Approximately 60% of PS forms a non-covalent 1:1 stoichiometric complex with C4b-binding protein, which results in loss of cofactor function for activated protein C [1,2].

PS and its structural homologue Gas6 are ligands for TAM receptors (Tyro3, Axl, and Mer) and are involved in various pathological conditions such as inflammation, cancer growth, and autoimmune disease [5]. Protein S is involved in the engulfment of phosphatidylserine-exposed apoptotic cells with Mer-expressing macrophages [6,7]. Mice lacking the PS gene show embryonic lethal coagulopathy and vascular defects [8,9].

VTE is a multifactorial disorder resulting from the interaction of acquired and genetic factors. Regarding the genetic factors, factor V Leiden (c.1601G>A, p.R534Q) and prothrombin G20210A mutations are well-known risk factors for VTE in Caucasians [10]. These two mutations do not exist in East Asian populations [11,12]. We and other researchers identified a missense mutation (c.586A>G, p.K196E) in the PS gene as a genetic risk factor for VTE with odds ratios between 3.74 and 8.56 [13–16].

The frequency of E-allele in the Japanese general population is approx. 0.009 [14,16,17]. PS K196E mutation is likely to be specific for Japanese, because it has not been identified in Chinese, Koreans and Caucasians [17,18]. This mutation is located in the second epidermal growth factor (EGF)-like domain of PS and is also called PS K155E mutation (using a nomenclature system of mature protein) or PS Tokushima mutation [19,20].

Heterozygous carriers for PS K196E mutation showed reduced anticoagulant activity within normal limits of antigen levels, indicating type II deficiency [13,16,19–21]. We reported that the PS anticoagulant activities in individuals with the heterozygous (KE) genotype in a Japanese general population were substantially overlapped with those in individuals with the wild-type (KK) genotype; the mean difference of PS anticoagulant activity was only 16% [22]. This finding suggests that PS anticoagulant activity is not a useful marker for the PS K196E mutation.

PS K196E carriers have been identified thus far by genetic analyses such as direct sequencing, genotyping (e.g., TaqMan), and restriction fragment length polymorphism analysis [19–22]. These analyses are accurate but expensive and time-consuming. In addition, they are not routinely available at clinical laboratories. A simple and rapid detection method for PS K196E carriers in the clinical setting remained to be established. In the present study, we developed a sandwich enzyme-linked immunosorbent assay (ELISA) system for detecting a PS K196E mutant in plasma, using a novel monoclonal antibody.

Materials and Methods

Ethics Statement

This study was approved by the Institutional Review Boards of the National Cerebral and Cardiovascular Center and the Kanazawa University Graduate School of Medical Science. Written informed consent was obtained from all individuals involved in the study.

Generation of PS K196E mutation-specific antibodies

Three GANP transgenic mice [23] were immunized with a keyhole limpet hemocyanin-conjugated synthetic 11-amino acid peptide (C^{186}KNGFVMLSNE^{196}, mutation underlined) with the K196E mutation. GANP mice express a high level of germinal center-associated nuclear protein and an increased frequency of somatic mutations in the Ig-variable region, and they are thus suitable for high-affinity antibody production [23].

Hybridoma cells were generated by a standard cell fusion technique and plated in 96-well plates by limiting dilution. Immunization and generation of hybridoma cells were performed by TransGenic Inc. (Kumamoto, Japan).

Expression and purification of recombinant PS proteins

Wild-type and K196E mutant forms of human full-length PS (Ala41–Ser676) (FL-PS-K and FL-PS-E, respectively) and those of four EGF-like domains of PS (Ile117–Glu283) (EGF-PS-K and EGF-PS-E, respectively) were expressed in HEK293S GnTI$^-$ cells obtained from the American Type Culture Collection (Manassas, VA) by our use of the mammalian expression vector with a mouse *Nid-1* signal sequence and a C-terminal His tag [24]. For the expression of FL-PS, vitamin K1 (10 mg L^{-1}) was added to the culture medium.

Recombinant PS proteins were purified from the culture supernatants by Ni-affinity chromatography. After dialysis in phosphate-buffered saline (PBS, pH 7.4), the proteins were concentrated and used for the binding assay of monoclonal antibodies, the Western blot analysis, and validation of the PS K196E ELISA system. The concentrations of the recombinant proteins were determined from the absorbance at 280 nm assuming an $E_{280}^{1\%} = 10.0$.

Screening of PS K196E-specific antibodies

The ELISA plates were coated with 50 μL of 1 μg mL^{-1} EGF-PS-K or EGF-PS-E for 1 h at room temperature (RT). The plates were washed three times with PBS with 0.05% Tween 20 and blocked with 200 μL of 0.5% gelatin in PBS overnight at 4°C. The plates were then incubated with 50 μL of culture supernatants of hybridoma cells for 1 h at RT. After being washed, the plates were incubated with 50 μL of 1 μg mL^{-1} horseradish peroxidase (HRP)-labeled anti-mouse IgG antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 1 h. After the plates were washed again, 0.5 mg mL^{-1} *o*-phenylenediamine was added to the plates, and absorbance was measured at 490 nm against a reference wavelength of 650 nm.

