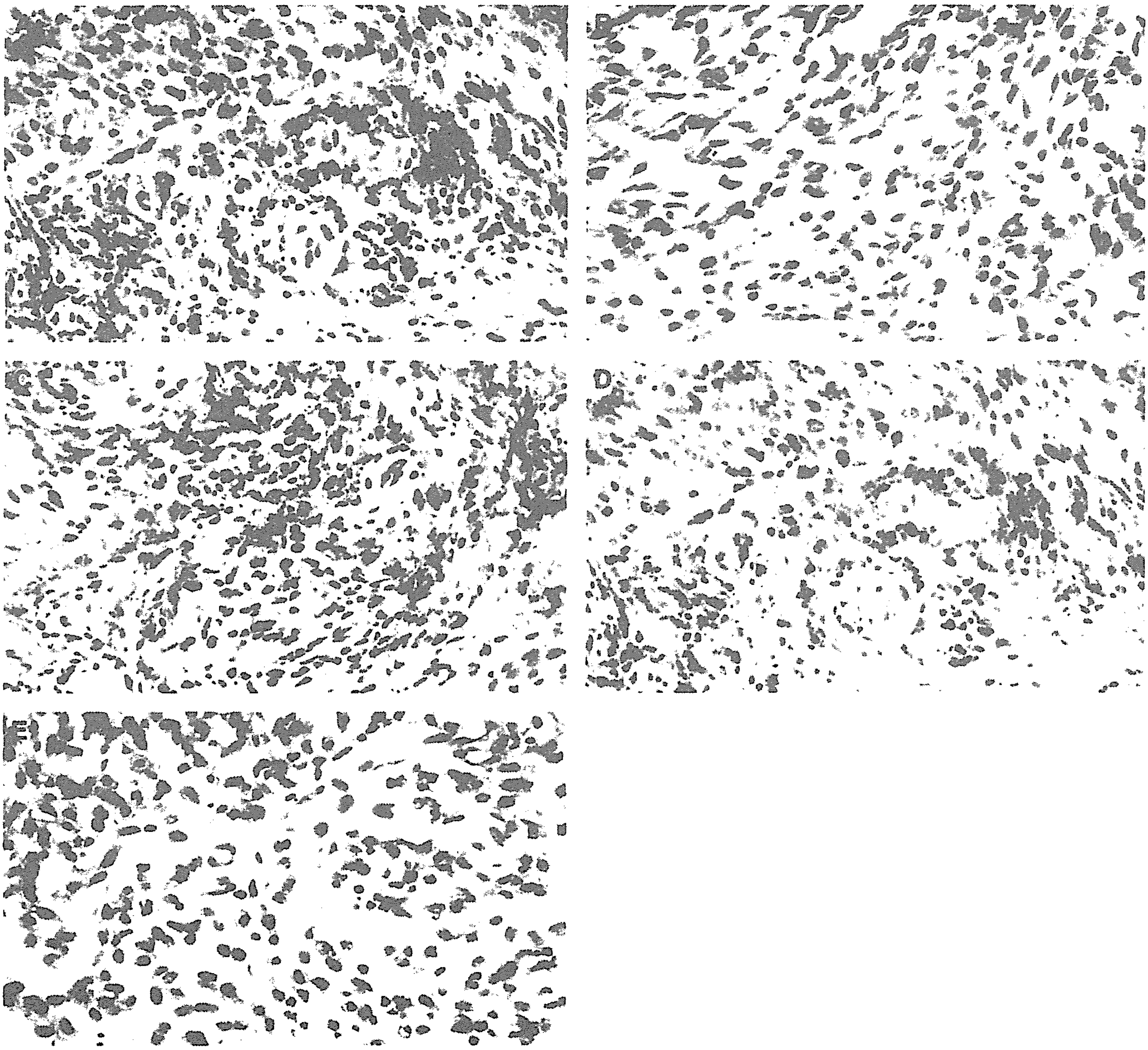

Fig. 8. Immunostaining of RA synovium (original magnification: ×100). Severe infiltration of macrophages was observed in RA synovial tissue (A) (CD68). oxLDL (C) and CRP (D) were detected in these cells. Positive staining of SR-PSOX (B) and the complexed form of β2GPI (E) was not obtained in the synovial macrophages.

