Original article

Increase in plasma thrombin-activatable fibrinolysis inhibitor (TAFI) may not contribute to thrombotic tendency in antiphospholipid syndrome because of inhibitory potential of antiphospholipid antibodies toward TAFI activation

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Running title: TAFI activation in antiphospholipid syndrome

Key words: antiphospholipid syndrome, thrombosis, thrombin-activatable fibrinolysis inhibitor, systemic lupus erythematosus

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Abstract

Background: The causes of thrombosis in antiphospholipid syndrome (APS) remain unknown, though several hypotheses in regard to hypofibrinolysis have been proposed. *Objective:* To clarify the mechanism, we measured plasma levels of thrombin-activatable fibrinolysis inhibitor (TAFI) in APS patients. *Methods and Results:* Both the TAFI antigen (TAFI:Ag) level measured with an ELISA, and thrombin-thrombomodulin-dependent TAFI activity (TAFI:Ac) were elevated in 68 APS patients as compared with those in 66 healthy controls, though they were lower than those in 46 patients with autoimmune diseases. As for the influence of antiphospholipid antibodies (aPL) on TAFI levels, the mean TAFI:Ac level in 39 SLE patients positive for APS was significantly lower than that in 27 SLE patients without APS, whereas there was no difference in TAFI:Ag between those groups. Furthermore, purified IgG from patients positive for aPL, and monoclonal aPL (EY2C9 and 23-1D) inhibited the activation of TAFI in a concentration dependent manner. *Conclusion:* These results suggest that aPL inhibits TAFI activation by affecting the function of thrombomodulin-thrombin complex through phospholipids. Although TAFI in plasma is elevated in autoimmune diseases including APS, we concluded that an elevated level is not likely a risk factor for thrombosis in APS patients, because of the inhibition of TAFI activation by aPL.

Introduction

Antiphospholipid syndrome (APS) is defined as the occurrence of arterial and/or venous thrombosis associated with persistently positive results for antiphospholipid antibodies (aPL), and is a cause of early pregnancy morbidity [1]. Laboratory detection of aPL can be performed with either a solid phase enzyme linked immunosolvent assay (ELISA) or fluid phase coagulation assay for lupus anticoagulant (LAC), which has been shown to prolong phospholipid-dependent clotting time. For aPL that can be detected by ELISA, anticardiolipin antibody (aCL), anti-β2-glycoprotein I (β2GPI) antibodies and phosphatidylserine-dependent anti-prothrombin antibody (aPS/PT) are useful for the diagnosis of APS [2, 3]. Although the mechanism of thrombosis in APS remains unclear, disturbances in fibrinolytic activity by aPL have been reported. In a previous study, the plasma concentration of plasminogen activator inhibitor (PAI)-1 was shown to be increased in patients with APS as compared to that in control subjects [4], while another suggested that the activity of tissue plasminogen activator (tPA) was reduced by the presence of antibodies against tPA in this syndrome [5].

In 1995, Bajzar [6] reported that the antifibrinolytic effect of thrombin during fibrinolysis was due to activation of a proenzyme, termed thrombin-activatable fibrinolysis inhibitor (TAFI). Later, amino-terminal sequencing demonstrated that TAFI, procarboxypeptide U, procarboxypeptide R, and plasma procarboxypeptide B are identical [7, 8, 9]. TAFI is activated *in vitro* by several trypsin-like enzymes, such as

thrombin, meizothrombin, plasmin, trypsin, and neutrophil elastase [6, 9, 10, 11], while another study found that the endothelial cell receptor thrombomodulin stimulated the activation of TAFI by thrombin by 1250-fold [12]. By removing the C-terminal lysine residues generated by the action of plasmin on fibrin, activated TAFI inhibits the enhanced cofactor activity of partially degraded fibrin and promotes the inhibition of plasmin by α_2-antiplasmin [13, 14, 15]. Furthermore, as long as activated TAFI (TAFIa) remains in plasma above a certain threshold level, fibrinolysis does not accelerate, but rather remains in the initial phase [16, 17].

TAFI attenuates fibrinolysis, thus it might contribute to the pathophysiology of arterial and venous thrombosis. Epidemiological studies have shown that elevated TAFI is a mild risk factor for development of first occurrence or recurrent venous thrombosis [18, 19, 20], though findings showing an association between TAFI levels and risk of arterial disease are inconsistent [21]. Several case-control studies have shown high TAFI levels to be associated with a risk of coronary artery disease [22, 23], whereas elevated TAFI was also reported to be related to a decreased risk of myocardial infarction [24]. In another case-control study, TAFI antigen levels were decreased, while high TAFI activity increased the risk of myocardial infarction in young patients [25]. Also, a recent study of patients with ischemic stroke found that TAFI was decreased significantly in the first 72 hours after stroke onset and then returned to the baseline level [26]. Recently, the levels of circulating TAFI were reported to be strongly influenced by polymorphisms in the promoter and 3’ untranslated region of the TAFI gene [27], and to have an effect on the risk of venous thrombophilia [28]. The

functional polymorphism in the promoter region is located at the amino acid positions 147 and 325 [28].

