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PAPER

Atherogenic oxidized low-density lipoprotein/β_2-glycoprotein I (oxLDL/β_2GPI) complexes in patients with systemic lupus erythematosus and antiphospholipid syndrome

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Oxidized low-density lipoprotein (oxLDL) interacts *in vitro* with β_2-glycoprotein I (β_2GPI) via LDL-derived specific ligands forming oxLDL/β_2GPI complexes. Circulating oxLDL/β_2GPI complexes have been demonstrated in patients with systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS). Autoimmune vascular inflammation and oxidative stress contribute to oxLDL/β_2GPI complex formation. Immunohistochemical staining of atherosclerotic lesions suggest that these complexes are formed in the arterial wall and released into circulation. The demonstration of antibodies to oxLDL/β_2GPI complexes indicates that these complexes are immunogenic, and the coexistence of complexes and antibodies suggest an active pro-thrombotic/pro-atherogenic role in the development of autoimmune vascular complications. Circulating oxLDL/β_2GPI complexes can be measured by ELISA using a monoclonal antibody specific to complexed human β_2GPI to capture β_2GPI bound to oxLDL. An enzyme-conjugated monoclonal antibody to human Apo B 100 allows the specific detection of oxLDL/β_2GPI complexes. OxLDL/β_2GPI complexes were common in SLE and APS patients suggesting an underlying process of inflammation and oxidation. Using oxLDL/β_2GPI complexes as capture antigen, antibodies to oxLDL/β_2GPI can be measured by ELISA. Serum levels of IgG anti-oxLDL/β_2GPI antibodies were significantly higher in SLE patients with APS compared to SLE controls without APS. Further, high titers of these IgG antibodies were observed in APS patients with a history of arterial thrombosis. The presence of circulating oxLDL/β_2GPI complexes and IgG antibodies to these complexes indicates significant vascular injury and oxidative stress as well as an active role in autoimmune-mediated atherothrombosis. *Lupus* (2006) **1**, 478–483.

Key words: antiphospholipid antibodies; autoimmunity; oxidized-LDL antibodies

Introduction

Dyslipoproteinemia, vascular inflammation and oxidative stress are important pathogenic mechanisms that contribute to the initiation and progression of atherosclerotic lesions.[1,2] Systemic autoimmune diseases are characterized by generalized vascular inflammation and active lipid peroxidation (oxidative stress) that generate pro-inflammatory and pro-atherogenic oxidized low-density lipoproteins (oxLDL).[3–5] Decreased anti-oxidant activity may also contribute the oxidative modification of LDL.[6,7] It is currently agreed that oxLDL plays an important pathogenic role in early events leading to atherosclerosis. OxLDL is a potent inflammatory agent as well as a chemotactic for macrophages and T lymphocytes,[8] and shown to be highly immunogenic.[9,10] OxLDL has been shown to co-localize in atherosclerotic lesions of rabbit and man with β_2GPI by immunohistochemical staining,[11,12] suggesting a role of β_2GPI (and antiphospholipid antibodies) in atherogenesis.[13,14] These findings have been implicated in the premature (or accelerated) development of atherosclerotic cardiovascular complications recently reported in autoimmune patients[15,16] that could not be fully explained by the traditional risk factors for atherosclerosis.[17]

We have previously reported that Cu^{2+}-oxidized LDL, unlike native LDL, binds *in vitro* to β_2GPI via oxLDL-derived ligands (oxLig-1) to form stable (non-dissociable) and likely pathogenic oxLDL/β_2GPI

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complexes.[18–20] The *in vitro* macrophage uptake of oxLDL/β_2GPI complexes was significantly increased in the presence of antiphospholipid antibodies, either β_2GPI-dependent anticardiolipin (aCL) or anti-β_2GPI antibodies, suggesting that macrophage Fcγ receptors were involved.[19] This mechanism may be relevant to the development of atherosclerosis in patients with SLE and APS. OxLDL/β_2GPI complexes have been demonstrated in patients with systemic autoimmune diseases and vascular complications (SLE and APS), representing a common lipoprotein abnormality contributing to inflammation but also implicated as atherogenic autoantigen.[21,22] High serum levels of oxLDL/β_2GPI complexes and autoantibodies to these complexes were associated with venous and arterial thrombosis in patients with APS.[23–25]