of SR-PSOX (Fig. 8B) and no presence of β2GPI (Fig. 8E) was observed. In contrast, oxLDL, CRP, and β2GPI 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/β2GPI complexes were associated with T-chol and HbA1c. However, there was no association between CRP/oxLDL/β2GPI 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 β2GPI and the presence of oxLDL/β2GPI 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/ β2GPI 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/β2GPI Complexes			CRP/oxLDL Complexes			CRP/oxLDL/β2GPI Complexes		
	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
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 Alc; 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*$^{-/-}$ and *ldlr*$^{-/-}$ genotypes, especially those fed a high-cholesterol diet (unpublished observations). These observations strongly suggested that increased serum levels of oxLDL/β2GPI complexes could represent a novel and clinically useful serologic marker for the assessment of atherosclerosis. In this study, we found CRP/oxLDL/β2GPI (and CRP/oxLDL) as well as oxLDL/β2GPI complexes in sera of patients with DM. CRP/oxLDL/β2GPI (and CRP/oxLDL) complexes were particularly present in patients with IMT-diagnosed atherosclerosis, and their levels strongly correlated with hsCRP but not with oxLDL/β2GPI complexes. Interestingly, LDL complexes containing β2GPI 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/β2GPI 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/β2GPI complexes are mainly, or possibly, only formed in atherosclerotic lesions based on the following observations: Immunohistochemistry of carotid artery plaques showed colocalization of oxLDL, β2GPI, 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^{2+}-oxLDL and β2GPI and that stable/non-dissociable complexes of oxLDL/β2GPI and CRP/oxLDL/β2GPI 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 ~10 min), due to its negative charge (17). Even though high concentrations of β2GPI (about 200 μg/ml) are present in the circulation, it seems impossible that oxLDL could form complexes with circulating β2GPI 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/β2GPI and oxLDL/β2GPI 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-atheroscelerotic 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 mechanisms 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/β2GPI 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/β2GPI complexes with these pro-atherosclerotic parameters, because CRP/oxLDL/β2GPI 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/β2GPI) 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/β2GPI complexes-positive DM sera, and one of them may be a spontaneously dissociated mononeric 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 ω-carboxyl function at the acyl chain of cholesteryl esters in oxLDL was responsible for the initial β2GPI 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/β2GPI 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/β2GPI 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 upregulated 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, β2GPI 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 β2GPI complexes with oxLDL or CRP/oxLDL complexes were not detected in the sera from these patients. We demonstrated that oxLDL, β2GPI, and CRP were colocalized with SR-PSOX-positive foamy macrophages and activated or transformed smooth muscle cells in carotid artery plaques, but not with β2GPI 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 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 β2GPI. 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 β2GPI 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/β2GPI 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/β2GPI and oxLDL/β2GPI) as well as CRP/oxLDL should be further elucidated in larger clinical studies.

