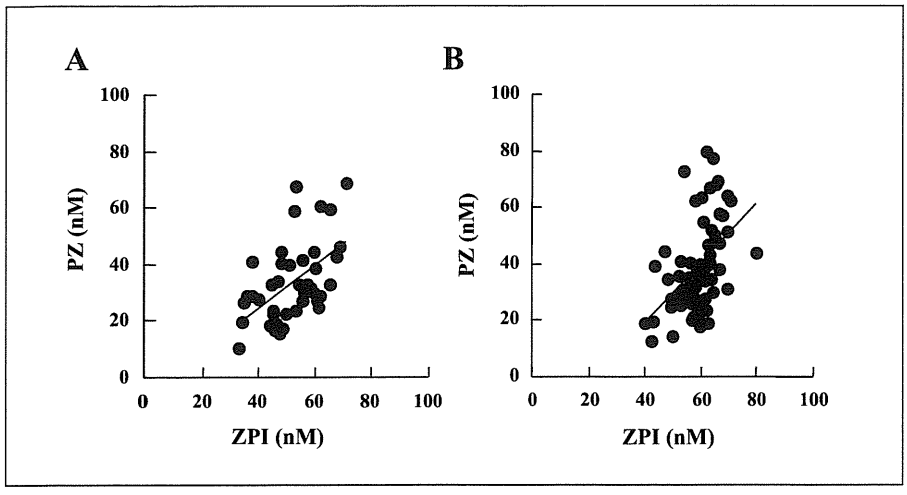

Figure 2: Correlation analysis between plasma ZPI and PZ levels in non-pregnant Japanese (A) and German (B) controls. Statistically significant correlations were obtained for normal Japanese (n=42) and German (n=64) controls (R^2=0.28 and 0.23, respectively; solid lines; p<0.001 and <0.0001, respectively).

Plasma ZPI levels and PZ concentrations in patients with RM

ZPI levels in non-pregnant patients with RM were similar to those in non-pregnant normal women (p=0.30) (Fig. 4A). In addition, plasma PZ levels in non-pregnant patients with RM were significantly lower, when compared with non-pregnant normal women (p=0.03) (Fig. 4B). Nevertheless, a strong linear relationship between the plasma levels of ZPI and PZ was demonstrated in patients with RM (R^2=0.34, p<0.0001; Suppl. Fig. 3B). Age did not affect ZPI and PZ levels (data not shown).

Discussion

Our present results indicate that the plasma ZPI levels are slightly lower in Japanese than in German individuals. In contrast, PZ levels were similar in Japanese and German individuals. It is important to note that the range of ZPI levels in the plasma samples of both Japanese and German control subjects was much narrower than the results for PZ ([20]; and the current study).

The strong positive correlation between ZPI and PZ levels was confirmed both in normal non-pregnant Japanese and German individuals by the current study. It was reported that the increase in plasma PZ after discontinuation of warfarin therapy was associated with a rise in the ZPI level (18). Al-Shanqeeti, et al. postulated that the rise in ZPI levels following discontinuation of warfarin may be related to a possible effect(s) of PZ, one of the vitamin K-dependent proteins, on ZPI catabolism including clearance of the ZPI-PZ complex, or the synthesis, secretion, or extra-vascular compartmentalisation of ZPI may be affected by PZ (18), or both. The exact mechanism of the inter-dependence of the plasma levels of ZPI and PZ will be explored in the future.

It is likely that the newly discovered increase in concentrations of ZPI during normal pregnancy is caused by the enhanced biosynthesis of this protein by the liver and/or its retarded clearance from the circulation, which might be related to a change in its post-

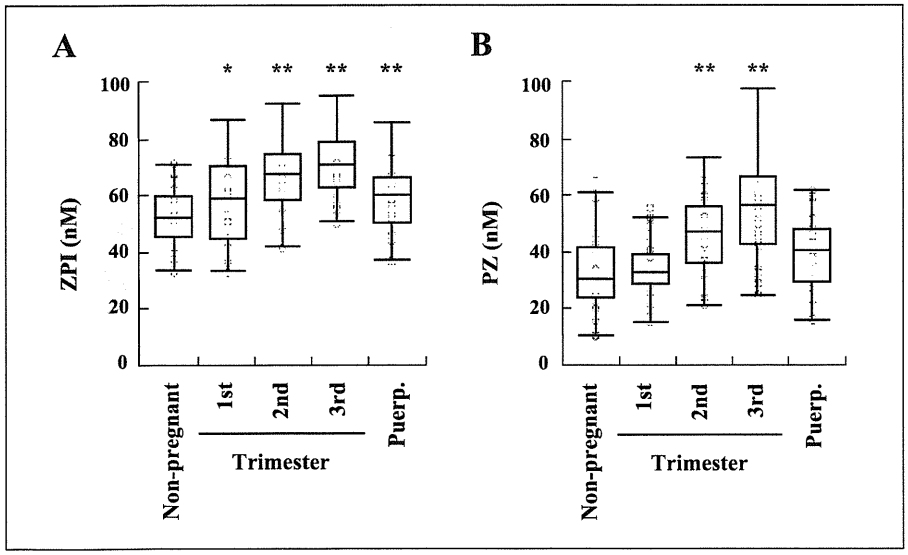

Figure 3: Plasma ZPI (A) and PZ (B) levels in Japanese women with normal pregnancy and in non-pregnant controls. Data are presented as described in Figure 1. Statistically significant differences were observed for both ZPI and PZ between each gestational period of women with normal pregnancy versus non-pregnant controls. *, p<0.05; **, p<0.01 (vs. non-pregnant control).

translational modification. The suggestion that the placenta could be the source of increased ZPI was excluded because no ZPI mRNA was detected by Northern blot analysis (10). Hormones which increase during pregnancy, such as oestrogen, progesterone, human placental lactogen, prolactin, etc., may enhance the production of ZPI. The placenta could be important as a source of placental hormones but not of ZPI itself, as discussed above.

Plasma concentrations of PZ also significantly increased as pregnancy advanced in the present study, which is in good agreement with previous reports in Caucasians (21, 22). A positive correlation between increased levels of plasma ZPI and PZ was also observed during all periods of normal pregnancy. It was reported that significantly higher levels of both ZPI and PZ were observed in women taking oral contraceptives (18), suggesting that oestrogen (and progesterone) has a positive effect on the synthesis of both ZPI and PZ, or that one of these two proteins affects the other. In our hands, however, hormone replacement therapy did not significantly influence either ZPI or PZ concentrations four weeks after the treatment in 15 cases (unpublished data).

In addition to their increased biosynthesis, the parallel change in ZPI and PZ levels may be attributable to the body's concomitant consumption of ZPI and PZ associated with the hyper-coagulability of normal pregnancy (19, 23). ZPI is consumed by FXa during coagulation, at least *in vitro*, and PZ is also digested by thrombin (20).

Nevertheless, our present study also demonstrates a more abrupt increase in ZPI over PZ, suggesting that distinct mechanisms are, at least in part, responsible for the elevation of these two gene products during normal pregnancy.

Since normal pregnancy is accompanied by increases in a number of coagulation factors including FX (24), which is one of the VKDPs and highly homologous to PZ (1), it was measured in pregnant and non-pregnant Japanese individuals. FX significantly increased during the 2nd and 3rd trimesters (see Suppl. Fig. 4, available online at www.thrombosis-online.com), as reported previously by Stirling et al. (25), the pattern of which closely resembles that of PZ. In contrast, FX did not significantly alter in RM cases (see Suppl. Fig. 5, available online at www.thrombosis-online.com). Accordingly, the increased ZPI and PZ levels during normal pregnancy may counterbalance the increased coagulation factors, especially FX, and regulate the hyper-coagulable state in pregnancy (24).

The fairly unchanged ZPI levels and slightly reduced PZ levels in non-pregnant RM was found in the present study. Inter-villous and/or spiral-artery thrombosis caused by ZPI or PZ deficiency may be associated with inadequate placental perfusion, resulting in complications of pregnancy. It is noteworthy that 35% of the offspring of ZPI-null were lost when their heterozygous adults were mated (26). These results suggest that ZPI deficiency would be a modest risk factor for miscarriage, at least in mice. These studies implicate the role(s) of the ZPI in the maintenance of normal pregnancy, in particular concerning placental function.

Enhanced clearance and/or hyper-consumption of ZPI and PZ caused by their autoantibodies could also lead to their secondary deficiency. Along this line, it is interesting that there was a good inverse correlation between anti-PZ IgM antibody levels and PZ concentrations in a subgroup of patients with recurrent embryo losses and PZ deficiency (27). However, anti-PZ antibody levels did not correlate with plasma PZ concentrations in controls and patients with pathologic pregnancies (27, 28). Most recently, it has also been reported that elevated anti-PZ IgG and IgM titers were seen in patients with idiopathic RM (29). In the case of ZPI, there is no report on its auto-antibodies, to our best knowledge.

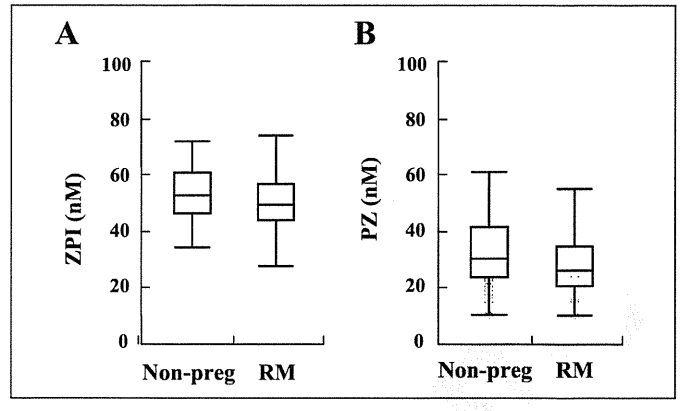

Figure 4: Plasma ZPI (A) and PZ (B) levels in patients with RM. No statistically significant difference was observed for ZPI (p=0.30), while there was a statistical difference in PZ (p=0.03) between patients with RM (n=134) vs. non-pregnant controls (n=42).