The presence of circulating oxLDL/β_2GPI complexes and IgG autoantibodies to oxLDL/β_2GPI by ELISA allowed further investigation of their clinical association and pathogenic role in vascular complication recognize with increasing frequency in autoimmune patients. Our results show that oxidation of LDL and its interaction with β_2GPI to form stable circulating complexes are common events in SLE and APS, and that autoantibodies to oxLDL/β_2GPI were also present in some SLE patients with secondary APS. These autoantibodies correlate with measures of disease activity suggesting a pathogenic role in the development of atherosclerosis in SLE and APS patients.

Subjects and methods

Serum samples were obtained from the following autoimmune populations: serum samples from 91 consecutive patients with SLE, 65 with SSc and 80 with RA. The diagnosis of SLE, SSc and RA were established according to American College of Rheumatology (ACR) Classification Criteria.[26] One-hundred and sixty-one healthy blood bank donors were included as controls. Serum samples from 51 selected SLE patients classified into two subgroups: 30 SLE patients without APS and no clinical history of antiphospholipid antibodies (controls) and 21 SLE patients with secondary APS. The clinical diagnosis was established according to the Sapporo Criteria for the classification of APS.[27] These patients were used to study the relationship between antibodies to oxLDL/β_2GPI and APS. Additional 93 serum samples from selected APS patients (20 with primary and 73 with secondary APS) were studied to assess the association between these antibodies and clinical manifestation of APS (ie, venous, arterial thrombosis and pregnancy morbidity). Forty of these APS patients had history of an arterial thrombotic event, 37 of venous thrombosis and 14 of pregnancy morbidity. Serum samples from 161 healthy blood donors were used as controls.

Monoclonal antibodies

The following monoclonal antibodies were used to develop the ELISA tests for measuring oxLDL/β_2GPI complexes and anti-oxLLD/β_2GPI antibodies: WB-CAL-1 monoclonal antibody reactive to β_2GPI (IgG2a, κ) derived from a NZW $\times$ BXSB F1 mouse, a model of spontaneous APS (33). This monoclonal antibody bind only to β_2GPI/negatively-charged phospholipid (or oxLDL) complexes, but not to monomeric (free) β_2GPI in solution.[28,29] 2E10 is an IgG murine monoclonal antibody specific to human Apo B-100, and equally reacts with both native and oxLDL.

Purification of human β_2GPI

Human β_2GPI was purified from fresh normal plasma as previously described[30] with slight modifications. Briefly, human plasma was first precipitated with 70% perchloric acid, extensively dialyzed against Tris/NaCl buffer (pH 8.0) and concentrated before loading into a heparin column (Amersham Biosciences, Piscataway, NJ). Pooled β_2GPI fractions were again dialyzed against sodium acetate/NaCl buffer (pH 4.8) and concentrated. This preparation was then loaded into a carboxy-methyl cellulose column (Sigma-Aldrich, St Louis, MO) and β_2GPI fractions were pooled, dialyzed against sodium acetate/NaCl buffer, concentrated at approximately 1 mg/mL and stored at –70°C until used. The β_2GPI preparation contains >95% of protein and a 50 kDa single diffuse band was demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In addition, the immunoreactivity of the purified β_2GPI was checked by an anti-β_2GPI ELISA procedure prior to use.

LDL purification and oxidation

LDL was isolated by ultracentrifugation of fresh normal human plasma in PBS/KBr solutions as described.[31] LDL (d = 1.019–1.063 g/mL) was adjusted to a concentration of 100 µg/mL based on protein concentration. The LDL fraction was oxidized with 5 µM CuSO4 in 10 mM Hepes, 150 mM NaCl, pH 7.4 (Hepes buffer) at 37°C for 12 hours.[32] Oxidation was terminated by the addition of EDTA (at a final concentration of 1 mM), and extensively dialyzed against Hepes buffer containing EDTA.

