



The presence of anti-phosphatidylserine/prothrombin antibodies as risk factor for both arterial and venous thrombosis in patients with systemic lupus erythematosus

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In an effort to clarify the clinical significance of anti-phospholipid antibodies (aPL) detected by enzyme-linked immunosorbent assay (ELISA), we examined the prevalence of anti-cardiolipin antibodies (aCL), anti- β 2-glycoprotein I antibodies (anti- β 2-GPI), anti-prothrombin antibodies (anti-PT), and anti-phosphatidylserine/prothrombin antibodies (anti-PS/PT) in 175 patients with systemic lupus erythematosus (SLE) comprising 67 patients with thrombotic complications. The present study showed that positive results of anti- β 2-GPI-ELISA and anti-PS/PT-ELISA could serve as markers of thrombotic complications in patients with SLE, whereas aCL and anti-PT are less reliable as markers of these complications. Furthermore, results of the anti-PS/PT-ELISA correlate best with the occurrence of both arterial and venous thrombosis in patients with SLE.

Key words: anti-phospholipid antibodies, systemic lupus erythematosus, anti- β 2-glycoprotein I antibodies, anti-phosphatidylserine/prothrombin antibodies,

Haematologica 2006; 91:699-702

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Anti-phospholipid antibodies (aPL) are a distinct group of autoantibodies that appear in a variety of autoimmune diseases, particularly systemic lupus erythematosus (SLE).^{1,2} They are associated with clinical events such as arterial and/or venous thrombosis, and obstetric complications.³ The term *anti-phospholipid syndrome* (APS) has been used to describe a condition in which these clinical manifestations are linked to the persistence of aPL.⁴⁻⁶ Detection of anti-cardiolipin antibodies (aCL) by enzyme-linked immunosorbent assay (ELISA) and detection of lupus anticoagulant (LA) activity by phospholipid-dependent coagulation assays have been standardized for the diagnosis of APS.

A number of clinical studies have established that aCL and LA activity are present in approximately 40% of patients with SLE and that the presence of these aPL constitutes a risk factor for arterial and/or venous thrombosis.⁷⁻⁹ Moreover, recent reports indicated that the presence of LA activity is the strongest risk factor for thrombotic events in patients with SLE.^{10,11} However, LA activity detected by a phospholipid-dependent coagulation assay is heterogeneous with respect to the specificities and functional capacities of the antibodies.¹² Therefore, the detection of LA activity requires a careful, sequential series of steps. Despite internationally accepted guidelines and many efforts to improve the standardization of LA assays, it is very difficult to standardize the laboratory diagnosis of LA.

Recent data suggested that some aPL-specific ELISA may help to confirm the presence of LA activity.¹³ Therefore, we examined the prevalence of aCL, anti- β 2-glycoprotein I antibodies (anti- β 2-GPI), anti-prothrombin antibodies (anti-PT), and anti-phosphatidylserine/prothrombin antibodies (anti-PS/PT) in 175 patients with SLE (164 females, 11 males; aged 10-75 years; mean 40.11 years; 32 with arterial thrombosis, 35 with venous thrombosis, 14 with fetal loss, 14 with thrombocytopenia, and 80 with no thrombotic complications) to investigate the role of these aPL in thrombotic complications. The concentrations of aCL, anti- β 2-GPI, anti-PT, and anti-PS/PT were measured by four different aPL-specific ELISA systems as reported previously.¹⁴⁻¹⁶ We judged the concentrations of aCL, anti- β 2-GPI, anti-PT, and anti-PS/PT in each absorbance level [milliabsorbance units (mAU)]. The absorbance of blank wells (i.e. coated only with TBS) was subtracted from the absorbance in the antigen-coated wells to account for non-specific binding. Monoclonal anti-human β 2-GPI and anti-human prothrombin were used in each assay as a positive control, and selected control plasma samples were used as negative controls. We also studied the concentrations of aCL, anti- β 2-GPI, anti-PT, and anti-PS/PT in 80 healthy control subjects (staff members of Osaka University Hospital; 74 women, 6 men; aged 22-60 years old; mean, 39.8 years). The concentrations of these antibodies in the 80 healthy controls subjects were log-transformed to approximate Gaussian

Table 1. Characteristics of the 175 patients with systemic lupus erythematosus.