To our knowledge, there is no report on the role of plasma TAFI levels in patients with aPL, thus we investigated whether a change in TAFI level contributes to thrombotic tendency in APS. In the present study, we measured the plasma levels of TAFI in patients with APS and compared the results with healthy controls, and also investigated whether the presence of aPL has an effect on the activation of TAFI through thrombin-thrombomodulin complexes.

Materials and Methods

Patients

We studied 68 Japanese patients with APS (mean age: 46.0 years old; 7 males, 61 females), who fulfilled the Sapporo criteria for APS [29], as shown in Table 1. Of them, 27 were patients with primary APS, while the others had secondary APS with mainly systemic lupus erythematosus (SLE) as the underlying disease. Clinical records were carefully reviewed retrospectively and/or the patients were interviewed at the time of sample collection. Thirty-one patients had arterial thrombosis, 26 had venous thrombosis, and 18 had experienced pregnancy morbidity. Arterial events included stroke, myocardial infarction, and iliac artery occlusion, which were confirmed by computed tomography scanning, magnetic resonance imaging, or angiography. Deep vein thrombosis and pulmonary thromboembolism were defined as venous thrombosis, and were confirmed by angiography or a scintigram. Furthermore, we studied 46 Japanese patients with autoimmune disorders (mean age: 42.4 years old; 8 males, 38 females), including 27 SLE patients. Of them, 2 patients were positive for aCL, though they had no APS symptoms. In addition, 66 healthy volunteers (mean age 45.8 years old; 14 males, 52 females) with normal coagulation test results were enrolled and served as controls.

Purification of IgG

Purified IgG was isolated from the peripheral blood of 8 patients, who were

positive for both aCL and aPS/PT, and 6 healthy donors by a protein G-sepharose (Pharmacia, Uppsala, Sweden) column (8 x 48 mm) chromatography method. The amount of purified IgG was measured using a BCA protein assay (Pierce, Rockford, IL, USA). We also used purified human IgM as control IgM, which was obtained from Sigma (St. Louis, MO, USA), to investigate the influence of monoclonal IgG and IgM on TAFI activation in the patients. Purified IgGs from the healthy subjects were used as control IgG.

Measurements of antiphospholipid antibodies

Anticardiolipin antibodies (aCL) were detected using ELISA, as previously described [30], and aCL sample titers were converted into Harris' standard titer, with values above 10.4 UPG and 29.8 UPM considered to be positive for IgG class and IgM class aCL, respectively. The levels of anti-phosphatidylserine/prothrombin antibodies (aPS/PT) were also determined by ELISA, as previously described [3]. IgG class and IgM class aPS/PT levels were determined in all patients and controls. APS/PT sample titers were converted into Atsumi's standard titer, with a cutoff of 2.0 units for IgG and 13.0 units for IgM [3].

Using an opto-mechanical coagulation analyzer (ST4; Diagnostica Stago, Asnieres, France), 3 clotting tests were performed for lupus anticoagulant (LAC) determination, according to the guidelines recommended by the Subcommittee on Lupus Anticoagulant/Phospholipid-Dependent Antibodies [31]. For activated partial thromboplastin time (aPTT), PTT-LA (Diagnostica Stago) was used for screening. A

mixing test (plasma samples: normal pooled plasma concentrations of 1:1, 1:4, and 1:9) was used to confirm the presence of LAC. A kaolin clotting time (KCT) test and confirmation via a mixing study were performed in the same manner. A dilute Russell's viper venom time (dRVVT) test was used to screen and confirm the presence of LAC, using a DVV-screen test and DVVT-confirm reagents (American Diagnostica, Greenwich, CT, USA).

Measurement of TAFI

The plasma level of TAFI antigen (TAFI:Ag) was measured using an ELISA kit (Affinity Biologicals, Ontario, Canada) according to the manufacturer's instructions. A standard curve was drawn up using diluted pool plasma obtained from 47 healthy donors. Since a large variation in average plasma concentration and range of TAFI:Ag (4.4-15.0 μg/ml) has been reported in healthy subjects [32], the data from the samples are shown as the percentage of standard pool plasma obtained from those 47 healthy donors.