The limitations of this study include the relatively small number of RM patients evaluated. Therefore, further studies are needed to elucidate the relevance of relative ZPI deficiency in abnormal pregnancy, e.g. by recruiting many more RM cases.

In conclusion, this is the first report on ZPI levels during normal pregnancy (as well as in cases of non-pregnant RM), and we postulate that the elevated plasma concentrations of ZPI may be important for restoring the shifted balance of fibrinolysis and coagulation toward hemostasis during normal pregnancy.

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What is known about this topic?

- Protein Z (PZ)-dependent protease inhibitor (ZPI) is a serine protease inhibitor which inactivates activated factor X.
- However, the clinical significance of low plasma ZPI levels in thrombosis and pregnancy abnormalities remains to be established.

What does this paper add?

- Plasma ZPI increases during normal pregnancies and quickly decreases after delivery.
- Plasma ZPI remains unaltered in non-pregnant women who had experienced recurrent miscarriage.

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Conflict of interest

None declared.

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Efficacy of the Antiphospholipid Score for the Diagnosis of Antiphospholipid Syndrome and Its Predictive Value for Thrombotic Events

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Objective. **To define the antiphospholipid score (aPL-S) by testing multiple antiphospholipid antibodies (aPL) and to evaluate its efficacy for the diagnosis of antiphospholipid syndrome (APS) and predictive value for thrombosis.**

Methods. **This study comprised 2 independent sets of patients with autoimmune diseases. In the first set of patients (n = 233), the aPL profiles were analyzed. Five clotting assays for testing lupus anticoagulant and 6 enzyme-linked immunosorbent assays (IgG/IgM anticardiolipin antibodies, IgG/IgM anti–β_2-glycoprotein I, and IgG/IgM phosphatidylserine-dependent antiprothrombin antibodies) were included. The association of the aPL-S with a history of thrombosis/pregnancy morbidity was assessed. In the second set of patients (n = 411), the predictive value of the aPL-S for thrombosis was evaluated retrospectively. Two hundred ninety-six of these patients were followed up for >2 years. The relationship between the aPL-S and the risk of developing thrombosis was analyzed.**

Results. **In the first set of patients, the aPL-S was higher in those with thrombosis/pregnancy morbidity than in those without manifestations of APS ($P < 0.00001$). For the aPL-S, the area under the receiver operating characteristic curve value was 0.752. In the second set of patients, new thromboses developed in 32 patients. The odds ratio (OR) for thrombosis in patients with an aPL-S of ≥30 was 5.27 (95% confidence interval [95% CI] 2.32–11.95, $P < 0.0001$). By multivariate analysis, an aPL-S of ≥30 appeared to be an independent risk factor for thrombosis (hazard ratio 3.144 [95% CI 1.383–7.150], $P = 0.006$).**

Conclusion. **The aPL-S is a useful quantitative index for diagnosing APS and may be a predictive marker for thrombosis in autoimmune diseases.**

Antiphospholipid antibodies (aPL) are a heterogeneous group of circulating immunoglobulins related to diverse clinical phenomena including arterial and venous thrombosis, pregnancy complications, livedo reticularis, valvular disease, nonthrombotic neurologic disorders, and thrombocytopenia. The term antiphospholipid syndrome (APS) is used to link thrombosis and/or pregnancy morbidity to the persistence of aPL as one of the most common causes of acquired thrombophilia (1).

In particular, anticardiolipin antibodies (aCL), anti–β_2-glycoprotein I (anti-β_2GPI), and lupus anticoagulant (LAC) are associated with APS. Assays for LAC are the most traditional laboratory method used to detect aPL. Lupus anticoagulants are immunoglobulins (IgG, IgM, IgA, or their combination) that interfere with in vitro phospholipid-dependent tests of coagulation (activated partial thromboplastin time [APTT], kaolin clotting time [KCT], dilute Russell's viper venom time [dRVVT]).

In the early 1980s, radioimmunoassays and enzyme-linked immunosorbent assays (ELISAs), which

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directly detected circulating aCL, were devised (2,3). Those aCL cross-reacted with negatively charged phospholipids, such as phosphatidylserine and phosphatidylglycerol (4). Thus, the term aCL was expanded to aPL. Further studies showed the requirement of a cofactor for the binding of autoimmune aCL to solid-phase phospholipids (5–7); β_2GPI was identified as that cofactor. Beta$_2$-glycoprotein I bears the epitopes for aCL binding that are exposed when β_2GPI binds to negatively charged phospholipids (8,9).

Prothrombin, another main phospholipid binding protein, has been reported to be a probable cofactor for LAC (10–13). An ELISA for the detection of antiprothrombin antibodies (APT) using prothrombin alone as the antigen coated onto irradiated plates (APT-alone assay) was described in 1995 (14). However, the association between APT alone and clinical manifestations of APS remains controversial (15). Our group (16) and other investigators (17,18) established an ELISA to detect antibodies against the phosphatidylserine/prothrombin complex (anti-PS/PT) and observed that IgG anti-PS/PT were highly prevalent in patients with APS compared with patients with other diseases (16). We also showed that the detection of anti-PS/PT strongly correlated with the clinical manifestations of APS and with the presence of LAC.

In consideration of this historical background and, moreover, the heterogeneity of the properties of aPL, we have performed multiple aPL assays, not only for research purposes but also as routine clinical practice in our autoimmune disease clinic. In the current study, we first tried to represent the aPL profile of each patient, using a quantitative score defined as the "antiphospholipid score" (aPL-S), and analyzed the value of the aPL-S for the diagnosis of APS. We then retrospectively analyzed the predictive value of the aPL-S for thrombotic events in patients with autoimmune diseases.

PATIENTS AND METHODS

Patients. This retrospective study included 2 sets of patients from our database. The first group comprised 233 consecutive patients with systemic autoimmune diseases who were examined at the Rheumatic and Connective Tissue Disease Clinic at Hokkaido University Hospital in 2006 (study 1).

Plasma and serum samples were obtained from the patients, and all testing for aPL was performed in our laboratory. The historical profiles, clinical manifestations, and diagnoses were carefully obtained by review of the medical records or by interviewing the patients (Table 1). Arterial thrombotic events comprised stroke, myocardial infarction, and iliac artery occlusion, as confirmed by computed tomography (CT) scanning, magnetic resonance imaging, or conventional angiography. Deep vein thrombosis and pulmonary thrombosis were defined as venous thrombosis and were confirmed by CT scanning, angiography, or scintigraphy. Pregnancy morbidity was defined by the revised Sapporo criteria for APS (1).

Table 1. Characteristics of the 233 patients in study 1*

Diagnosis and manifestations	No. men/ no. women	Total
APS	5/32	37
Primary APS	1/12	13
APS with SLE	3/13	16
APS with other collagen disease	1/7	8
SLE	4/73	77
Rheumatoid arthritis	7/24	31
Sjögren's syndrome	0/18	18
Systemic sclerosis	5/9	14
Vasculitis syndrome	3/8	11
Polymyositis/dermatomyositis	0/8	8
Behçet's disease	1/5	6
Others	10/21	31
Clinical manifestations of APS	6/40	46
Thrombosis	6/32	38
Arterial thrombosis	3/24	27
Venous thrombosis	5/10	15
Pregnancy morbidity	0/14	14
Total	35/198	233

* APS = antiphospholipid syndrome; SLE = systemic lupus erythematosus.

The second group comprised 411 consecutive patients who were examined at the Rheumatic and Connective Tissue Disease Clinic between January 1, 2002 and December 31, 2003 (study 2). Among these 411 patients, those who were followed up for <2 years were excluded from the study. The final population eligible for analysis of thrombosis risk comprised 296 patients. The median followup period for the eligible patients was 72 months. The clinical profiles of these patients are described in Table 2. The study was performed in accordance with the Declaration of Helsinki and the Principles of Good Clinical Practice.

Table 2. Characteristics of the 296 patients in study 2*

Diagnosis and manifestations	No. men/ no. women	Total
APS	3/40	43
Primary APS	2/17	19
APS with SLE	1/23	24
SLE without APS	10/79	89
Rheumatoid arthritis	8/42	50
Sjögren's syndrome	0/16	16
Systemic sclerosis	4/21	25
Vasculitis syndrome	3/2	5
Polymyositis/dermatomyositis	2/8	10
Behçet's disease	4/7	11
Others	9/39	48
Newly developed thrombosis	6/26	32
Arterial thrombosis	2/20	22
Venous thrombosis	4/10	14
Total	43/253	296

* APS = antiphospholipid syndrome; SLE = systemic lupus erythematosus.

Plasma samples. Venous blood was collected into tubes containing a one-tenth volume of 0.105*M* sodium citrate and was centrifuged immediately at 4°C. Plasma samples were depleted of platelets by filtration and then stored at −80°C until used.

Determination of LAC. Three clotting tests were performed for LAC determination, using a semiautomated hemostasis analyzer (STart 4; Diagnostica Stago) according to the guidelines recommended by the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis (19). For measurement of the APTT, a sensitive reagent with a low phospholipid concentration (PTT-LA test; Diagnostica Stago) was used for screening and mixing tests, and the results were confirmed with the use of a StaClot LA kit (Diagnostica Stago). The dRVVT was used to screen for the presence of LAC, and the results were confirmed with a Gradipore LAC test. The KCT was measured using a kaolin solution (Dade-Behring) according to a standard protocol. The cutoff level of positivity for the LAC tests was previously established as above the 99th percentile of levels in 40 healthy subjects, as used for our routine laboratory assays. For defining the aPL-S, the results of the 3 mixing procedures and the 2 confirming tests were used.

