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aCL/β_2GPI and aPS/PT show synergic thrombogenic effects in suppressing anticoagulant activity of APC and stimulating tissue factor expression and TNF-α secretion by mononuclear cells

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ABSTRACT

Introduction: Patients with systemic lupus erythematosus (SLE) possessing anti-phospholipid antibodies (aPLs) are often complicated by thrombotic vascular events. aPLs commonly associated with the complications are anti-cardiolipin/β_2-glycoprotein I antibodies (aCL/β_2GPI) and anti-phosphatidylserine/prothrombin antibodies (aPS/PT). However, the pathological mechanisms leading to thrombosis remain unclear. We explored clinical features of SLE patients with aCL/β_2GPI and aPS/PT and investigated thrombogenic effects of their IgG fractions.
Materials and methods: We enrolled 97 SLE patients and 38 healthy control volunteers and performed activated protein C (APC) resistance screening test using their plasma samples. To detect the direct effect of aPLs IgG on APC, we developed an APC sensitivity ratio assay. Effects of aPLs IgG on monocytes were studied by measuring the surface expression of tissue factor (TF) and excretion of TNF-α from peripheral blood mononuclear cell culture.
Results and conclusion: Thrombotic complications among SLE patients were closely associated with aCL/β_2GPI or aPS/PT, with higher prevalence in patients with both antibodies. Addition of aPLs$^{(+)}$-IgG to the APC sensitivity ratio assay led to significant suppression of the anticoagulant activity of APC. The suppression was more pronounced in double-positive cases. TF expression on monocytes and concentration of TNF-α in culture medium were increased by aPLs, again more pronounced in double-positive cases. These results indicate that the effects of aCL/β_2GPI and aPS/PT are synergic both for APC anticoagulant activity and for production of TF and TNF-α from mononuclear cells. These modes of thrombogenic action of aPLs could be an important target for developing specific measures to prevent complications of SLE.

1. Introduction

Anti-phospholipid antibodies (aPLs) are a heterogeneous group of autoantibodies that are frequently found in the plasma of patients with systemic lupus erythematosus (SLE) [1–3]. The presence of aPLs is associated with arterial and/or venous thrombotic complications in SLE patients [4–8]. Anti-phospholipid syndrome (APS) is currently diagnosed by both laboratory evidence of persistent aPLs and clinical findings such as recurrent arterial and/or venous thrombosis and obstetric complications [9]. Cerebral infarction is the most common arterial thromboembolic complication, and deep vein thrombosis is the most frequent venous thromboembolic event in APS [7,10,11]. It is now generally accepted that aPLs do not bind directly to the negatively charged phospholipid itself but rather to complexes of the phospholipid and phospholipid-binding proteins and that the most common and well-characterized antigenic targets are β_2-glycoprotein I (β_2GPI) and prothrombin. Recent studies have also indicated that the epitopes for aPLs expressed on β_2GPI or prothrombin change conformationally by interaction with anionic phospholipid surfaces such as cardiolipin and phosphatidylserine [5,8,12,13].

Presently, aPLs suggested to be clinically useful are anti-cardiolipin/β_2GPI antibodies (aCL/β_2GPI), anti-phosphatidylserine/prothrombin antibodies (aPS/PT), and lupus anticoagulant activity [9,15]. aCL/β_2GPI and aPS/PT are detected with solid-phase immunoassays, typically enzyme-linked immunosorbent assays (ELISAs) [16]. Lupus anticoagulant activity is currently detected as the inhibitory effect of aPLs

Abbreviations: SLE, systemic lupus erythematosus; aPLs, anti-phospholipid antibodies; aCL/β_2GPI, anti-cardiolipin/β_2-glycoprotein I antibodies; aPS/PT, anti-phosphatidylserine/prothrombin antibodies; APC, activated protein C; TF, tissue factor; APS, anti-phospholipid syndrome; ELISAs, enzyme-linked immunosorbent assays; PBMCs, peripheral blood mononuclear cells; APTT, activated partial thromboplastin time; OR, odds ratio; CI, confidence interval; CMCI, cell-mediated coagulation induction

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on certain in vitro phospholipid-dependent coagulation reactions [17]. Several clinical studies have established that aPLs are present in approximately 40% of patients with SLE and that the presence of aPLs apparently constitutes a risk factor for arterial and/or venous thrombotic complications [1,9–11]. However, the precise mechanisms responsible for arterial or venous thrombotic complications in these patients remain unclear.

In our previous studies, we found that SLE patients frequently possess various subclasses of aPLs in the plasma [6], and the occurrence of recurrent arterial and/or venous thrombotic complications depends on variable combinations of these subclasses of aPLs [18]. Furthermore, we reported two possible mechanisms by which aPLs cause arterial and/or venous thrombotic complications. For venous thrombotic complications, we postulated that aPLs cause an acquired activated protein C (APC) resistance by inhibiting the phospholipid-dependent reactivity of the APC pathway. For arterial thrombosis, we presumed that aPLs cause persistently high tissue factor (TF) expression and inflammatory cytokine production by interacting with peripheral blood monocytes and lymphocytes.

