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Detection of serum β_2-GPI–Lp(a) complexes in patients with systemic lupus erythematosus

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ABSTRACT

Background: Circulating β_2-glycoprotein-I-oxidized low-density lipoprotein (β_2-GPI–ox-LDL) complexes have been found in patients with systemic lupus erythematosus (SLE) and other autoimmune diseases as a contributor to the development of autoimmune-mediated atherosclerosis. *In vitro* study showed that β_2-GPI also bound with high affinity to atherogenic lipoprotein (a) [Lp(a)] which shares structural similarity to LDL. We examined the existence and clinical significance of serum complexes of β_2-GPI with Lp(a) in SLE patients.
Methods: A "sandwich" ELISA was developed for measuring serum concentrations of β_2-GPI–Lp(a) complexes, using rabbit anti-human β_2-GPI antibody as capturing antibody, and quantitating with antibody against apo(a). Forty-seven SLE patients and 42 healthy controls were studied.
Results: Both Lp(a) (400 ± 213 mg/l *vs.* 181 ± 70 mg/l) and ox-Lp(a) (27.07 ± 22.30 mg/l *vs.* 8.20 ± 4.55 mg/l) concentrations were higher in SLE patients than in controls ($P < 0.0001$). β_2-GPI–Lp(a) complexes were detectable in both controls and SLE. The complexes levels in SLE were higher than in controls (0.96 ± 0.41 U/ml *vs.* 0.59 ± 0.20 U/ml, $P < 0.0001$) and was positively correlated with ox-Lp(a) ($P < 0.001$).
Conclusions: We report the existence of β_2-GPI–Lp(a) complexes in both controls and SLE patients. The complexes levels increase in SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a multifactorial polysystemic autoimmune disorder and is associated with premature atherosclerosis. It has been shown that premature or accelerated atherosclerosis is an important cause of morbidity and mortality in patients with SLE [1,2]. However, traditional cardiovascular risk factors do not fully explain the excessive cardiovascular events observed in these patients [2,3]. Therefore, exploration and evaluation of new risk factors are needed to further expand our capacity to predict atherothrombotic events when these factors are included along with the traditional ones in the assessment of cardiovascular risk in SLE patients.

The prothrombotic state of SLE is associated with the presence of antiphospholipid antibodies. β_2-GPI is the major autoantigen for anticardiolipin antibodies found in the sera of SLE patients and other autoimmune diseases and is characterized by its ability to bind to negative charged molecules, including lipoproteins [4–7]. A small amount of plasma β_2-GPI is bound to the low-density lipoprotein (LDL) molecule in circulation [8]. Recently, it was shown that β_2-GPI specifically interacted with oxidized LDL (ox-LDL), a principal lipoprotein contributing to the development of atherosclerosis, and formed complexes with ox-LDL in the intima of atherosclerotic lesions and the complexes were taken up avidly by macrophages via anti-β_2-GPI autoantibody-mediated phagocytosis [9]. More recently, it has been demonstrated that these complexes can be found in the bloodstream of patients with autoimmune, such as SLE and antiphospholipid syndrome [10–14]. These results indicate that serum β_2-GPI–ox-LDL complexes contribute to the development of autoimmune-mediated atherosclerosis in SLE [9–14].

Lipoprotein(a) [Lp(a)] is a key molecule in the fibrinolytic and atherogenic systems that structurally resembles LDL particle but contains a molecule of apolipoprotein(a) [apo(a)] attached to apoB-100 by a disulfide bond [15]. Elevated plasma concentrations of Lp(a) have been shown to be one of the independent risk factor for premature atherosclerosis in SLE and other diseases [16–19]. *In vitro* studies demonstrated that β_2-GPI bound with high affinity to Lp(a) [20,21], suggesting that β_2-GPI might bind to Lp(a) to form complexes *in vivo*. Therefore, in this study, we developed an enzyme-linked immunosorbent assay (ELISA) for measuring serum β_2-GPI–Lp(a) to make sure the existence of the complexes in circulation, and to investigate their value as a risk factor for the development of premature atherosclerosis in SLE.

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2. Materials and methods

2.1. Subjects and blood collection

Forty-seven consecutive patients with an average age of 31.8 ± 10.7 y (male 9, female 38) were randomly selected from the Nephrology Department, Jinling Hospital. All patients fulfilled ≥4 of the revised American College of Rheumatology criteria for the classification of SLE [22]. Forty-two healthy subjects matched for age and sex (mean age 31.0 ± 8.3 y, male 8, female 34) with no history of autoimmune, infectious or thrombotic diseases were selected for comparison. The blood was sampled at least 12 h after fasting and serum was separated immediately and stored at −70 °C until analysis. This study was approved by the Ethics Committee of Jinling Hospital and all the subjects had given their informed consent.

2.2. Purification of β_2-GPI and the production of polyclonal antibody against β_2-GPI

β_2-GPI was purified from normal human plasma as described previously with slight modifications [23]. Briefly, pooled plasma from healthy donors was precipitated with perchloric acid, followed by ion-exchange chromatography on a DEAE-cellulose-52 column. β_2-GPI was then further purified with a heparin-Sepharose 6B column. The final β_2-GPI fraction was delipidated by extensive washing with *n*-butanol. The β_2-GPI sample was checked by SDS-PAGE, Western blot and amino acid sequencing [24]. Rabbit antisera against β_2-GPI from Millipore (CHEMICON/Upstate/Linco) were used as antibody standard.

Antibodies to β_2-GPI were obtained by immunization of New Zealand white female rabbits with purified β_2-GPI. Both reactivity and specificity of the resulting rabbit antisera were evaluated with ELISA and Western blot.

