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Nicked β2-glycoprotein I binds angiostatin 4.5 (plasminogen kringle 1-5) and attenuates its antiangiogenic property

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Angiostatin was first discovered as a plasminogen fragment with antitumor/antiangiogenic property. One of the angiostatin isoforms, that is, angiostatin 4.5 (AS4.5), consisting of plasminogen kringle 1 to 4 and a most part of kringle 5, is produced by autoproteolysis and present in human plasma. β2-glycoprotein I (β2GPI) is proteolytically cleaved by plasmin in its domain V (nicked β2GPI), resulting in binding to plasminogen. Antiangiogenic properties have been recently reported in nicked β2GPI as well as in intact β2GPI at higher concentrations. In the present study, we found significant binding of nicked β2GPI to AS4.5 ($K_D = 3.27 \times 10^6\ M^{-1}$). Via this binding, nicked β2GPI attenuates the antiangiogenic functions of AS4.5 in the proliferation of arterial/venous endothelial cells, in the extracellular matrix invasion and the tube formation of venous endothelial cells, and in vivo angiogenesis. In contrast, intact β2GPI does not bind to AS4.5 or inhibit its antiangiogenic activity. Thus, nicked β2GPI exerts dual effects on angiogenesis, that is, nicked β2GPI promotes angiogenesis in the presence of AS4.5, whereas nicked β2GPI inhibits angiogenesis at concentrations high enough to neutralize AS4.5. Our data suggest that plasmin-nicked β2GPI promotes angiogenesis by interacting with plasmin-generated AS4.5 in sites of increased fibrinolysis such as thrombus. (Blood. 2009;114:2553-2559)

Introduction

Angiogenesis is the formation of a new capillary network from preexisting vessels and is essential in many physiologic and pathologic states, such as reproduction, development, wound healing, tumorigenesis, rheumatoid arthritis, diabetic retinopathy, and thrombosis.[1,2] Angiogenesis is tightly controlled by activators, such as fibroblast growth factors (FGFs) and vascular endothelial growth factor (VEGF), and by inhibitors, such as thrombospondin-1, interferon-α/β, platelet factor-4, and angiostatin.[3] Angiostatin was discovered in urine from mice with low-metastatic Lewis lung carcinoma as a kringle-containing fragment of plasminogen.[4] This first-reported angiostatin consists of 4 kringle domains (K1-K4) and possesses antitumor/antiangiogenic properties. Later, K1 to K3 was revealed to be a more potent inhibitor of angiogenesis.[5] Although several isoforms have been reported, angiostatin 4.5 (AS4.5, also referred to as K1-K5) is the only naturally occurring isoform identified in human plasma that consists of kringles 1 to 4 and approximately 85% of kringle 5.[6-8] Plasma concentrations of in vivo–generated AS4.5 were measured in cancer patients who received tissue plasminogen activator and mesna in a clinical trial.[9] In this study, generation of 2 isoforms of AS4.5 in human plasma was observed, namely, Lys-AS4.5 and Glu-AS4.5. Whereas Lys-AS4.5 is the originally reported natural AS4.5, Glu-AS4.5 is a larger form that obtains intact N-terminal domain of the precursor protein. Approximately 20 nM Lys-AS4.5 was detected even in the patients before treatment. After infusion of tissue plasminogen activator with mesna, Lys-AS4.5 levels increased to approximately 40 nM.

β2-glycoprotein-I (β2GPI), also known as apolipoprotein H, is a phospholipid-binding plasma protein that is one of the major autoantigens in patients with antiphospholipid syndrome, an autoimmune disorder characterized by thrombosis and pregnancy morbidity.[10-13] β2GPI is a single chain plasma glycoprotein at a concentration of approximately 4 μM composed of 5 homologous short consensus repeats, designated as domains I to V. β2GPI is cleaved by plasmin between Lys-317 and Thr-318 in domain V (nicked β2GPI), being unable to bind phospholipids.[14] This cleavage was first observed in vivo by Horbach et al[15] in plasma of patients with disseminated intravascular coagulation and in plasma with patients treated with streptokinase. In these cases, up to 12 μg/mL nicked β2GPI (~ 6% of intact β2GPI) was present. In other pathologic contexts, approximately 0.1% and approximately 1.5% of intact β2GPI are cleaved to nicked β2GPI in patients with leukemia and in patients with lupus anticoagulant, respectively.[16] In addition, in our previous study in which patients with history of stroke were investigated, up to 0.5%, mostly from 0.1% to 0.2% of intact β2GPI, was converted to nicked β2GPI in the stable state of their disease.[17] Instead of losing phospholipid-binding properties, nicked β2GPI gains the binding capacity to plasminogen and mildly suppresses the plasmin generation in the presence of tissue plasminogen activator and fibrin.[17] Because this binding is mediated by the interaction between lysine binding site on K5 of plasminogen and the lysine cluster on domain V of nicked β2GPI, we speculated on the interaction between nicked β2GPI and AS4.5, which still possesses the most part of kringle 5.

In the present study, we show that nicked β2GPI does bind AS4.5 and attenuates its antiangiogenic property in an endothelial cell proliferation assay, in an invasion assay, in a tube-formation assay, and in an in vivo angiogenesis assay.

Submitted November 27, 2008; accepted July 4, 2009. Prepublished online as *Blood* First Edition paper, July 22, 2009; DOI 10.1182/blood-2008-12-190629.

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Methods

Proteins

β2GPI and nicked β2GPI were prepared as previously reported.[17] AS4.5 (K1-K5) was prepared from Glu-plasminogen (Technoclone GmbH) treated with plasmin (Calbiochem Novabiochem Corp), followed by purification steps using a lysine-Sepharose column and a Sephadex G-75 column (GE Healthcare), as previously reported.[8] Purified AS4.5 was reduced using 2-mercaptoethanol and subjected to polyacrylamide gel electrophoresis (PAGE). In some experiments, AS4.5 was treated with PNGase F or Endo H (New England Biolabs Inc) to determine whether our preparation of AS4.5 undergoes glycosylation. Purified materials were tested to exclude the possibility of lipopolysaccharide contamination using Limulus ES II Single Test (Wako).

Cells

Human aortic endothelial cells (HAECs) and human umbilical vein endothelial cells (HUVECs) were obtained from Kurabo. These cells were cultured in the moisturized chamber at 37°C with 5% CO_2, using provided cell-culture medium "EGM2" which include 2% fetal calf serum (FCS), human epidermal growth factor (10 ng/mL), human FGF-B (5 ng/mL), heparin (10 μg/mL), hydrocortisone (1 μg/mL), amphotericin B (50 ng/mL), and gentamicin (50 μg/mL). HAECs/HUVECs from 2 to 6 passages were used in the following experiments.

Kinetic assay for molecular interaction between nicked β2GPI and AS4.5

Real-time analysis for molecular interaction between intact/nicked β2GPI and AS4.5 was performed using an optical biosensor, BIACORE X (Biacore AB). Biotinylated AS4.5 was immobilized onto the streptavidin-coupled sensor chip (Biacore AB). After blocking, various concentrations (0.125, 0.25, 0.5, 1.0, and 2.0 μM) of intact or nicked β2GPI were injected and ligands bound to the surface were detected. Obtained data were used to determine the association rate constant (k_{ass}) and dissociation rate constant (k_{diss}). K_D and K_A were determined as follows: $K_D = k_{diss}/k_{ass}$ and $K_A = k_{ass}/k_{diss}$.

Inhibition ELISA

To see the fluid-phase binding between AS4.5 and nicked β2GPI, enzyme-linked immunosorbent assay (ELISA) was performed in a similar way that we previously reported.[17] Briefly, Glu-plasminogen was immobilized onto a Sumilon Type S microtiter plate (Sumitomo Bakelite). After blocking, 0.25 μM nicked β2GPI with or without AS4.5 (0.4, 0.8, or 1.6 μM) dissolved in 1% bovine serum albumin–phosphate-buffered saline (PBS) was added to the wells and plasminogen-bound nicked β2GPI molecules were detected by Cof-22 mouse monoclonal anti-β2GPI antibody.

Cell proliferation assay

To see the effect of nicked β2GPI on the proliferation of aortic endothelial cells in the presence of AS4.5, which is a potent inhibitor of endothelial cell growth, we used tetrazolium/formazan assay. After wash with PBS, 5000 HAECs in 50 μL Opti-MEM I medium (Invitrogen) were added to each wells of Celltiter 96 proliferation Assay Kit (Promega). Different concentrations of intact/nicked β2GPI with or without approximately 50 nM AS4.5 were added to the medium. After a 72-hour incubation at 37°C, 100 μL Dye Solution was added to each well and incubated for another 4 hours at 37°C. Reaction was terminated using Stop Solution and optical density at 570 nm was measured. Cell proliferation assays were also performed using HUVECs in the same manner with or without 2.5 ng/mL human recombinant VEGF (Kurabo). These assays were done in a triplicate manner for 3 times. Because the effects of intact/nicked β2GPI on HAEC proliferation and HUVEC proliferation were similar regardless of the presence or absence of AS4.5, we performed the following in vitro angiogenesis experiments using HUVECs.

Matrigel cell-invasion assay

Biocoat invasion chambers containing Matrigel-coated membranes with 8-μm pores (BD Biosciences) were used to evaluate the effect of intact/nicked β2GPI on HUVECs to migrate through a basement membrane-like extracellular matrix, in the presence or absence of AS4.5. This assay was done as previously reported, with some alterations.[18] Briefly, after removal of FCS and growth factors by washing with PBS, 5×10^4 of HUVECs in 0.5 mL Opti-MEM I medium were placed in the top chamber of each well of a 24-well culture dish. Different concentrations of intact or nicked β2GPI were applied to the top chamber in the presence or absence of AS4.5 (final concentration, ~ 50 nM). Opti-MEM I medium containing 10% FCS was added to the lower chamber of each well as chemo-attractant. After an 8-hour incubation at 37°C, the noninvading cells were removed from the top chamber and the cells that extravasated through the extracellular matrix were stained with Diff-Quick (Kokusai Shiyaku). The number of cells that migrated through the membrane's 8-μm pores were counted under Olympus IX71 inverted microscope (Olympus) equipped with a 10×/0.30 ph1 objective at a final magnification of 100×, using WinROOF image processing software (Mitani Corp). Each assay was performed in a triplicate manner 4 times.