ELISA procedure for oxLDL/β_2GPI complexes

Monoclonal antibody against β_2GPI (WB-CAL-1) was coated onto Immulon 2HB microplates (Dynex

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Technologies, Inc., Chantilly, VA) by incubating overnight at 2–4°C. WB-CAL-1 is an IgG murine monoclonal antibody against human β_2GPI used in this assay to capture oxLDL/β_2GPI complexes via its reactivity with β_2GPI. The plate was blocked and stabilized. One hundred μL of samples diluted 1 : 100 in diluent containing 2 mM $MgCl_2$ were added to the appropriate microwells and incubated for one hour at room temperature. $MgCl_2$ dissociates intermediate oxLDL/β_2GPI complexes (ie, electrostatically bound) allowing the specific detection of non-dissociable and covalently bound complexes present in serum samples. The microwells were washed four times with PBS-0.05% tween-20. HRP-conjugated 2E10 monoclonal antibody (anti-human Apo B-100) was added to the microwells and incubated for one hour at room temperature. Color was developed with tetramethylbezydine (TMB)/H_2O_2, and the reaction stopped with 0.36 N sulfuric acid. Optical density was read at a wavelength of 450 nm (650 nm reference). The intra-assay precision (% CV) for the assay ranged from 3.0% to 4.8% for high and 5.1–7.5% for low reactive samples. Serum oxLDL/β_2GPI complex concentration (expressed in units) was calculated against a reference curve built with two-fold serial dilutions of oxLDL/β_2GPI complex solution. The complexes were prepared in advance by incubating equal amounts of Cu^{2+}-oxLDL and purified human β_2GPI at pH 7.4 for 18 hours at 37°C. The unit value was arbitrarily derived from the protein concentration of the oxLDL/β_2GPI complex used in the reference curve. The detection range for the assay was from 1.5 to 100 units with a normal range of <7.0 units, established by testing 99 serum samples from healthy blood donors (95th percentile). Oxidized (not native) LDL reacted with exogenous human β_2GPI and only oxLDL/β_2GPI complexes were detected by the assay.[23]

ELISA for IgG anti-oxLDL/β_2GPI antibodies

OxLDL/β_2GPI complexes were coated onto Immulon 1B microplates by overnight incubation, blocked and stabilized. The oxLDL/β_2GPI complexes were prepared as described above and used as antigenic substrates to capture patient's antibodies. One-hundred μL of samples diluted 1 : 100 were subsequently added to the microwells and incubated for one hour at room temperature. The microwells were washed four times with PBS-0.05% tween-20. HRP-conjugated anti-human IgG antibody was added to the microwells and incubated for one hour. Color was developed with TMB/H_2O_2 and the reaction stopped with 0.36 N sulfuric acid. Optical density was read at a wavelength of 450 nm (650 nm reference). The intra-assay precision (% CV) for this assay ranged from 3.5% to 6.7% for high and low reactive samples. A strong reactive sample(s) were selected and used as controls or to build a reference curve. IgG oxLDL/β_2GPI antibody concentration of patient's samples (expressed in units) was calculated against a reference curve prepared with serial dilutions of a pre-selected positive sample. A normal range for the assay was established at <20 units by testing 100 serum samples from healthy blood donors (95th percentile).

Statistical analysis

Statistical analysis was performed with a SigmaStat program (SPSS Science, Inc., Chicago, IL). Student's *t*-test was performed to compare the results between different groups and Fisher's exact test to assess the relationship between antibodies and clinical manifestations. Sensitivity, specificity, positive predictive value (PPV) and odds ratio of anti-oxLDL/β_2GPI antibodies were calculated by 2×2 contingency table analysis. A *P*-value of 0.05 or less was considered as significant.

Results

OxLDL/β_2GPI complexes in consecutive autoimmune patients

The mean oxLDL/β_2GPI complex level of 91 consecutive SLE patients (55.9 units) and 65 SSc patients (30.03 units) were significantly higher than the mean level of 80 RA patients (4.6 units) and 161 healthy controls (3.6 units). The mean level of RA patients was not statistically different to healthy controls (Table 1). Sixty-three percent of SLE, 98% of SSc patients reacted above the normal range of the oxLDL/β_2GPI complex assay (seven units). In contrast, only 6.3% of RA patients had positive levels of oxLDL/β_2GPI complexes. Serum levels of oxLDL/β_2GPI complexes of 21 patients with 2ry APS (21.2 units) and 30 SLE controls (28.8 units) were not different, indicating that dyslipoproteinemia and oxidative stress is not unique to APS. Further, as previously reported, complex levels did not correlate with any of the clinical manifestation of APS.[24]