In the present study, to investigate the clinical significance of aPLs detected by ELISA, we measured concentrations of aCL/β_2GPI and aPS/PT in 155 patients with SLE including those with arterial and venous thrombotic complications. To evaluate the validity of the above hypotheses, we conducted in vitro studies to test for the direct effect of purified IgG-aCL/β_2GPI and/or IgG-aPS/PT on the anticoagulant activity of APC and the cell-surface expression of TF in healthy peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1. Patients

We studied plasma samples from 155 patients (144 females, 11 males; age range 8–82 years, mean 44.0 years) with SLE. Diagnosis of SLE was made according to the revised criteria of the American College of Rheumatology Criteria for Classification of Systemic Lupus Erythematosus. All 155 patients with well-defined SLE had been clinically followed regularly for at least 10 years. All of the thromboembolic complications occurred either at SLE onset or during follow-up. Clinical histories revealed that thromboembolic complications were observable in 55 of the 155 SLE patients: arterial thrombosis in 33 patients and venous thrombosis in 22 patients. All incidences of thrombosis had been documented by venography, arteriography, angiography, Doppler ultrasound, and/or computed tomography scan. Blood samples were drawn into vacuum tubes (5.0 mL total volume, SEKISUI, Japan) containing 0.5 mL of 3.13% trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), and platelet-poor plasma was prepared by double centrifugation at 2800 $\times g$ for 15 min at 18 °C. The plasma samples were then frozen at −80 °C until batch assays could be performed. This study was reviewed and approved by the medical research ethics committee of Yamaguchi University Graduate School of Medicine, Faculty of Health Sciences (approval no. 363), and informed consent was obtained from all patients and control subjects.

2.2. APC resistance screening test

We conducted an APC resistance screening test using plasma samples from 38 normal healthy volunteers (35 females, 3 males; age range 23–58 years, mean, 39.7 years) collected by APS standardization workshop and plasma samples from 97 SLE patients (92 females, 5 males; age range 10–82 years, mean 43.7 years) provided by Osaka University Hospital by use of the commercially available Coatest APC Resistance reagent kit (CHROMOGENIX, Tokyo, Japan).

Blood samples were drawn into vacuum tubes (SEKISUI) containing 0.5 mL of 3.13% trisodium citrate, and platelet-poor plasma was prepared by double centrifugation at 2800 $\times g$ for 15 min at 15 °C. The plasma samples were frozen at −80 °C until batch assays could be performed. Written informed consent was obtained from all study participants.

Table 1
Characteristics of IgG fractions from 27 patients examined in this study.

IgG	LA activity	aCL/β2GPI (U/mL)	aPS/PT (U/mL)
$aPLs^{(+/+)}$-IgG−1	+	22.8	30.2
$aPLs^{(+/+)}$-IgG−2	+	13.5	14.1
$aPLs^{(+/+)}$-IgG−3	+	13.5	21.5
$aPLs^{(+/+)}$-IgG−4	+	31.3	38.9
$aPLs^{(+/+)}$-IgG−5	+	156.8	48.3
$aPLs^{(+/+)}$-IgG−6	+	257.0	18.1
$aPLs^{(+/+)}$-IgG−7	+	1226.9	32.2
$aPLs^{(+/+)}$-IgG−8	+	87.5	17.4
$aPLs^{(+/-)}$-IgG−1	+	48.1	< 1.56
$aPLs^{(+/-)}$-IgG−2	+	53.5	< 1.56
$aPLs^{(+/-)}$-IgG−3	+	158.4	< 1.56
$aPLs^{(+/-)}$-IgG−4	−	24.5	< 1.56
$aPLs^{(+/-)}$-IgG−5	+	81.1	< 1.56
$aPLs^{(+/-)}$-IgG−6	−	12.6	< 1.56
$aPLs^{(+/-)}$-IgG−7	+	49.8	< 1.56
$aPLs^{(-/+)}$-IgG−1	−	< 1.30	41.4
$aPLs^{(-/+)}$-IgG−2	+	< 1.30	24.1
$aPLs^{(-/+)}$-IgG−3	+	< 1.30	29.5
$aPLs^{(-/+)}$-IgG−4	+	< 1.30	129.0
$aPLs^{(-/+)}$-IgG−5	+	< 1.30	52.0
$aPLs^{(-/+)}$-IgG−6	+	< 1.30	258.0
$aPLs^{(-/+)}$-IgG−7	+	< 1.30	25.0
$aPLs^{(-/-)}$-IgG−1	+	< 1.30	< 1.56
$aPLs^{(-/-)}$-IgG−2	+	< 1.30	< 1.56
$aPLs^{(-/-)}$-IgG−3	+	< 1.30	< 1.56
$aPLs^{(-/-)}$-IgG−4	+	< 1.30	< 1.56
$aPLs^{(-/-)}$-IgG−5	+	< 1.30	< 1.56
$aPLs^{(-/-)}$-IgG−6	+	< 1.30	< 1.56

$aPLs^{(-/-)}$-IgG fractions were obtained from plasma of 6 APS patients double negative for aCL/β_2GPI and aPS/PT aPLs; $aPLs^{(+/-)}$-IgG fractions from plasma of 7 APS patients single positive for aCL/β_2GPI aPLs; $aPLs^{(-/+)}$-IgG fractions from plasma of 7 APS patients single positive for aPS/PT aPLs; and $aPLs^{(+/+)}$-IgG fractions from plasma of 8 APS patients double-positive for aCL/β_2GPI and aPS/PT aPLs.

2.3. IgG purification

IgG fractions were purified from 8 plasma samples from SLE patients with both aCL/β_2GPI and aPS/PT [$aPLs^{(+/+)}$-IgG] and 6 plasma samples from SLE patients without aPLs [$aPLs^{(-/-)}$-IgG] by use of an MAbTrap kit (GE Healthcare Japan, Tokyo, Japan). All 14 IgG fractions were purified from the plasma of individual SLE patients. Furthermore, aCL/β_2GPI$^{(+)}$-IgG fractions [$aPLs^{(+/-)}$-IgG] were purified from plasma samples of 7 aCL/β_2GPI single-positive patients, and aPS/PT$^{(+)}$-IgG fractions [$aPLs^{(-/+)}$-IgG] were purified from plasma samples of 7 aPS/PT single-positive patients (Table 1). Non-APS-IgG fractions as a control were purified from plasma samples of a SLE patient who had no aPLs.