Capillary tube formation of HUVECs cocultured with fibroblasts

The capillary tube formation of HUVECs was evaluated using an angiogenesis kit (Kurabo), according to the manufacturer's instructions. In vitro HUVEC cord formation can be observed when cocultured with human primary fibroblasts, being suitable for quantification of cumulative sprout length.[19] Evaluation of HUVEC tube formation using this coculture system is reproducible using in vitro angiogenesis kits from Kurabo or TCScell-works.[20] The culture medium in each well of a 24-well cluster dish seeded with HUVECs and human skin fibroblasts was replaced by the fresh medium containing 2.5 ng/mL human recombinant VEGF at days 1, 4, 7, and 9. We reduced the concentration of VEGF to maximize the antiangiogenic effect of AS4.5, although the recommended concentration of VEGF for the positive growth control was 10 ng/mL. At day 11, the capillary tubes formed were detected by immunostaining using anti–human CD31 antibody supplied by the manufacturer. For scoring the capillary tube formation, tube length was measured quantitatively using an Olympus IX71 inverted microscope equipped with a 4×/0.13 PhL objective and angiogenesis measuring software (KURABO Angiogenesis Image Analyzer, Version 2; Kurabo).[21] This experiment was done in a duplicate manner 3 times.

Directed in vivo angiogenesis assay

The directed in vivo angiogenesis assay was obtained from Trevigen Inc and performed as previously described,[22,23] with modifications. Briefly, sterile 0.15-cm × 1-cm-long semiclosed surgical silicone tubing (angioreactors) was filled with 18-μL high-concentration basement membrane extract, including VEGF and FGF (Trevigen Inc). Various concentrations of nicked β2GPI (0-0.4 μM) were added to angioreactors containing AS4.5. The angioreactors were then inverted and incubated at 37°C for 1 hour to allow gel formation. The angioreactors were then implanted subcutaneously into the dorsal flank of 6- to 8-week-old athymic nude female mice. Blood vessels generated in the angioreactors were quantified by staining of the recovered cell pellets with fluorescein isothiocyanate-lectin on day 10. This experiment was performed in a triplicate manner.

Statistical analysis

Statistical evaluation was performed by Student *t* test. *P* values less than .05 were considered statistically significant.

Results

Purification of nicked β2GPI and AS4.5

Purified nicked β2GPI and intact β2GPI appeared as a single band with appropriate size under sodium dodecyl sulfate-PAGE with

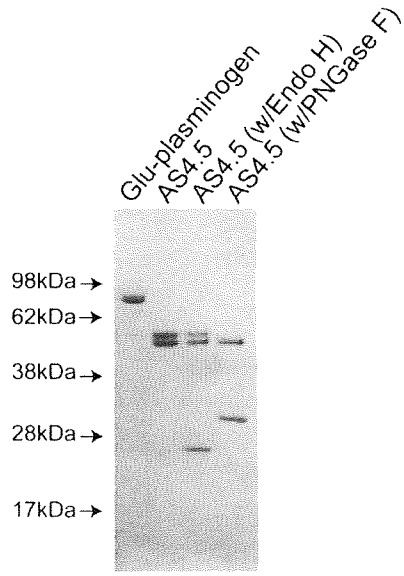

Figure 1. Preparation of nicked β2GPI and AS4.5. AS4.5 was prepared from Glu-plasminogen by plasmin digestion followed by purification using lysine-Sepharose column and Sephadex G-75 column. Purified AS4.5 was treated with PNGase F or Endo H to determine whether AS4.5 undergoes glycosylation. Glu-plasminogen, purified product (AS4.5), AS4.5 treated with PNGase F, and AS4.5 treated with Endo H were subjected to sodium dodecyl sulfate-PAGE under reduced conditions.

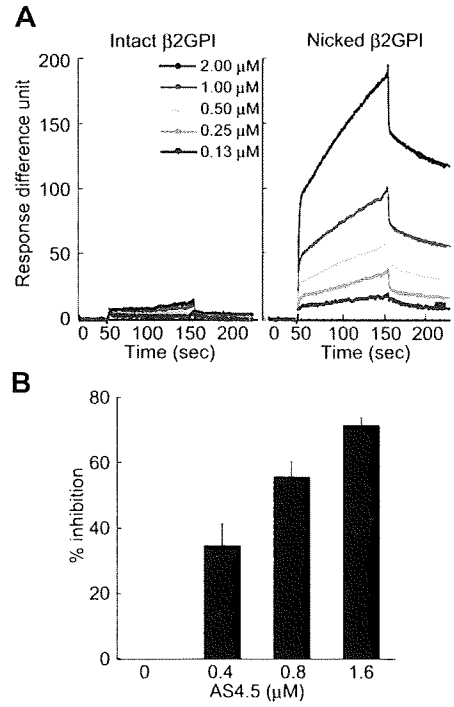

Figure 2. Binding of intact/nicked β2GPI to AS4.5. (A) Kinetic curves showing molecular interaction between AS4.5 and intact or nicked β2GPI. Intact β2GPI or nicked β2GPI binding to immobilized AS4.5 was detected using Biacore X, an optical biosensor as described in "Kinetic assay for molecular interaction between nicked B2GPI and AS4.5." Binding curve was compared between intact (left panel) and nicked β2GPI (right panel). Binding constants (K_D and K_A) between AS4.5 and nicked β2GPI were determined. (B) Binding of nicked β2GPI to immobilized Glu-plasminogen was tested in the presence or absence of AS4.5 in the fluid, using ELISA. The abilities of AS4.5 to inhibit the binding between fluid-phase nicked β2GPI and solid-phase Glu-plasminogen were shown as percentage inhibition. Error bars represent SE.

reduced conditions. Purified AS4.5 showed clear main band at expected size (52-55 kDa) with slightly larger minor band (Figure 1A). Treatment of purified AS4.5 with PNGase F resulted in the reduction of molecular weight to that of the main band, whereas treatment with Endo H had scarce effect on the size of AS4.5 (Figure 1B). According to the susceptibility to PNGase F and resistance to Endo H, at least some portion of purified AS4.5 is suggested to undergo glycosylation with complex oligosaccharides. Plasminogen undergoes glycosylation at Asn-289 and Thr-346, and additional site Leu-532, such glycosylation, can alter interaction between plasmin kringle domains and integrin αVβ3 expressed on endothelial cells.[24]

Binding of nicked β2GPI to AS4.5

Molecular interaction between intact or nicked β2GPI and AS4.5 was analyzed using an optical biosensor. Nicked β2GPI showed a large extent of binding to immobilized AS4.5, whereas intact β2GPI did not show any specific binding (Figure 2A). The data at different concentrations of nicked β2GPI were regressed, determining k_{ass} as 2.99×10^3 $M^{-1}s^{-1}$, and k_{diss} as $9.14 \times 10^{-4}s^{-1}$. Accordingly, K_D and K_A were determined as 3.05×10^{-7} M and 3.27×10^6 M^{-1}, respectively. To confirm this binding in the fluid phase, we performed inhibition ELISA. In this system, AS4.5 inhibited the binding of nicked β2GPI to immobilized Glu-plasminogen, even in the presence of excess amount of albumin (Figure 2B).

Effect of intact/nicked β2GPI on the proliferation of HAECs/HUVECs in the presence or absence of AS4.5

AS4.5 exhibited suppressive effect on the proliferation of HAECs to approximately 15% inhibition at the concentration of 50 nM, compared with the HAEC proliferation in the absence of AS4.5 (Figure 3A). In this growth factor-removed system, intact β2GPI up to the final concentration of 0.4 μM did not have any effect on the proliferation of HAECs both in the presence and absence of AS4.5. In contrast, nicked β2GPI reversed the suppressive effect on HAEC proliferation by AS4.5 ($P = .021$ at 0.4 μM of nicked β2GPI**, compared with the point without nicked β2GPI*). However, this form of β2GPI again had no effect on proliferation in the absence of AS4.5 at concentrations up to 0.4 μM. When HUVEC proliferation was examined in the same system, intact/nicked β2GPI exerted similar effect both in the presence and in the absence of AS4.5 (Figure 3B). When VEGF was added to this proliferation system, HUVEC proliferation was suppressed by lower concentrations of intact/nicked β2GPI in the absence of AS4.5 (Figure 3C). Suppressive effect of 50 nM AS4.5 on HUVEC proliferation was neutralized by approximately 0.1 μM nicked β2GPI. Higher concentrations (1.0-4.0 μM) of intact/nicked β2GPI suppressed HAEC/HUVEC proliferation regardless of the existence of VEGF/AS4.5 (data not shown).

Effect of intact/nicked β2GPI on the invasion of HUVECs in the presence or absence of AS4.5

Without AS4.5, intact or nicked β2GPI had no effect on the migration of HUVECs in concentrations up to 0.4 μM (Figure 4), whereas both forms of β2GPI significantly suppressed HUVEC migration in higher concentrations (from 1 to 4 μM) with dose dependency (~ 40% and 60% inhibition by 4 μM of intact and nicked β2GPI, respectively). Migration of HUVECs was down-regulated by 50 nM of AS4.5 to approximately 30% inhibition. Nicked β2GPI reversed this suppressive effect of AS4.5 at lower concentrations from 0.2 to 0.4 μM ($P = .027$ at 0.4 μM of nicked β2GPI**, compared with the point without nicked β2GPI*),

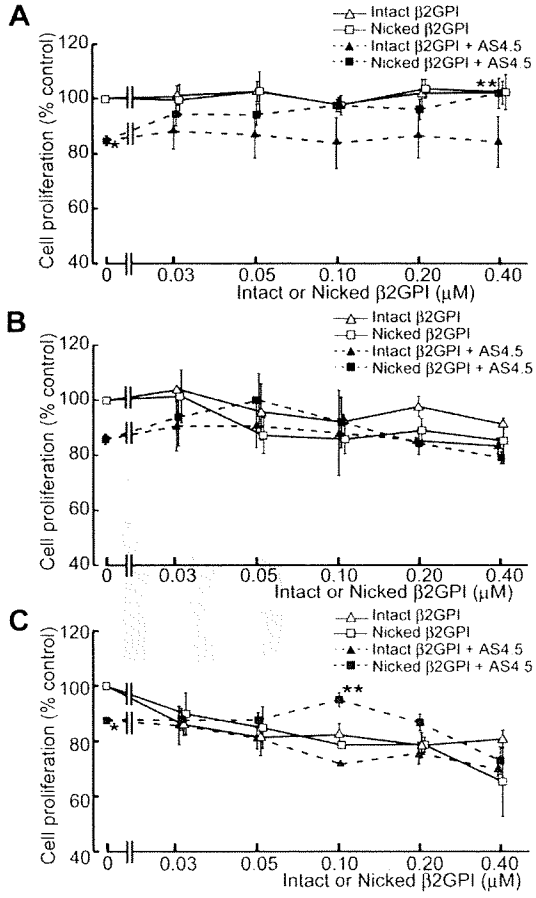

Figure 3. Effect of intact/nicked β2GPI on the proliferation of HAECs in the presence or absence of AS4.5. (A) HAECs were subjected to cell-proliferation assay using tetrazolium/formazan-based method. A total of 5000 HAECs were placed onto each wells of 96-well plate and incubated for 72 hours. The effect of serial concentrations of intact or nicked β2GPI was tested in the presence or absence of 50 nM AS4.5. HAEC proliferation in the presence of AS4.5 alone (50 nM)* was compared with that in the presence of both AS4.5 (50 nM) and nicked β2GPI (0.4 μM; **P = .021; Student t test). (B) Proliferations of HUVECs were tested using the same proliferation assay. (C) HUVEC proliferation was tested in the presence of VEGF. HUVEC proliferation in the presence of AS4.5 alone (50 nM)* was compared with that in the presence of both AS4.5 (50 nM) and nicked β2GPI (0.1 μM; **P = .030; Student t test). Error bars represent SE.