TAFI activity (TAFI:Ac) was determined using an Actichrome TAFI kit (American Diagnostica). Briefly, TAFI in plasma samples was activated by the addition of thrombin-thrombomodulin complex, after which TAFI:Ac was determined with a chromogenic substrate. The levels of TAFI:Ac in the samples were obtained by subtracting the level of activated TAFI (TAFIa) retained in the sample from that level after thrombin-thrombomodulin complex was added to the sample. Data from the samples are shown as the percentage of standard pooled plasma.

Influence of antiphospholipid antibodies on activation of TAFI

To investigate the influences of aPL on the activation of TAFI, monoclonal aPL (EY2C9 and 23-1D) were mixed with pooled normal plasma from 6 of the healthy plasma donors at final concentrations of 16.0, 32.0, and 64.0 μg/ml, after which TAFI:Ac levels in the mixtures were determined using an Actichrome TAFI kit. Those levels were then compared with those in pooled normal plasma after adding normal human IgG, which was isolated from 6 healthy donors. This examination was repeated 6 times with each normal IgG. EY2C9 is a human monoclonal aCL established from the peripheral blood lymphocytes of an APS patient with a high titer of aCL [33]. We also raised a mouse monoclonal aPS/PT, termed 23-1D, by immunization of human prothrombin into Balb/c mice, which showed a high binding activity to phosphatidylserine-prothrombin complex, but little binding to prothrombin directly immobilized on an irradiated ELISA plate.

We also investigated the influence of purified IgG obtained from the plasma of 8 APS patients on the activation of TAFI in pooled normal plasma. Plasma samples were isolated from patients positive for both aCL and aPS/PT, whose volume was adequate for purification of IgG using a chromatography technique. In addition, control IgG samples were purified from the plasma of 2 healthy subjects. The IgG samples from the 8 patients and 2 control subjects were added to pooled normal plasma from 6 of the healthy plasma donors at final concentrations of 84.5, 168.9, and 337.8 μg/ml, after which TAFI:Ac levels in the mixtures were determined using an Actichrome TAFI kit.

Those levels were then compared with that in the pooled normal specimen in the absence of purified IgG.

Statistics

All data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using an unpaired Student's t-test or Welch's t-test. P-values <0.05 were considered to be statistically significant.

Results

TAFI levels in plasma samples from APS patients

The level of TAFI:Ag in APS patient plasma samples was 127.4 ± 28.8%, which was a significant increase as compared with that in the control group (101.5 ± 26.1%, $p<0.001$) (Fig. 1A). There was no significant difference in TAFI:Ag level between the PAPS (124.3 ± 25.6%) and SAPS (130.9 ± 30.9%) patients. The level of TAFI:Ag in patients with autoimmune diseases (AI) was 141.5 ± 37.1%, which was significantly higher than that in the APS patients ($p=0.024$) and the control group ($p<0.001$). The levels of TAFI:Ac in patients with APS and AI were 110.4 ± 15.3% and 122.8 ± 17.6%, respectively (Fig. 1B), which were both significantly higher than that in the control group (99.6 ± 15.5%, $p<0.001$ vs. APS, $p<0.001$ vs. AI). Furthermore, the level of TAFI:Ac in patients with AI was significantly higher than that in the APS patients ($p<0.001$). Although there was no significant difference in TAFI:Ac level between PAPS (111.3 ± 12.6%) and SAPS (109.9 ± 17.1%) patients, TAFI:Ac in those with SAPS was significantly lower than that in the AI patients ($p<0.001$).

Influence of aPL on TAFI levels in SLE patients

TAFI:Ag and TAFI:Ac were investigated in 27 SLE patients without APS as well as in 39 SLE patients with APS. There was no significant difference in TAFI:Ag between those patient groups (SLE with APS: 131.4 ± 31.8%, SLE without APS: 147.3 ± 38.8%) (Fig. 2A). However, the TAFI:Ac level in SLE patients without APS (126.3 ±

18.3%) was significantly higher than that in those with APS (109.5 ± 16.8%, $p<0.001$) (Fig. 2B).

To investigate the influence of the presence of aPL on the plasma level of TAFI:Ag and TAFI:Ac, we divided the SLE patients with and without APS into those positive and negative for aPL, and compared their TAFI levels, with the results shown in Table 2. The level of TAFI:Ag was not affected by the presence of aPL, however, TAFI:Ac in patients positive for aPS/PT (n=27; 111.1 ± 14.1%) was significantly lower than in those negative for aPS/PT (n=39; 119.9 ± 16.3%, $p<0.05$). Also, TAFI:Ac in the patients with LAC (n=41) was significantly lower (108.4 ± 17.6%) than in those without LAC (n=25; 124.8 ± 18.6%, $p<0.001$). Furthermore, the level in patients with aCL (n=47) was lower than that in those without aCL (n=19), though the difference was not statistically significant.