2.4. Detection of anti-CL/β_2GPI and anti-PS/PT

The presence of aPLs was determined on the basis of measurements of anti-CL/β_2-GPI and anti-PS/PT. The concentrations of anti-CL/β_2-GPI were measured with an anti-CL/β_2-GPI ELISA Kit (Yamasa Shoyu Co., Ltd., Tokyo, Japan) as reported previously [7,19]. Anti-PS/PT levels were measured with an anti-PS/PT IgG ELISA Kit (Cosmic Corporation, Tokyo, Japan) as reported previously [20].

2.5. Measurement of APC sensitivity ratio

We developed a new testing method to measure the APC sensitivity ratio by use of an activated partial thromboplastin time (APTT) assay using a KC-4 coagulometer. APTT was measured in the presence and absence of APC, and the APC sensitivity ratio was expressed as the ratio

of APTT in the presence and absence of APC (APTT with APC/APTT without APC). An APC sensitivity ratio of < 2.40, which was the mean − 2SD in normal controls, was chosen as the normal cut-off value in the APC resistance assay. We studied the in vitro effects of IgG fractions ($aPLs^{(+/+)}$-IgG, n = 8; $aPLs^{(-/-)}$-IgG, n = 6; $aPLs^{(+/-)}$-IgG, n = 7; and $aPLs^{(-/+)}$-IgG, n = 6) on the APC sensitivity ratio.

2.6. Isolation and preparation of PBMCs

Peripheral venous blood was collected by BD Vacutainer® CPT™ (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) from a healthy donor and was centrifuged at 1700 × *g* for 15 min. After centrifugation, we collected the PBMC layer and transferred it to a tube with cap containing PBS. After 3 washings with PBS, we resuspended it in RPMI-1640 medium (Gibco Life Technologies Japan Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies Japan Ltd.) containing penicillin and streptomycin. Cells were seeded and incubated for 1.5 h in medium at 37 °C in a humidified atmosphere containing 5% CO_2.

2.7. Stimulation of PBMCs

For the flow cytometric analysis, the stimulation was performed with $aPLs^{(+/+)}$-IgG, $aPLs^{(+/-)}$-IgG, $aPLs^{(-/+)}$-IgG, or non-APS-IgG on 1.0×10^6 cells/mL. LPS (20 ng/mL) was used for the positive control, and unstimulated cells using PBS served as the negative control. Cells were stimulated for 6 h. For the purpose of cytokine assays, after the 6 h of cell stimulation, we collected the supernatant and stored it at −80 °C.

2.8. Measurement of monocyte surface expression of human TF by flow cytometer

We examined TF expression on the IgG-stimulated monocyte surface in the 6 normal healthy volunteers. Monocyte TF expression was assessed by measurement of TF on the surface of CD14-positive cells by flow cytometric analysis as reported previously with a MAQSQuant Analyzer 10 Flow Cytometer (Miltenyi Biotec, Tokyo, Japan). Monocytes were defined as CD14-positive cells. TF expression on monocytes was measured as the percentage of CD14-positive cells with TF staining. This was achieved by plotting the side scatter characteristics vs. CD14. The negative and positive delineators were determined by gating 1% background staining in the FITC-conjugated mouse IgG1 control. The percentage of FITC (TF)-positive events in the population was then determined.

2.9. Measurement of TNF-α

Secreted protein concentrations of TNF-α in cell culture supernatants were measured using an ELISA kit (Human TNF-alpha Quantikine ELISA Kit; R&D SYSTEMS, Boston, MA, USA).

2.10. Statistical analysis

We derived the cutoff value for the APC sensitivity ratio from receiver operating characteristic curve analysis. An APC sensitivity ratio of < 2.40, which was the mean −2SD in normal controls, was determined as the normal cut-off value in the study of the APC resistance screening test. The Mann-Whitney test was used to compare the levels of TF expression and production of TNF-α between SLE and healthy groups. A P value of < 0.05 was considered to indicate statistical significance.

Table 2
Independent utility of aCL/β_2GPI and aPS/PT in distinguishing thrombotic events.

		Thrombosis n = 55 % (n)	No thrombosis n = 100 % (n)	OR (95% CI)
aCL/β_2GPI	(+) (n = 61)	63.9% (39)	36.1% (22)	8.6 (4.1–18.3)
	(−) (n = 94)	17.0% (16)	83.0% (78)	
aPS/PT	(+) (n = 42)	69.0% (29)	31.0% (13)	7.5 (3.4–16.4)
	(−) (n = 113)	23.0% (26)	77.0% (87)	

aCL/β_2GPI, anti-cardiolipin β_2-glycoprotein I antibodies; aPS/PT, anti-phosphatidylserine prothrombin antibodies; OR, odds ratio; CI, confidence interval. OR was considered statistically significant when the lower limit of the 95% CI was > 1.0.

3. Results

3.1. Utility of aCL/β_2GPI and aPS/PT for diagnosing thrombosis in patients with SLE

We calculated the prevalence of aCL/β_2GPI and aPS/PT among the 155 patients with SLE (Table 2). In total, 55 patients suffered thrombotic complications: 22 with venous and 33 with arterial complications. Without distinction between venous and arterial complications, the prevalence of positive aCL/β_2GPI was 63.9% (39/61 patients) in those with thrombosis and 36.1% (22/61 patients) without thrombosis. The prevalence of positive aPS/PT was 69.0% (29/42) in those with thrombosis and 31.0% (13/42) in those without thrombosis. The odds ratio and its 95% confidence interval of each antibody for distinguishing thrombotic complications were 8.6 (4.1–18.3) for aCL/β_2GPI and 7.5 (3.4–16.4) for aPS/PT.