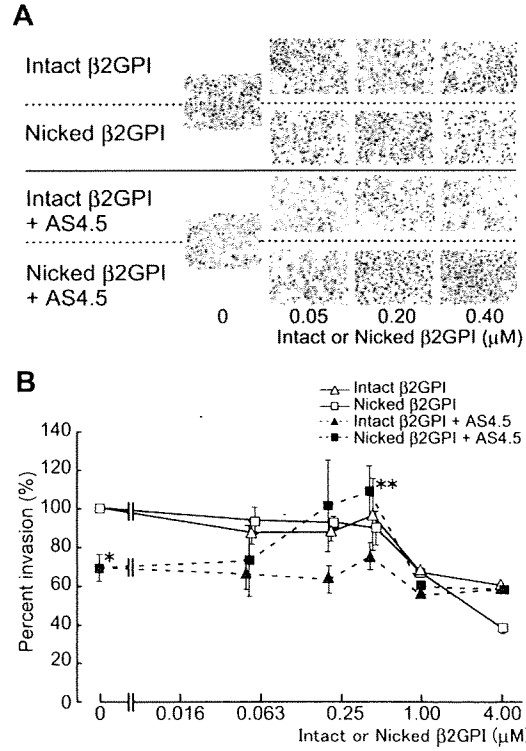

Figure 4. Effect of intact/nicked β2GPI on extravasation of HUVECs using a Matrigel-cell invasion assay in the presence or absence of AS4.5. Effect of intact or nicked β2GPI on the ability of HUVECs to migrate through a basement membrane-like extracellular matrix was evaluated in the presence or absence of AS4.5. HUVECs were added to the top wells of each chamber, and 10% FCS-enriched culture medium was added to each bottom chamber as a source of chemotactic factors. (A) In the top 2 lines of the panels, assays were done without AS4.5 AS4.5 was added to the wells in the bottom 2 lines of the panels. Serial concentrations of intact β2GPI were added in lines 1 and 3, whereas nicked β2GPI was added in lines 2 and 4. Similar results were obtained in other 3 experiments (original magnification, ×100). (B) HUVECs migrated through the Matrigel, and 8-μM pores on the membrane were stained and counted using image processing software. Ratios of the numbers of the HUVECs migrated under treatment with reagents against the number of those cells without any additional reagents were plotted on the graph. Concentrations of intact/nicked β2GPI were as follows: 0.05, 0.2, 0.4, 1.0, or 4.0 μM. Error bars represent SE. Invaded cell counts in the presence of AS4.5 alone (50 nM)* were compared with those in the presence of both AS4.5 (50 nM) and nicked β2GPI (0.4 μM; **P = .027; Student t test).

although increment of concentration diminished this reverse effect and, in turn, resulted in inhibition of HUVEC migration (~ 40% inhibition by 4 μM nicked β2GPI). Thus, depending on the concentration, nicked β2GPI shows dual effect on the mobility of HUVECs in the presence of AS4.5. Intact β2GPI had no additional or reverse effect on HUVEC migration in the presence of AS4.5 at concentrations up to 0.4 μM.

Effects of nicked β2GPI on angiogenesis (in vitro tube formation assay)

Formation of capillary-like structure by HUVECs was evaluated in the VEGF-dependent tube formation assay system. Fifty nanomolar of AS4.5 suppressed the function of HUVECs in this assay (Figure 5). Capillary tube formation was disrupted by intact β2GPI in a dose-dependent manner, in the presence or absence of AS4.5. Nicked β2GPI again reversed the inhibitory effect of AS4.5 on tube formation by HUVECs in a dose-dependent manner at concentrations up to 0.4 μM (P = .044 at 0.4 μM nicked β2GPI**, compared with the point without nicked β2GPI*). In the absence of AS4.5, nicked β2GPI had no significant effect on tube formation at concentrations less than 0.4 μM. In this assay, higher concentrations of intact/nicked β2GPI suppressed tube formation, the latter being more potent (4 μM of nicked β2GPI suppressed tube area to ~ 40 × 10^3 pixels).

Effects of nicked β2GPI on in vivo angiogenesis

Generation of blood vessels into angioreactors was suppressed by 0.2 μM AS4.5 (Figure 6). Addition of nicked β2GPI to this system up to 0.4 μM significantly recovered angiogenesis in a dose-dependent manner (P = .01).

Discussion

In the first part of this study, we demonstrated that nicked β2GPI binds AS4.5 with similar kinetics found in the interaction between nicked β2GPI and plasminogen.[17] Whereas intact β2GPI does not show any binding to plasminogen, the binding between nicked β2GPI and plasminogen was mediated via interaction between the lysine cluster of β2GPI domain V and the lysine binding site on the plasminogen K5.[17] In addition, in the present study, intact β2GPI

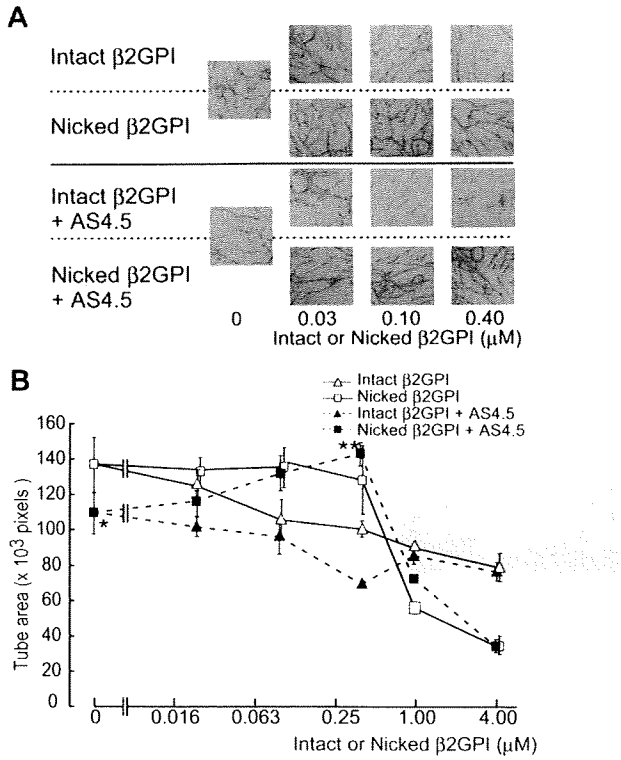

Figure 5. Effect of intact/nicked β2GPI on the VEGF-dependent tube formation of HUVECs cocultured with fibroblasts in the presence or in the absence of AS4.5. VEGF-dependent tube formation of HUVECs cocultured with primary human fibroblasts was evaluated in the presence or absence of AS4.5 (A) HUVECs were visualized by immunostaining with anti–human CD31 antibodies. In the top 2 lines of the panels, the assay was done without AS4.5 AS4.5 was added in the bottom 2 lines of the panels. Serial concentrations of intact β2GPI were added in lines 1 and 3, whereas nicked β2GPI was added in lines 2 and 4. Similar results were obtained in the second and third experiments (original magnification, ×40). (B) Capillary tube formation was quantified using KURABO Angiogenesis Image Analyzer, Version 2. Obtained data (pixels) were plotted onto the graph. Concentrations of intact/nicked β2GPI were as follows: 0.025, 0.1, 0.4, 1.0, or 4.0 μM. Error bars represent SE. Tube areas in the presence of AS4.5 alone (50 nM)* were compared with those in the presence of both AS4.5 (50 nM) and nicked β2GPI (0.4 μM; **P = .044; Student t test).

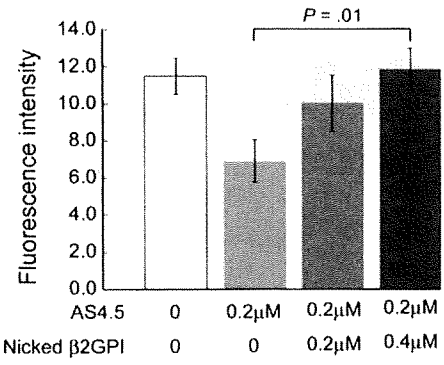

Figure 6. Nicked β2GPI suppresses the antiangiogenesis effect of AS4.5 in in vivo angiogenesis assay. Directed in vivo angiogenesis assay was performed. Semiclosed surgical silicone tubings (angioreactors) were prefilled with extracellular matrices containing VEGF and FGF alone, VEGF, FGF, and AS4.5, or VEGF, FGF, AS4.5, plus various concentrations of nicked β2GPI, then implanted subcutaneously into the dorsal flank of athymic nude mice. Blood vessels generated in the angioreactors were quantified by staining of the recovered cell pellets with fluorescein isothiocyanate-lectin. Error bars represent SE. *Fluorescence values in the presence of AS4.5 (0.2 μM) alone were compared with those in the presence of both AS4.5 and nicked β2GPI (0.4 μM) by Student t test (P = .01).

did not show any specific binding to AS4.5. This phenomenon indicates that 85% of K5 still functions enough for the binding with nicked β2GPI or that nicked β2GPI gains accessibility to other kringle domain(s) even when 15% of K5 is lost, the latter being less probable because neither K1 to K3 nor K4 disrupted the binding between nicked β2GPI and plasminogen in our previous inhibition assay.[17]

Next, we investigated the functional aspect of the interaction between nicked β2GPI and AS4.5 on the vascular endothelial cell biology. The antiangiogenic function of angiostatin is mediated by its binding onto the endothelial cell surface. Angiostatin inhibits adenosine triphosphate synthase F1F0 by direct binding to the extracellular portion of this enzyme, resulting in caspase-mediated apoptosis of endothelial cells.[25,26] Angiostatin also binds other endothelial cell surface molecules, such as integrin αVβ3 and angiomotin, although the latter binding was shown using original angiostatin K1 to K4.[27] Thus, it is speculated that nicked β2GPI interferes with the binding of AS4.5 onto the endothelial cells, resulting in attenuation of the antiangiogenic function of AS4.5. Indeed, 25 nM nicked β2GPI starts to compete with 50 nM AS4.5 and 0.1 to 0.2 μM nicked β2GPI completely abolished the antiangiogenic effect of the given AS4.5 in our HAEC proliferation assay (Figure 3A). In the HUVEC proliferation assay, 50 nM nicked β2GPI neutralized the same molar of AS4.5 (Figure 3B). In the HUVEC invasion assay and tube-formation assay, 0.1 to 0.2 μM nicked β2GPI abolished antiangiogenic properties of 50 nM AS4.5 (Figures 4,5). Based on the previous studies,[9,15,16] plasma concentrations of both nicked β2GPI and AS4.5 used in this study are physiologically available at least in thrombotic status. Moreover, it is predicted that local concentrations of nicked β2GPI and AS4.5 increase at sites of thrombosis where plasmin generation is up-regulated.

