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The inhibition of protein C anticoagulant activity by anti-β_2-glycoprotein I (β_2GPI) antibodies isolated from patients with antiphospholipid syndrome by chromatography methods

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Abstract Antiphospholipid antibodies (aPL) are associated with an increased risk of thrombosis; however, the mechanism remains unknown. Recent studies have focused on the impediment of protein C anticoagulant activity by anti-β_2-glycoprotein I (β_2GPI) antibodies (aβ_2GPI Ab). We purified IgG fractions containing a high concentration of aβ_2GPI Ab from patients with antiphospholipid syndrome (APS) and then investigated the effect of purified aβ_2GPI Ab on the activity of activated protein C (APC). Using a three-step chromatography method (DEAE-sepharose column, phosphatidylserine polyacrylamide gel column dependent on the presence of β_2GPI, and protein G column chromatography), we successfully isolated anti-β_2GPI IgG from nine patients with APS. Seven of nine samples inhibited APC activity in a concentration-dependent manner only in the presence of β_2GPI, as observed by a chromogenic assay that was able to determine thrombin activity even in the presence of APC. The extent of APC inhibition by these fractions appeared to be related to aβ_2GPI Ab titers of the purified IgG. However, the inhibitory effect of IgG from patients was not detected in the absence of β_2GPI. IgG purified from three normal subjects did not affect APC activity. Herein, we show a useful method for the isolation of IgG containing a high concentration of aβ_2GPI Ab. Moreover, the present findings indicate that inhibition by aβ_2GPI Ab on APC anticoagulant activity could explain one of the mechanisms for the thrombotic state in APS.

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Introduction

Antiphospholipid antibodies (aPL) are a heterogeneous group detected in patients with antiphospholipid syndrome (APS), well known to be associated with venous and arterial thrombosis and recurrent spontaneous abortion.[1–3] aPL are usually detected as anticardiolipin antibodies (aCL) by solid-phase immunoassays, or as a lupus anticoagulant when measured as an activity that prolongs lipid-dependent coagulation. It has been shown that β_2-glycoprotein I (β_2GPI) appears to provide the actual aCL-binding site(s) in major cases of APS.[4–6] Most aCL from patients with APS are autoantibodies to β_2GPI on anionic phospholipids, i.e., anti-β_2GPI antibodies (aβ_2GPI Ab). β_2GPI is a 50-kDa plasma glycoprotein first described in 1961.[7] This protein inhibits several hemostatic reactions in vitro[8–11]; however, its physiological role remains obscure.

For the development of thromboembolic events with APS, several hypotheses have been described. One possible mechanism is interference with the protein C-protein S system. Protein C is a vitamin K-dependent zymogen and can be activated by thrombin. When thrombin binds to the endothelial cell receptor thrombomodulin,[12] its activity toward protein C is greatly enhanced. Activated protein C (APC) acts as an anticoagulant factor by degrading factors Va and VIIIa.[13] Interference of aPL with the anticoagulant activity of the protein C system has been reported in several studies. Gali et al.[14] noted that aβ_2GPI Ab was associated with thrombotic events in APS and had a relationship with impairment of the anticoagulant activity of the protein C system. Recently, we developed a chromogenic assay that can determine thrombin activity even in the presence of APC by the addition of a soybean trypsin inhibitor (SBTI) as an inhibitor of protein C activity[15] and reported that monoclonal aβ_2GPI Ab, which was established from the periph-

eral blood lymphocytes of a patient with APS, can inhibit APC activity only in the presence of β_2GPI. Our next area of interest has been focused on whether the immunoglobulin G (IgG) in patients with APS inhibits the activity of protein C. We investigated the effect of aβ_2GPI Ab, which was isolated from the plasma of patients with APS by a chromatography method, on APC activity.