The utility of using aCL/β_2GPI and aPS/PT in combination was also evaluated by partitioning the SLE patients into four groups according to their positivity to the two aPLs (Table 3): group A, aCL/β_2GPI$^{(+)}$/aPS/PT$^{(+)}$; group B, aCL/β_2GPI$^{(+)}$/aPS/PT$^{(-)}$; group C, aCL/β_2GPI$^{(-)}$/aPS/PT$^{(+)}$; and group D, aCL/β_2GPI$^{(-)}$/aPS/PT$^{(-)}$. The prevalence of thrombosis was 75.8% (25/33) in group A, 51.9% (14/27) in group B, 20.0% (1/5) in group C, and 16.7% (15/90) in group D. The results clearly indicated that the prevalence of thrombotic complications was obviously higher in patients with double-positive results than in those with double-negative or single-negative results for either aCL/β_2GPI or aPS/PT.

3.2. Relationship between the presence of aPLs and acquired APC resistance

3.2.1. APC resistance screening test

We performed the APC resistance screening test with a commercial kit. In plasma samples from 38 healthy volunteers and 97 patients with SLE (Fig. 1), 26 (27%) of the SLE patients showed positive results. Factor V Leiden mutation was examined in these 26 SLE patients with

Table 3
Utility of aCL/β_2GPI, aPS/PT, and their combinations in distinguishing thrombotic events.

Groups	Thrombosis n = 55 % (n)	No thrombosis n = 100 % (n)	OR (95% CI)
A: aCL/β_2GPI(+)/aPS/PT(+) (n = 33)	75.8% (25)	24.2% (8)	9.6 (4.3–21.6)
B: aCL/β_2GPI(+)/aPS/PT(−) (n = 27)	51.9% (14)	48.1% (13)	2.3 (1.0–5.2)
C: aCL/β_2GPI(−)/aPS/PT(+) (n = 5)	20.0% (1)	80.0% (4)	0.4 (0.1–3.9)
D: aCL/β_2GPI(−)/aPS/PT(−) (n = 90)	16.7% (15)	83.3% (75)	0.1 (0.1–0.3)

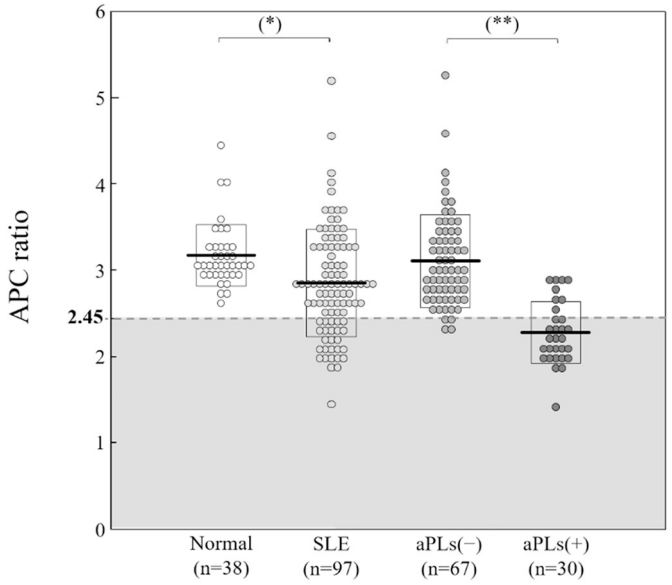

Fig. 1. Comparison of APC ratios between healthy subjects and three subgroups of SLE patients. An APC ratio of 2.45 or less was regarded as resistance-positive, indicating low affinity to APC. In SLE patients as a whole, APC ratios were significantly reduced to 2.83 (2.43–3.26) (median [25%–75%]) compared to 3.08 (2.96–3.29) in the healthy volunteers. However, after partitioning according to the status of aPLs, the APC ratios of aPLs-positive patients were significantly decreased to 2.24 (2.01–2.58), whereas those without aPLs were 3.00 (2.75–3.39), which were not different from those of the healthy volunteers. Mann-Whitney test: *Normal vs. SLE, $P < 0.001$; ** $aPLs^{(-)}$ vs. $aPLs^{(+)}$, $P < 0.001$. OR was considered statistically significant when the lower limit of the 95% CI was > 1.0. APC, activated protein C; SLE, systemic lupus erythematosus; aPLs, anti-phospholipid antibodies.