To make the situation complex, intact/nicked β2GPI itself has been reported as an inhibitor of angiogenesis. In the first report, Beecken et al[28] identified β2GPI from transitional cell carcinoma cell line, which inhibits the growth of a tumor implant in severe combined immunodeficiency mice. They demonstrated that β2GPI together with plasmin, but neither β2GPI alone nor plasmin alone, inhibits proliferation and tube formation of HUVECs, concluding that the nicked form of β2GPI is an inhibitor of angiogenesis. However, lack of using purified material makes it difficult to assume the concentration of nicked β2GPI generated in this study. Sakai et al[29] reported that 4 μM nicked β2GPI inhibited endothelial cell migration, proliferation, and neovascularization into subcutaneous implants that contain VEGF. Intraperitoneal injection of nicked β2GPI also inhibited growth of orthotopically injected tumors in a murine prostate cancer model. Only in the neovascularization study, which is a VEGF-dependent system, the same amount of intact β2GPI had similar antiangiogenic properties. Although the authors found no binding between nicked β2GPI and commercially available angiostatin (Sigma-Aldrich) using immunoprecipitation, this preparation of angiostatin does not include any portion of kringle 5. Recently, Yu et al[30] have shown that β2GPI inhibits VEGF- and FGF-induced proliferation, migration, and tube formation of HUVECs. This antiangiogenic property was found in intact β2GPI, nicked β2GPI, and also in deletion mutant lacking domain V of β2GPI, but not in β2GPI lacking domain I, concluding that this antiangiogenic property is mediated via domain I of β2GPI. This group also demonstrated that 0.5 to 2 μM intact β2GPI down-regulates mRNA expression of the VEGF receptor.

In our HAEC/HUVEC proliferation assay in which EGF and FGF were removed (Figure 3A-B), intact/nicked β2GPI had almost no effect on the proliferation of arterial endothelial cells in lower concentrations, up to 0.4 μM. Our result does not contradict that

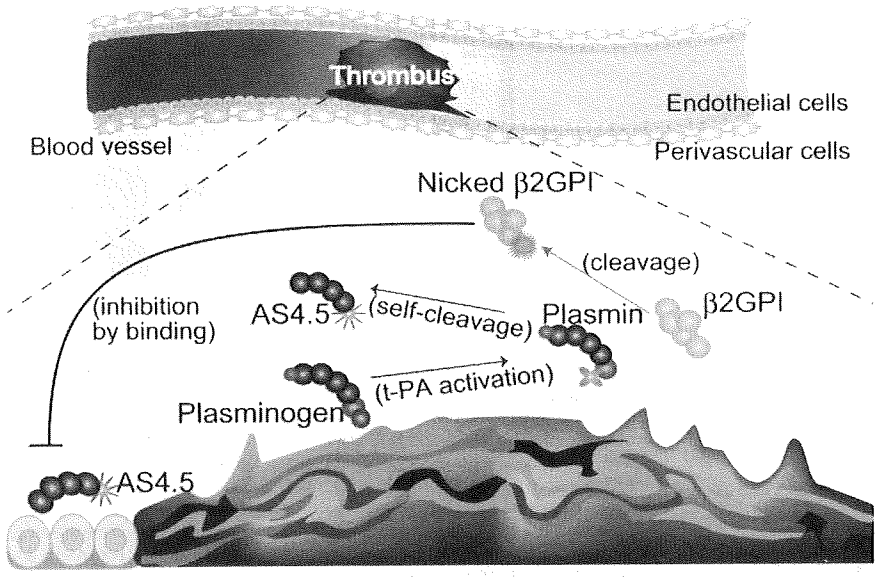

Figure 7. Beneficial effect of nicked β2GPI on thrombotic ischemia (hypothesis). (1) If thrombus is formed in the artery, up-regulation of fibrinolysis occurs and plasminogen is converted into plasmin. (2) Plasmin cleaves β2GPI into nicked β2GPI, whereas angiostatin is generated via autoproteolysis of plasminogen. (3) Nicked β2GPI binds angiostatin and attenuates its antiangiogenic property, resulting in promoted angiogenesis. tPA indicates tissue plasminogen activator.

from the Yu et al study[25] in which the antiangiogenic property of intact/nicked β2GPI was dependent on VEGF and FGF. Indeed, in our HUVEC proliferation assay with VEGF (Figure 3C) and in our VEGF-dependent tube formation assay (Figure 5), both intact and nicked β2GPI exerted antiangiogenic properties at lower concentrations. At relatively high concentrations more than 1 μM, both intact and nicked β2GPI inhibited HUVEC migration and tube formation (Figures 4B,5B), being relevant to the previous reports.[29,30] In these functional assays, nicked β2GPI exerted dual effect in the presence of AS4.5; at the lower concentrations, nicked β2GPI works as an AS4.5 inhibitor, whereas it works as an angiogenesis inhibitor at the higher concentrations. In the last part of this study, we confirmed that nicked β2GPI suppresses the antiangiogenic effect of AS4.5 in vivo (Figure 6).

Although intact β2GPI in human plasma is abundant, plasma levels of nicked β2GPI in steady-state human is up to 0.5%, 1.5% of intact form even in patients with a history of stroke[17] and those with lupus anticoagulant,[16] respectively. Thus, the antiangiogenic/antitumor effect of β2GPI in steady state is probably dependent on the intact form. On the other hand, both nicked β2GPI and angiostatin are proteolytically processed by plasmin, thus being produced at sites where fibrinolysis is up-regulated. When ischemic thrombus is formed in the artery, collateral blood flow needs to be generated by angiogenesis. In such situations, plasmin is generated, leading to the production of both nicked β2GPI and angiostatin. Those products might be stuck near the lesion resulting from the stagnant blood flow. Nicked β2GPI may exert its property as antiangiostatin in such situations, resulting in promoted angiogenesis (Figure 7). In other situations, such as tumor and diabetic retinopathy where angiostatin works beneficially, however, nicked β2GPI might be a disease-worsening factor, being a candidate for the treatment target.

In conclusion, (1) we have demonstrated the binding between nicked β2GPI and AS4.5; and (2) we propose that nicked β2GPI is a physiologic inhibitor of angiostatin both of which are produced only when fibrinolytic system is activated.

Acknowledgments

The authors thank Drs Hiroko Ogawa and Satoshi Hirohata (Department of Molecular Biology and Biochemistry, Okayama University School of Medicine) for great suggestions and discussions.

This work was supported in part by grants from the Japanese Ministry of Health, Labor and Welfare and the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Authorship

Contribution: H.N. performed the research and analyzed data; S.Y. designed and performed the research and wrote the paper; E.M. and K.K. contributed analytical tools; M.I. contributed vital reagents; H.K. and T.H. analyzed data; and T.A. and T.K. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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ARTHRITIS & RHEUMATISM
Vol. 60, No. 8, August 2009, pp 2457–2467
DOI 10.1002/art.24708

The Effects of Phosphatidylserine-Dependent Antiprothrombin Antibody on Thrombin Generation

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Objective. **Antibodies to prothrombin (APTs) and to β_2-glycoprotein I are the major autoantibodies responsible for lupus anticoagulant (LAC) activity. APTs comprise antibodies against prothrombin alone as well as antibodies against phosphatidylserine/prothrombin complex (anti-PS/PT), the latter being highly associated with the antiphospholipid syndrome (APS). The effect of anti-PS/PT on thrombin generation has not been elucidated, and the paradoxical effect of LAC (an anticoagulant in vitro, but a procoagulant in vivo) remains an enigma. The purpose of this study was to investigate the effects of anti-PS/PT on thrombin generation and to examine the LAC paradox.**

Methods. **We evaluated 36 anti-PS/PT–positive APS patients and 127 healthy subjects. Markers of in vivo thrombin/fibrin generation, including prothrombin fragment F_{1+2}, thrombin–antithrombin III complex, soluble fibrin monomer, D-dimer, and fibrin degradation products, were measured. Mouse monoclonal anti-PS/PT antibody 231D was established, and its effects on in vitro thrombin generation were investigated by chromogenic assay.**

Results. **Significantly elevated levels of markers of thrombin/fibrin generation were observed in anti-PS/PT–positive patients, regardless of the presence or absence of anticardiolipin antibodies, as compared with healthy subjects. In the presence of low concentrations of human activated factor V (FVa), monoclonal antibody 231D increased thrombin generation in a dose-dependent manner. In contrast, when high concentrations of FVa were added, monoclonal antibody 231D decreased thrombin generation. Under a constant concentration of FVa, a high concentration of human FXa enhanced the effect of 231D.**

Conclusion. **The presence of anti-PS/PT greatly correlated with increased thrombin generation in APS patients. The in vitro effects of monoclonal antibody 231D on thrombin generation are "biaxial" according to the FVa/FXa balance. These data may serve as a clue to understanding the LAC paradox and the thrombogenic properties of anti-PS/PT.**

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

It has been shown that despite their name, aPL are not directed against anionic phospholipids, as was previously thought, but are part of a large family of autoantibodies against phospholipid-binding plasma proteins or phospholipid–protein complexes (2). The most common and best characterized antigenic target of these antibodies is β_2-glycoprotein I (β_2GPI) (3–5), a phospholipid binding protein that has been extensively studied and has been shown to play a prominent role in the binding of aPL to phospholipid. Anticardiolipin

Supported by the Japanese Ministry of Health, Labor and Welfare and by the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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Submitted for publication July 18, 2008; accepted in revised form May 1, 2009.

antibodies (aCL), which are associated with APS, are not directed against cardiolipin alone, but require β_2GPI as a cofactor for the binding of cardiolipin in enzyme-linked immunosorbent assay (ELISA) plates. Beta$_2$-glycoprotein I bears the epitopes for aCL binding that are exposed when β_2GPI binds to negatively charged phospholipids (6,7).

Prothrombin, another main phospholipid binding protein, was first reported by Loeliger in 1959 (8) to be a probable cofactor for the lupus anticoagulant (LAC). Fleck et al (9) subsequently confirmed that antiprothrombin antibodies (APTs) are responsible for the LAC activity, and in 1991, Bevers et al (10) emphasized the importance of prothrombin in generating LAC activity. Some years later, the inhibitory effect of LAC on endothelial cell–mediated prothrombinase activity was reported, and it was also demonstrated that the IgG fraction containing LAC activity bound to the phospholipid–prothrombin complex (11). Therefore, prothrombin was recognized as another target for autoantibodies with LAC activity. Accordingly, it is widely accepted that APTs and anti-β_2GPI antibodies are the 2 major autoantibodies responsible for LAC activity: APTs for prothrombin-dependent LAC and anti-β_2GPI antibodies for β_2GPI-dependent LAC.