Complication	Number of patients	Female/Male	Median age	Age range
Thrombotic complications	67	61/6	42.9	26-71
Arterial thrombosis	32	27/5	43.3	29-67
Venous thrombosis	35	34/1	42.6	26-71
Fetal loss	14	14/0	31.4	24-40
Thrombocytopenia	14	13/1	41.7	26-70
Without above complications	80	76/4	39.0	10-75

distribution with the Stat Flex program (Ver. 4.2, Artech Inc.) before statistical analysis was performed. We chose the mean + 3SD of each aPL concentration in the 80 controls as the cut-off point. The cut-off values for aCL, anti- β 2-GPI, anti-PT, and anti-PS/PT were 398.0, 392.1, 500.8, and 403.2 mAU, respectively. We regarded a result as positive when the absorbance exceeded each cut-off value. In plasma of SLE patients, aCL, anti- β 2-GPI, anti-PT, anti-PS/PT, and LA activity was detected in 65 (37.1%), 55 (31.4%), 95 (54.3%), 76 (43.4%), and 89 (50.9%) of 175 patients, respectively. The prevalence of anti-PS/PT was most strongly correlated with the prevalence of LA activity [odds' ratio (OR) = 11.4, 95% confidence interval (CI) = 4.64-27.9]. In our anti-PS/PT ELISA system, purified human prothrombin was added to the phosphatidylserine-coated well. We measured the anti-PS/PT levels using both phosphatidylserine-coated prothrombin(+) wells and phosphatidylserine-coated prothrombin(-) wells. The absorbance of each sample in the prothrombin(+) wells and prothrombin(-) wells was compared for the evaluation of prothrombin dependency of antibodies. In this experiment, all of the samples in the prothrombin(+) wells showed clearly higher absorbance levels compared to the prothrombin(-) wells. These results suggest that the anti-PS/PT detected by our ELISA system was not directed against phosphatidylserine but recognized the complex of phosphatidylserine and prothrombin.

Multivariate logistic analysis confirmed that both anti-PS/PT and anti- β 2-GPI were significant, independent risk factors for arterial thrombosis [anti-PS/PT, OR=9.36, 95%CI=2.67-32.8; anti- β 2-GPI, OR=4.23, 95%CI=1.02-17.5] and venous thrombosis (anti-PS/PT, OR=7.33, 95%CI=2.51-21.4; anti- β 2-GPI, OR=4.10, 95%CI=1.19-14.2) in patients with SLE. It is important to note that the odds ratio for the presence of anti-PS/PT was much higher than that for the presence of anti- β 2-GPI. The prevalence of anti-PT was increased in SLE patients with arterial thrombosis and venous thrombosis. However, multivariate logistic regression analysis indicated that the presence of anti-PT was not a risk factor for these thrombotic complications (OR=2.15, 95%CI=0.60-7.70 for arterial thrombosis; OR=1.66, 95%CI=0.58-4.75 for venous thrombosis) because anti-

PT was detected in a large number of SLE patients without thrombotic complications (32/95 cases, 40.0%). The prevalence of aCL was significantly higher in SLE patients with arterial thrombosis and venous thrombosis, suggesting that the presence of aCL is an important risk factor for these thrombotic complications. Although a significant correlation was observed between the concentrations of aCL and anti- β 2-GPI, 21 (32.3%) of the 65 aCL-positive patients had no anti- β 2-GPI. Because the vast majority of aCL-positive patients who had no anti- β 2-GPI (16/21 cases, 76.2%) did not have thrombotic complications, the presence of aCL was not reliable as a risk factor for thrombotic complications. Multivariate logistic analysis also indicated that the only significant risk factor for thrombocytopenia was the presence of aCL (OR=2.36, 95%CI=0.56-9.91). It has been suggested that the antibodies detected by the standard aCL-ELISA differ from those detected by an anti- β 2-GPI-specific ELISA. The anti- β 2-GPI ELISA detects antibodies that are specific for a conformationally changed epitope of β 2-GPI whereas the standard aCL-ELISA may also detect antibodies that react with some components of human plasma and/or bovine serum proteins bound to the cardiolipin-coated wells.¹⁷ The prevalence of anti- β 2-GPI was increased in SLE patients with thrombotic complications but not significantly increased in the SLE patients with thrombocytopenia.

Much effort has been made to clarify the mechanisms underlying arterial and/or venous thrombosis and thrombocytopenia in SLE patients with aPL, including activation of platelets, promotion of the blood coagulation system, and impairment of the endothelial system. However, the precise mechanisms responsible for thrombotic and thrombocytopenic events in these patients remain unclear. Two possible mechanisms by which aPL could cause thrombotic events have been proposed. The first possibility is that aPL bind to phospholipid/plasma protein complexes on the membranes of activated platelets and influence platelet activation and aggregation. The second possibility is that aPL inhibit phospholipid-dependent reactivity of the activated protein C pathway, such as activation of protein C by thrombin/thrombomodulin and/or degradation of factor Va by activated protein C/protein S.

In previous studies,^{16,18} we found that SLE patients with both anti-CL/ β 2-GPI and anti-PS/PT had a quite high prevalence of cerebral infarction, as compared with those with either anti-CL/ β 2-GPI or anti-PS/PT. Furthermore, when we studied the *in vitro* effects of anti-CL/ β 2-GPI and/or anti-PS/PT on the enhancement of platelet activation induced by stimulation with a low concentration of adenosine diphosphate (ADP), we found that the purified IgG containing both anti-CL/ β 2-GPI and anti-PS/PT significantly enhanced ADP-induced platelet activation. However, the purified IgG containing either anti-CL/ β 2-GPI or anti-PS/PT alone did not enhance ADP-induced platelet activation. These results indicate that anti-CL/ β 2-GPI and anti-PS/PT may co-operate to promote platelet activation, and may be involved in the pathogenesis of cerebral

Table 2. Multivariate logistic analysis.