2.3. Isolation and oxidation of Lp(a)

Lp(a) was purified from plasma of healthy donors by sequential density ultracentrifugation, followed by a gel-filtration chromatography as described by Albers et al. [25]. Purified Lp(a) was diluted in phosphate-buffered saline (PBS) to a protein concentration of 0.5 mg/ml and incubated with 30 μmol/l $CuSO_4$ for 12 h at 37 °C, followed by extensive dialysis against 0.01 mol/l PBS with 0.1 mmol/l EDTA in pH 7.4, respectively [26]. The degree of oxidation was quantified by relative electrophoretic mobility (REM) and the generation of thiobarbituric acid-reactive substances (TBARS).

2.4. Production of polyclonal antibody against apo(a)

Polyclonal antibodies to apo(a) were obtained by immunization of sheep with Lp(a), and then the cross-reactivity in these Lp(a) antisera against LDL, plasminogen and other apolipoproteins was removed by absorbing on an affinity chromatographic column of Sepharose 4B (Pharmacia) coupled with plasminogen and pooled sera obtained from Lp(a)-negative subjects as previously described [27]. The obtained polyclonal sheep anti-apo(a) reacts with both native and oxidized Lp(a), but not with plasminogen and LDL [27].

2.5. Sandwich ELISA for β_2-GPI–Lp(a)

Five hundred microliters of serum was firstly incubated with $MgCl_2$ (final concentration 10 μmol/l) at 37 °C for 2 h, and then polyethyleneglycol (PEG)-6000 (Sigma) was added to isolate β_2-GPI–Lp(a) from endogenous free form of β_2-GPI to eliminate the possible influence of endogenous free β_2-GPI to the assay. The samples, were incubated overnight at 4 °C and then centrifuged at 10,000 rpm for 20 min. The precipitates were resuspended in 500 μl washing solution containing 0.5% gelatin in 0.01 mol/l PBST (0.01 mol/l PBS containing 0.05% Tween-20).

We developed a sandwich ELISA method to measure the complexes of β_2-GPI with Lp(a). Microtitre plates were coated for 2 h at 37 °C and then overnight at 4 °C with 100 μl/well anti-human β_2-GPI antibody in 0.05 mol/ml, pH 9.6, sodium carbonate/bicarbonate buffer (8 μg/ml, 100 μl). After washed three times with 0.01 mol/l PBS, the plates were quenched with 1% gelatin in 0.01 mol/l PBS for 1 h at 37 °C. One hundred microliters of diluted (1:6) serum samples (resuspended with gelatin in PBST) or serial reference sera were added to the wells and incubated for 2 h at 37 °C. After washing, 100 μl volume of HRP-labeled polyclonal antibody against apo(a) was added to each well and incubated for 2 h at 37 °C. The wells were washed and color was developed by adding 100 μl *o*-phenylenediamine and H_2O_2. After 15 min at room temperature, the enzyme reaction was stopped with 100 μl 2 mol/l sulfuric acid and the absorbance was measured at 450 nm.

A pooled fresh-frozen serum sample (from 50 healthy subjects) was used as reference serum of β_2-GPI–Lp(a). Reference serum was also precipitated every time as serum sample. The two SD above the mean optical density (OD) of our studied 42 samples from healthy blood donors was arbitrarily expressed as 1 U/ml. The value of reference serum was then assigned (0.6 U/ml). A sample was considered to be positive when its level was higher than 1.00 U/ml.

2.6. Sandwich ELISA for Lp(a) and ox-Lp(a)

Lp(a) was measured by a sandwich ELISA using monoclonal anti-apo(a) as the capture antibody and detected with polyclonal anti-apo (a) enzyme conjugates as previously described [28]. Reference serum of Lp(a) was from Immuno AG Vienna. Ox-Lp(a) was measured by a sandwich ELISA using polyclonal antibody against ox-LDL as the capture antibody and detected with monoclonal anti-apo(a) enzyme conjugate as previously described [27]. Antibodies to ox-LDL were obtained by immunization of New Zealand white female rabbit with ox-LDL as described by Virella et al. [29]. The resulted rabbit antisera were first fractionated by affinity chromatography with immobilized protein G, and the IgG fractions were then absorbed in a column of immobilized native LDL. The washout from the column of immobilized native LDL, containing antibodies to ox-LDL and irrelevant IgG, was used to capture ox-LDL, which had no reactivity with native LDL [27]. Calculation of the concentration of ox-Lp(a) was based on the concentration of ox-Lp(a) as the standard [27].

2.7. Determinations of other lipids

Serum levels of total cholesterol (TC), triacyglycerols (TG) (RANDOX, England), LDL-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), and apoB (Daiichi Pure Chemicals, Japan) were measured on a Hitachi 7600 analyzer.

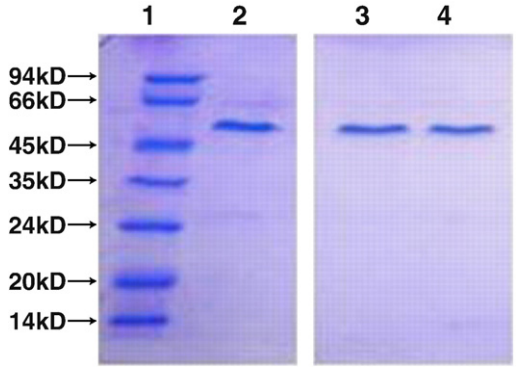

Fig. 1. SDS-PAGE analysis of prepared β_2-GPI protein. Lane 1: marker proteins (molecular weight from 14 kDa to 94 kDa); lane 2: aliquots of fractions recovered from DEAE-cellulose-52 ion exchange; lane 3: fractions recovered from heparin-Sepharose 6B affinity chromatography one time; lane 4: fractions recovered from heparin-Sepharose 6B affinity chromatography 2 times.