An ELISA for the detection of APTs using prothrombin alone as the antigen coated onto irradiated plates (APT-alone assay) was described in 1995 (12). Since then, a number of studies have investigated the clinical relevance of testing APT alone; nevertheless, the association between APT alone and clinical manifestation of APS is still a subject of controversy (13). In 1996, antibodies against the phosphatidylserine/prothrombin complex (anti-PS/PT; or phosphatidylserine-dependent APTs) were described in LAC-positive patients (14). Moreover, the ELISA using phosphatidylserine-bound prothrombin as antigen was reported to be more sensitive for detecting the presence of APTs than the ELISA using prothrombin alone as antigen (15). Our group assessed the anti-PS/PT ELISA in a large population of patients with autoimmune diseases and found that IgG anti-PS/PT were highly prevalent in patients with APS as 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 APS patients, the LAC paradox, that is, the behavior of LAC as an anticoagulant in vitro but a procoagulant in vivo, remains unresolved. In addition, the effects of anti-PS/PT on thrombin generation, whether in vitro or in vivo, have not been clarified. In order to investigate the effects of anti-PS/PT on thrombin generation, we evaluated markers of thrombin generation and fibrinolytic turnover in plasma samples from APS patients with anti-PS/PT antibodies. Furthermore, we established a mouse monoclonal anti-PS/PT antibody (231D) and used this monoclonal antibody to analyze thrombin generation in vitro.

PATIENTS AND METHODS

Patients. Plasma and serum samples were obtained from 36 APS patients with IgG and/or IgM anti-PS/PT antibodies (32 women and 4 men with a mean age of 46 years [range 22–74 years]) who fulfilled the revised Sapporo criteria for APS (1). Fifteen patients were diagnosed as having primary APS, and 21 patients had APS in association with other connective tissue diseases. Twenty-six patients (72%) had experienced arterial thrombotic events, such as stroke, myocardial infarction, and iliac artery occlusion, as confirmed by computed tomography scanning, magnetic resonance imaging, or conventional angiography. Deep vein thrombosis and pulmonary thrombosis were defined as venous thrombosis (12 of 36 patients [33%]) and were confirmed by angiography or scintigraphy. Thirteen women (36%) had pregnancy morbidity as defined by the APS criteria. Anti-PS/PT antibodies of IgG, IgM, and both isotypes were detected in 47%, 22%, and 31% of patients, respectively.

None of the patients had thrombotic events or pregnancy complications within 3 months before blood collection. Signs of acute thrombosis were not detected in any patient at the time blood was drawn. The time since the latest manifestation of APS varied from 4 months to 6 years. Therefore, our data correspond to the baseline of thrombin generation in anti-PS/PT–positive patients. When blood was drawn for this study, no patients were receiving heparin; some were taking warfarin, but there had been no modification of any medications within the 3 previous months. None of the patients had a tendency toward bleeding.

Blood samples were also collected from 127 apparently healthy subjects who consented to join the study. There were a total of 51 women and 76 men with a mean age of 34 years (range 18–65 years).

The study was performed in accordance with the Declaration of Helsinki and the Principles of Good Clinical Practice. Approval was obtained from the Local Ethics Committee, and informed consent was obtained from each study subject before enrollment.

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 then stored at −80°C until they were used in the experiments.

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, Tokyo, Japan) were coated with 30 μl of a 50 μg/ml preparation of phosphatidylserine (Sigma, St. Louis, MO) and dried overnight at 4°C. To avoid nonspecific binding of proteins, the wells were blocked with 150 μl of Tris buffered

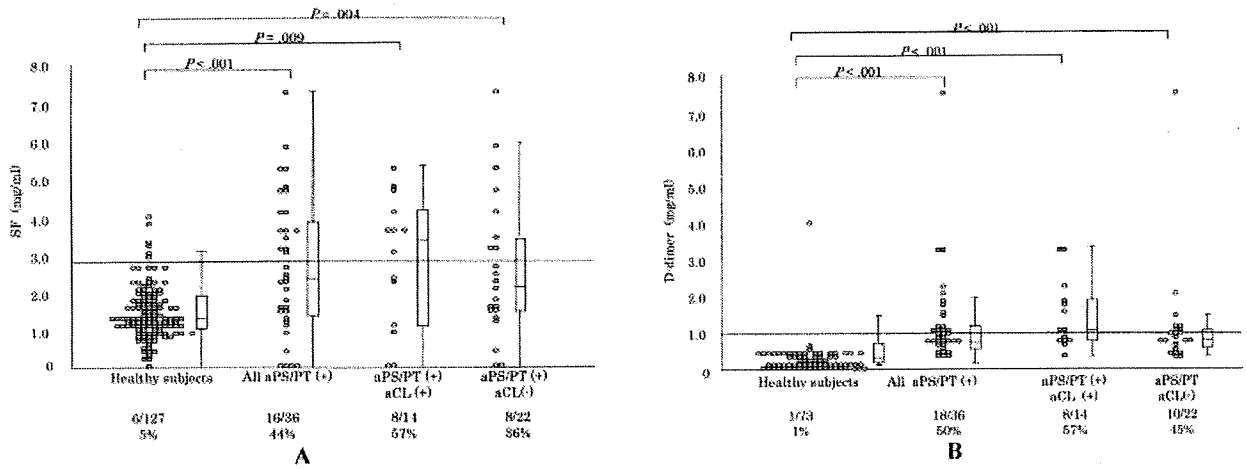

Figure 1. Distribution of plasma levels of soluble fibrin antigen (SF) and D-dimer in patients with antiphospholipid syndrome (APS) and in healthy individuals. Plasma levels of **A**, soluble fibrin antigen and **B**, D-dimer were measured in healthy controls, in all APS patients with anti–phosphatidylserine/prothrombin complex (anti-PS/PT) antibody, and in anti-PS/PT antibody–positive APS patients with or without anticardiolipin antibody (aCL). Horizontal line shows the cutoff level of positivity, which was defined as the mean ± 2SD of the level in control subjects. Data are shown as individual results as well as box plots, where each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. Values across the bottom are the number of subjects positive/total number tested, as well as the percentages.

saline (TBS) containing 1% fatty acid–free bovine serum albumin (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* $CaCl_2$ (TBS–Tween–$CaCl_2$), 50 μl of a 10 μg/ml preparation of human prothrombin (Diagnostica Stago, Asnières-sur-Seine, France) 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, and alkaline phosphatase–conjugated goat anti-human IgG or IgM and substrate were added. The optical density of wells coated with phosphatidylserine alone was subtracted from that of wells containing phosphatidylserine/prothrombin. The anti-PS/PT antibody titer of each sample was derived from the standard curve according to dilutions of the positive control.

Determination of aCL and LAC. IgG and IgM aCL were measured according to a standard aCL ELISA, as described elsewhere (17).

Two 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 (18). For measurement of the activated partial thromboplastin time (APTT), a sensitive reagent with a low phospholipid concentration (test PTT-LAC; Diagnostica Stago) was used for screening, and the results were confirmed with the use of a StaClot LAC kit (Diagnostica Stago). The dilute Russell's viper venom time (dRVVT) was screened for and confirmed by use of a Gradipore LAC test (Sydney, New South Wales, Australia). LAC was considered positive when at least 1 of these tests confirmed its presence.

Assessment of markers of thrombin and plasmin generation in vivo. Plasma levels of soluble fibrin antigen (Mitsubishi Kagaku Iatron, Tokyo, Japan), prothrombin fragment F_{1+2} (Enzygnost F1+2 Micro; Dade-Behring, Marburg, Germany) and thrombin–antithrombin III complex (TAT test Kokusai F; International Reagent Corporation, Kobe, Japan) were assessed as markers of thrombin generation. Among them, F_{1+2} was not measured in patients receiving warfarin. We also evaluated D-dimer (D-dimer test-F; International Reagent Corporation) and fibrin/fibrinogen degradation products (Nonapia p-FDP; Daiichi Kagaku, Tokyo, Japan) as markers of fibrinolytic turnover.

Establishment of a mouse monoclonal anti-PS/PT antibody using prothrombin as antigen. Eight-week-old female BALB/c mice were immunized intraperitoneally and were given 2 booster injections with 50 μg of human prothrombin (Enzyme Research Laboratories, Swansea, UK) emulsified with Freund's complete adjuvant and with Freund's incomplete adjuvant (Difco, Detroit, MI), respectively. The spleens were excised from the mice, and spleen cells were fused with P3U1 mouse myeloma cells (19). Cells producing antibodies against the phosphoserine/prothrombin complex were screened by anti-PS/PT ELISA. Antibody-producing hybridomas were cloned by serial limiting dilution and injected intraperitoneally into pristane-pretreated BALB/c nude mice to obtain ascitic fluid. Monoclonal antibody 231D was sequentially purified by protein G–Sepharose affinity chromatogra-

phy (MabTrap-TMGII; Amersham Pharmacia Biotech, Uppsala, Sweden).

Establishment of a mouse monoclonal anti-PS/PT antibody using prethrombin 1 as antigen. Prothrombin (1 mg/ml in TBS) was digested for 3 hours at 37°C with 10 units of bovine thrombin (Sigma). The reaction was stopped by the addition of 1 m*M* p-ABSF and p-APMSF. Prethrombin 1, which lacks the prothrombin domain 1 that comprises the phospholipid-binding site (Gla-domain), was purified from the solution by ion-exchange chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of prethrombin 1 fractions revealed a single band at 50 kd under nonreducing conditions.

To obtain a monoclonal APT that binds to prothrombin but does not interact with the phospholipid-binding site of prothrombin, a BALB/c mouse was immunized with prethrombin 1. Hybridomas were screened using an anti-PS/PT ELISA, and monoclonal antibody 51A6 was established and purified in the same manner as described for 231D.

APT-alone assay for activity of the monoclonal anti-PS/PT antibody. An APT-alone assay was performed as described previously (20), with some modifications. Briefly, either irradiated microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark) or nonirradiated plates (Sumilon Type S) were coated overnight at 4°C with 10 μg/ml of purified human prothrombin in TBS containing 5 m*M* $CaCl_2$. Wells were blocked for 1 hour at 37°C with 0.5% gelatin. After 3 washes with TBS–Tween–$CaCl_2$, 50 μl of sample (monoclonal antibodies, control mouse IgG, or serum from mouse immunized with human prothrombin), diluted in BSA–$CaCl_2$ as appropriate, was added to duplicate wells. Plates were incubated for 1 hour at room temperature, followed by the addition of alkaline phosphatase–conjugated goat anti-mouse IgG and substrate. Optical density at 405 nm was then measured.