Anticardiolipin antibody–anti-β_2GPI ELISA. IgG and IgM aCL were assayed according to a standard aCL ELISA (20). Normal ranges for IgG aCL (>18.5 IgG phospholipid units) and IgM aCL (>7.0 IgM phospholipid units) were previously established, using the 99th percentile of the levels in 132 healthy controls as the cutoff level of positivity.

IgG and IgM anti-β_2GPI antibodies were determined by ELISA, as previously reported (21). Purified human β_2GPI was purchased from Yamasa. Irradiated microtiter plates (MaxiSorp; Nunc) were coated with 4 μg/ml of purified β_2GPI in phosphate buffered saline (PBS) at 4°C and washed twice with PBS. To avoid nonspecific binding of proteins, wells were blocked with 150 μl of 3% gelatin (BDH Chemicals). After 3 washes with PBS containing 0.05% Tween 20 (PBS–Tween 20; Sigma), 50 μl of serum diluted with PBS containing 1% bovine serum albumin (PBS–1% BSA; Sigma) in a 1:50 dilution was added in duplicate. Plates were incubated for 1 hour at room temperature and washed 3 times with PBS–Tween 20. Fifty microliters per well of the appropriate dilution of alkaline phosphatase–conjugated goat anti-human IgG and IgM (Sigma) in PBS–1% BSA was added. After 1 hour of incubation at room temperature and after 4 washes in PBS–Tween 20, 100 μl/well of 1 mg/ml of *p*-nitrophenyl phosphate disodium (Sigma) in 1*M* diethanolamine buffer (pH 9.8) was added. Following color development, optical density at 405 nm was measured by a Multiskan Ascent plate reader (ThermoElectron Corporation). Normal ranges for IgG (>2.2 units/ml) and IgM (>6.0 units/ml) anti-β_2GPI were established, using the 99th percentile of the levels in 132 nonpregnant healthy controls as the cutoff level of positivity.

ELISA for the detection of anti-PS/PT. Anti-PS/PT antibodies were detected by ELISA, as previously described (16). Briefly, nonirradiated microtiter plates (Sumilon Type S; Sumitomo Bakelite) were coated with 30 μl of a 50-μg/ml preparation of phosphatidylserine (Sigma) and dried overnight at 4°C. To avoid nonspecific binding of proteins, wells were blocked with 150 μl of Tris buffered saline (TBS) containing 1% fatty acid–free BSA (catalog no. A6003; Sigma) and 5 m*M* $CaCl_2$ (BSA–$CaCl_2$). After 3 washes in TBS containing 0.05% Tween 20 (Sigma) and 5 m*M* $CaCl_2$, 50 μl of a 10-μg/ml preparation of human prothrombin (Diagnostica Stago) in BSA–$CaCl_2$ was added to half of the wells in the plates, and the same volume of BSA–$CaCl_2$ alone (as sample blank) was added to the other half.

After 1 hour of incubation at 37°C, the plates were washed, and 50 μl of serum diluted 1:100 in BSA–$CaCl_2$ was added to duplicate wells. Plates were incubated for 1 hour at room temperature, followed by the addition of alkaline phosphatase–conjugated goat anti-human IgG or IgM and substrate. The anti-PS/PT antibody titer of each sample was derived from the standard curve according to dilutions of the positive control. Normal ranges for IgG (>2.0 units/ml) and IgM (>9.2 units/ml) anti-PS/PT antibodies were established, using the 99th percentile of the levels in 132 nonpregnant healthy controls as the cutoff level of positivity.

Statistical analysis. Statistical analysis was performed by Mann-Whitney U test, Fisher's exact test, or chi-square test, as appropriate. *P* values less than 0.05 were considered significant. The diagnostic accuracy of the aPL-S was assessed by receiver operating characteristic (ROC) curve analysis. The Kaplan-Meier approach was used to estimate the probability of thrombosis developing after aPL testing was performed. The risk of thrombosis was evaluated using multivariate Cox regression analysis. All statistical analyses were performed using SPSS software.

RESULTS

Definition of the aPL-S. To define the aPL-S, we used the first group of patients (n = 233) with autoimmune disease. In this population, the relative risks (approximated by odds ratios [ORs]) of having clinical manifestations of APS (thrombosis and/or pregnancy morbidity) were calculated for each aPL test. Furthermore, in each test, the specificity and sensitivity for the diagnosis of APS were calculated (Table 3). To define the aPL-S, we devised an original formula in which the aPL-S was determined by the OR, as follows: aPL-S = 5 × exp([OR] − 5)/4. Consequently, an OR of 5 corresponds to an aPL-S of 5. The upper limit of the score for each aPL test was determined as 20.

In the aCL, anti-β_2GPI, and anti-PS/PT ELISAs, a second cutoff level was defined to separate patients with high antibody levels from those with medium or low levels of antibodies. The definition of high titers was established as more than the median levels of antibody-positive patients in each of the tests in the entire population studied. We observed that high levels of IgG aCL, anti-β_2GPI, and anti-PS/PT antibodies were closely related to the clinical manifestations of APS. In contrast, no relationship between clinical manifestations and titers of antibodies was observed in the IgM ELISAs.

Table 3. Relative risk of clinical manifestations of APS for each aPL test*

Test	Cutoff	Sensitivity, %	Specificity, %	OR (95% CI)	aPL score
APTT mixing	>49 sec.	39.1	89.3	5.36 (2.53–11.4)	5
Confirmation test, ratio	>1.3	19.6	95.2	4.81 (1.79–12.9)	2
	>1.1	30.4	90.9	4.38 (1.96–9.76)	1
KCT mixing	>29 sec.	45.6	88.8	6.64 (3.17–13.9)	8
dRVVT mixing	>45 sec.	28.2	90.9	3.93 (1.74–8.88)	4
Confirmation test, ratio	>1.3	17.4	94.7	3.72 (1.38–10.1)	2
	>1.1	28.3	90.4	3.7 (1.65–8.27)	1
IgG aCL, GPL					
High titers	>30	15.2	98.4	11 (2.72–44.5)	20
Medium/low titers	>18.5	19.5	94.6	4.31 (1.63–11.3)	4
IgM aCL, MPL	>7	6.52	96.3	1.79 (0.45–7.22)	2
IgG anti-β_2GPI, units					
High titers	>15	23.9	98.4	19.3 (5.11–72.7)	20
Medium/low titers	>2.2	30.4	92.5	5.4 (2.35–12.4)	6
IgM anti-β_2GPI, units	>6	8.7	91.4	1.02 (0.32–3.20)	1
IgG anti-PS/PT, units					
High titers	>10	19.6	97.8	11.1 (3.25–38.1)	20
Medium/low titers	>2	28.3	95.7	8.81 (3.39–22.9)	13
IgM anti-PS/PT, units	>9.2	6.52	98.9	6.45 (1.05–39.8)	8

* APS = antiphospholipid syndrome; aPL = antiphospholipid antibody; OR = odds ratio; 95% CI = 95% confidence interval; APTT = activated partial thromboplastin time; KCT = kaolin clotting time; dRVVT = dilute Russell's viper venom time; aCL = anticardiolipin antibody; GPL = IgG phospholipid units; MPL = IgM phospholipid units; anti-β_2GPI = anti–β_2-glycoprotein I; anti-PS/PT = anti–phosphatidylserine/prothrombin complex.

Therefore, the aPL scores for the IgG aCL, anti-β_2GPI, and anti-PS/PT antibody tests were separately defined.

For the determination of LAC, APTT, dRVVT, and KCT mixing tests were performed. In case of a positive APTT or dRVVT result, a complementary confirmation test was carried out, and an additional score was given. If the result of the confirmation test was >1.3, a score of 2 was added, and if the result was >1.1, a score of 1 was added. The aPL-S for each patient was calculated as the total scores for positive aPL tests and represents the complete aPL-S.

The partial aPL-S was defined using aPL tests that were included in the updated classification criteria for APS (1) and according to the guidelines recommended by the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis (19) and included tests for IgG/IgM aCL, IgG/IgM anti-β_2GPI, and LAC (only the APTT and dRVVT).

Correlation between the aPL-S and clinical manifestations. Among the first group of 233 patients, the aPL-S ranged from 0 to 86. Forty-six patients had experienced at least 1 of the clinical manifestations of APS (thrombosis and/or pregnancy morbidity), and the scores for these patients were higher than the scores for patients who did not have such manifestations (Figure 1A).

The prevalence of APS manifestations increased in accordance with increasing antiphospholipid scores. Patients were subdivided into 5 groups according to the aPL-S as follows: score of 0, scores of 1–9, scores of 10–29, scores of 30–59, and scores of ≥60. The prevalence of APS manifestations in the 5 groups was 10%, 26%, 29%, 56%, and 89%, respectively.

The partial aPL-S was also evaluated in the same population of patients and ranged from 0 to 56. When patients were subdivided into groups according to the partial aPL-S, the prevalence of APS manifestations was 13%, 23%, 36%, 44%, and 88% for a score of 0, scores of 1–9, scores of 10–19, scores of 20–39, and scores of ≥40, respectively.