Materials and methods

Patients

Subjects comprised nine patients (Pt-1–Pt-9) found positive for aβ_2GPI Ab, as detected by enzyme-linked immunosorbent assay (ELISA). Seven of the nine patients were also positive for lupus anticoagulant (LA), as determined by a diluted Russell's viper venom test (American Diagnostica, Greenwich, CT, USA). Six patients had exhibited episodes of thromboembolism, and three had mild to severe thrombocytopenia (Table 1). Plasma from three healthy donors and pooled normal plasma (PNP) from 20 healthy adults with normal coagulation test results were used as controls.

Detection of anti-β_2GPI antibodies

Detection of β_2GPI-dependent aCL was determined using a standardized ELISA as previously described.[16] Activities of aβ_2GPI Ab in plasma diluted at 1:200 or in isolated IgG fractions diluted at 1:100 were determined. Absorbance at 405 nm was measured with a microtiter plate reader (MTP-120; Corona, Katsuta, Japan).

Purification of aβ_2GPI Ab

To isolate the IgG fraction containing aβ_2GPI Ab, citrate-treated plasma (2 ml) from the nine patients was diluted with Tris buffer saline (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.6), and diluted plasma was applied to a DEAE-sepharose (Pharmacia, Uppsala, Sweden) column (10 × 130 mm). The fraction eluted with Tris buffer saline containing 0.5 M NaCl was dialyzed with Tris buffer saline and applied to a phosphatidylserine (Sigma, St. Louis, Mo, USA) polyacrylamide gel column (8 × 80 mm) with the same volume of 0.2 mg/ml purified human β_2GPI (Yamasa Shoyu, Choshi, Chiba, Japan). The fraction eluted with 0.5 M NaCl was dialyzed and then applied to a protein G-sepharose (Pharmacia) column (8 × 48 mm) to concentrate the IgG as well as to exclude β_2GPI from the applied sample. The fraction eluted with 0.2 M glycine (pH 2.5) was dialyzed and concentrated with a Centriprep 30 filtration cell (Amicon, Beverly, MA, USA). The flow rate for each chromatography step was 40 ml/h. The amount of purified IgG was determined using a BCA protein assay (Pierce, Rockford, IL, USA). Control IgG was also isolated from healthy donors (three individuals and PNP) using DEAE-sepharose column and protein G-sepharose column chromatography. The purity of the isolated IgG was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblot analysis confirmed that β_2GPI was not contained in the isolated IgG fractions from the plasma samples (Fig. 1) or purified human coagulation factors used in this assay.

A phosphatidylserine polyacrylamide gel column was prepared as described by McNeil et al.,[17] with slight modifications. A solution of dissolved phosphatidylserine (5 mg) was evaporated under a stream of nitrogen, and the residue was dispersed in 1 ml Tris buffer saline by vigorous mixing with plastic beads. A portion (500 µl) of the resulting suspension was transferred to a glass tube, and 5 ml of a solution containing 15% acrylamide and 5% bis-acrylamide

Table 1. Characteristics of patients with antiphospholipid syndrome and normal controls

Subject	LA	aβ_2GPI Ab	Thrombotic events
1	(−)	L	(+) Thrombocytopenia
2	(−)	M	(+)
3	(+)	H	(+) Thrombocytopenia
4	(+)	H	(+) Thrombocytopenia
5	(+)	M	(+)
6	(+)	H	(+)
7	(+)	H	(+)
8	(+)	M	(+)
9	(+)	M	(+)
PNP	(−)	(−)	(−)
NC-1	(−)	(−)	(−)
NC-2	(−)	(−)	(−)
NC-3	(−)	(−)	(−)

LA, lupus anticoagulant; PNP, pooled normal plasma; NC, normal control; +, positive; −, negative; L, low positive (OD, 0.125–0.434); M, moderate positive (OD, –0.744); H, high positive (OD, 0.745–)