Detection of LAC activity in normal plasma using monoclonal anti-PS/PT antibody. Blood samples from 4 healthy donors were collected in precooled tubes containing a one-tenth volume of 0.105*M* sodium citrate and were immediately centrifuged at 2,000*g* for 15 minutes. Platelets were removed by filtration, and the platelet-free plasma was stored at −80°C. Different concentrations of monoclonal antibodies (50–3.1 μg/ml) were spiked into the pooled normal plasma, and clotting times were determined using the Start 4 system. Measurements of dRVVT and APTT were performed as described above. In addition, the kaolin clotting time (KCT) was measured with a kaolin solution (Dade-Behring) according to standard protocols.

Competitive ELISAs. IgG from 9 APS patients with high titers of anti-PS/PT antibodies was purified using protein G–Sepharose affinity chromatography (MabTrap-TMGII). Monoclonal antibody 231D or 51A6 (200 or 20 ng/ml) was added to plates that had been coated with PS/PT complex, and the plates were incubated for 1 hour at room temperature. Purified IgG (1 mg/ml) was added to the wells, and binding to PS/PT complex was determined by anti-PS/PT ELISA. The inhibition of IgG binding by monoclonal antibodies was calculated by comparing the optical density values with the values for IgG binding in the absence of monoclonal antibodies.

An additional competitive ELISA was performed in which 200 ng/ml of either 231D or 51A6 was coincubated with several concentrations (200, 50, 12.5, and 3.1 μg/ml) of 2 representative purified IgG from APS patients.

Measurement of in vitro thrombin generation. The effects of anti-PS/PT antibodies on thrombin generation were evaluated with a chromogenic assay, using the prothrombinase complex phospholipid, $CaCl_2$, purified human activated factor V (FVa; Haematologic Technologies, Essex Junction, VT), and FXa (Enzyme Research Laboratories). The thrombin generation assays used in this study were based on our previous analyses (21). Thrombin generation was measured by using a specific substrate for thrombin, D-Phe-pipecolyl-Arg-paranitroaniline (S-2238; Chromogenix Instrumentation Laboratory, Milan, Italy). Cephalin (PTT-Reagent RD; Roche Diagnostics, Basel, Switzerland), a phospholipid from rabbit brain extract, was used as the source of phospholipid. Cephalin was used at a dilution of 1:63 in assay buffer (1% BSA, 0.1 m*M* $CaCl_2$, TBS).

Ten microliters of a 10 μg/ml preparation of purified human prothrombin diluted in assay buffer, 10 μl of diluted phospholipid, and 40 μl of 231D at various concentrations was transferred into each well of a 96-well microtiter plate and then incubated at 37°C for 20 minutes. Ten microliters of FVa (0–1 ng/ml) and FXa (0.5–5 μg/ml) was added to the preincubated mixture, and the plate was left at room temperature for 2 minutes. The coagulation reaction was initiated by adding 25 μl of a 50 m*M* concentration of $CaCl_2$, followed by 25 μl of 2 m*M* concentration of S-2238. After incubation at 37°C, the absorbance of the mixture was measured at 405 nm with a Multiscan Ascent plate reader (Thermo Electron Corporation, Waltham, MA).

Statistical analysis. Statistical evaluation was performed by Mann-Whitney U test, Fisher's exact test, or Student's *t*-test, as appropriate. *P* values less than 0.05 were considered significant.

RESULTS

Plasma levels of thrombin generation and markers of fibrinolytic turnover. Levels of all markers of thrombin generation and fibrinolytic turnover were higher in APS patients with anti-PS/PT antibodies as compared with those in healthy control subjects. The distribution of representative markers, soluble fibrin antigen, and D-dimer are displayed in Figure 1. Plasma levels of soluble fibrin antigen and D-dimer were higher in both aCL subgroups of anti-PS/PT–positive patients as compared with those in healthy controls.

The cutoff level of each marker was defined as the mean ± 2SD of the levels in control subjects. A higher prevalence of elevation in the levels of markers of thrombin/plasmin generation (F_{1+2}, thrombin–antithrombin III complex, soluble fibrin antigen, D-dimer, and fibrin/fibrinogen degradation products) was found in all anti-PS/PT–positive patients, in anti-PS/PT–positive patients with aCL, and in anti-PS/PT–positive patients without aCL as compared with the levels in healthy subjects ($P < 0.05$ for each comparison) (Table 1).

Table 1. Prevalence of markers of increased thrombin/plasmin generation in patients and healthy controls*

	All patients	Anti-PS/PT+ patients		Healthy controls
		aCL+	aCL−	
Prothrombin fragment F_{1+2}	10/28 (36)	1/7 (14)	9/21 (43)	3/60 (5)
Thrombin–antithrombin III complex	10/36 (28)	2/14 (14)	8/22 (36)	7/73 (10)
Soluble fibrin antigen	16/36 (44)	8/14 (57)	8/22 (36)	6/127 (5)
D-dimer	18/36 (50)	8/14 (57)	10/22 (45)	1/73 (1)
Fibrin/fibrinogen degradation products	10/36 (28)	6/14 (43)	4/22 (18)	3/74 (4)

* Values are the number positive/total number tested (%). All values were statistically significant as compared with those in the controls ($P < 0.05$). Anti-PS/PT = anti–phosphatidylserine/prothrombin complex; aCL = anticardiolipin antibody.

Binding activity of mouse monoclonal anti-PS/PT antibody. Two anti-PS/PT antibody clones, 231D and 51A6, were obtained. The 231D antibody was established from a mouse that had been immunized with human prothrombin, and the 51A6 antibody was established from a mouse that had been immunized with

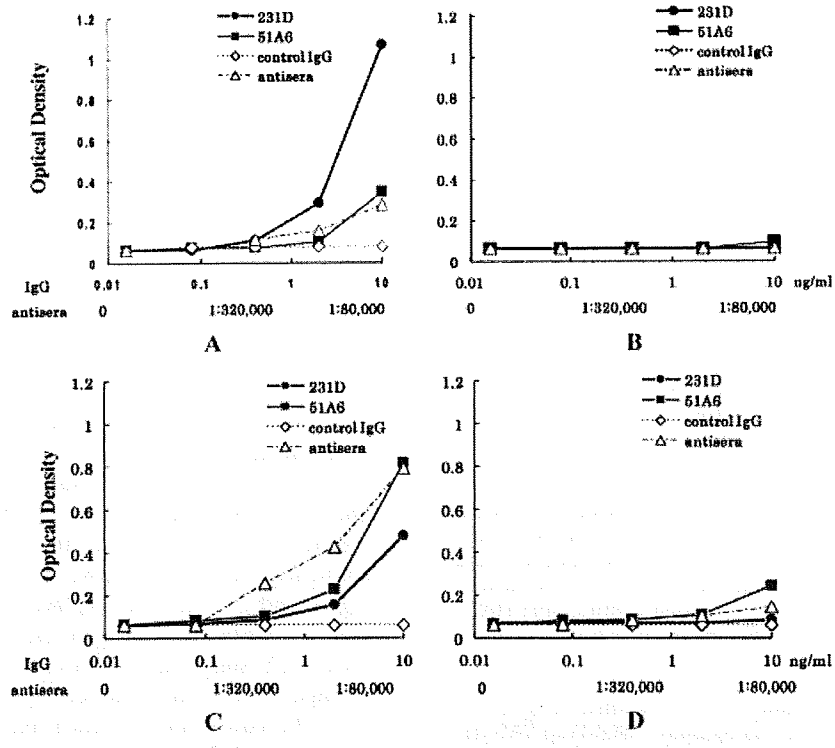

Figure 2. Binding activity of mouse monoclonal anti–phosphatidylserine/prothrombin complex (anti-PS/PT) antibodies 231D and 51A6. **A** and **B**, Binding activity of 231D and 51A6 for the PS/PT complex (**A**) and for phosphatidylserine alone (**B**) was determined by enzyme-linked immunosorbent assay (ELISA) using phosphatidylserine-coated plates. **C** and **D**, Binding activity of 231D and 51A6 for antiprothrombin antibody (APT), using prothrombin alone as the antigen coated onto either irradiated (**C**) or nonirradiated (**D**) plates, was determined by ELISA. Monoclonal antibody 51A6 bound to prothrombin coated on both irradiated and nonirradiated plates, whereas 231D showed little binding to prothrombin under both conditions. In all experiments, control IgG, consisting of purified mouse IgG from pooled normal mouse serum, was used at the indicated concentrations (ng/ml), and antisera, consisting of sera from mice that had been immunized with human prothrombin, were used at the indicated dilutions. Values are from a representative experiment.

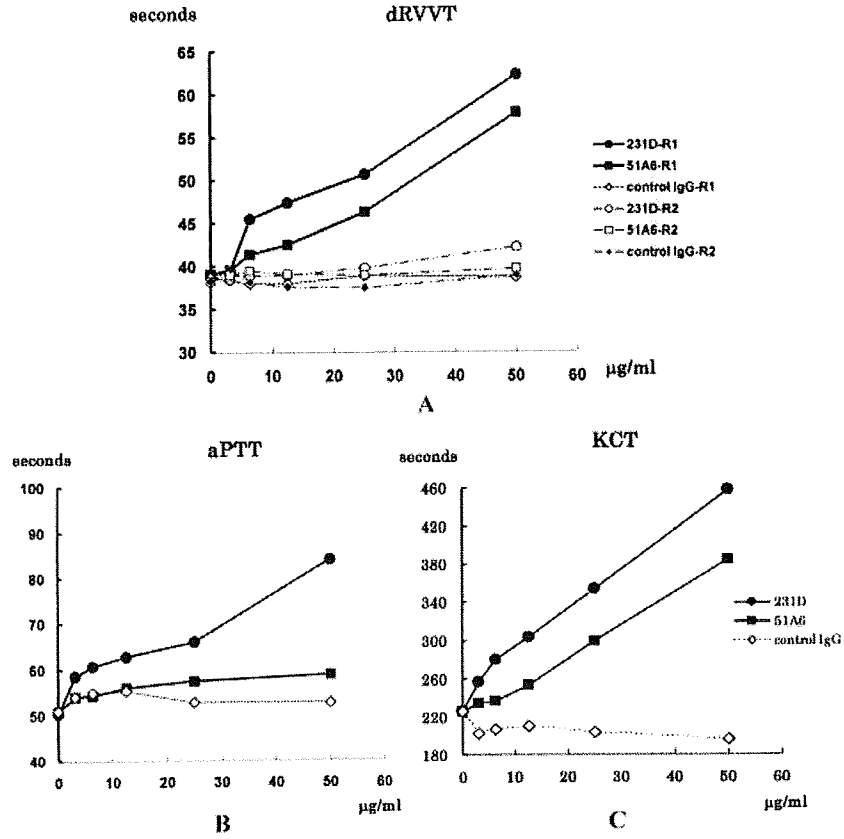

Figure 3. Lupus anticoagulant (LAC) activity of mouse monoclonal anti–phosphatidylserine/prothrombin complex antibodies 231D and 51A6, as determined by **A**, the dilute Russell's viper venom time (dRVVT), **B**, the activated partial thromboplastin time (APTT), and **C**, the kaolin clotting time (KCT). For measurement of dRVVT, purified 231D or 51A6 monoclonal antibody was added to normal plasma, and the dRVVT was determined. Reagent 1 (R1) contains a low concentration of phospholipid, and reagent 2 (R2) contains a high concentration of phospholipid. The APTT and KCT were determined in plasma that had been spiked with either 231D or 51A6. In all experiments, control IgG consisted of purified mouse IgG from pooled normal mouse serum. Numbers across the bottom are the concentration (in µg/ml) of 231D, 51A6, and control IgG tested. Values are from a representative experiment.

human prethrombin 1. Both clones bound strongly to the PS/PT complex, but not to phosphatidylserine alone (Figures 2A and B). Both murine monoclonal antibodies are of IgG1 isotype.