Diagnostic value of the aPL-S for APS. The ROC curves for the aPL-S, the partial aPL-S, and the revised Sapporo criteria for APS showed a hyperbolic pattern, implying that the aPL-S is a potential quantitative marker for diagnosing APS (Figure 1B). The area under the curve (AUC) values were 0.752 for the aPL-S, 0.692 for the partial aPL-S, and 0.686 for the revised Sapporo criteria. ROC analysis was performed for each of the clinical manifestation of APS. ROC curves for either arterial thrombosis, venous thrombosis, or pregnancy

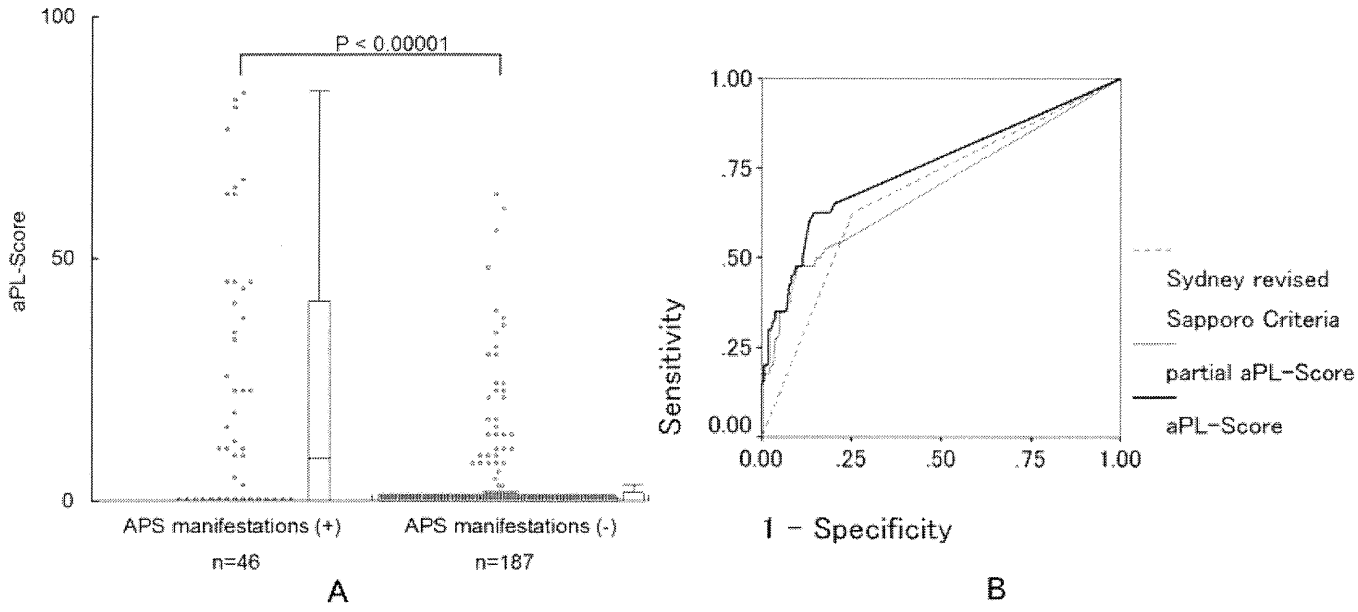

Figure 1. A, Distribution of the antiphospholipid scores (aPL-S) for patients in study 1 (n = 233). The aPL-S was defined as described in Patients and Methods. Data are shown as individual results as well as box plots, where each box represents the 25th to 75th percentiles; lines inside the box represent the median. The whisker represents the highest data still within 1.5 times the upper interquartile range. The scores for patients with antiphospholipid syndrome (APS) manifestations were significantly higher than those for patients without APS manifestations ($P < 0.00001$ by Mann-Whitney U test). **B,** Receiver operating characteristic (ROC) curves for the aPL-S, the partial aPL-S, and the revised Sapporo criteria for APS. Sensitivity and the specificity were calculated according to the presence of a history of clinical manifestations of APS. The area under the ROC curve values for the aPL-S and the partial aPL-S were 0.752 (95% confidence interval [95% CI] 0.656–0.849) and 0.692 (95% CI 0.588–0.795), respectively.

morbidity showed a hyperbolic pattern, and the AUC for each of them was larger than that for the revised Sapporo criteria (data not shown).

When the cutoff levels for the aPL-S and the partial aPL-S were defined as 30 and 20, respectively, the OR for the aPL-S (13.6 [95% confidence interval (95% CI) 4.81–38.7]) was higher than that for the revised Sapporo criteria (4.91 [95% CI 2.36–10.2]) and the partial aPL-S (7.85 [95% CI 2.99–20.7]). The sensitivity and specificity of an aPL-S of <30 were 35% and 96%, respectively, compared with 26% and 95%, respectively, for a partial aPL-S of <20 and 63% and 75%, respectively, for the revised Sapporo criteria.

Development of new thrombotic events. In the second group of patients, we retrospectively evaluated the relationship between the aPL-S and the risk of new thrombosis. This analysis included all thrombotic events that developed since the day the aPL-S was determined until the last followup in 2009.

During the followup period, new thromboses developed in 32 patients (22 arterial thrombotic events and 14 venous thrombotic events; some patients had both events). The aPL-S among patients in whom thromboses developed was significantly higher than that among those without thrombotic events during the followup (median score 5.5 versus 0; $P = 0.012$ by Mann-Whitney U test). This was also the case for the partial aPL-S (median score 5 versus 0; $P = 0.001$ by Mann-Whitney U test).

Predictive value of the aPL-S for APS manifestations. Patients with a higher aPL-S had a stronger risk of thrombosis compared with patients with lower scores. The ORs for newly developed thrombosis in patients with an aPL-S of ≥10, ≥30, and ≥50 were 2.86 (95% CI 1.33–6.6, $P = 0.006$), 5.27 (95% CI 2.32–11.95, $P < 0.0001$), and 5.31 (95% CI 1.81–15.53, $P = 0.0008$). The positive predictive values of an aPL-S of ≥10, ≥30, and ≥50 were 20%, 31%, and 35%, respectively, whereas the negative predictive values were 92%, 92%, and 91%, respectively. For the partial aPL-S, the positive predictive values of scores ≥10, ≥20, and ≥40 were 21%, 16%, and 25%, respectively, and the negative predictive values were 92%, 91%, and 91%, respectively (Figure 2A).

Effect of antithrombotic therapy. The effect of treatment on the aPL-S was evaluated in patients with an aPL-S of ≥30. This group included 39 patients (14 with primary APS, 15 with APS and SLE, and 10 with other autoimmune diseases), and 34 (87%) received

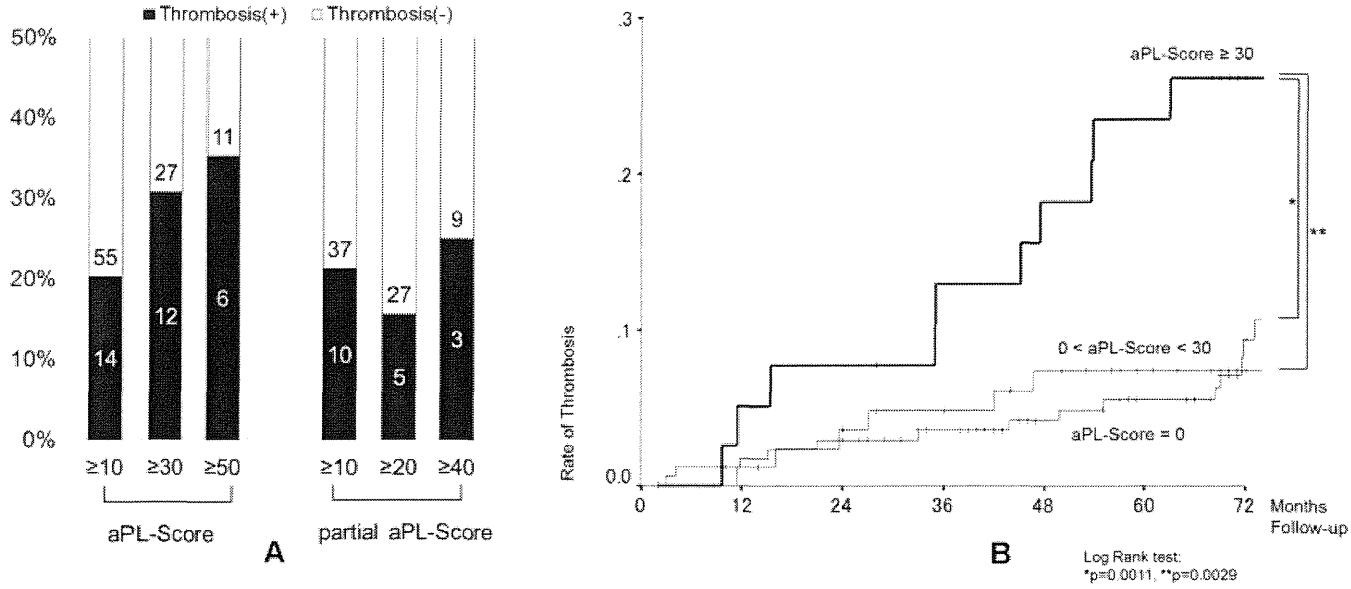

Figure 2. A, Positive predictive values (PPVs) of the antiphospholipid score (aPL-S) and the partial aPL-S for thrombosis in study 2. The numbers inside the bars represent the numbers of patients. The PPVs of an aPL-S of ≥10, ≥30, and ≥50 were 20.3%, 30.8%, and 35.3%, respectively. The PPVs of the aPL-S gradually increased in accordance with the cutoff value of the aPL-S, though the PPVs of the partial aPL-S did not. The negative predictive values of the aPL-S and the partial aPL-S were similar (90.5–92.2%). **B,** Kaplan-Meier analysis of the rate of thrombosis among patients in study 2, according to the aPL-S.

some antithrombotic medications. In 12 (31%) of these 39 patients, 15 new thromboses developed during the followup period despite antithrombotic therapy. The prevalence of thromboses among patients with an aPL-S of ≥30 was higher than that among those with a lower aPL-S (OR 5.40, 95% CI 2.38–12.23, $P = 0.00015$). The incidence rate of thrombosis among patients with an aPL-S of ≥30 was 5.144/100 person-years, whereas the rate among those with an aPL-S of 0 (no aPL) was 1.455/100 person-years. The rate of thrombosis among patients with an aPL-S of ≥30 was significantly higher than that among those with lower scores ($P = 0.0011$ for patients with an aPL-S of 0 and $P = 0.0029$ for patients with an aPL-S of 1–29, by log-rank test) (Figure 2B). In contrast, the partial aPL-S did not show significant correlation with the development of thrombosis.