Influence of aPL on activation of TAFI

We also investigated the influences of 8 purified IgG samples from patients with APS and monoclonal aPL (EY2C9 and 23-1D, respectively) on the activation of TAFI. Although normal IgG and IgM did not have an influence on the activation of TFAI, 32.0 μg/ml and 62.0 μg/ml of EY2C9 (monoclonal aCL) significantly inhibited the formation of TAFI:Ac (93.9 ± 3.3% and 92.5 ± 1.1%, respectively) (Fig. 3). In addition, TAFI:Ac was significantly reduced by the addition of 32.0 μg/ml and 62.0 μg/ml of 23-1D (monoclonal aPS/PT) to 91.3 ± 2.6% and 85.0 ± 2.7%, respectively. Furthermore, 7 of the 8 IgG samples purified from patients positive for both aCL and

aPS/PT inhibited the activation of TAFI in pooled normal plasma in a concentration-dependent manner, whereas the 2 control IgG samples did not have an effect on TAFI activation (Table 3).

Discussion

There is increasing evidence that aPL play a role in the hyper-coagulable state of APS patients, as these antibodies are able to inhibit anticoagulant activity, such as the protein C-protein S system via phospholipid-binding proteins [34, 35, 36], and fibrinolytic function [37, 38, 39]. aPL activate endothelial cells by recognizing the complex of phospholipid-binding proteins and phospholipids on the cell surface, and induce procoagulant substances such as tissue factor [40, 41]. However, the responsible mechanisms remain unclear.

APS symptoms include both venous and arterial thrombosis, thus there is a possibility that the concentration of TAFI in plasma is related to thrombotic tendency in APS patients. Although the association between TAFI level and risk of arterial disease has not been established, several epidemiological studies have indicated a relationship between TAFI plasma concentration and thrombotic tendency. In the present study, we found increased levels of plasma TAFI:Ag in APS patients. However, elevated TAFI:Ag is not specific to APS, as TAFI:Ag levels in patients with AI are also increased as compared with healthy subjects. Previously, TAFI:Ag levels in patients with Behçet's disease were reported to be significantly higher than those in healthy controls regardless of thrombosis manifestation [42]. It has also been suggested that TAFI is an acute phase protein [43, 44] and plays a role in regulating inflammation [45], and that regulation of TAFI mRNA expression is mediated by cytokines such as interleukin (IL)-6 and IL-1β [46]. In APS as well as AI, the plasma concentrations of the inflammatory cytokines

IL-6 and IL-1β are higher than those in healthy subjects [47, 48]. Thus, it is possible that these findings explain the increased TAFI levels in patients with autoimmune diseases, including those with APS.

The present results suggest that a high level of plasma TAFI:Ag is one of the causes of thrombogenic state in APS patients. However, plasma TAFI:Ag in patients with AI was significantly higher than that in APS patients. Furthermore, TAFI:Ag levels in patients with SAPS, who had AI as an underlying disease, were similar to those in PAPS patients. Thus, we investigated TAFI levels in the present SLE patients after dividing them into groups based on positive or negative aPL findings. Our results showed that TAFI:Ac in SLE patients positive for aPL was significantly lower than that in those negative, though there was no difference in TAFI:Ag levels between the 2 groups (Fig. 2). Although the discrepancy between TAFI:Ag and TAFI:Ac was also found in the AI group, for which we have no reasonable explanation, we observed a statistically significant decrease of TAFI:Ac in patients with aPL as compared to those without aPL. Considering that TAFI:Ac is formed in the presence of thrombin-thrombomodulin complex, these results suggest that aPL may exert an inhibitory effect on the activation of TAFI caused by that complex.

In subjects with aPL, especially with aPS/PT and/or LAC, the inhibition of fibrinolysis by TAFI may be weaker than expected, in spite of an increased plasma TAFI concentration (Table 2). In addition, the inhibitory capacity of TAFI activation by monoclonal aCL was only 6.1-15.0% of TAFI activation in the controls, as shown in Figure 3. Furthermore, 168.9 and 337.8 μg/ml of purified IgG including both aCL and

aPS/PT decreased TAFI activation by only 14.5% and 21.7%, respectively. Such inhibition may have an effect on fibrinolytic activity, because changes in TAFI level, reported as a risk factor in a previous report [32], also ranged from about 10-50% of that in the controls.