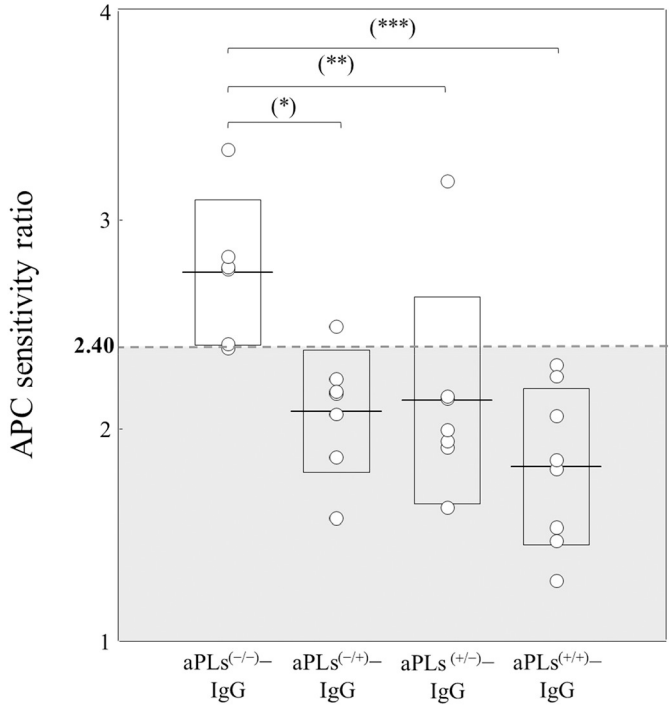

Fig. 2. Effect of four categories of aPLs-IgG on the anticoagulant activity of APC. We distinguished four IgG fractions of aPLs according to the presence of aCL/β_2GPI and aPS/PT as listed below and examined the effect on anticoagulant activity of APC by adding each to the APC resistance assay. As a result, the addition of $aPLs^{(+/+)}$-IgG, $aPLs^{(+/-)}$-IgG, and $aPLs^{(-/+)}$-IgG led to a significant reduction in the APC sensitivity ratio, indicating potent suppression of the anticoagulant activity of APC. $aPLs^{(-/-)}$-IgG: double-negative for aCL/β_2GPI and aPS/PT aPLs, (n = 6); $aPLs^{(+/-)}$-IgG: single-positive for aCL/β_2GPI aPLs, (n = 7); $aPLs^{(-/+)}$-IgG: single-positive for aPS/PT aPLs, (n = 7); and $aPLs^{(+/+)}$-IgG: double-positive for aCL/β_2GPI and aPS/PT aPLs, (n = 8). By *t*-test: * $aPLs^{(-/-)}$-IgG vs. $aPLs^{(-/+)}$-IgG, $P < 0.005$; ** $aPLs^{(-/-)}$-IgG vs. $aPLs^{(+/-)}$-IgG, $P < 0.05$; *** $aPLs^{(-/-)}$-IgG vs. $aPLs^{(+/+)}$-IgG, $P < 0.001$.

APC resistance, and all of them tested negative for the mutation. Therefore, all 26 SLE patients with APC resistance were considered to have acquired APC resistance. Furthermore, we divided the SLE patients into two groups according to their aPLs status: 30 with aPLs and 67 without aPLs. As a result, the prevalence of APC resistance was 73% (22/30 patients) in those with aPLs and 6% (4/67 patients) in those without aPLs.

3.2.2. *Effect of $aPLs^{(+)}$-IgG on APC sensitivity ratio*

Under normal conditions, the addition of APC to the APTT assay usually causes a 3- to 4-fold increase in clotting time. We quantitate this change as an APC sensitivity ratio of 3.0 or 4.0 in comparison to the control testing without APC. However, in patient plasma with resistance to APC, the prolongation of clotting time is suppressed, which thus decreases the APC sensitivity ratio. Using this assay of APC sensitivity ratio measurement, we examined whether aPLs suppress the anticoagulant activity of APC. For this objective, we used IgG fractions from 28 SLE patients: eight double-positive for aCL/β_2GPI and aPS/PT [$aPLs^{(+/+)}$-IgG], seven single-positive for aCL/β_2GPI [$aPLs^{(+/-)}$-IgG], seven single-positive for aPS/PT [$aPLs^{(-/+)}$-IgG], and six double-negative for aCL/β_2GPI and aPS/PT [$aPLs^{(-/-)}$-IgG]. As shown in Fig. 2, we confirmed that the addition of $aPLs^{(+/-)}$-IgG, $aPLs^{(-/+)}$-IgG, or $aPLs^{(+/+)}$-IgG to the APTT assay resulted in significant suppression of the anticoagulant activity of APC, compared to the case with addition of $aPLs^{(-/-)}$-IgG. The degree of suppression seemed to be more pronounced by the IgG with double-positive aPLs [$aPLs^{(+/+)}$], although the difference was not statistically significant with the small sample sizes.

3.2.3. *Association of positive aPLs and TF expression on monocyte*

We examined TF expression on monocytes in relation to the presence of aPLs. As shown in Fig. 3, the rate of TF expression compared to the control was significantly increased by stimulation of $aPLs^{(+/+)}$-IgG, $aPLs^{(+/-)}$-IgG, and $aPLs^{(-/+)}$-IgG. The expression of TF was more pronounced with the use of $aPLs^{(+/+)}$-IgG. In contrast, stimulation by non-APS-IgG or treatment with PBS did not cause any noticeable changes in the expression of TF on monocytes.

3.2.4. *Association of positive aPLs with release of TNF-α into the culture medium*

We also used the ELISA to measure the concentration of TNF-α in cell culture medium of PBMCs 6 h after stimulation by use of various aPLs IgG. The addition of $aPLs^{(+/+)}$-IgG caused significant enhancement of the production of TNF-α compared to the control (Fig. 4), and that of $aPLs^{(+/-)}$-IgG and $aPLs^{(-/+)}$-IgG also caused the production of TNF-α.