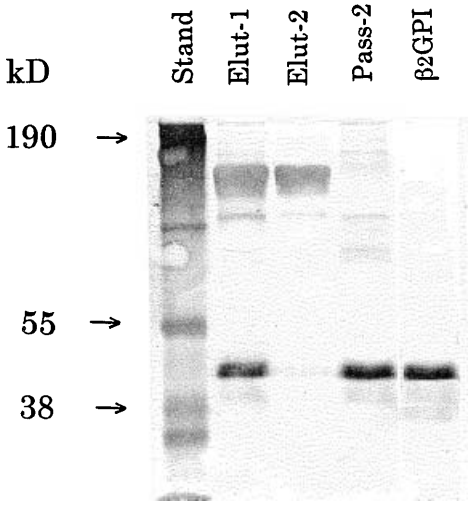

Fig. 1. Western blot analysis of anti-β_2-glycoprotein I (β_2GPI) from purified IgG. The purified IgG from patient 2 (Pt-2) was investigated. *β_2GPI* was contained in the elution fraction (*Elut-1*) from phosphatidylserine polyacrylamide gel column chromatography but not in the elution fraction (*Elut-2*) from protein G sepharose column chromatography. This protein was contained in the pass-through fraction (*Pass-2*) from protein G column chromatography. *St*, standard; *kD*, kDa

(Bio-Rad, Hercules, CA, USA) was added. After vigorous mixing and addition of 100 µl ammonium persulfate (100 mg/ml) and 4 µl *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), the mixture was allowed to polymerize for 3 h at room temperature. The gel was removed from the tube, rinsed with distilled water, and homogenized with a hand-operated loose-fitting Teflon pestle. After washing three times with distilled water, the homogenized gel was allowed to settle for 5 min and then transferred to a column and equilibrated with a solution containing 20 mM Tris-HCl (pH 7.4), 10 mM NaCl, and 1 mM $CaCl_2$ at a flow rate of 40 ml/h.

Determination of APC activity in thrombin generation

Measurements of APC activity were performed according to the method described previously.[15] Platelin (Organon Teknika, Durham, NC, USA), a phospholipid substitute for platelets, was used at a dilution of 1:15 in the assay buffer as the source of phospholipid.[18] Diluted phospholipid (20 µl), 20 µl of either purified human β_2GPI (final concentration, 3.4 µM) or assay buffer [20 mM Tris-HCl (pH 7.3), 150 mM NaCl, 1 mM $CaCl_2$, 0.1% bovine serum albumin (BSA)], and purified IgG (17.5, 35.0, 87.5, and 175.0 µg/ml) were transferred into each well of a 96-well microtiter plate (Costar), which was then incubated at 37°C for 20 min. Twenty microliters of protein S (25.9 nM) (Enzyme Research Laboratories, South Bend, IN, USA), 20 µl factor Xa (1.4 nM) (Hematologic Technologies, Essex Junction, VT, USA), 20 µl factor V (0.024 nM) (Hematologic Technologies), 20 µl prothrombin (1.2 µM) (Hematologic Technologies), and 20 µl of either APC (3.2 nM) (Enzyme Research Laboratories) or assay buffer were added to the preincubation mixture; the plate was then incubated at room temperature for 2 min. Coagulation was initiated by the addition of 50 µl 50 mM $CaCl_2$ to the mixture. After incubation at 37°C for 10 min, 50 µl 100 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4) and 25 µl of soybean trypsin inhibitor (20 mg/ml) were added to the mixture. A portion (100 µl) of the reaction mixture was then transferred to another well to which 50 µl 1.8 mM synthetic substrate S-2238 (Chromogenix AB, Möndal, Sweden) was also added. After incubation at 37°C for 5 min, the chromogenic reaction was stopped by adding 50 µl 50% acetic acid. Absorbance of the mixture was measured at 405 nm with a microtiter plate reader.

Thrombin activity difference, which was considered to be an activity of APC, was calculated by subtracting thrombin activity in the presence of APC from that in the absence of APC. Inhibition of APC activity by test samples was initially expressed as a percentage of that obtained with IgG purified from PNP.

Results

Isolation of aβ_2GPI Ab

IgG was purified from the plasma of nine patients, as well as from three healthy donors and PNP. The purified IgG was tested by ELISA for aβ_2GPI Ab. Absorbance units of aβ_2GPI Ab/mg purified IgG are shown in Fig. 2. All IgG samples from patients with APS were positive for aβ_2GPI Ab whereas those from the normal controls and PNP were negative.