aPL can bind with complexes of phospholipid binding proteins (such as prothrombin or β2GPI) and phospholipids, which also bind with thrombomodulin-thrombin complex. Thus, it seems that aPL inhibits the function of the thrombomodulin-thrombin complex and phospholipids toward the activation of TAFI in the in vitro assay, though definitive proof of a causal relationship has not been shown. A number of reports have shown that aPL can inhibit the function of thrombomodulin [49]. Keeling [50] reported that β2GPI inhibits thrombomodulin-thrombin dependent activation of protein C in the presence of phospholipids. Furthermore, β2GPI inhibits the function of phospholipids and the antibody to β2GPI may enhance the inhibitory activity of β2GPI on phospholipids [34]. (Also, it has been reported that certain pathogenic aPL may cross-react with thrombomodulin and induce down-regulation of thrombomodulin on endothelial cells [51].) Oosting [52] previously reported that autoantibodies against thrombomodulin were transiently present in sera from SLE patients with aPL. IgG fractions of those serum samples inhibited the activation of protein C and bound to a recombinant version of the EGF-like portion of thrombomodulin. In addition, it has been demonstrated that the levels of expression of thrombomodulin on endothelial cells are reduced by internalization of the thrombomodulin-thrombin complex in the presence of the antibody to thrombomodulin

[53].

In the present study, the plasma level of TAFI was found to be elevated in APS patients, which may produce a condition in which thrombosis easily occurs, though that elevation is not specific to APS. However, it is also possible that aPL exert an inhibitory effect on the activation of TAFI by the thrombin-thrombomodulin complex. Therefore, we concluded that an elevated level of TAFI is not a definite risk factor for thrombosis development in APS patients.

Acknowledgements

We would like to thank Hiroko Juraku for the technical assistance as well as Yayoi Ishida for help in preparation of the manuscript.

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Figure Legends

Fig. 1. Plasma levels of TAFI antigen (TAFI:Ag) and TAFI activity (TAFI:Ac) in antiphospholipid syndrome.

[A] The level of TAFI antigen (TAFI:Ag) in patients with APS was significantly higher than that in the healthy controls ($p<0.001$), whereas it was lower than that in patients with AI ($p<0.05$). There was no significant difference in TAFI:Ag levels between PAPS and SAPS patients. [B] The level of TAFI activity (TAFI:Ac) in patients with APS was higher than that in the healthy controls ($p<0.001$) and was significantly lower than in AI ($p<0.001$). Furthermore, the level in SAPS was lower than that in AI ($p<0.001$), whereas there was no difference in level between PAPS and SAPS patients.

TAFI:Ag: TAFI antigen, TAFI:Ac: TAFI activity, APS: antiphospholipid syndrome, PAPS: primary APS, SAPS: secondary APS, AI: autoimmune diseases. *: $p<0.001$ vs. control, NS: not significant.

Fig. 2. Plasma levels of TAFI:Ag and TAFI:Ac in SLE patients with or without APS.

[A] There was no significant difference in TAFI:Ac level between SLE patients with (n=39) and without APS (n=27). [B] The level of TAFI:Ac in patients with APS was significantly higher than that in those without APS ($p<0.001$).

APS: antiphospholipid syndrome. *: $p<0.001$ vs. control, **: $p<0.005$ vs. control.

Fig. 3. Influences of monoclonal antiphospholipid antibodies on activation of TAFI.

IgG (open circles) and IgM (open squares) from healthy subjects did not have an effect on the activation of TAFI. In contrast, addition of the monoclonal anticardiolipin antibody (EY2C9; closed squares) and

monoclonal antibody to phosphatidylserine-prothrombin complex (23-1D; closed circles) inhibited the activation of TAFI in a concentration dependent manner.

*: $p<0.05$ vs. control, **: $p<0.01$ vs. control, #: $p<0.01$.

Table 1. Subject characteristics

1. Antiphospholipid syndrome (APS)	68
Male : Female = 7 : 61, 46.0 years old (27 - 73)	
(1) Primary APS (PAPS)	27
(2) Secondary APS (SAPS)	41
SLE: 39, RA: 1, MCTD: 1	
2. Autoimmune diseases (AI)	46
Male : Female = 8 : 38, 42.4 years old (22 - 66)	
SLE: 27, PSS: 7, SjS: 6, RA: 4, others: 2	
3. Healthy control	66
Male : Female = 14 : 41, 45.8 years old (19 - 71)	

SLE: systemic lupus erythematosus, RA: rheumatoid arthritis, MCTD: mixed connective tissue disease, PSS: progressive systemic sclerosis, SjS: Sjögren's syndrome.