Table 4. Risk factors for thrombosis in autoimmune disease, as determined using multivariate analysis

Risk factor	Hazard ratio (95% CI)*
Glucocorticoid treatment	1.979 (0.809–4.842)
History of thrombosis	1.401 (0.640–3.068)
Hypertension	1.621 (0.750–3.504)
Hyperlipidemia	1.917 (0.927–3.966)
Diabetes	0.963 (0.394–2.355)
Age	1.017 (0.987–1.047)
Male sex	1.002 (0.385–2.606)
Systemic lupus erythematosus	1.052 (0.480–2.303)
Rheumatoid arthritis	0.470 (0.101–2.181)
Antiphospholipid score ≥30	3.144 (1.383–7.150)†

* 95% CI = 95% confidence interval.
† $P = 0.006$.

To analyze the risk of thrombosis, multivariate Cox regression tests were conducted using the following data: aPL-S ≥30, age, sex, treatment with glucocorticoids, and the presence of hypertension, hyperlipidemia, diabetes, systemic lupus erythematosus, or rheumatoid arthritis at the time the aPL assays were performed. An aPL-S of ≥30 appeared to be an independent risk factor for thrombosis (hazard ratio [HR] 3.144, 95% CI 1.383–7.150, $P = 0.006$) (Table 4). A partial aPL-S of ≥20 was also analyzed using the same statistics but was not revealed to be an independent risk factor for thrombosis (HR 1.525, 95% CI 0.581–4.007, $P = 0.391$).

DISCUSSION

In this study, we demonstrated that the profile of aPL can be successfully quantitated as the aPL-S. The aPL-S level correlated with a history of thrombosis or pregnancy morbidity, suggesting that the aPL-S is a potential quantitative marker of APS. Therefore, the

current aPL-S can be unified and become a marker of the probability of having APS. Furthermore, we confirmed that the aPL-S had predictive value for recurrence and/or new onset of thrombotic events in the autoimmune disease setting. This fact suggests that treatment of APS can be modified considering the aPL-S.

Although aPL, as a group of autoantibodies sharing their properties in the phospholipid-associated molecules or reactions (22–27), have a strong link to thrombosis/pregnancy morbidity, the value of each aPL determination as a marker of APS is still not elucidated (28–32). Antiphospholipid antibodies are significantly prevalent in patients with infectious diseases, autoimmune diseases, malignant diseases, or hepatic diseases and even in healthy elderly individuals (33–37). One of the major issues involving the classification of APS has been avoiding overdiagnosis of APS by not accepting a positive result of a nonspecific aPL test as diagnostic (38). According to the APS criteria, aPL must be detected on 2 occasions not less than 12 weeks apart to determine that the presence of aPL is not transient. A low titer of aCL is not considered to be a marker of APS, although a "low positive" titer is a statistically abnormal laboratory phenomenon. However, efforts have not been successful enough, because aPL are found in many settings other than APS. In addition, updated diagnostic algorithms for catastrophic APS have been proposed, but no particular aPL has been proven to be associated with that syndrome (39).

In addition, standardization of each aPL assay has been extremely difficult. The presence of aPL defines the APS; thus, the greatest efforts have been made since the mid 1980s, when aCL were described (40,41). However, a number of variables in the assay, such as techniques, reagents, and standards, have hampered achievement of consensus (2), as described by de Groot et al in their article "Twenty-two years of failure to set up undisputed assays to detect patients with the antiphospholipid syndrome" (42). Considering the history of standardization, the establishment of a single aPL to define APS is unlikely in the near future.

In contrast, the premise that aPL represent the risk of thrombotic events and/or pregnancy morbidity either in the past or in the future would not be disputed (38,43–45). Accordingly, it would be more sensible to use aPL tests to establish an aPL profile as a marker of thrombotic risk rather than using these tests for diagnosis. Furthermore, combining multiple aPL tests would compensate for or reduce the disadvantage of each single aPL. From this point of view, our definition of the aPL-S has been proven to represent the "probability" or "likelihood" of having APS, depending on the level of the score; higher antiphospholipid scores were associated with higher risks of thrombotic events or pregnancy morbidity.

In the second part of the study, we retrospectively evaluated the value of the aPL-S for predicting the development of APS-related events in patients with autoimmune diseases. Despite receiving standard antithrombosis prophylaxis, many patients developed thrombosis during the followup period. In this cohort, the aPL-S showed a positive correlation with the risk of thrombotic events and had a significant predictive value. Those data would lead to a potential therapeutic strategy in which the intensity of antithrombotic treatment could be determined according to the aPL-S.

In clinical practice, all aPL tests are not available to all physicians. Therefore, we also defined a partial aPL-S that corresponds to the total score for the aPL tests included in the classification criteria for definite APS (1). For calculation of the partial aPL-S, the KCT mixing test and the anti-PS/PT IgG and IgM tests were excluded. The results for the complete aPL-S derived from the full battery of tests were compared with those for the partial aPL-S. A partial aPL-S seems to be a useful tool with which to evaluate the risk of thrombosis in patients with aPL (diagnostic value). However, although the aPL-S showed a positive predictive value for thrombosis that gradually increased in accordance with increasing scores, this increasing tendency was not observed with the partial aPL-S. None of the combinations of aPL tests used to define the aPL-S showed better relevance for the diagnosis of APS or for the prediction of thrombosis than the original complete aPL-S (data not shown). Inclusion of anti-PS/PT antibodies in the battery of aPL tests allows better quantification of the thrombosis risk.

Recently, Pengo et al (46) reported that in their cross-sectional study, patients with triple positivity for aCL, LAC, and anti-β_2GPI had a greater risk of thrombotic events than those who were positive for only 1 or 2 of these antibodies, which supports, in part, our findings. In the Pengo study, triple positivity was categorical (i.e., either present or absent), but our criteria were more quantitative, as proven by the ROC curves. Further, in the study of Pengo et al, anti-PS/PT antibodies were not considered. In their analysis, patients with prothrombin-dependent LAC and anti-PS/PT antibody positivity could be classified as single-positive for LAC, although this group of patients had a higher risk of APS than those with aPL positivity alone (47,48). In any case, the

combination of aPL tests should be considered when discussing the risk of thrombosis/pregnancy morbidity.

In the current study, we proved the efficacy of the aPL-S as a marker of the "probability" of APS and its value for predicting thrombosis in the setting of autoimmunity. This study is the first to attempt scoring the aPL profile, and the aPL-S successfully correlated with the risk of thrombotic events. However, the score could have other definitions, according to the population, and obviously the "true" predictive value should be validated in prospective studies. Higher accuracy of the aPL-S is obtained when all aPL tests are included. However, in clinical practice and trials, if all of the tests are not accessible, a partial aPL-S will provide important information regarding the thrombosis risk for each patient and consequently will help clinicians in making decisions about the therapeutic approach.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Atsumi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Otomo, Atsumi, Amengual, Fujieda, Kato, Oku, Horita, Yasuda, Koike.

Acquisition of data. Otomo, Atsumi, Amengual, Fujieda, Kato, Oku, Horita, Yasuda, Koike.

Analysis and interpretation of data. Otomo, Atsumi, Amengual, Fujieda, Kato, Oku, Horita, Yasuda, Koike.

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REVIEW

Pathophysiology of thrombosis and pregnancy morbidity in the antiphospholipid syndrome

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ABSTRACT

In patients with the antiphospholipid syndrome (APS), the presence of a group of pathogenic autoantibodies called antiphospholipid antibodies causes arteriovenous thrombosis and pregnancy complications. To date, the pathogenicity of the antiphospholipid antibodies has been the focus of analysis. Recently, the antibodies were reported to be capable of direct cell activation, and research on the underlying mechanism is ongoing. The antiphospholipid antibodies bind to the membranes of vascular endothelial cells, monocytes and platelets, provoking tissue factor expression and platelet aggregation. This activation functions as intracellular signalling, independent of the cell type, to activate p38MAPK and the transcription factor NFκB. Currently, there are multiple candidates for the membrane receptors of the antiphospholipid antibodies that are being tested for potential in specific therapy. Recently, APS was reported to have significant comorbidity with complement activation, and it was proposed that this results in placental damage and cell activation and, therefore, could be the primary factor for the onset of pregnancy complications and thrombosis. The detailed mechanism of complement activation remains unknown; however, an inflammation-inducing substance called anaphylatoxin, which appears during the activation process of the classical complement pathway, is thought to be a key molecule. Complement activation occurs in tandem, regardless of the pathology of APS or the type of antiphospholipid antibody, and it is thought that this completely new understanding of the mechanism will contribute greatly to comprehension of the pathology of APS.

Keywords Antiphospholipid syndrome, cellular activation, complement activation.

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Introduction

Antiphospholipid syndrome (APS) is an autoimmune disorder defined by the persistent presence of antiphopholipid antibodies (aPL) in plasma of patients with vascular thrombosis and/or pregnancy morbidity. The clinical features and laboratory manifestations associated with aPL have considerably broadened since the first description of APS in 1983 [1] including thrombocytopenia, haemolytic anaemia, cardiac valve disease, pulmonary hypertension, nephropathy, skin ulcers, livedo reticularis, cognitive dysfunction and atherosclerosis [2].

An international consensus on classification criteria for APS was stated in Sapporo (Sapporo criteria) [3], and they were revised in 2006 in Sydney [4]. Definition of APS is made when at least one of the two clinical criteria (vascular thrombosis or pregnancy morbidity) occurs in a patient whose laboratory tests for aPL are positive (Table 1).