	LA activity Sensitivity Specificity OR (95%CI) p	Arterial thrombosis Sensitivity Specificity OR (95%CI) p	Venous thrombosis Sensitivity Specificity OR (95%CI) p	Fetal loss Sensitivity Specificity OR (95%CI) p	Thrombocytopenia Sensitivity Specificity OR (95%CI) p
aCL	60.7% 87.2% 5.60 (2.04-15.4) < 0.001	75.0% 71.3% 2.36 (0.59-9.91) 0.240	42.9% 64.3% 0.64 (0.18-2.20) 0.475	21.4% 61.5% 1.24 (0.19-8.04) 0.819	57.1% 64.6% 5.76 (1.35-24.5) < 0.05
Anti-β2GPI	49.4% 87.2% 1.53 (0.52-4.54) 0.442	71.9% 77.6% 4.23 (1.02-17.5) < 0.05	48.6% 72.9% 4.10 (1.19-14.2) < 0.05	7.1% 66.5% 0.55 (0.05-6.29) 0.630	21.4% 67.7% 0.46 (0.08-2.52) 0.368
Anti-PT	67.4% 59.3% 0.88 (0.36-2.13) 0.773	78.1% 51.0% 2.15 (0.60-7.70) 0.240	74.3% 50.7% 1.66 (0.58-4.75) 0.346	14.3% 42.2% 0.25 (0.05-1.25) 0.092	71.4% 47.2% 3.66 (0.90-14.8) 0.069
Anti-PS/PT	71.9% 86.1% 11.4 (4.64-27.9) < 0.001	84.4% 65.7% 9.36 (2.67-32.8) < 0.001	68.6% 62.9% 7.33 (2.51-21.4) < 0.001	21.4% 54.7% 1.49 (0.26-8.56) 0.653	50.0% 57.1% 1.84 (0.45-7.46) 0.393

aCL, anti-cardiolipin antibodies; anti- β 2GPI, anti- β 2glycoprotein I antibodies; anti-PT, anti-prothrombin antibodies; anti-PS/PT, anti-phosphatidylserine/prothrombin antibodies; LA, lupus anticoagulant; OR, odds' ratio; CI, confidence interval. LA activity was detected by both dilute Russell viper venom time and STACLOT LA tests. Statistical analysis was performed using univariate and multivariate logistic analysis. An OR was considered statistically significant when the lower limit of the 95% CI was > 1.0. A value of $p < 0.05$ was considered to be statistically significant to indicate a risk factor.

infarction in patients with SLE. On the other hand, when we examined the *in vitro* effects of anti-CL/ β 2-GPI and/or anti-PS/PT on the anticoagulant activity of activated protein C, we found that purified IgG containing anti-CL/ β 2-GPI or anti-PS/PT significantly hampered the anticoagulant activity of activated protein C.¹⁵ Our results indicate that anti-CL/ β 2-GPI and anti-PS/PT independently cause activated protein C resistance, which may contribute as an independent risk factor for venous thrombotic events such as pulmonary embolism, deep vein thrombosis, and thrombophlebitis. These findings raise the possibility that the high frequency of thrombotic complications in SLE patients with anti-CL/ β 2-GPI and/or anti-PS/PT might be due to enhancement of platelet activation and inhibition of anticoagulant systems.

As mentioned above, several clinical studies have established that the presence of LA activity is the strongest risk factor for thrombotic events in patients with SLE.^{10,11} In this study, we confirmed that the prevalence of anti-PS/PT was most strongly correlated with the presence of LA activity. It is thus reasonable to spec-

ulate that the presence of anti-PS/PT is the most significant risk factor for both arterial thrombosis and venous thrombosis. However, our finding that anti-PS/PT and anti- β 2-GPI were significant independent risk factors for thrombotic events in patients with SLE suggests that not only anti-PS/PT-ELISA but also anti- β 2-GPI -ELISA should be performed for the diagnosis of APS.

Although β 2-GPI and PT are the major protein targets involved in the binding of aPL to phospholipids, other phospholipid-binding proteins such as protein C, protein S, annexin V, and high- and low-molecular-weight kininogens have also been described as target proteins for aPL. ELISA for aPL against other phospholipid-binding proteins are still under development and will need standardization and extensive evaluation.

All authors contributed equally to the writing and conception of the manuscript. The authors declare they have no potential conflicts of interest. This work was supported by the Kurozumi Medical Foundation.

Manuscript received September 21, 2005. Accepted February 17, 2006.

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