Table 2. Relationship between aPL and TAFI in SLE

(n=66)

	aPL	TAFI:Ag (%)	TAFI:Ac (%)	
aCL	positive (47)	132.8 ± 29.2	111.2 ± 15.3	
	negative (19)	141.3 ± 37.3	118.4 ± 16.4	
aPS/PT	positive (27)	138.6 ± 30.0	111.1 ± 14.1	*
	negative (39)	138.5 ± 37.6	119.9 ± 16.3	
LAC	positive (41)	130.9 ± 32.6	108.4 ± 17.6	*
	negative (25)	147.4 ± 38.3	124.8 ± 18.6	
Control (66)		101.5 ± 26.1	99.6 ± 15.5	

aCL: anticardiolipin antibodies, aPS/PT: Phosphatidylserine dependent antiprothrombin antibodies, LAC: lupus anticoagulant

*: $p<0.05$ between aPL positive and aPL negative.

Table 3. Inhibition of TAFI activity by purified IgG from patients with APS

Purified IgG		TAFI:Ac (%)		
	(μg/ml)	84.5	168.9	337.8
APS	No. 3	91.5	90.1	82.5
	6	83.8	78.7	74.3
	7	88.7	79.5	73.0
	17	96.1	93.9	78.4
	24	95.9	89.2	64.9
	29	102.7	96.0	98.7
	30	80.0	78.2	70.3
	33	94.8	86.6	84.6
	(mean±SD	91.7±7.3	86.5±7.0	78.3±10.4)
Control	No. 1	98.1	102.7	99.5
	2	104.6	102.9	106.3

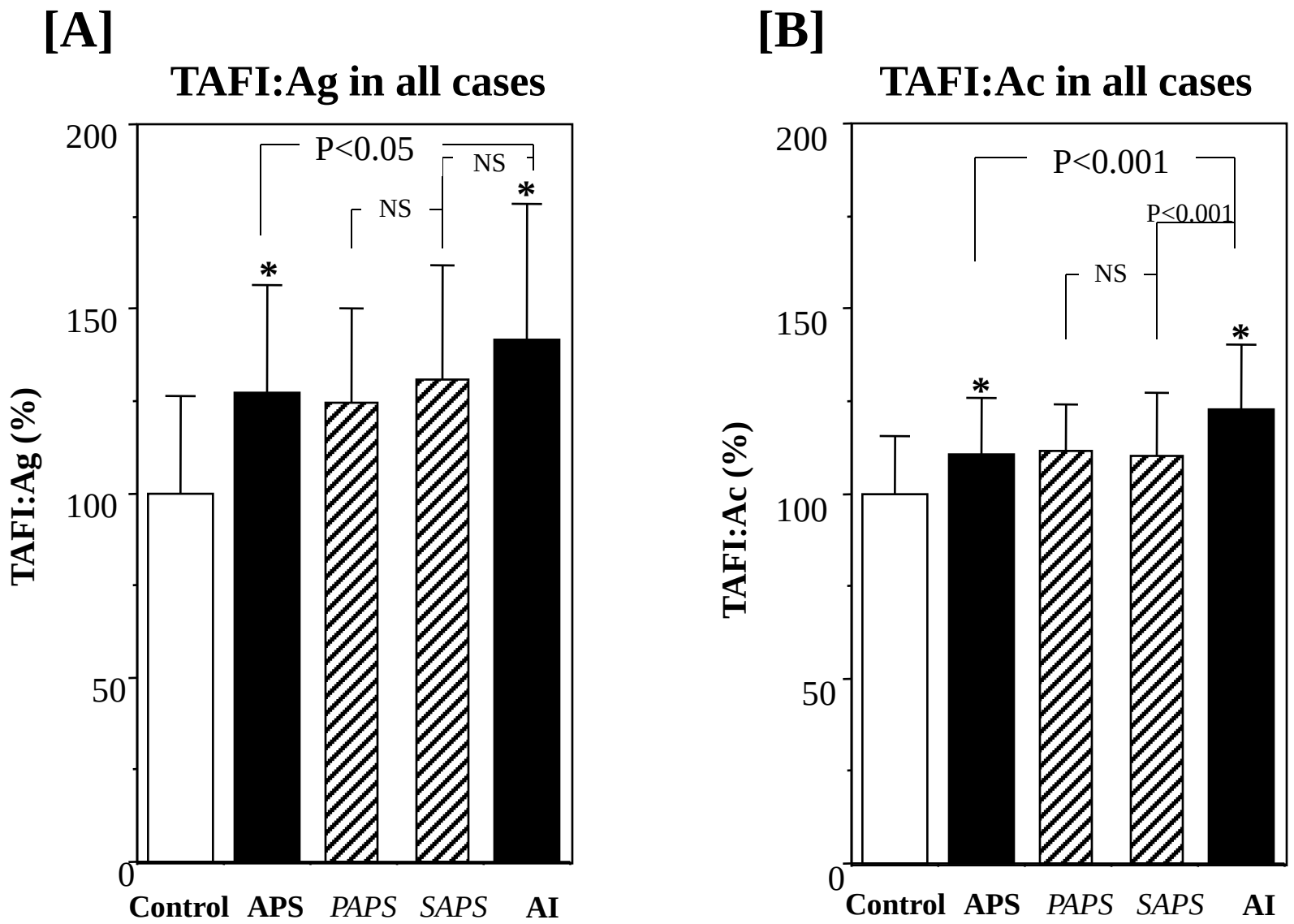
[A]
TAFI:Ag in all cases
TAFI:Ag (%)
200
150
100
50
0
P<0.05
NS
NS
*
*
*
Control
APS
PAPS
SAPS
AI
[B]
TAFI:Ac in all cases
TAFI:Ac (%)
200
150
100
50
0
P<0.001
P<0.001
NS
*
*
Control
APS
PAPS
SAPS
AI