4. Discussion

SLE is a typical systemic autoimmune disease characterized by the appearance of a wide variety of autoantibodies and multiple organ lesions. It has long been known that deep vein thrombosis such as pulmonary embolism and arterial thrombosis such as cerebrovascular disorders occur preferentially in SLE patients [10]. This susceptibility to thrombotic complications is apparently related to the emergence of various types of aPLs in these patients' sera. The pathophysiological mechanisms of aPLs leading to the thrombotic events are currently under active investigation. Recent studies have shown that there are several types of aPLs against epitopes on a variety of phospholipid molecules. Among well-known aPLs, aCL/β_2GPI and aPS/PT are of

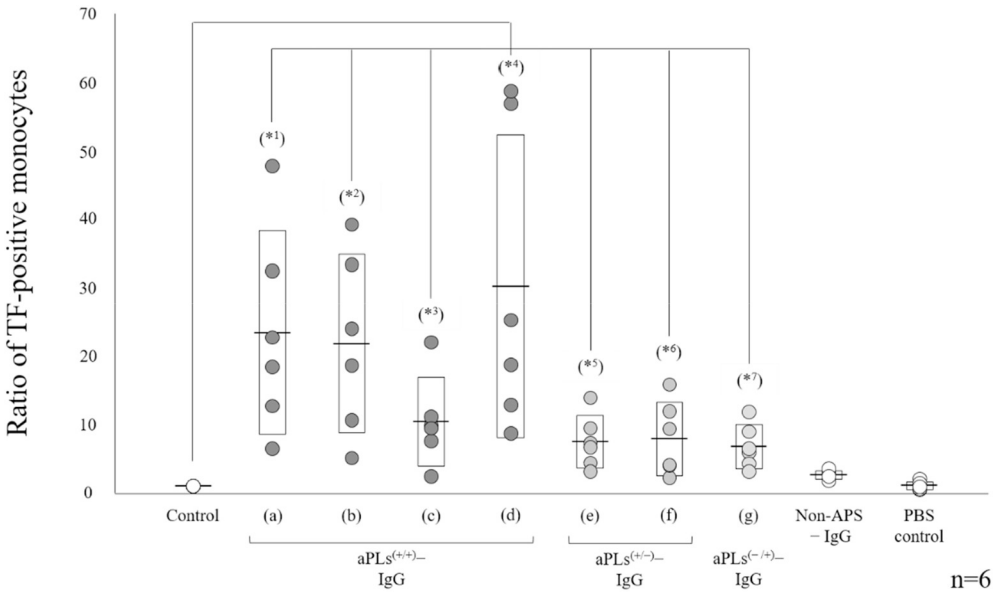

Fig. 3. Comparison of TF expression on monocytes from healthy donors after stimulation by various IgG fractions and control specimens. We measured TF-positive monocytes by flow cytometric analysis after stimulation by various IgG-fractions. The level of TF expression was expressed as a ratio to the control specimen without IgG. The ratios of TF expression were significantly increased after stimulation by aPLs$^{(+/+)}$-IgG compared to the control. TF: tissue factor; aPLs$^{(+/+)}$-IgG: double-positive for aCL/β_2GPI and aPS/PT aPLs; aPLs$^{(+/-)}$-IgG: single-positive for aCL/β_2GPI aPLs; aPLs$^{(-/+)}$-IgG: single-positive for aPS/PT. *t*-Test: $^{*1-4}$ Control vs. aPLs$^{(+/+)}$-IgG, $P < 0.005$, ^{*5} Control vs. aPLs$^{(+/-)}$-IgG, $P < 0.005$, ^{*6} Control vs. aPLs$^{(+/-)}$-IgG, $P < 0.05$, ^{*7} Control vs. aPLs$^{(-/+)}$-IgG, $P < 0.05$.

primary importance due to their close association with thrombotic complications [6]. The purpose of this study was to investigate the thrombogenic effect of aCL/β_2GPI and aPS/PT.

In our recent study [6], we found the incidence of arterial/venous thrombosis to be significantly higher in patients with APS who possessed both aCL/β_2GPI and aPS/PT in combination (Tables 1, 2). Although APC resistance by Factor V Leiden mutation is known to cause severe venous thrombosis, the mutation is unique to Westerners [21–24]. In SLE patients, the occurrence of unexplained acquired APC resistance is known, but its prevalence and relationship to the event of venous thrombosis are not clear [19,25]. In the present study, we postulated that both aCL/β_2GPI and aPS/PT would induce acquired APC resistance, thereby triggering the thrombotic event. Therefore, we investigated whether aPLs specifically suppress the anticoagulant activity of APC by use of IgG fractions of two representative varieties of aPLs: aCL/β_2GPI and aPS/PT.

As a result, we found that both aCL/β_2GPI and aPS/PT exerted an independent suppressive effect on the anticoagulant activity of APC with a tendency for greater suppression when the two varieties coexisted. This inhibitory action of aPLs on the blood coagulation control mechanism of APC is now regarded as contributory to the high prevalence of venous thrombosis in SLE patients.

Secondarily, we examined whether aPLs promote TF expression on monocytes and the release of TNF-α in cell culture medium. It is known that arteriosclerotic lesions develop rapidly in APS patients almost invariably despite the absence of any traditional risk factors such as dyslipidemia, hypertension, diabetes, obesity, and smoking [26]. In our previous studies, we reported that the IgG fraction of aPLs (double-positive for aCL/β_2GPI and aPS/PT) acted on monocytes to enhance cellular surface expression of TF and the production of inflammatory cytokines (TNF-α, IL-1β, IL-6), both of which were closely related to clinical features of arteriosclerosis such as cerebral infarction and myocardial infarction [27]. However, we were not certain which of the two types of aPLs contributes more to the stimulation of monocytes. In the present study, we utilized the same in vitro experimental system and examined