IgG anti-oxLDL/β_2GPI antibodies in consecutive autoimmune patients

Mean IgG anti-oxLDL/β_2GPI levels of 92 consecutive SLE patients (19.1 units) and 79 RA patients (17.6 units) were significantly higher that 100 healthy

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Table 1 Mean serum level and prevalence of oxLDL/β_2GPI complexes measured by ELISA in systemic autoimmune populations

Subjects (n)	*Mean oxLDL/β_2GPI complex + SD (units)*	*% positive* (>7 units)*
SLE (n = 91)	55.9 + 59.2	63.7
SSc (n = 65)	30.3 + 22.9	98.4
RA (n = 80)	4.6 + 2.2	6.3
Healthy controls (n = 161)	3.6 + 0.9	0.6
2ry APS (n = 21)	21.2 + 17.3	76.2
SLE control (n = 30)	28.8 + 24.5	80.0

SLE = systemic lupus erythematosus, SSc = systemic sclerosis, 2ryAPS = secondary antiphospholipid syndrome, RA = rheumatoid arthritis.
*Normal range less than seven units (95th percentile of 99 healthy controls).

Table 2 Mean serum level and prevalence of IgG anti-oxLDL/β_2GPI antibodies measured by ELISA in systemic autoimmune populations

Subjects (n)	*Mean IgG anti-oxLDL/β_2GPI antibody level + SD (units)*	*% positive* (>20 units)*
SLE (n = 92)	19.1 + 13.2	30.4
RA (n = 79)	17.6 + 11.6	28.0
Healthy controls (n = 100)	11.4 + 4.7	5
2ryAPS (n = 21)	41.4 + 29.4	81.0
SLE control (n = 32)	21.1 + 7.5	53.0

SLE = systemic lupus erythematosus, 2ryAPS = secondary antiphospholipid syndrome, RA = rheumatoid arthritis.
*Normal range <20 units (95th percentile of 99 healthy controls).

controls (11.4 units) (Table 2). Thirty percent of SLE and 28% RA patients reacted above the normal range of the assay compared to 5% of the controls.

IgG anti-oxLDL/β_2GPI antibodies in APS patients

Mean IgG anti-oxLDL/β_2GPI antibody levels of 21 SLE patients with secondary APS (41.4 units) was significantly higher compared to SLE controls without APS (21.1 units) (Table 2). Eighty-one percent of SLE patients with secondary APS had positive antibody levels, compared to 53% SLE controls without APS. The clinical performance of IgG anti-oxLDL/β_2GPI antibodies was compared against IgG aCL antibodies to predict APS in these SLE patients (2 × 2 contingency table analysis). IgG anti-oxLDL/β_2GPI antibodies were 45% sensitive and 93.7% specific for APS with a positive predictive value of 90% ($P < 0.001$) while IgG aCL antibodies were 62.5% sensitive and 80% specific with a positive predictive value of 71.4%.

Ninety-three selected APS patients were classified into subgroups according to their clinical history of venous thrombosis, arterial thrombosis and pregnancy morbidity. Mean IgG anti-oxLDL/β_2GPI antibody level of APS patients with history of arterial thrombosis was 30.3 units with 34% reacting above the normal range. The mean antibody level of APS patients with history of venous thrombosis was 24.7 units with 24% reacting positive, and the mean antibody level for APS patients with history of pregnancy morbidity was 11.9 units with 7% reacting positive (Table 3). The difference between the subgroups with arterial and venous thrombosis did not reach statistical significance. The clinical performance of IgG anti-oxLDL/β_2GPI antibodies to predict thrombosis (arterial and venous), arterial only or venous only in these patients with APS was studied (2 × 2 contingency table analysis). The positive predictive value of IgG anti-oxLDL/β_2GPI antibodies for total (arterial and venous) thrombosis was 96% ($P = 0.049$), for arterial thrombosis was 93.7% ($P = 0.014$), for venous thrombosis was 90% ($P = 0.082$), and for pregnancy morbidity was only 50%.