Effects of aβ_2GPI Ab on APC activity

Addition of APC (1.6, 6.3, and 25.0 µg/ml) to the thrombin generation assay resulted in dose-dependent inhibition of thrombin formation (data not shown). The IgG from nor-

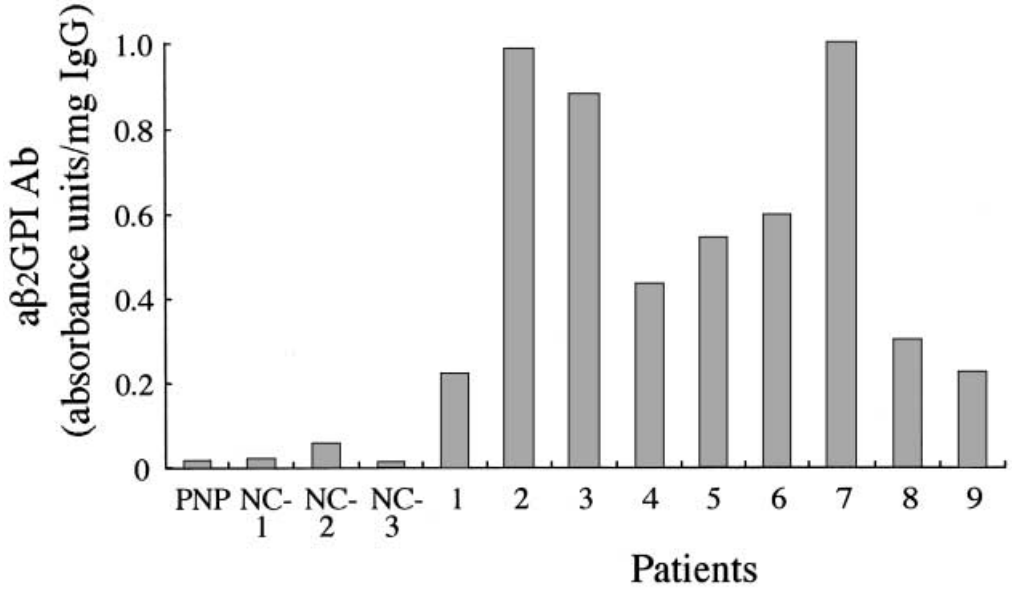

Fig. 2. Absorbance units of anti-β_2GPI antibodies (*aβ_2GPI Ab*) per purified milligram IgG in nine patients, three normal controls (*NC-1, -2, -3*), and pooled normal plasma (*PNP*). All IgG samples from patients with antiphospholipid syndrome (APS) were positive for aβ_2GPI Ab; however, those from normal controls and PNP were negative

mal controls did not affect APC activity. When IgG samples from normal plasma, including PNP at concentrations of 17.5, 87.5, and 175 μg/ml, were added to the assay system in the presence of 1.6 μg/ml APC, the APC activities were 99.5 ± 13.4, 114.0 ± 15.7, and 98.0 ± 15.7% (mean ± SD), respectively. When the purified aβ_2GPI Ab from patients was added, seven (Pt-2, -3, -4, -6, -7, -8, and -9) of these samples inhibited 1.6 μg/ml APC in a concentration-dependent manner (Table 2). The APC activity under the value subtracted SD from mean in normal controls was considered to be a significant decrease. At an added concentration of 17.5 μg/ml, the purified IgG from four (Pt-2, -3, -6, and -7) of these seven patients with a high titer of aβ_2GPI Ab (Fig. 2) reduced APC activity to 69.2%–82.7% of control values. At the added concentrations of 87.5 and 175 μg/ml, the purified aβ_2GPI Ab from these four patients reduced APC activity to 48.8%–74.0% and to 17.2%–59.0% of control values, respectively. The purified IgG from the other three patients (Pt-4, -8, and -9), which had a moderate aβ_2GPI Ab titer, had a less marked effect, reducing APC activity to 70.7%–78.9% of control values at an added concentration of 175 μg/ml. The purified IgG from Pt-5 showed a moderate aβ_2GPI Ab titer but did not inhibit APC activity. The purified IgG from Pt-1 showed a low aβ_2GPI Ab titer and only slightly increased APC activity.