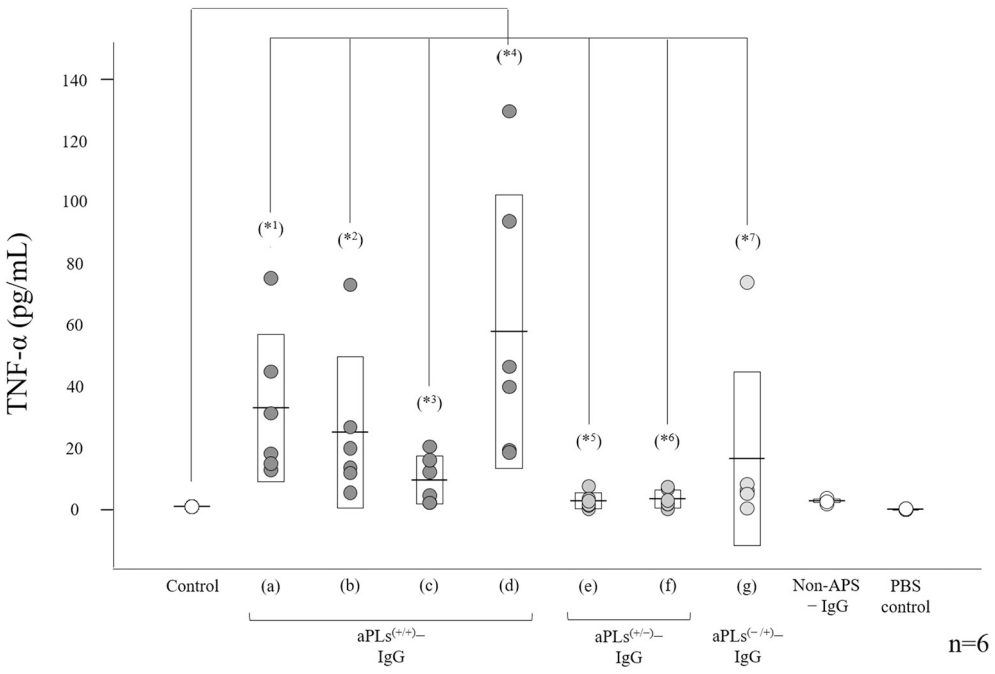

Fig. 4. Comparison of TNF-α released in cell culture medium of healthy peripheral blood mononuclear cells (PBMCs) after stimulation by various IgG fractions or control specimens. We stimulated PBMCs by use of various aPLs IgG and measured TNF-α by ELISA kit. The production of TNF-α was significantly enhanced by aPLs$^{(+/+)}$-IgG stimulation compared to the control. aPLs$^{(+/+)}$-IgG: double-positive for aCL/β_2GPI and aPS/PT aPLs, aPLs$^{(+/-)}$-IgG: single-positive for aCL/β_2GPI aPLs, aPLs$^{(-/+)}$-IgG: single-positive for aPS/PT. *t*-Test: $^{*1-4}$ Control vs. aPLs$^{(+/+)}$-IgG, $P < 0.05$, $^{*5-6}$ Control vs. aPLs$^{(+/-)}$-IgG, $P < 0.1$, ^{*7} Control vs. aPLs$^{(-/+)}$-IgG, $P < 0.5$.

the independent effect of the two aPLs (aCL/β_2GPI and aPS/PT) by administering IgG fractions of either single- or double-positive ones. As a result, we revealed that TF expression and TNF-α production from mononuclear cells were enhanced both by aCL/β_2GPI and by aPS/PT independently of each other. Furthermore, the stimulatory action was more pronounce with use of the IgG double-positive fraction for both antibodies. These experimental results matched well with our clinical findings of a high incidence of arterial/venous thrombosis in APS patients who possess both aCL/β_2GPI and aPS/PT together, which is clearly documented in our case series (Tables 1, 2). This synergistic action of aCL/β_2GPI and aPS/PT promotes the production of TF and proinflammatory cytokines from mononuclear cells and plays a central role in thrombus formation both in arteries and in veins.

In understanding the roles of aPLs in triggering thrombus formation, it is important to consider the concept of a "cell-based model" [28] for the induction of coagulation, which has recently been regarded as playing a key role in pathological intravascular thrombus formation. For ease of discussion, we call this "cell-mediated coagulation induction" (CMCI). CMCI involves the activation of monocytes and/or endothelial cells with cell-surface expression of TF. A wide range of recent studies indicate that aPLs commonly found in SLE patients are a potent trigger of CMCI, with the resultant promotion of thromboembolic complications at relatively young ages. In the schematic illustration in Fig. 5, we propose a hypothetical mechanism with respect to how aPLs trigger enhanced thrombus formation. First, we confirmed in this study that IgG fractions of aPLs, especially aCL/β2GPI and aPS/PT, stimulated the expression of TF on monocytes and the release of TNF-α (#1). TF then triggers a cascade activation of coagulation factors: i.e., TF/FVIIa complex activates FXa (#2) and promotes the formation of initial thrombin (#3) [29]. As a chain reaction, the initial thrombin subsequently triggers positive feedback activation of FV-FVIII-FXI and platelets (#4). The activated platelets then induce the formation of Xase and prothrombinase complex on their cell-surface phospholipid (#5). As a net effect of this series of triggering events, the coagulation process is enhanced tens of thousands of times, culminating in a so-called thrombin burst (#6). In this complicated network of CMCI events, APC is known to exert its anticoagulatory function through selective inhibition of cofactors Va and VIIIa, with subsequent control of coagulation through the reduced formation of Xase and prothrombinase (#7). Furthermore, as confirmed in the present study, this pathologically activated coagulation process is further promoted by aPLs, especially aCL/β2GPI and aPS/PT, with the suppressive action of APC. We hypothesize that the suppression of APC by antiphospholipid antibodies is exerted by inhibiting selective degradation of factors Va and VIIIa in Xase and prothrombinase (#8). As an additional pro-coagulatory action of aPLs, in our recent study, we reported that aPLs is also involved in CMCI as a potent activator of platelets (#9) [7]. In summary, we surmise that aPLs are involved in multiple key triggering events leading to a thrombin burst, thus causing unusual thromboembolic complications in relatively young patients possessing aPLs.