Fig. 1

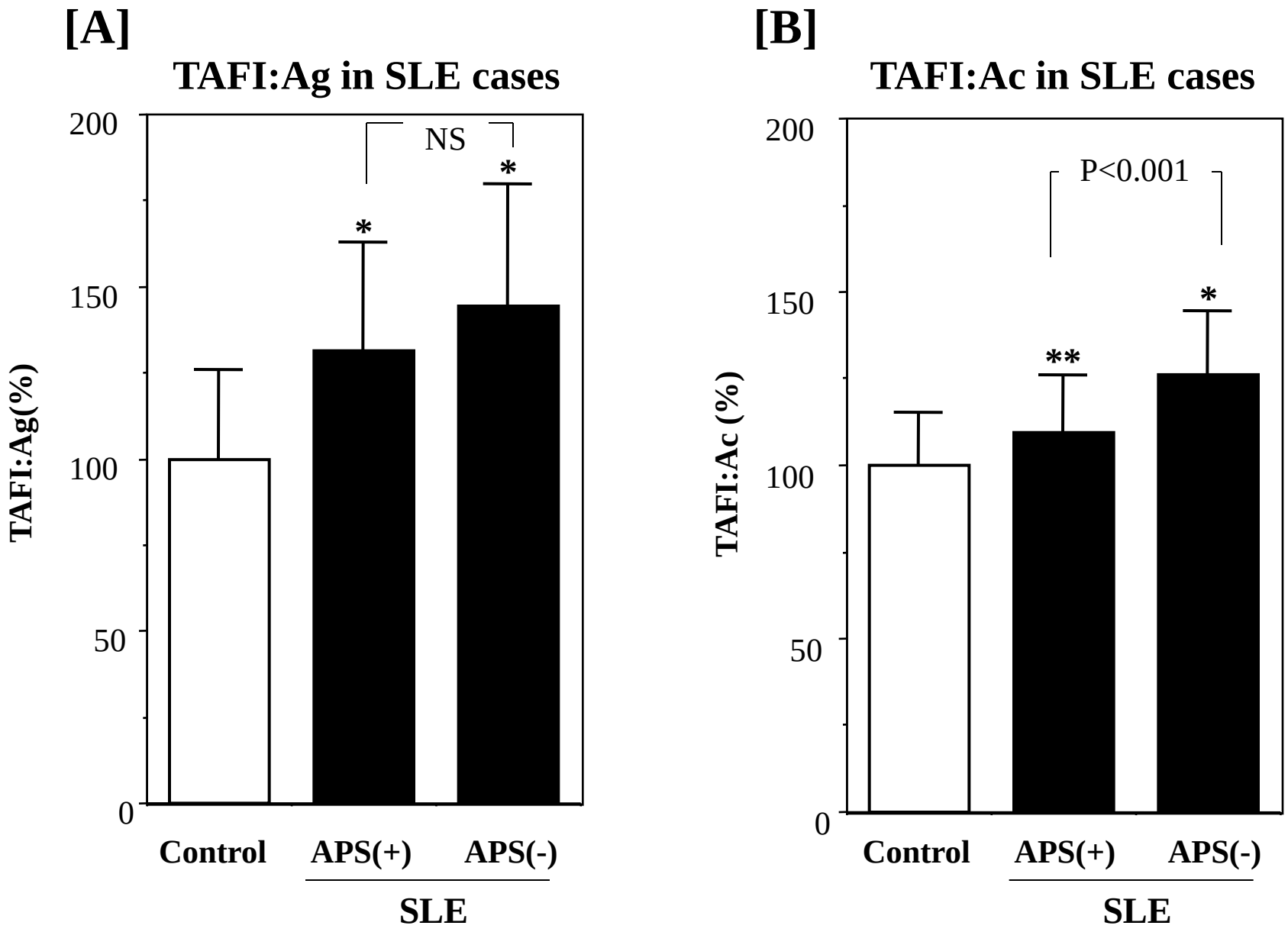
[A]
TAFI:Ag in SLE cases
TAFI:Ag(%)
200
150
100
50
0
NS
*
*
Control
APS(+)
APS(-)
SLE
[B]
TAFI:Ac in SLE cases
TAFI:Ac (%)
200
150
100
50
0
P<0.001
**
*
Control
APS(+)
APS(-)
SLE