Discussion

These results demonstrate significantly higher serum levels of oxLDL/β_2GPI complexes in patients with SLE and SSc, compared to RA and healthy controls. We have previously shown that high serum levels of oxLDL/β_2GPI complexes are frequent in SLE patients with vascular complications, and proposed that these complexes play a pathogenic role in autoimmune-mediated thrombosis and atherosclerosis.[22,25] Unlike

Table 3 Mean serum level, prevalence and positive predictive value of IgG anti-oxLDL/β2GPI antibodies measured by ELISA in APS patients classified according to their history of thrombosis (Tx)

APS subjects (n)	*Mean IgG anti-oxLDL/β_2GPI Ab level + SD (units)*	*% positive* (> 20 units)*	*Positive predictive value (P value)*
Arterial Tx (n = 45)	30.3 + 37.1	33	93.7% (P = 0.014)
Venous Tx (n = 37)	24.7 + 40.2	24	90% (P = 0.082)
Pregnancy morbidity (n = 15)	11.9 + 5.1	7	50% (P = 1.0)
SLE without APS (n = 20)	9.3 + 4.1	5	

APS = antiphospholipid syndrome, Tx = thrombosis.
*Normal range <20 units (95th percentile of 100 healthy controls).

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RA patients, both SLE and SSc are characterized by generalized vascular complications.

Increased urinary isoprostane-$F_{2\alpha}$ production indicating lipid peroxidation (oxidative stress)[33,34] and decreased antioxidant activity of the enzyme paraoxonase (PON) have been reported in patients with SLE, SSc and APS.[6,7] Furthermore, *in vivo* markers of oxidative stress measured in various systemic autoimmune diseases showed that SLE patients had the most active oxidation, followed by SSc, RA and healthy controls.[4] The degree of oxidation in RA patients was similar to healthy controls. If serum levels of oxLDL/β_2GPI complexes are the reflection of oxidative stress, our results correspond to the reported degree of autoimmune oxidation. Widespread chronic autoimmune vascular inflammation together with decreased PON activity may contribute to oxidative stress, dyslipoproteinemia reflected by LDL modification (oxLDL) and oxLDL/β_2GPI complex formation.

OxLDL/β_2GPI complexes have been implicated as atherogenic antigens.[22] SLE and SSc had higher serum levels of IgG anti-oxLDL/β_2GPI antibodies than RA and healthy controls. These antibodies were also significantly higher in SLE patients with secondary APS with a positive predictive value for APS of 90% whereas IgG anticardiolipin (aCL) antibodies had a predictive value of only 71.4%. APS patients with arterial and venous thrombosis also had higher IgG antibody levels than pregnancy morbidity or SLE controls without APS. The predictive value of IgG anti-oxLDL/β_2GPI antibodies for arterial thrombosis was 94%, better than 90% for venous thrombosis.[25]

IgG anti-oxLDL antibodies have been detected in patients with cardiovascular diseases with predictive value for atherosclerosis. However, these antibodies have also been found in healthy individuals. Experimental models of atherosclerosis have yielded contrasting results suggesting a dual role for these antibodies.[35,36] Natural antibodies (IgM isotype) may be anti-atherogenic, so their absence (or decreased levels) may indirectly facilitate the development of atherosclerosis. We have reported that young SLE patients with increased carotid IMT also had elevated levels of oxLDL/β_2GPI complexes and IgG (but not IgM) anti-oxLDL/β_2GPI antibodies.[37]

Atherosclerotic plaque formation in autoimmune diseases requires macrophage uptake of immune complexes[10] which may include oxLDL/β_2GPI/IgG complexes. The *in vitro* macrophage uptake of oxLDL/ β_2GPI complexes increased in the presence of anti-β_2GPI antibodies, suggesting the participation of Fcγ receptors.[18–20] Other autoantibodies may be produced by unidentified antigenic complexes created during chronic oxidative stress. These antibodies along with a range of metabolic and inflammatory dyslipoproteinemia may further enhance platelet and endothelial activation resulting in severe autoimmune-mediated atherothrombotic disease. OxLDL/β_2GPI complexes and autoantibodies to these complexes may not only play a pathogenic role, but be used as serologic markers to assess the patient's risk for vascular complications. It also provides a rationale to develop vascular preventive programs and/or more targeted therapeutic intervention.

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