As limitations of this study, only a small number of APS patients with known antibody characteristics were available for analyzing the in vitro effects of the IgG fraction of aPLs. Therefore, we need to confirm the reproducibility of our present findings. Besides, we evaluated the in vitro action of aPLs IgG against mononuclear cells only. As a next investigation, it will be necessary to examine the possible effects of aPLs IgG on other types of cells involved in thrombus formation, such as endothelial cells and/or lymphocytes, by setting up a co-culture system composed of monocytes, lymphocytes, and/or endothelial cells to mimic an in vivo situation more closely.

5. Conclusion

In this study enrolling 97 SLE patients and 38 healthy volunteers, aPLs were positive in 31% (30/97) and 0.0%, respectively, and 27% (26/97) of the SLE patients had a positive APC resistance screening test. Thromboembolic complications among the SLE patients were closely associated with the existence of aCL/β_2GPI and/or aPS/PT, with an increased prevalence seen among those possessing both antibodies together. In in vitro assays, IgG fractions of aPLs(+) patients led to

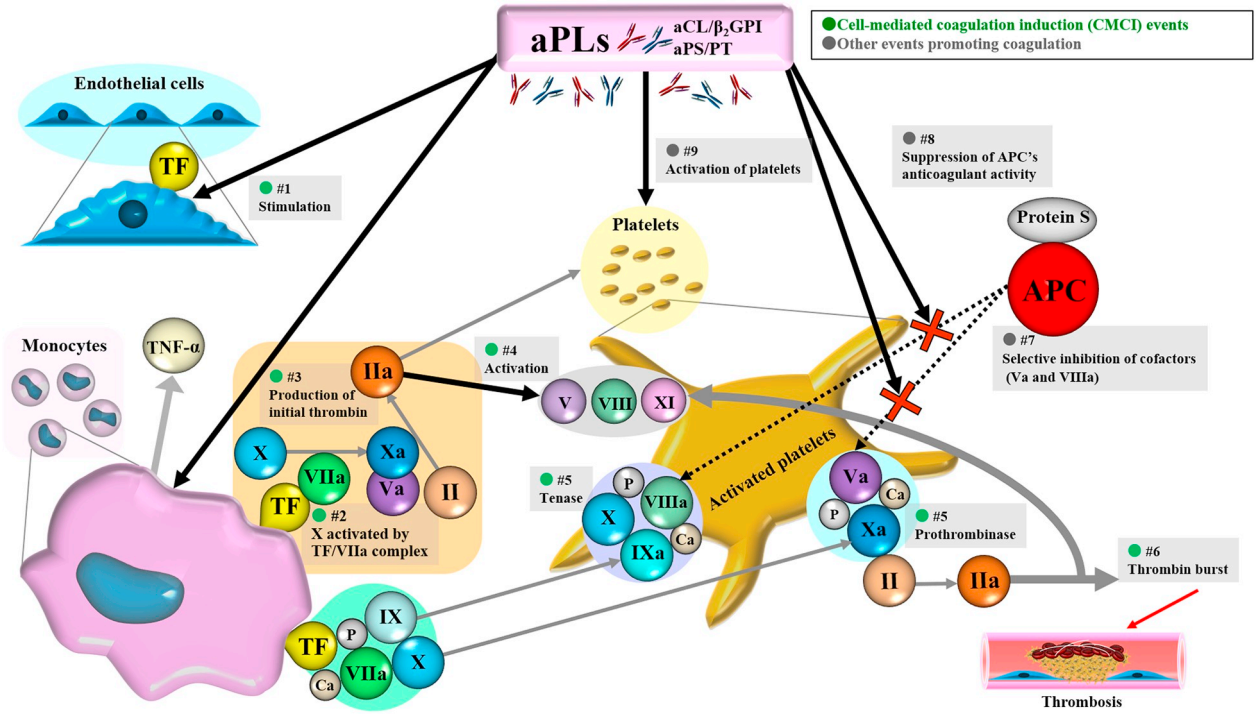

Fig. 5. Roles of aPLs in cell-mediated coagulation induction (CMCI) and suppression of APC. CMCI [28] is regarded as a key mechanism of pathological intravascular thrombus formation. See text for explanations in reference to the gray shaded numbers (#1–#9). APC: activated protein C; TF: tissue factor; aPLs: anti-phospholipid antibodies; aCL/β2GPI: anti-cardiolipin/β2-glycoprotein I antibodies; aPS/PT: anti-phosphatidylserine/prothrombin antibodies.

enhanced TF expression on monocytes and release of TNF-α in culture medium, and the suppression of APC in an APTT-based APC resistance assay. These effects of aPL IgG fractions were more pronounced among aCL/β_2GPI and aPS/PT double-positive cases, implying their synergic action.

We assume that aPLs are involved in multiple pro-coagulatory processes, including CMCI (through activation of monocytes, platelets, and endothelial cells) and the inhibition of APC, and thus lead to unusual thromboembolic complications in SLE patients. These modes of the thrombogenic actions of aPLs could be an important target for developing specific measures to prevent complications of thrombosis.

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Declaration of Competing Interest

We declare that we have no conflicts of interest.

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