The relevant antibodies found in APS are directed against specific plasma proteins that possess an affinity for anionic phospholipids, such as β2 glycoprotein I (β2GPI) and prothrombin [5,6]. APL can be categorised into those antibodies detected by solid-phase enzyme-linked immunosorbent assays (ELISA) such as anticardiolipin antibodies, antiβ2GPI antibodies or those that prolong phospholipid-dependent coagulation time, called lupus anticoagulant.

There are two aspects of APS, vascular manifestations and pregnancy complications. Thrombus formation is the key event of vascular manifestations in APS, and many pathogenic mechanisms have been proposed to explain the thrombotic predisposition in this syndrome. However, obstetrical complications in patients with APS cannot be caused solely by thrombosis in the uteroplacental vasculature, and additional pathways have been raised to pregnancy problems in APS [7].

The mechanisms of thrombosis production in patients with APS are not completely clarified. However, the interaction between aPL and cells involved in the regulation of haemostasis is one of the mechanisms responsible of the thrombophilic state in APS. The aPL-cell interaction induces a perturbation in

Table 1 Revised classification criteria for the antiphospholipid syndrome [3]

Clinical Criteria
Vascular thrombosis
≥ 1 clinical episodes of arterial, venous or small vessel thrombosis, in any tissue or organ confirmed by objective validated criteria by imaging or histopathology in the absence of significant evidence of inflammation in the vessel wall
Pregnancy morbidity
≥ 1 unexplained deaths of a morphologically normal foetus at or beyond the 10th week of gestation, or
≥ 1 premature births of a morphologically normal neonate before the 34th week of gestation owing to eclampsia, severe pre-eclampsia or placental insufficiency, or
≥ 3 unexplained consecutive spontaneous abortions before the 10th week of gestation (maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded)
Laboratory Criteria
Lupus anticoagulant present in plasma, on ≥ 2 occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Haemostasis
IgG and/or IgM anticardiolipin antibodies present in medium or high titre in serum or plasma, on ≥ 2 occasions at least 12 weeks apart, measured by a standardised ELISA
IgG and/or IgM antiβ2 glycoprotein I antibodies present in titre >99th percentile, in serum or plasma, on ≥ 2 occasions at least 12 weeks apart, measured by a standardised ELISA

Antiphospholipid syndrome is present if at least one of the clinical criteria and one of the laboratory criteria are met.
ELISA, enzyme-linked immunosorbent assay.

the cells that results in a pro-thrombotic/pro-inflammatory response and subsequently thrombosis.

Complement activation, one of the mechanisms related to obstetric complications in APS has also been involved in the production of thrombosis in patients with aPL. In this manuscript we discuss the aPL-cell interaction and the role of complement in the aPL-associated complications as the major pathogenic mechanism (Table 2).

Antiphospholipid antibody-associated thrombosis

The association between aPL and thrombotic events is well established. Evidence from animal models of APS indicates that aPL may play a causal role in the vascular abnormalities in both the venous and arterial territories [8,9]. In an animal model of photochemically induced arterial thrombosis, monoclonal antibodies raised against human β2GPI promoted thrombus formation [8]. Ramesh *et al.* [9] demonstrated that aPL inhibit the activation of nitric oxide and that the resulting decline in nitric oxide production underlies the promotion of leucocyte–endothelial cell (EC) adhesion and arterial thrombosis in mice. Injection of aPL in mice increased thrombus formation, carotid artery tissue factor (TF) activity, as well as peritoneal macrophage TF activity and expression [10]. Furthermore, enhanced thrombus formation was observed in femoral vein of mice treated with aPL [11]. Vega-Ostertarg *et al.* [12] found that mice injected with aPL have an enlargement in the thrombus size in the postcapillary venular endothelium in the cremaster muscle. Rapid endothelial deposition of fibrinogen and intravascular platelet–leucocyte aggregates were detected by intravascular microscopy on the mesenteric vessels of rats receiving an intraperitoneal injection of bacterial lipopolysaccharide followed by infusion of immunoglobulin G (IgG) purified from patients with APS [13].

Despite the persistent presence of aPL in circulation, thrombotic events in patients with aPL only occur occasionally, suggesting that the presence of aPL is necessary but not sufficient for clot formation *in vivo*. The 'two-hit hypothesis' has been proposed in which aPL (first hit) can only exert their prothrombotic influence in the presence of another thrombophilic condition (second hit). This 'two-hit hypothesis' was shown in an animal model of APS in which the injection of aPL in rats only resulted in increased thrombus formation when rats were pretreated with lipopolissacharide, but not when were injected with buffer [13].

Antiphospholipid antibodies and cell interactions

The major antigen structures recognised by aPL in patients with APS are phospholipid–binding proteins, β2GPI and prothrombin, expressed on the membranes of different cell types. The antibody forms a complex with the corresponding antigen,

Table 2 Antiphopholipid antibodies (aPL)-mediated pathogenic mechanisms

aPL-mediated thrombosis
Interference with the components of the coagulation cascade
Protein C pathway
Protein Z pathway
Contact activation pathway
β2GPI-thrombin interaction
Impairment of fibrinolysis
Cell interaction
Induction of proinflammatory phenotype on endothelial cells
Induction of procoagulant activity on endothelial cells and monocytes
Release of membrane-bound microparticles
Pro-coagulant effects on platelets
Disruption of the annexin V shield
Complement activation
aPL-mediated foetal loss
Intraplacental thrombosis
Inflammation
Inhibition of syncitium-trophobalst differentiation
Disruption of the annexin V shield
Complement activation

β2GPI, β2 glycoprotein I.

leading to the cell perturbation, the activation of cell signalling pathways, the transcription of procoagulant substances, adhesion molecules and subsequently thrombus formation.

Studies on the pathogenicity of aPL have been carried out mainly on the corresponding target molecules especially on the function of β2GPI and their modifications by aPL. However, to evaluate whether antiβ2GPI antibodies can block the function of β2GPI is difficult as true physiological role of β2GPI in coagulation cascade is not elucidated. Individual with complete β2GPI deficiency does not have any particular phenotype [14]. Thus, the recent trend is to favour the hypothesis that the function of aPL on prothrombotic cells, via β2GPI, is more important than the function of β2GPI.

Membranes of activated platelets with negatively charged phospholipids are an important source of catalytic surface for blood coagulation. Activated factor *X* and thrombin are generated on activated platelets, and procoagulant microparticles shed by platelet activation. Platelets are prone to agglutinate and aggregate after exposed to aPL [15], and circulating activated platelets are found in patients with APS [16]. β2GPI binds to membranes of activated platelets and inhibit the generation of activated factor *X*. Antiβ2GPI antibodies interfere with this inhibition [17]. Thus, activated platelets may be a predominant immune target of antiβ2GPI antibodies and direct action of aPL in platelets contribute to APS-related thrombosis.

The endothelium is a predominant target of aPL. Pathogenic aPL binding to β2GPI cause the up-regulation of adhesion molecules [18], TF [19] and endothelin-1 [20] causing a pro-inflammatory and prothrombotic EC phenotype. Prothrombin also binds to ECs, and this binding is enhanced by a human monoclonal IgG antiprothrombin antibody, IS6. IS6 up-regulates expression of TF and E-selectin on ECs [21].

Antiphospholipid antibodies exert also effect in the stimulation of the release of microparticles from ECs [22]. Microparticle production is a hallmark of cell activation, but the role of microparticle in the pathophysiology of thrombosis has not been elucidated. Antiphospholipid antibodies bind to the negatively charged membrane of monocytes and induce TF up-regulation [23,24]. Monocytes are the source of most majority of circulating TF-bearing microparticles [25] and TF up-regulation is a major feature of monocyte activation in the APS [26].

Cell receptors for antiphosphospholipid antibody interactions

The cell activation mediated by aPL might require an interaction between phospholipid-binding plasma protein and a specific cell receptor(s). A number of potential receptors for the binding of β2GPI to cellular membranes have been identified including annexin A2, apolipoprotein E receptor 2 (ApoER2'), low-density-lipoprotein receptor (LDL-R) -related protein, megalin, Toll-like receptor (TLR) 2, TLR 4, the very-LDL-R and P-selectin glycoprotein (GP) ligand-1. β2GPI also directly binds to the platelet adhesive receptor GPIbα and to the platelet factor 4 (PF4) [27–32]. Most of these receptors are expressed on various cell types and whether those different receptors are involved in the pathophysiology of thrombosis is still matter of debate.

Annexin A2 is a receptor for tissue-type plasminogen and its ligand plasminogen. Annexin A2 is a membrane-bound protein found on the surface of ECs and monocytes, and on the brush-border membrane of placental syncytiotrophoblasts [33]. Annexin A2 interacts with the β2GPI-antiβ2GPI antibody complex on the ECs and monocyte surfaces, mediating cell activation [27,28]. The involvement of annexin A2 in aPL-mediated pathogenic effects has been reported *in vitro* and *in vivo* models [34]. However, it is unlikely that annexin A2 *per se* is actually involved in cellular activation because it lacks transmembrane domain. The activation of signalling responses requires the presence of another transmembrane adaptor protein(s) that associates with annexin A2 on the ECs surface [29]. TLR-4 was

identified as a potential putative adaptor protein for annexin A2 [28].

Several groups reported that TLR-2 and TLR-4 are involved in aPL-mediated cell activation [30,35,36]. TLR-4 signalling was shown in ECs after the incubation with aPL [29], but a direct interaction between TLR4 and β2GPI remains to be confirmed. Binding of β2GPI to TLR2 on endothelial surface has been reported [37].