To clarify the influence of β_2GPI on the inhibitory effect of APC by aβ_2GPI Ab, the same assay as just described was performed with a sample buffer added in place of β_2GPI. As a result, none of the aβ_2GPI Ab from the patients had an effect on APC activity. Figure 3 shows typical results obtained by the addition of purified IgG from Pt-3 and Pt-7.

Table 2. Effects of anti-β_2GPI antibodies from patients on activated protein C (APC) activity

Sample	APC activity (% control) Concentrations of IgG (μg/ml)			
	17.5	35.0	87.5	175
1	110.6	ND	142.6	146.0
2	69.2	65.4	55.4	37.2
3	82.7	69.6	68.2	59.0
4	92.8	80.8	83.1	70.7
5	101.5	ND	101.3	95.2
6	75.1	74.6	74.0	57.0
7	72.2	50.5	48.8	17.2
8	ND	ND	111.8	71.2
9	ND	80.1	109.9	78.9
NC-1	118.0	88.9	100.7	89.0
NC-2	87.1	84.8	128.1	93.2
NC-3	92.9	ND	127.0	109.9

ND, not determined

Discussion

Clarification of the mechanism of thrombosis in APS is important for the treatment of patients; however, this mechanism remains not clearly understood. Many possible mechanisms have been described, such as the inhibition of protein C activation by aPL,[19,20] the activation of platelets by aPL,[21,22] the activation of vascular endothelial cells by aPL,[23,24] a disturbance in the formation of thrombin–antithrombin III complexes by aPL,[25] the inhibition of fibrinolytic activity by aPL,[26,27] and the inhibition of annexin V