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REFERENCES

1. Steinberg, D. 1997. Low density lipoprotein oxidation and its pathobiological significance. *J. Biol. Chem.* **272:** 20963–20966.
2. Heinecke, J. W. 1997. Mechanisms of oxidative damage of low density lipoprotein in human atherosclerosis. *Curr. Opin. Lipidol.* **8:** 268–274.
3. Hughes, G. R. V., E. N. Harris, and A. E. Gharavi. 1986. Anticardiolipin syndrome. *J. Rheumatol.* **13:** 486–489.
4. Matsuura, E., K. Kobayashi, M. Tabuchi, and L. R. Lopez. 2006. Oxidative modification of low-density lipoprotein and immune regulation of atherosclerosis. *Prog. Lipid Res.* **45:** 466–486.
5. Matsuura, E., Y. Igarashi, T. Yasuda, D. A. Triplett, and T. Koike. 1994. Anticardiolipin antibodies recognize β2-glycoprotein I structure altered by interacting with an oxygen modified solid phase surface. *J. Exp. Med.* **179:** 457–462.
6. Kasahara, H., E. Matsuura, K. Kaihara, D. Yamamoto, K. Kobayashi, J. Inagaki, K. Ichikawa, A. Tsutsumi, S. Yasuda, T. Atsumi, et al. 2005. Antigenic structures recognized by anti-β2-glycoprotein I auto-antibodies. *Int. Immunol.* **17:** 1533–1542.
7. Hasunuma, Y., E. Matsuura, Z. Makita, T. Katahira, S. Nishi, and T. Koike. 1997. Involvement of β2-glycoprotein I and anticardiolipin antibodies in oxidatively modified low-density lipoprotein uptake by macrophages. *Clin. Exp. Immunol.* **107:** 569–573.
8. Kobayashi, K., E. Matsuura, Q. Liu, J. Furukawa, K. Kaihara, J. Inagaki, T. Atsumi, N. Sakairi, T. Yasuda, D. R. Voelker, et al. 2001. A specific ligand for β2-glycoprotein I mediates autoantibody-dependent uptake of oxidized low density lipoprotein by macrophages. *J. Lipid Res.* **42:** 697–709.
9. Liu, Q., K. Kobayashi, J. Furukawa, J. Inagaki, N. Sakairi, A. Iwado, T. Yasuda, T. Koike, D. R. Voelker, and E. Matsuura. 2002. ω-Carboxyl variants of 7-ketocholesteryl esters are ligands for β2-glycoprotein I and mediate antibody-dependent uptake of oxidized LDL by macrophages. *J. Lipid Res.* **43:** 1486–1495.
10. Kobayashi, K., M. Kishi, T. Atsumi, M. L. Bertolaccini, H. Makino, N. Sakairi, I. Yamamoto, T. Yasuda, M. A. Khamashta, G. R. V. Hughes, et al. 2003. Circulating oxidized LDL forms complexes with β2-glycoprotein I: implication as an atherogenic autoantigen. *J. Lipid Res.* **44:** 716–726.
11. Shoenfeld, Y., R. Wu, L. D. Dearing, and E. Matsuura. 2004. Are anti-oxidized low-density lipoprotein antibodies pathogenic or protective? *Circulation.* **110:** 2552–2558.
12. Shoenfeld, Y., R. Gerli, A. Doria, E. Matsuura, M. M. Cerinic, N. Ronda, L. J. Jara, M. Abu-Shakra, P. L. Meroni, and Y. Sherer. 2005. Accelerated atherosclerosis in autoimmune rheumatic diseases. *Circulation.* **112:** 3337–3347.
13. Lopez, L. R., B. L. Hurley, D. F. Simpson, and E. Matsuura. 2005. Oxidized low-density lipoprotein/β2-glycoprotein I complexes and autoantibodies in patients with type 2 diabetes mellitus. *Ann. N. Y. Acad. Sci.* **1051:** 97–103.
14. Kasahara, J., K. Kobayashi, Y. Maeshima, Y. Yamasaki, T. Yasuda, E. Matsuura, and H. Makino. 2004. Clinical significance of serum oxidized low-density lipoprotein/β2-glycoprotein I complexes in patients with chronic renal diseases. *Nephron Clin. Pract.* **98:** c15–c24.
15. Lopez, D., I. Garcia-Valladares, C. A. Palafox-Sanchez, I. G. De La Torre, K. Kobayashi, E. Matsuura, and L. R. Lopez. 2004. Oxidized low-density lipoprotein/β2-glycoprotein I complexes and autoantibodies to oxLig-1/β2-glycoprotein I in patients with systemic lupus erythematosus and antiphospholipid syndrome. *Am. J. Clin. Pathol.* **121:** 426–436.
16. Lopez, L. R., M. Salazar-Paramo, C. Palafox-Sanchez, B. L. Hurley, E. Matsuura, and I. Garcia-De La Torre. 2006. Oxidized low-density lipoprotein and β2-glycoprotein I in patients with systemic lupus erythematosus and increased carotid intima-media thickness: implications in autoimmune-mediated atherosclerosis. *Lupus.* **15:** 80–86.
17. Van Berkel, T. J., Y. B. De Rijke, and J. K. Kruijt. 1991. Different fate in vivo of oxidatively modified low density lipoprotein and acetylated low density lipoprotein in rats. Recognition by various scavenger receptors on Kupffer and endothelial liver cells. *J. Biol. Chem.* **266:** 2282–2289.
18. Volanakis, J. E. 2001. Human C-reactive protein: expression, structure, and function. *Mol. Immunol.* **38:** 189–197.
19. Paffen, E., and M. P. Demaat. 2006. C-reactive protein in atherosclerosis: a causal factor? *Cardiovasc. Res.* **71:** 30–39.
20. Pepys, M. B., and G. M. Hirschfield. 2003. C-reactive protein: a critical update. *J. Clin. Invest.* **111:** 1805–1812.
21. Chang, M-K., C. J. Binder, M. Torzewski, and J. L. Witztum. 2002. C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: phosphorylcholine of oxidized phospholipids. *Proc. Natl. Acad. Sci. USA.* **99:** 13043–13048.
22. Binder, C. J., S. Horkko, A. Dewan, M. K. Chang, E. P. Kieu, C. S. Goodyear, P. X. Shaw, W. Palinski, J. L. Witztum, and S. G. Jilverman. 2003. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. *Nat. Med.* **9:** 736–743.
23. Mold, C. 1999. Role of complement in host defense against bacterial infection. *Microbes Infect.* **1:** 633–638.