Megalin/gp33 is an endocytic receptor that internalises multiple ligands including apolipoprotein E and B100. Megalin was shown to behave as a receptor of β2GPI and β2GPI-phospholipid complex [38]. Pennings *et al.* [39] demonstrated that dimeric β2GPI can interact with LDL-R family members, including megalin.

Apolipoprotein E receptor 2 is a member of the LDL-R family expressed in many cell types. Studies on platelets suggested ApoER2′ as a receptor of β2GPI [40]. The blockage of the platelet ApoER2′ using a receptor-associated protein abrogated the increased adhesion of platelets to collagen induced by β2GPI-anti-β2GPI antibody complex [41]. Using a recombinant soluble form of LDL-binding domain 1 of ApoER2′, it was shown that the interaction between β2GPI and ApoER2′ mediated the aPL action in endothelium [9]. The importance of ApoER2′ in the induction of prothrombotic state mediated by aPL was confirmed *in vivo* in a murine model of thrombosis and using ApoER2′ deficient mice [42]. Injection of aPL caused a significant increase in thrombus formation, vascular TF activity and monocyte activation in the murine model of thrombosis, which were significantly reduced in the ApoER2′ deficient mice. Those data support the role of ApoER2′in thrombus formation in APS; however, the role of other potential receptors cannot be excluded as demonstrated by the partial protection from thrombogenic effects of aPL in ApoER2′-deficient mice.

β2 glycoprotein I directly binds to GPIbα subunit of the platelet adhesion receptor GPIb/IX/V *in vitro* [35,36]. The platelet GPIbα subunit has the von Willebrand factor as the most important ligand, but also serves to localise factor XI and thrombin on the platelet surface. Binding of β2GPI to GPIbα enables antiβ2GPI antibodies, directed against domain I, to activate platelets, resulting in thromboxane production and also to the activation of the phosphoinositol-3 kinase (PI3-kinase)/Akt pathway [31] contributing to the platelet adhesion and aggregation.

The involvement of Fcγ receptor on cellular activation has been investigated *in vivo* [8] and *in vitro* studies on platelets [26], monocytes [26] and ECs [28]. Results suggest that this receptor is not strictly necessary for cellular activation.

The direct binding of β2GPI to PF4 derived from platelet granules has been reported [43]. PF4 is a member of the C–X–C chemokine family secreted by activated platelets and has ability to bind to the platelets surfaces. PF4 contributes to the natural dimerisation of β2GPI, leading to the stabilisation of β2GPI binding onto the phospholipid cell surfaces which facilitates the antibody recognition. The β2GPI-PF4 complex is strongly recognised by serum of patients with APS [43]. Moreover, platelets may be activated by β2GPI-antiβ2GPI antibody-PF4 or β2GPI-PF4 complexes. Almost every cell type can be a source of PF4 especially under some stimulation. Both, β2GPI and PF4 are abundant in plasma; thus, the preformed β2GPI-PF4 complexes may prime several pro-coagulants cells culminating in coagulation.

Those potential receptors proposed to be involved in the aPL-mediated cell activation have significantly increased in the last years, and additional studies are needed to clarify their biological and pathological roles.

Signalling pathways of cell activation

The signal transduction mechanisms involved in aPL-mediated cell activation have been the centre of interest for many researchers. How pathogenic aPL recognition of phospholipid-binding proteins on the cell surface elicits a transmembrane signal to modify intracellular events is not completely understood.

The adapter molecule myeloid differentiation protein (MyD)88-dependent signalling pathway and the nuclear factor kappa B (NFkB) have been involved in the ECs activation by aPL [44,45]. Incubation of ECs with antiβ2GPI antibodies resulted in a redistribution of NFkB from the cytoplasm to the nucleus, and this effect was accompanied by an increased expression of TF and leucocyte adhesion molecules [46]. The p38 mitogen-activated protein kinase (MAPK) pathway is an important component of intracellular signalling cascades that initiate various inflammatory responses. It is recognised that the p38 MAPK pathway has a crucial role in mediating the effect of aPL in different cell types [24,47,48]. Activation of p38 MAPK increases activities of cytokines such as tumour necrosis factor (TNF) alpha, IL-1β and macrophage inflammatory cytokine 3β [24,36]. Monocytes stimulated by monoclonal antiβ2GPI antibodies from patients with APS induce phosphorylation of p38 MAPK, a locational shift of NFkB into the nucleus and up-regulation of TF expression. Such activation was not seen in the absence of β2GPI, indicating that the disturbance of monocyte by anti-β2GPI antibodies is started by interaction between the cell and the autoantibody-bound β2GPI [24,44]. The implication of p38 MAPK in cell activation has been also demonstrated in platelets [47] and ECs [48]. Pretreatment of platelets with p38 MAPK-specific inhibitor, SB203580, completely abrogated aPL-mediated platelet aggregation. The induction of TF expression was also reported through the simultaneous activation of NFkB via the MAPK pathway and of the MEK-1/ERK pathway, but an inhibitor of the MEK-1/ERK pathway could not suppress the TF

expression, implying the main role of p38 MAPK in those reactions [49].

Purified IgG from APS patients with venous thrombosis, without pregnancy morbidity, caused phosphorylation of NFkB and p38MPK and up-regulation of TF in monocytes. These effects were not seen with IgG fractions from patients with obstetric APS alone, suggesting that aPL from patients with different clinical aspects of APS may trigger different signalling responses [44]. Figure 1 shows the procoagulant cell activation as one of the pathogenic mechanisms of thrombosis mediated by aPL.

Recently, two major findings in the antigenic structures recognised by aPL have been reported: first, the structural changes in β2GPI. β2GPI can exist in two different conformations, plasma β2GPI circulates in a circular (closed) conformation, whereas after interaction with antiβ2GPI antibodies undergoes a major conformational change into a fishhook-like (open) structure [50]. Second, the finding that β2GPI can be reduced by thioredoxin 1 (TRX-1). β2GPI treated with TRX-1 generate free thiols within β2GPI, a process that may affect the function of β2GPI, and may have a regulatory role in platelet adhesion [51]. Those novel biochemical findings into the structural changes that can occur within β2GPI and the consequences of these changes for the function of β2GPI might be relevant to our better understanding of the APS, but further studies are necessary to clarify their roles in the pathogenesis of APS.

Complement activation in APS

Several genes involved in SLE susceptibility have recently been identified and confirmed. Of those, particularly *IRF5* [52] and *STAT4* [53] have been confirmed in several studies to be clearly associated with primary APS. In a study by our institution on gene polymorphisms of *STAT4* in Japanese primary patients with APS, the polymorphism rs7574865G/T was related to APS and *STAT4* was considered a disease-susceptibility gene for both SLE and APS regardless of race [54]. Of the similarities between SLE and APS, the focus in recent years has turned to complement activation as a common point that has been observed in the mechanism of pathology of both diseases [55].

Hypocomplementemia caused by complement activation is commonly observed in SLE and is thought to be well correlated with disease activity. Many proposed hypotheses have explained the involvement of the hypocomplementemiathis in the disease onset mechanism in SLE. Understanding the mechanism of hypocomplementemia and complement activation in

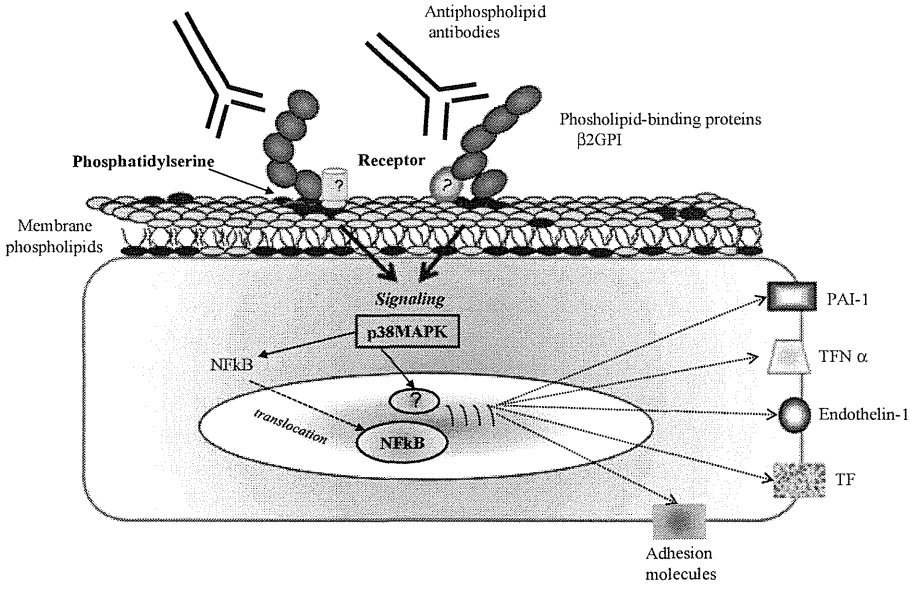

Figure 1 Pathogenic mechanisms of cell activation mediated by antiphosphopholipid antibodies. Antiphospholipid antibodies interact with monocytes or endothelial cells through binding to phospholipid-binding protein (β2GPI or prothrombin) on cell surface. This interaction might require a specific cell receptor (s) and results in p38MAPK phosphorylation, nuclear translocation of NFkB and up-regulation of procoagulant substances and adhesion molecules, and subsequently thrombus formation. p38 MAPK, p38 mitogen-activated protein kinase; NFkB, nuclear factor kappa B; β2GPI, β2 glycoprotein I; PAI-1, plasminogen activator inhibitor-1; TNFα, tumour necrosis factor alpha; TF, tissue factor.