APT-alone activity was also investigated in the monoclonal anti-PS/PT antibodies. We found that 51A6 bound to prothrombin coated onto both irradiated and nonirradiated ELISA plates, but 231D showed a lower level of binding to prothrombin under both conditions (Figures 2C and D). Normal mouse IgG and pooled sera obtained from mice that had been immunized with human prothrombin were used as the negative control and the positive control, respectively.

LAC activity of monoclonal anti-PS/PT antibody. Purified 231D or 51A6 monoclonal antibody was added to normal plasma, and the dRVVT was measured in the monoclonal anti-PS/PT antibody–spiked plasma (Figure 3). With reagent 1 of the dRVVT test, which has a low phospholipid concentration, the clotting time of 231D-spiked plasma was prolonged in a dose-dependent manner. The clotting time was largely more prolonged with reagent 1 of the dRVVT test than with reagent 2, which

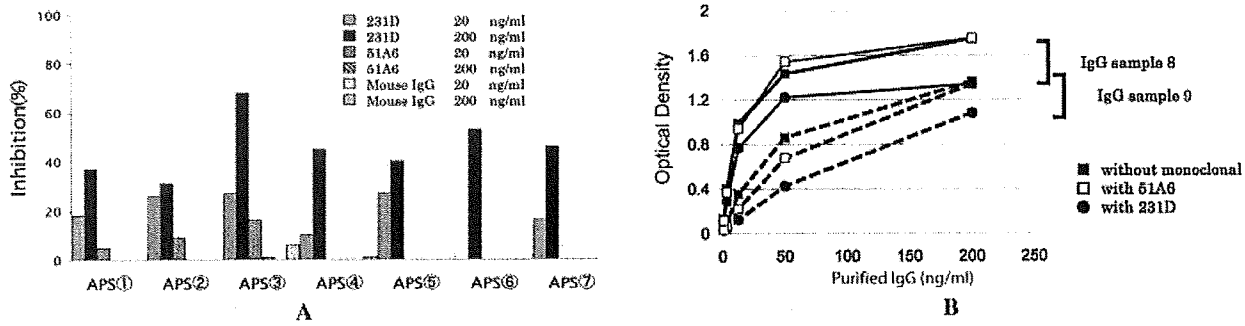

Figure 4. Competitive enzyme-linked immunosorbent assay (ELISA). IgG was purified from serum samples obtained from 9 patients with antiphospholipid syndrome (APS) who had anti–phosphatidylserine/prothrombin complex (anti-PS/PT) antibodies. **A,** Percentage inhibition of IgG binding in the presence of monoclonal antibody 231D, 51A6, or mouse IgG. Monoclonal antibody 231D, 51A6, or purified mouse IgG from pooled normal mouse serum (20 or 200 ng/ml) was preincubated on plates that had been coated with PS/PT complex, and 1 mg/ml of purified IgG from 7 of the APS patients was added. The percentage inhibition of IgG binding was calculated by comparing the optical density values in the presence of 231D, 51A6, or mouse IgG with the optical density values in the absence of 231D, 51A6, or mouse IgG, respectively. **B,** Inhibition curves following coincubation of monoclonal antibody 231D or 51A6 with the indicated concentrations of purified IgG from APS patients 8 and 9. As controls, purified IgG from the 2 APS patients was also incubated without monoclonal antibody.

contains a high concentration of phospholipids. Similar results were obtained with the 51A6-spiked plasma, but the clotting time was not as prolonged as with the 231D-spiked plasma.

Dose-dependent prolongations of the clotting time in both the APTT and the KCT tests were also found in 231D-spiked plasma and in 51A6-spiked plasma. Plasma containing 231D showed stronger anticoagulant properties than did plasma containing 51A6.

Findings of competitive ELISAs. The binding of purified IgG from anti-PS/PT–positive APS patients to the PS/PT complex was inhibited by 231D (35–70%). In contrast, there was no significant effect of 51A6 on the binding of IgG fractions to PS/PT complex (Figure 4A). Coincubation of 231D with purified IgG from APS patients also produced dose-dependent inhibition (Figure 4B).

Effects of monoclonal anti-PS/PT antibody on thrombin generation. The effect of monoclonal anti-PS/PT antibody on thrombin generation in vitro was evaluated by chromogenic assay using purified human clotting factors (Figures 5A–C). In the absence or in the presence of a very low concentration of FVa (0.1 ng/ml), the 231D monoclonal antibody increased thrombin generation by as much as 87% and in a dose-dependent manner. In contrast, when a high concentration of FVa (1 ng/ml) was added, 231D decreased thrombin generation by as much as 35%. The 51A6 monoclonal antibody displayed a lower level of inhibition of thrombin generation regardless of the concentration of FVa.

We also examined whether various concentrations of FXa altered the effects of 231D on thrombin generation (Figure 5D). Under 2 different constant concentrations of FVa, the effects of 231D on thrombin generation were increased in the presence of increasing concentrations of FXa. Again, we found that the 51A6 monoclonal antibody exhibited little inhibition of thrombin generation under any condition examined.

DISCUSSION

In this study, we demonstrated that the plasma levels of markers of thrombin generation/fibrinolysis turnover were elevated in patients with anti-PS/PT antibody, regardless of the coexistence of aCL. The mouse monoclonal anti-PS/PT antibody 231D, which has binding properties similar to those of anti-PS/PT found in patients with APS, showed "bipolar" effects on thrombin generation triggered by FXa.

Despite the proposal by some investigators of a possible correlation between APT and thrombosis, no clinical data have reported a link between increased thrombin/plasmin generation and antibodies against prothrombin. This study is the first to show the up-regulation of thrombin/plasmin generation in patients with anti-PS/PT antibody regardless of the presence of aCL. We also tested anti-β_2GPI antibodies in this study (data not shown), and the results were almost identical to those found in aCL. (None of our patients were positive for anti-β_2GPI antibodies but negative for aCL.)

There are several reports showing enhanced thrombin generation and fibrinolytic turnover in APS

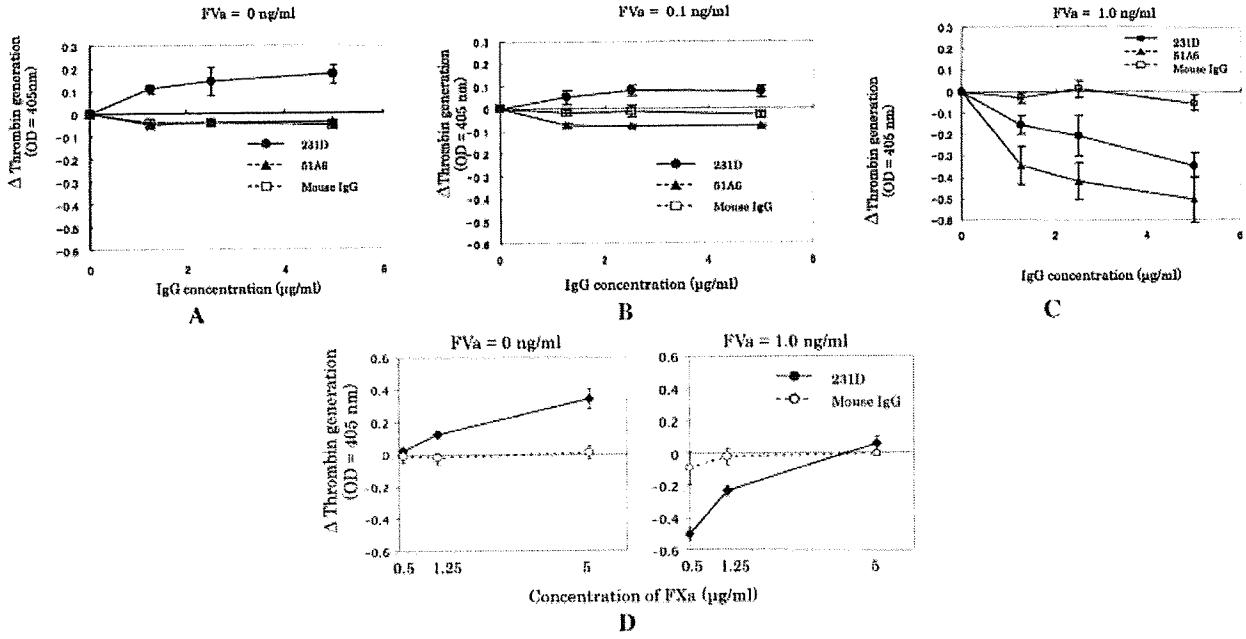

Figure 5. Evaluation of the effects of monoclonal anti–phosphatidylserine/prothrombin complex (anti-PS/PT) antibodies on thrombin generation in vitro, as determined by chromogenic assay. Purified human clotting factors were used in these experiments. **A–C,** Thrombin generation was measured in the absence of activated factor V (FVa) (**A**), in the presence of 0.1 ng/ml of FVa (**B**), and in the presence of 1 ng/ml of FVa (**C**), using a constant concentration of 1.25 μg/ml of FXa. **D,** Thrombin generation was measured in the absence of FVa (left) and in the presence of 1 ng/ml of FVa (right), using 0.5, 1.25, and 5 μg/ml of FXa. A constant concentration of 2.5 μg/ml of 231D or control IgG (purified mouse IgG from pooled normal mouse serum) was used. Values are the mean ± SEM difference in thrombin generation, as determined by the optical density at 405 nm (OD_{405}) value minus the OD_{405} value in the absence of monoclonal antibodies.

patients with aCL (22,23). In addition, de Laat et al (24) showed that β_2GPI-dependent LAC is highly correlated with thrombosis in patients with APS. Those reports clearly indicated that antibodies against β_2GPI, represented by aCL, anti-β_2GPI antibodies, or β_2GPI-dependent LAC, are correlated with high levels of thrombin generation. In contrast, there has been no report showing a correlation between antibodies against prothrombin, represented by prothrombin dependent LAC or anti-PS/PT. De Laat et al (24) failed to demonstrate increased thrombin generation in patients with β_2GPI-independent LAC, but such LAC would comprise antibodies against prothrombin and antibodies against nonspecific (or undetermined) proteins. Our data revealed that in the absence of antibodies against β_2GPI, levels of anti-PS/PT antibody correlated with elevated levels of markers of thrombin generation in APS patients, providing evidence that the increased thrombin/fibrin generation in these patients is related to anti-PS/PT itself.