24. Ji, S-R., Y. Wu, L. A. Potempa, Q. Qiu, and J. Zhao. 2006. Interactions of C-reactive protein with low-density lipoproteins: implications for an active role of modified C-reactive protein in atheroscierosis. *Int. J. Biochem. Cell Biol.* **38:** 648–661.
25. Libby, P., and P. M. Ridker. 2004. Inflammation and atherosclerosis: role of C-reactive protein in risk assessment. *Am. J. Med.* **116 (Suppl. 6A):** 9–16.
26. Verma, S., P. E. Szmitko, and P. M. Ridker. 2005. C-reactive protein comes of age. *Nat. Clin. Pract. Cardiovasc. Med.* **2:** 29–36.
27. Pasceri, V., J. T. Willerson, and E. T. H. Yeh. 2000. Direct proinflammatory effect of C-reactive protein on human endothelial cells. *Circulation.* **102:** 2165–2168.
28. Pasceri, V., J. S. Cheng, J. T. Willerson, and E. T. H. Yeh. 2001. Modulation of C-reactive protein-mediated monocyte chemoattractant protein-1 induction in human endothelial cells by anti-atherosclerosis drugs. *Circulation.* **103:** 2531–2534.
29. Verma, S., S. Devaraj, and I. Jialal. 2006. Is C-reactive protein an innocent bystander or proatherogenic culprit? C-reactive protein promotes atherothrombosis. *Circulation.* **113:** 2135–2150.
30. WHO Study Group. 1985. Diabetes mellitus. *World Health Organ. Tech. Rep. Ser.* **727:** 1–113.
31. Arnett, F. C., S. M. Edworthy, D. A. Bloch, D. J. McShane, J. F. Fries, N. S. Cooper, L. A. Healey, S. R. Kaplan, M. H. Liang, H. S. Luthra, et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* **31:** 315–324.
32. Hashimoto, Y., M. Kawamura, K. Ichikawa, T. Suzuki, T. Sumida, S. Yoshida, E. Matsuura, S. Ikehara, and T. Koike. 1992. Anticardiolipin antibodies in NZW x BXSB F1 mice. A model of antiphospholipid syndrome. *J. Immunol.* **149:** 1063–1068.
33. Igarashi, M., E. Matsuura, Y. Igarashi, H. Nagae, K. Ichikawa, D. A. Triplett, and T. Koike. 1996. Human β2-glycoprotein I as an anticardiolipin cofactor determined using mutants expressed by a baculovirus system. *Blood.* **87:** 3262–3270.
34. Minami, M., N. Kume, T. Shimaoka, H. Kataoka, K. Hayashida, Y. Akiyama, I. Nagata, K. Ando, M. Nobuyoshi, M. Hanyuu, et al. 2001. Expression of SR-PSOX, a novel cell-surface scavenger receptor for phosphatidylserine and oxidized LDL in human atherosclerotic leisions. *Arterioscler. Thromb. Vasc. Biol.* **21:** 1796–1800.
35. Krauss, R. M., and D. J. Burke. 1982. Identification of multiple subclasses of plasma low density lipoproteins in normal humans. *J. Lipid Res.* **23:** 97–104.
36. Ozaki, K., K. Inoue, H. Sato, A. Iida, Y. Ohnishi, A. Sekine, H. Sato, K. Odashiro, M. Nobuyoshi, M. Hori, et al. 2004. Functional variation in LGALS2 confers risk of myocardial infarction and regulates lymphotoxicin-α secretion in vitro. *Nature.* **429:** 72–75.