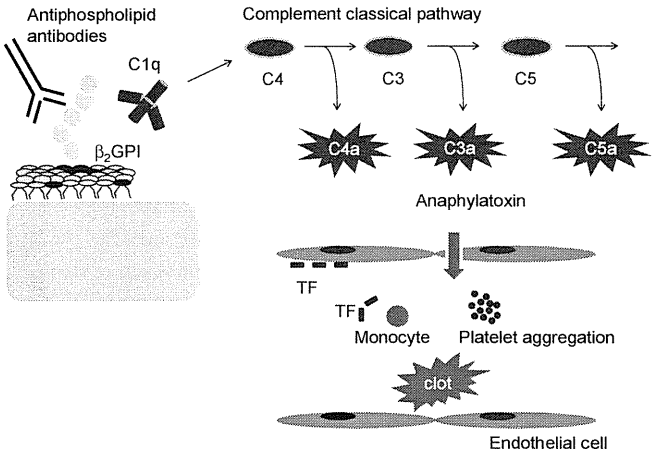

Figure 2 Pathogenic mechanisms of complement activation in antiphospholipid syndrome. Complement classical pathway which is initiated by C1q protein is significantly activated in the serum of patients with antiphospholipid syndrome. Activation of the pathway proceeds as cascade reaction producing anaphylatoxins, the fragments of the complement proteins that amplify the activation of monocytes, platelets or endothelial cells. Activation of these cells and molecules induces expression of TF or adhesion molecules and platelet aggregation. β2GPI, β2 glycoprotein I; TF, tissue factor.

APS is important because it has the potential to be helpful not only in understanding the pathology of APS but also in clarifying the involvement of the mode of complement activation in SLE as well.

Complement system

'Complement' is a general term for over 20 serum proteins that function in innate immunity. They would attach to the membrane of organisms and would be activated sequentially in cascade process. They are activated by pattern recognition receptors that have evolved to recognise specific molecular patterns. The complement system has three known activation pathways according to the differences of activation elements: the classical pathway activated by an antigen–antibody complex; the lectin pathway activated by lectin, recognising glycans on microorganisms; and the alternative pathway, in which C3 is bound without recognition molecules. These three pathways culminate in activation of C3, the central step of complement activation. Activation of C3 leads to the production of pro-inflammatory fragment C3a which is called anaphylatoxin and sequentially activates C5. Activation of C5 also produces anaphylatoxin C5a which has especially strong inflammation-inducing effect. C4, a classical pathway protein, also produces C4a on its activation which is another member of anaphylatoxin but has little effect on inducing inflammation. C5b, an another fragment of activated C5, forms a complex with C6, C7, C8 and C9 which is called membrane attack protein (MAC). MAC produces a trans-membrane channel on the membrane of microorganism that disrupts the phospholipid bilayer, leading to cell lysis and death.

Complement activation in APS pregnancy morbidity

Complement activation was first thought to be involved in the pathophysiology of pregnancy complication in APS. Initially, it was believed that thrombosis in placentas was an important mechanism of pregnancy complication in APS. Obstructed blood flow in placenta caused by thrombosis may impair foetal growth because of exchange failure of blood between mother and foetus [56]. Placental thrombosis has been reported, and *in vitro* studies have confirmed that aPL have the ability to disrupt the annexin A5 anticoagulant shield on trophoblast and EC monolayers [57]. However, other reports were unable to confirm the occurrence of multiple thrombosis in the placentas of patients with APS; in fact, placental thrombosis imaging was either virtually absent or only mild in the analysis of many cases [58].

A recent study pinpointed the trophoblastic basement membrane as a target of aPL, where the aPL were thought to complicate pregnancy by directly inducing localised inflammation. Multiple peritoneal injections of human IgG with aPL activity to pregnant naïve mice enabled embryo implantation and induced considerable placental damage that resulted in foetal resorption and growth retardation. Deposits of human-derived IgG and complement were observed in a pathological study of the placentas of these mouse models, accompanied with local TNF secretion and a temporary but a clear increase in serum TNF levels [59–61].

Obstetrical problems were markedly suppressed in these models when complement was deficient or when antibodies against the complement were administered, such that complement activation was thought to be an important mechanism in pregnancy complications by aPL [62].

The anaphylatoxin C5a is especially important and causes placental damage, having a strong inflammation-inducing effect that causes localised placental inflammation and promotes TF expression in neutrophils infiltrated to the placenta [63,64]. In addition, anti-complement activation is involved in the effect of heparin preventing aPL-related pregnancy complications [65]. However, considering the fact that the mouse models were given large doses (10 mg each) of IgG fractions containing aPL in human serum, it is unclear whether they adequately reflect the behaviour of aPL *in vivo* in human.

The involvement of complement activation in pregnancy morbidities in patients with APS has been vigorously studied, with a series of reports suggesting a clear involvement

in mouse models. In one retrospective study, complement deposition was found in the placental tissues of women positive for aPL [66]. There are some reports discussing the dysfunction of complement-regulating factor. Complement-regulating factor mutations are related to preeclampsia in patients with SLE or APS [67]. Decreased expression of decay-accelerating factor (CD55, a complement regulatory factor) in the endometrium has been confirmed in aPL-positive pregnancies (by endometrial biopsy) [68]. However, there remains a lack of conspicuous evidence that complement activation is directly related to the APS pregnancy morbidity outcome. Preliminary data from recent reports indicate that the histology of placental specimens from patients with APS shows evidence of complement activation compared with control placental specimens; however, complement deposition can be detected both in abortive specimens and in placentas at term without a clear relationship with either pregnancy outcome or therapy [45].

Although large prospective analyses are needed to demonstrate definite conclusions about the involvement of complement in APS-related pregnancy morbidity, the potential role of complement in aPL-mediated clinical manifestations should not be neglected. In addition to causing acute local inflammation, complement components are able to modulate the functions of procoagulant cells (monocyte, ECs) and decidual or trophoblast cells [69].

Complement activation in APS thrombosis

According to the positive results showing the relationship between complement activation and pregnancy morbidity in the APS murine model, an intensive study was carried out that investigated the relationship between thrombosis and complement activation in this model.

Mice deficient in C3 ($C3^{-/-}$) and C5 ($C5^{-/-}$) were used to investigate the role of complement activation in APS thrombosis. Each was administered aPL–IgG or control IgG, and thrombosis was induced via standardised pinch injury to the femoral vein [70]. The sizes of the thrombi in $C3^{-/-}$ and $C5^{-/-}$ mice were significantly reduced compared to those of wild-type mice. Additionally, mice treated with monoclonal anti-C5 antibody developed smaller thrombi compared with mice that did not receive the monoclonal antibody [71,72]. Complement activation using C5a production observed in the aPL-administered mice was found to induce TF expression on neutrophils, resulting in modified prothrombin time [71]. These phenomena suggest a possible mechanism by which aPL activation of complement pathway can initiate coagulation. Finally, antiβ2GP1 antibodies were found to initiate thrombus formation, with decreased thrombotic occlusions in C6-deficient rats and in mice treated with anti-C5 antibody [21].

A recent report investigated the significance of complement activation in patients with primary APS [72]. From the analysis of serum complement levels (C3, C4, CH50) and anaphylatoxins (C3a, C4a, C5a) in patients with primary APS, non-SLE connective tissue disease and healthy subjects, it revealed that complement levels were significantly lower in patients with primary APS compared with those with the other groups. Most patients with primary APS showed elevated serum levels of C3a and C4a related to hypocomplementemia. Among the patients with primary APS, no correlation was found between any particular clinical manifestation and hypocomplementemia. Hypocomplementemia is frequently found in patients with primary APS, reflecting complement activation and consumption rather than deficiency as suggested by the correlation between high serum C3a concentrations and low serum C3 levels. This conclusion strengthens the recognition of crosstalk between complement activation and prothrombotic status in APS (Fig. 2).

Crosstalk between complement and coagulation pathways

There is lesser evidence of APS thrombosis and complement activation than pregnancy complications, but various aspects of involvement have become known for complement activation and thrombosis. Complement activation is increasingly being recognised as a major contributor of vascular inflammation [73]. Complement deposition has been frequently observed in atherosclerotic lesions [74], and accumulating evidence suggests that complement plays a significant role in ischaemia/reperfusion injury [75]. C3a and C5a enhance leucocyte recruitment and support the host inflammatory response [76]. C5a level elevations have been associated with increased cardiovascular risk in patients with advanced atherosclerosis [77].

Recently, the complement pathway has also been identified as having an effect on the coagulation pathway itself. Both have highly substrate-specific reactions that proceed with the cascading activation of many different serine proteases, but some reactions also cross from one cascade to the other. For example, activated factor XII (FXIIa), an initiator of the intrinsic coagulation pathway, degrades and activates C1, an initiator of the classical complement pathway [78], while thrombin directly degrades C5 in the absence of C3 to produce the anaphylatoxin C5a [79]. In addition, C5a increases the expression of TF [80], and the membrane attack complex degrades prothrombin to thrombin [81]. Thus, the complement and coagulation pathways have a close relationship. This mechanism is very effective at the site of trauma by causing anaphylatoxin production at place of haemostasis; complement activation induces inflammation at the trauma site and effectively prevent microorganism infiltration. It is possible that a complex combination occurs

in the involvement of complement activation in APS thrombosis, such as with vascular injury, direct activation of the coagulation pathway or cellular activation.

Conclusion

Ongoing research focused on cell receptors and intracellular signalling pathways involved in the cell activation mediated by aPL substantially advance the understanding of the thrombotic mechanism in APS. Further studies are needed to clarify the biological role of the numerous potential receptors proposed for aPL–cell interaction.

Complement activation seems to be an essential factor for disease manifestation in pregnancy morbidity in patients with aPL from the results of the experiments in *in vivo* models. Accumulating evidences are offering promising prospects on the involvement of complement activation in thrombosis related to aPL. Although the definite conclusion that complement activation is a part of the process of disease manifestation can only be induced from the result of large prospective studies, there is no doubt that the clarification of the mechanism of complement activation in APS would be a key to a better understanding of pathogenesis of APS.

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