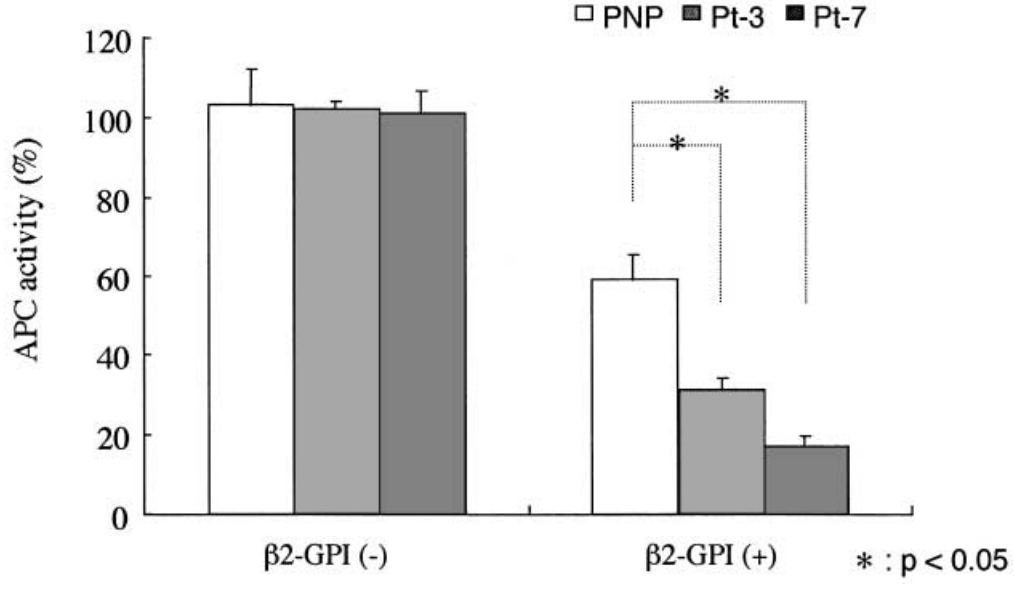

Fig. 3. Effect of aβ_2GPI Ab from Pt-3 and Pt-7 on protein C activity in the absence or presence of β_2-GPI. Values are means ± SD ($n = 4$) and are expressed as a percentage of activated protein C (APC) activity in the presence of purified IgG from PNP (in the absence or presence of β_2GPI, respectively). In the absence of β_2GPI, neither purified IgG from PNP (*open bars*) nor aβ_2GPI Ab from patients (Pt-3, *shaded bars*; Pt-7, *black bars*) exerted an influence on APC activity. However, in the presence of β_2GPI, APC activity decreased (to 59.6% of that in the absence of β_2GPI), even with PNP IgG. Furthermore, 175 μg/ml aβ_2GPI Ab from Pt-3 and Pt-7 inhibited APC activity to 31.7% and 17.8% IgG, respectively, when 3.4 μM β_2GPI was present

activity by aPL.[28] Moreover, some studies have suggested that a prothrombic state may be induced by the inhibition of protein C activity in the presence of aPL.[29–31]

As it has been clarified that β_2GPI serves as a major antigenic target of thrombosis-associated aPL,[4–6] the effect of aPL with β_2GPI has also been considered for thrombotic mechanisms. We have shown in this study that aβ_2GPI Ab inhibited the degradation of factor Va by APC. Furthermore, the extent of the inhibitory effect of IgG from different patients was proportional to the titer of the antibodies. Some studies have attempted to isolate aPL IgG from patients with APS by protein A or protein G column chromatography.[29–31] However, it is doubtful whether these isolated IgG fractions contain a high concentration of aPL, especially the antibody to β_2GPI. Therefore, it is difficult to conclude that isolated aPL can have an effect on coagulation reactions. We were able to isolate IgG contained at a high concentration relative to aβ_2GPI Ab with a technique that first used DEAE-sepharose column chromatography, followed by phosphatidylserine polyacrilamide gel column chromatography in the presence of β_2GPI, and finally by protein G-sepharose column chromatography. This isolation method was complicated and time consuming; however, it was apparently good for the isolation of an aβ_2GPI Ab.

Interference of aPL with the protein C anticoagulant system has been proposed as a potential factor predisposing thrombosis in individuals with these antibodies. Using a one-stage factor V clotting assay, Malia et al.[29] showed that factor Va degradation by APC and protein S in the presence of IgG purified from patients with aPL was 14%, compared to 52% with normal IgG. This condition was reported as an acquired activated protein C resistance and may partially explain the increased risk of venous thromboembolism in patients with aPL. Some studies have implicated antibodies to β_2GPI in activated protein C resistance.[14] However, at present it is unclear whether the activated protein C resistance is a common property of all aPL or pertains to only a few antibody subsets. Although it was reported that aPL hampered the function of protein S and β_2GPI interfered with the binding of protein S to its inhibitor, C4b-binding protein,[32] we believe that β_2GPI also inhibited the activity of protein C whereas the aβ_2GPI Ab enhanced the inhibitory effect of β_2GPI on protein C activity. In our previous study,[15] we noted that β_2GPI inhibits not only thrombin generation but also protein C activity and that the monoclonal aβ_2GPI Ab antibodies EY1C8 and EY2C9, which were established from peripheral blood lymphocytes from a patient with APS, inhibited the activity of protein C only in the presence of β_2GPI. Monoclonal aβ_2GPI Ab appeared to increase the inhibitory potential of β_2GPI on APC activity. In the present study, it was shown that aβ_2GPI Ab isolated from patients with APS also inhibited the activity of protein C only in the presence of β_2GPI. That is, in patients with aPL, the presence of aPL containing a large amount of aβ_2GPI Ab may result in thrombotic events by impeding APC anticoagulant activity.

In summary, we succeeded in isolating IgG, which contained a rich source of aβ_2GPI Ab, from patients with APS using a three-step chromatography method. Furthermore, aβ_2GPI Ab may contribute to thrombosis in APS through aβ_2GPI Ab-dependent inhibition of APC activity.

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