37. Shi, S-R., M. E. Key, and K. L. Kalra. 1991. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating tissue sections. *J. Histochem. Cytochem.* **39:** 741–748.
38. Devaraj, S., S. Venugopal, and I. Jialal. 2006. Native pentameric C-reactive protein displays more potent pro-atherogenic activities in human aortic endothelial cells than modified C-reactive protein. *Atherosclerosis.* **184:** 48–52.
39. Tsujimoto, M., K. Inoue, and S. Nojima. 1983. Purification and characterization of human serum C-reactive protein. *J. Biochem. (Tokyo).* **94:** 1367–1373.
40. Buttari, B., E. Profumo, V. Mattei, A. Siracusano, E. Ortona, P. Margutti, B. Salvati, M. Sorice, and R. Rigano. 2005. Oxidized β2-glucoprotein I induces human dendritic cell maturation and promotes a T helper type 1 response. *Blood.* **106:** 3880–3887.
41. George, J., D. Harats, B. Gilburd, A. Afek, Y. Levy, J. Schneiderman, I. Barshack, J. Kopolovic, and Y. Shoenfeld. 1999. Immunolocalization of β2-glycoprotein I (apolipoprotein H) to human atherosclerotic plaques: potential implications for lesion progression. *Circulation.* **99:** 2227–2230.
42. Pasceri, V., H. D. Wu, J. T. Willerson, and E. T. H. Yeh. 2000. Modulation of vascular inflammation in vitro and in vivo by peroxisome proliferator-activated receptor-gamma activators. *Circulation.* **101:** 235–238.
43. Steinberg, D., and A. Lewis. 1997. Conner memorial lecture. Oxidative modification of LDL and atherosclerosis. *Circulation.* **95:** 1062–1071.
44. Venugopal, S. K., S. Devaraj, and I. Jialal. 2005. Macrophage conditioned medium induces the expression of C-reactive protein in human aortic endothelial cells: potential for paracrine/autocrine effects. *Am. J. Pathol.* **166:** 1265–1271.
45. Yasojima, K., C. Schwab, E. G. McGeer, and P. L. McGeer. 2001. Generation of C-reactive protein and complement components in atherosclerotic plaques. *Am. J. Pathol.* **158:** 1039–1051.
46. Calabro, P., J. T. Willerson, and E. T. H. Yeh. 2003. Inflammatory cytokines stimulated C-reactive protein production by human coronary artery smooth muscle cells. *Circulation.* **108:** 1930–1932.
47. James, M. J., D. van Reyk, K. A. Rye, R. T. Dean, L. G. Cleland, P. J. Barter, and W. Jessup. 1998. Low density lipoprotein of synovial fluid in inflammatory joint disease is mildly oxidized. *Lipids.* **33:** 1115–1121.
48. Dai, L., Z. Zhang, P. G. Winyard, K. Gaffney, H. Jones, D. R. Blake, and C. J. Morris. 1996. A modified form of low-density lipoprotein with increased electronegative charge is present in rheumatoid arthritis synovial fluid. *Fee Radic. Res.* **22:** 705–710.