Fig .2

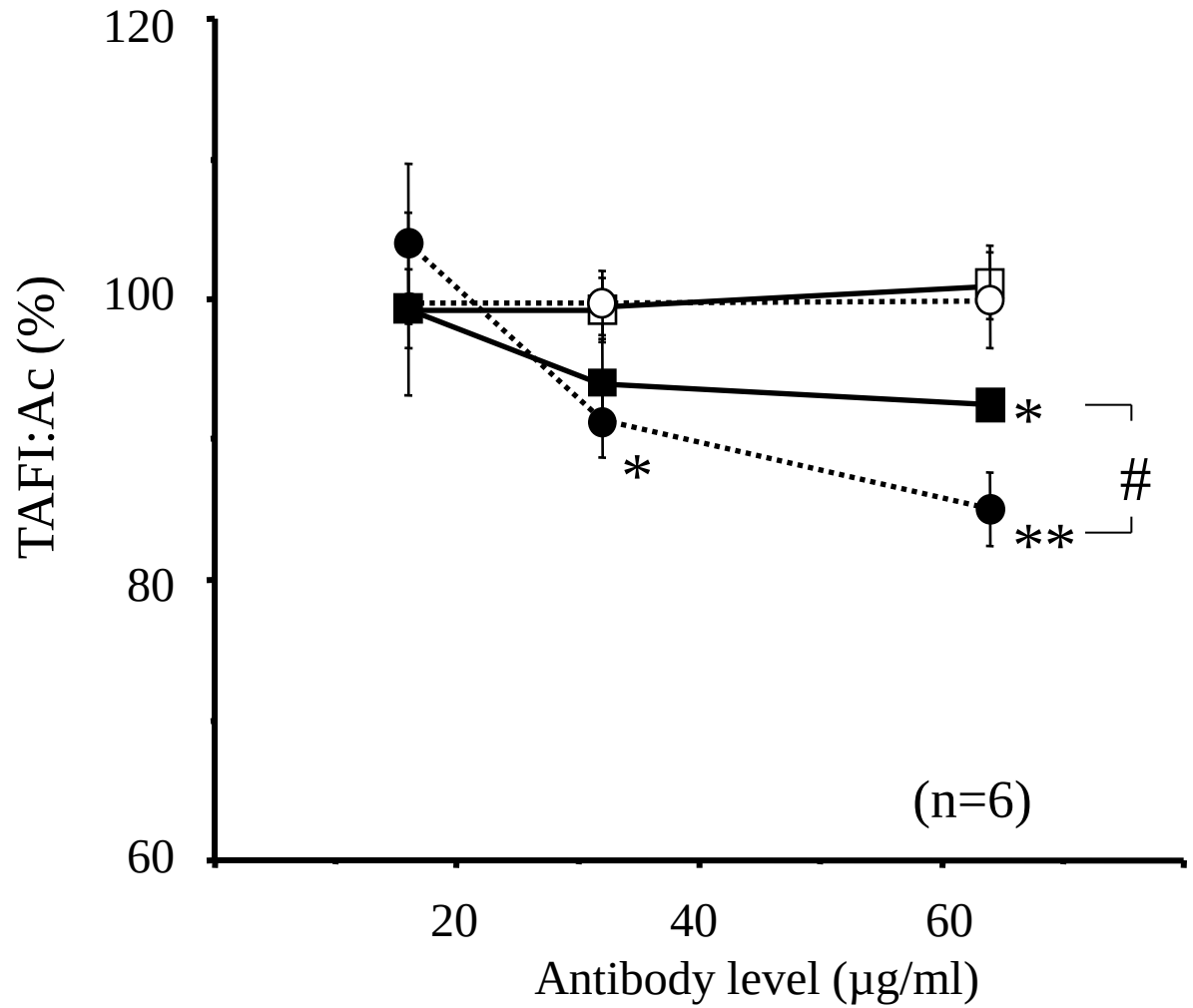

Fig. 3