The plasma samples were collected at least 3 months after the last thrombotic event, suggesting that patients with anti-PS/PT antibody are basically in a thrombophilic state. Some prothrombotic triggers may alter the balance between thrombin generation and regulators of thrombin generation, eventually leading to thrombosis.

The antibody responsible for prothrombin-dependent LAC activity is closely related to APTs detected by anti-PS/PT assay. In the setting of autoimmune disease, both anti-PS/PT and APT alone have been shown to be correlated with the presence of LAC, but anti-PS/PT had a markedly stronger relative risk for the presence of LAC than did APT alone (16). Many patients in that study had both anti-PS/PT and APT alone, but no correlation of their titers was found, even though some patients had very high levels of anti-PS/PT antibody in the absence of APT alone and vice versa.

To clarify the characteristics and properties of anti-PS/PT in thrombin generation or in the prothrombotic state observed in patients with anti-PS/PT, we successfully established 2 monoclonal antibodies. The

231D monoclonal antibody, which was obtained by immunizing mice with whole prothrombin, showed strong anti-PS/PT activity. Prothrombin was digested with thrombin, and the prethrombin 1 fraction, which lacks the phospholipid-binding domain of prothrombin, was used as immunogen to establish monoclonal APTs with phosphatidylserine-independent binding activity. We established monoclonal antibody 51A6, which as we expected, had strong APT-alone activity but lower anti-PS/PT activity. Monoclonal antibody 231D had minor APT-alone activity as compared with the 51A6 monoclonal antibody. The presence of calcium did not affect APT-alone activity in either of these monoclonal antibodies. The affinity constant (K_a) of 51A6 to prothrombin, as determined by liquid-phase inhibition ELISA, was $5.49 \times 10^{-9}M$ (data not shown), suggesting that 51A6 had moderate or strong affinity to the prothrombin molecule. The binding affinity of 231D to the PS/PT complex may be comparable to that of 51A6 according to the results of the anti-PS/PT ELISA.

Competitive ELISA revealed that 231D partially inhibited the binding to the PS/PT complex of the autoimmune anti-PS/PT antibody derived from patients with APS, implying that 231D shared the epitope(s) on phosphatidylserine-bound prothrombin with autoimmune anti-PS/PT. In contrast, 51A6 did not display any interaction in the binding between autoimmune anti-PS/PT and the PS/PT complex; thus, the 51A6 epitope on prothrombin is independent of those of autoimmune anti-PS/PT antibody.

LAC activity of monoclonal APTs alone has previously been reported (25). However, our data showed that the 231D monoclonal antibody had a stronger inhibitory effect in the APTT test than did the 51A6 monoclonal antibody, suggesting that 231D represents immunologic and hematologic properties of autoimmune anti-PS/PT antibody found in patients with APS.

Prothrombin is a single-chain glycoprotein composed of 3 structural regions as follows: fragment 1, which contains the Gla domain and kringle 1 domain, fragment 2, which mainly contains the kringle 2 domain, and a serine protease precursor domain (26,27). Prothrombin is activated and cleaved into α-thrombin in a membrane-dependent process that includes the actions of FXa, its cofactor FVa, and divalent calcium ions assembled into a complex on the membrane.

To investigate the direct effect of anti-PS/PT on thrombin generation in vitro, we prepared a chromogenic thrombin generation assay, and the effect of the 231D monoclonal antibody was explored in the presence of different concentrations of FVa and FXa. In the presence of a low concentration of FVa, 231D increased thrombin generation in a dose-dependent manner. In contrast, when a high concentration of FVa was added, 231D diminished thrombin generation. In the second set of experiments, a high concentration of FXa was found to enhance the effect of 231D in the presence of a constant concentration of FVa. When FXa was added at a high concentration, the relative FVa concentration was low, resulting in increased thrombin generation by 231D. Taken together, the balance of FVa/FXa was the determinant of the behavior of 231D with regard to the generation of thrombin, antithrombin, or prothrombin.

In the presence of sufficient amounts of FVa, the 231D monoclonal antibody decreased thrombin generation, and the 51A6 monoclonal antibody showed a similar effect, although its potential was lower. A previous study has also shown inhibitory effects of APTs on thrombin generation. Church et al (28) produced 5 monoclonal antibodies to prothrombin kringle 2, and 2 of them inhibited FVa-dependent prothrombin activation. In terms of the phospholipid-dependency of LAC-like activity shown by the phospholipid-neutralizing test in the LAC assay, the interpretation of the behavior of monoclonal APTs may be as follows: under in vitro conditions, the higher the amount of phospholipid the more prothrombinase and/or prothrombin are available, leading to the acceleration of thrombin generation in the presence of APTs.

The in vitro effects of the 231D monoclonal antibody on thrombin generation, on the other hand, are different according to the balance of FVa and FXa. Zhao et al (29) generated and characterized a human monoclonal antiprothrombin antibody with strong LAC activity that enhanced prothrombin binding to phospholipid and shortened the plasma coagulation times (29). Their data provide an explanation for the LAC paradox, showing that a single, highly purified aPL can behave as LAC and can paradoxically increase coagulation in endothelial cell–based coagulation assays. Our current findings support the hypothesis stated above, showing that the mouse monoclonal anti-PS/PT antibody 231D, which carries strong LAC activity, increased thrombin generation in the absence of FVa as well as in the presence of very low concentrations of FVa. The 231D monoclonal antibody may allow prothrombin to bind more firmly to phospholipid, assembling prothrombin on the phospholipid and subsequently increasing thrombin generation. When abundant FXa is present, FXa is able to act on prothrombin to generate thrombin. This may not be the situation when large amounts of FVa are

present, since the coenzyme activity of FVa is sufficiently potent to overcome the antithrombin generation effects of 231D. The 51A6 monoclonal antibody, needing no phospholipid involvement for its binding to prothrombin, would not play a role in the augmentation of thrombin generation in this mechanism.

This is the first study to show that monoclonal antibodies against prothrombin do not exclusively have LAC-like thrombin reduction potential, but are also able to increase thrombin generation. However, the phenomena we observed are evidently not the only mechanism of thrombosis in patients with APS. Recently, great interest has arisen with regard to the binding of aPL to procoagulant cells and how this binding mediates cell activation related to the clinical manifestations of APS. Within the last few years, studies examining the mechanism of signal transduction implicated in the induction of procoagulant substances by aPL have been performed. There is now clear evidence that the p38 MAPK pathway of cell activation plays an important role in anti-β_2GPI antibody–mediated cell activation (30–32). Considering the similarities of the properties of anti-β_2GPI and APT, procoagulant cell activation may be a major event in the generation of thrombosis in APS patients who have anti-PS/PT antibody. Thrombin may serve as a trigger for cells to express phosphatidylserine on their surface via protease-activated receptors, which are present on many types of procoagulant cells, and with glycoprotein Ib–IX–V complex on the surface of platelets, leading to platelet aggregation and activation (33).

Thrombin is a key enzyme in hemostasis and is a multipotential enzyme in the coagulation/inflammation system. The direct involvement of anti-PS/PT antibody in thrombin generation may be a clue to the pluripathologic process that occurs in patients with APS (34). Although further clarification of the roles of anti-PS/PT antibodies in APS is essential, we believe that the findings of this study contribute to the understanding of the pathophysiology of thrombophilia in patients with APS.

AUTHOR CONTRIBUTIONS

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 design. Atsumi, Ieko, Koike.

Acquisition of data. Sakai, Atsumi, Ieko, Amengual, Furukawa, Furusaki, Bohgaki, Kataoka, Horita, Yasuda, Koike.

Analysis and interpretation of data. Sakai, Atsumi, Ikeo, Amengual, Koike.

Manuscript preparation. Sakai, Atsumi, Amengual, Koike.

Statistical analysis. Sakai, Atsumi.

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Immunological Reconstitution after Autologous Hematopoietic Stem Cell Transplantation in Patients with Systemic Sclerosis: Relationship Between Clinical Benefits and Intensity of Immunosuppression

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ABSTRACT. ***Objective.*** To analyze the relationship between clinical benefits and immunological changes in patients with systemic sclerosis (SSc) treated with autologous hematopoietic stem cell transplantation (HSCT).

Methods. Ten patients with SSc were treated with high-dose cyclophosphamide followed by highly purified CD34+ cells (n = 5) or unpurified grafts (n = 5). Two groups of patients were retrospectively constituted based on their clinical response (good responders, n = 7; and poor responders, n = 3). As well as clinical findings, immunological reconstitution through autologous HSCT was assessed by fluorescence-activated cell sorter analysis, quantification of signal joint T cell receptor rearrangement excision circles (sjTREC), reflecting the thymic function, and *foxp3*, a key gene of regulatory T cells, mRNA levels.

Results. Patients' clinical and immunological findings were similar between good and poor responders, or CD34-purified and unpurified groups at inclusion. The sjTREC values were significantly suppressed at 3 months after autologous HSCT in good responders compared with poor responders (p = 0.0152). Reconstitution of CD4+CD45RO- naive T cells was delayed in good responders compared with poor responders. The phenotype of other lymphocytes, cytokine production in T cells, and *foxp3* gene expression levels after autologous HSCT did not correlate with clinical response in good or poor responders. Clinical and immunological findings after autologous HSCT were similar between CD34-purified and unpurified groups.

Conclusion. Our results suggest that immunosuppression intensity, sufficient to induce transient suppression of thymic function, is attributable to the feasible clinical response in patients with SSc treated with autologous HSCT. Appropriate monitoring of sjTREC values may predict clinical benefits in transplanted SSc patients after autologous HSCT. (First Release May 15 2009; J Rheumatol 2009;36:1240–8; doi:10.3899/jrheum.081025)

Key Indexing Terms:
SYSTEMIC SCLEROSIS HEMATOPOIETIC STEM CELL TRANSPLANTATION
IMMUNOLOGICAL RECONSTITUTION

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Supported by a grant from the Japanese Ministry of Health, Labor and Welfare.

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Accepted for publication January 8, 2009.

Systemic sclerosis (SSc) is an autoimmune disease characterized by the presence of skin sclerosis, organ fibrosis, and autoantibodies[1]. Despite extensive research on autoimmunology and endotheliology, its pathophysiology has been far from conclusive[2,3]. The skin and organ manifestations of SSc are, in general, slowly progressive and chronically disabling. In some patients, however, they can be rapidly progressive and fatal due to organ involvements such as interstitial pneumonia, arrhythmia, and renal failure. Severe organ involvement frequently occurs within the first 3 years of disease[1]. These clinical features affect daily living activity and life expectancy in patients with SSc.

Autologous hematopoietic stem cell transplantation (HSCT) has been indicated for patients with autoimmune diseases, resulting in great success particularly in patients with SSc[4-10]. Autologous HSCT is one of the treatments in