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Lectin-like oxidized LDL receptor-1 (LOX-1) expression is associated with atherosclerotic plaque instability—analysis in hypercholesterolemic rabbits

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Abstract

Lectin-like oxidized LDL receptor-1 (LOX-1), a cell-surface receptor for oxidized LDL (Ox-LDL), has been implicated in vascular cell dysfunction related to atherosclerotic plaque instability, according to cell culture experiments. In the present study, we investigated the relationship between LOX-1 expression and plaque instability in hypercholesterolemic rabbits by immunohistological analyses in vivo. We prepared thirty series of cross sections of the thoracic aorta from six myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits (12–24 months), in which seventy atherosclerotic plaques were observed. LOX-1, matrix metalloproteinase-9 (MMP-9), monocyte chemoattractant protein-1 (MCP-1) expression, apoptotic events, plaque instability index (an index of the morphological destabilization of atherosclerotic plaques) and fibromuscular cap thickness in each atherosclerotic plaque were determined by immunohistochemical staining, TUNEL staining and Azan–Mallory staining. LOX-1 expression was positively correlated with the plaque instability index and MMP-9 expression. LOX-1 expression was more prominent in atherosclerotic plaques with thinner fibromuscular cap (<100 μm). Furthermore, LOX-1 expression was shown in the macrophage-rich lipid core area where MCP-1 expression and apoptotic events were prominent. These results indicate that enhanced LOX-1 expression was associated with histologically unstable atherosclerotic plaques in hypercholesterolemic rabbits, suggesting the involvement of LOX-1 in the destabilization of atherosclerotic plaques in vivo.

Keywords: LOX-1; MMP-9; MCP-1; Apoptosis; WHHLMI rabbits

1. Introduction

The disruption of unstable atherosclerotic plaques and subsequent formation of occlusive thrombi are currently recognized as the primary cause of acute coronary syndrome [1–3]. Atherosclerotic plaques with large lipid cores, thin fibromuscular caps, enhanced mononuclear leukocyte accumulation and proinflammatory responses, and expression of matrix metalloproteinases (MMPs) have been suggested to be more susceptible to rupture [4].

Lectin-like oxidized LDL receptor-1 (LOX-1), a type II membrane glycoprotein belonging to the C-type lectin family, acts as a cell-surface receptor for oxidized LDL (Ox-LDL) and mediates several biological effects of Ox-LDL [5]. Previous studies with cultured cells suggest that LOX-1 may play important roles in the pathogenesis of atherosclerosis, such as induced expression of adhesion molecules and chemokines

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for monocytes [6,7], transformation of macrophages into foam cells [8–10], smooth muscle cells apoptosis [11,12] and degradation of extracellular matrix proteins by induction of MMPs [13]. These biological effects mediated by Ox-LDL–LOX-1 interactions would collectively enlarge the lipid core, weaken the fibromuscular cap, and induce proinflammatory responses, resulting in destabilization of atherosclerotic plaques; however, roles of LOX-1 in plaque instability in vivo remain to be determined.

As a hypercholesterolemic rabbit model of spontaneous atherosclerosis, myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits (previous strain; Watanabe heritable hyperlipidemic (WHHL) rabbits) have been widely used because the histological characteristics of their atherosclerotic lesions appear to be similar to those in humans [14–17]. In addition, a monoclonal antibody directed to rabbit LOX-1 has been established, and LOX-1 expression by endothelial cells and macrophages in fatty streak lesions has already been reported in WHHL rabbit aortas [18]. LOX-1 expression in advanced atherosclerotic plaques of human carotid arteries has been observed in intimal smooth muscle cells, as well as macrophages and endothelial cells [19].

In the present study, we examined the relationship between LOX-1 expression and histological markers of plaque instability, such as the plaque instability index (an index of morphological destabilized characteristics of atherosclerotic plaques), MMP-9 expression, MCP-1 expression, apoptotic events, and fibromuscular cap thickness in this hypercholesterolemic rabbit model.

2. Methods

2.1. Animals

Six WHHLMI rabbits (12–24 months old: 3.0–3.25 kg body weight) bred at Kobe University were used in the present study. Rabbits were fed standard rabbit chow (type CR-3; Clea Japan Inc., Tokyo, Japan: 120 g/day) and were given water ad libitum. As a control, a Japanese White rabbit (3 month old; Biotec Inc., Saga, Japan: 3.0 kg body weight) was used. All experimental procedures were approved by the Kyoto University Animal Care Committee.

2.2. Preparation of histological sections

Rabbits were sacrificed with an overdose of sodium pentobarbital. The descending thoracic aorta was divided into 5 portions (2 cm segments), after adjacent fat and connective tissue had been removed. These aortic segments were immediately fixed in a solution containing L-(+)-lysine hydrochloride (75 mmol/L) and 4% paraformaldehyde in phosphate buffer (37.5 mmol/L, pH 7.4), and embedded in paraffin. Consecutive 5 μm thick slices were prepared at 1 cm from the end of the 2 cm segments.

2.3. Preparation of a monoclonal antibody directed to rabbit LOX-1

A monoclonal antibody directed to rabbit LOX-1 was established using a standard hybridoma technique. Polypeptide corresponding to the amino acid numbers between 150 and 171 of the rabbit LOX-1 (extracellular domain) was used as an antigen to immunize mice. A monoclonal antibody by which COS-7 cells transfected with rabbit LOX-1 cDNA, but not untransfected wild-type COS-7 cells, were able to be immunochemically stained, was utilized.

2.4. Histological analysis

Serial sections were subjected to immunostaininig for LOX-1, MMP-9, MCP-1 and cell type marker antigens. A monoclonal antibody for MMP-9 (mouse IgG) was obtained from Daiichi Fine Chemical Corp., Toyama, Japan. A monoclonal antibody for MCP-1 (mouse IgG) was obtained from BD Biosciences Inc., San Jose, CA, USA [20]. Monoclonal antibodies for a rabbit macrophage-specific antigen (RAM-11, mouse IgG) and smooth muscle actin (1A4, mouse IgG) were obtained from Dako Corp., Santa Barbara, CA, USA. Immunohistostaining was carried out using a Dako Envision + kit (Dako) with hematoxylin counterstaining. Immunohistochemical staining with an anti-von Willebrand factor (vWf) polyclonal antibody (goat IgG, Atlantic Antibodies, Stillwater, MN), which cross-reacts with rabbit vWf, was carried out to identify rabbit endothelial cells by use of a Dako Envision/AP kit (Dako) followed by hematoxylin counterstaining. Immunostaining with subclass-matched irrelevant IgG served as a negative control.

Azan–Mallory staining was performed with standard procedures. Apoptotic nuclei were determined by terminal deoxyribonucleotide transferase (TdT)-mediated nick-end labeling (TUNEL) using a commercially available kit (in situ apoptosis detection kit, Trevigen Inc., Gaithersburg, MD, USA).

2.5. Definition of atherosclerotic plaques

In atherosclerotic plaques, the lipid core was defined as a layer more than 100 μm thick consisting of macrophage foam cells and extracellular lipid deposits, which was measured using a VHX Digital Microscope (Keyence Corp., Osaka, Japan). Fibromuscular caps were defined as areas consisting of smooth muscle cells and collagen fibers, which cover the lipid core. The systematic structure of an atherosclerotic plaque is shown in Fig. 1. Typical unstable plaques contained predominantly macrophages or large lipid cores, with a thin or scarcely perceivable fibromuscular cap consisting of smooth muscle cells and collagen fibers. On the other hand, typical stable plaques contained scattered macrophages and lipid cores in deep intimal areas with a thicker fibromuscular cap. The atherosclerotic plaques were classified into two groups based on fibromuscular cap thickness (cut-off value, 100 μm)

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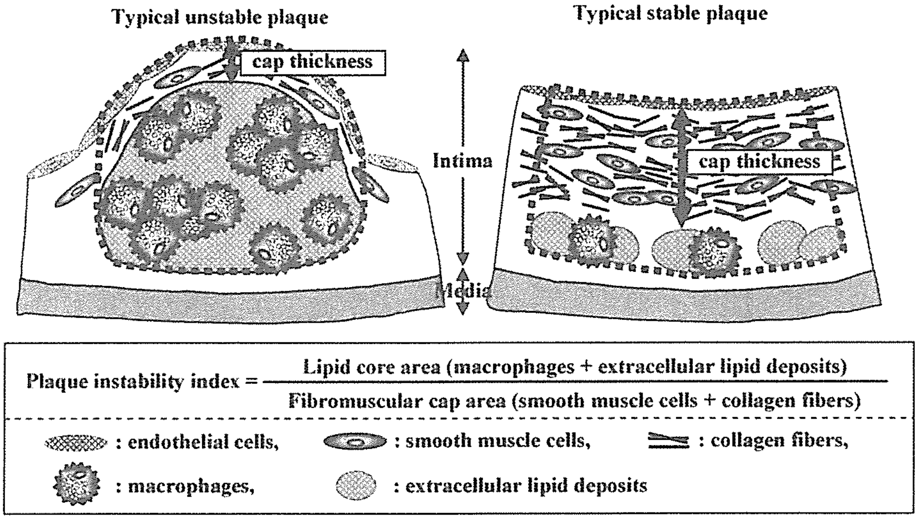

Fig. 1. Schematic illustration of a typical atherosclerotic plaque and its components described in the text. The area within dotted lines shows typical unstable plaques (left) and typical stable plaques (right). In each atherosclerotic plaque, the ratio of the lipid core area (macrophages and extracellular lipid deposits) to the fibromuscular cap area (smooth muscle cells and collagen fibers covering the lipid core area) was defined as a 'plaque instability index'. Fibromuscular cap thickness was measured at the point of the thickest lipid core with clear demarcation of the fibromuscular cap-lipid core interface.

measured at the point of the thickest lipid core with a clear demarcation of the fibromuscular cap-lipid core interface as shown in Fig. 1.

2.6. Quantitative analysis

Lesion component areas were quantitatively evaluated with the microscope. In each section with Azan–Mallory staining, we examined all atherosclerotic plaques and measured the ratio of the LOX-1-positive area to the entire area of the plaque (LOX-1 expression density). Using the same method, MMP-9 expression density was determined. As an index of morphologically destabilized characteristics, the ratio of the lipid core area (macrophages and extracellular lipid deposits) to the fibromuscular cap area (smooth muscle cells and collagen fibers covering the lipid core area) was calculated from the results of Azan–Mallory staining, as well as immunostaining for macrophages (RAM-11) and smooth muscle cells (1A4). This ratio (lipid core area/fibromuscular cap area) was defined as a 'plaque instability index' in each atherosclerotic plaque. We measured blue region as collagen fibers or other extracellular matrix proteins, and extracellular vacuoles and lacunae as extracellular lipid deposits in Azan–Mallory-stained sections [21,22], and RAM-11-positive areas as macrophages and 1A4-positive areas as smooth muscle cells in immunostained sections. In each section, the plaque instability index, MMP-9 expression density and fibromuscular cap thickness were compared with LOX-1 expression density.

2.7. Statistical analyses

Values were expressed as the mean ± S.D. Statistical analysis was performed with the Mann–Whitney *U* test for comparing LOX-1 expression density. Correlation coefficients were assessed with Spearman rank correlation coefficients. Statistical significance was defined as $P < 0.0001$.

3. Results

3.1. Composition of atherosclerotic plaque and MMP-9 expression

In the thoracic aorta of WHHLMI rabbits, we analyzed seventy atherosclerotic plaques found in thirty series of sections from six animals (five segments per rabbit). Fig. 2 shows typical images of unstable (left two columns) and stable (right two columns) atherosclerotic plaques with Azan–Mallory and immunohistochemical staining. Fig. 3 shows higher magnification images of each staining corresponding to the area indicated as a square in Fig. 2 V. The unstable plaques (left two columns) looked similar to rupture-prone unstable human plaques which consist of a large lipid core with abundant macrophages and extracellular lipid deposits, accompanied by a thin fibromuscular cap with relatively few smooth muscle cells scattered in the superficial region (Figs. 2 and 3). MMP-9 expression was mainly observed in RAM-11-positive areas (Figs. 2E, F, I, J and 3B, C). On the other hand, stable plaques (right two columns) showed typical characteristics such as few macrophages, less extracellular lipid deposits and a thicker fibromuscular cap (Fig. 2). Less prominent MMP-9 expression, as well as a larger number of smooth muscle cells and collagen fibers or more extracellular matrix was observed (Fig. 2G, H, O, P, W, X). MMP-9 signals were undetectable in aortic sections from normocholesterolemic control rabbits (data not shown).

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