Antibody purification and labeling

Three hybridoma cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum. The monoclonal antibodies were purified from the culture supernatants using an Ex-Pure Protein G kit (Kyoto Monotech, Kyoto, Japan). Purified antibodies were labeled with HRP using the Peroxidase Labeling Kit NH_2 (Dojindo Laboratories, Kumamoto, Japan). The concentrations of the monoclonal antibodies were determined from the absorbance at 280 nm assuming an $E_{280}^{1\%} = 10.0$.

Binding assay of monoclonal antibodies

For the antibody binding assay, the plates were coated with 50 μL of 0.05 μg mL^{-1} EGF-PS-K or EGF-PS-E or 50 μL of 1.0 μg mL^{-1} FL-PS-K or FL-PS-E for 1 h at RT. After blocking, the plates were incubated with 50 μL of 1.0 μg mL^{-1} monoclonal antibody for 1 h at RT. After the plates were washed, 50 μL of HRP-labeled anti-mouse IgG antibody was added and the plates were incubated for 1 h. After the plates were washed again, 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Kirkegaard & Perry Laboratories) was added. After a 10-min incubation, 100 μL of 1 N HCl was added and the absorbance was measured at 450 nm against a reference wavelength of 650 nm.

Western blot analysis

The recombinant PS proteins with a C-terminal His tag were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%–20% gradient gel) and transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). After blocking with 5% skim milk in PBS, the membrane was incubated with 1.0 μg $mL^{-1}$of monoclonal mouse anti-His tag antibody (Cat. No. D291-3, MBL, Nagoya, Japan) or the PS K196E mutation-specific

monoclonal antibody 15C8 for 1 h, washed with PBS, and incubated with 0.1 μg mL^{-1} HRP-labeled anti-mouse IgG (Cat. No. 474–1802, Kirkegaard & Perry Laboratories). The chemiluminescence signal was detected by using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) and an LAS-3000 imager (Fujifilm, Tokyo).

Plasma samples

We collected DNA and plasma samples from patients with VTE and normal individuals to evaluate the hereditary or acquired risks for the development of VTE, at the Kanazawa University Graduate School of Medical Science. Although DNA samples were obtained from all of the participants of the study, plasma samples were obtained from only 133 individuals consisting of 11 with the mutant KE genotype and 122 with the wild-type KK genotype. Of the 11 individuals with the KE genotype, three had a history of adverse pregnancy outcome, two had a history of a thrombotic event, one was a rheumatoid arthritis patient, and the remaining five were healthy. Of the two individuals with a history of thrombotic events, one suffered from pulmonary embolism and warfarin was administered. The other developed thrombophlebitis in pregnancy, and heparin was administered. None of the individuals with the KK genotype had a history of thrombotic events.

Blood samples were drawn from an antecubital vein through a needle into disposable, siliconized, evacuated plastic tubes containing a 1:10 volume of 3.2% (wt/vol) trisodium citrate. The plasma fractions were separated by centrifugation at 1,710 × *g* for 10 min, and genomic DNA was isolated from peripheral blood leukocytes. The plasma samples were stored at −80°C until measurement.

Genotyping

The K196E mutation was genotyped using a polymerase chain reaction-restriction fragment length polymorphism (PCR-FFLP) analysis. The mutagenic primers 5′-AAT GGT TTT GTT ATG CTT TCA CAT-3′ (mutation underlined) and 5′- TGT TAG TAT AAG CAC TTA CAT ATC-3′ were employed. Two fragments (27 bp and 128 bp) of the Hsp 92II (Promega, Madison, WI)-digested PCR product from the mutant allele were separated by 8% Synergel (Diversified Biotech, Dedham, MA) electrophoresis.

The PS K196E ELISA system for the detection of a PS K196E mutant in plasma

The plates were coated with 100 μL of 10 μg mL^{-1} polyclonal rabbit anti-human PS antibody (Cat. No. A0384, Dako, Glostrup, Denmark) in 50 mM carbonate bicarbonate buffer (pH 9.6) and left overnight at 4°C. The plates were washed with Tris-buffered saline (TBS, pH 7.4) with 0.1% Tween 20 and blocked with 200 μL of 1% bovine serum albumin in TBS for 1 h at RT. After blocking, the plates were incubated with TBS alone or with 100 μL of patients' plasma samples diluted 20-fold using TBS for 1–2 h at RT.

After the plates were washed, the plates were then incubated with 100 μL of 5 μg mL^{-1} HRP-labeled PS K196E mutation-specific monoclonal antibody 15C8 for 1 h. After the plates were washed, 100 μL of TMB substrate solution was added to the plates. After a 10-min incubation, 100 μL of 1 N HCl was added and the absorbance was measured at 450 nm against a reference wavelength of 650 nm. The assay result of each sample was obtained by subtracting the absorbance value of TBS alone from that of each sample.

Validation of PS K196E ELISA system

We tested our newly developed ELISA system by determining the intra-assay variability, inter-assay variability, dilution linearity, and spiking recovery. Intra- and inter-assay variations were determined as the coefficients of variation (CV). The CV was determined by dividing the standard deviation by the mean of absorbance at 450 nm against a wavelength of 650 nm for each plasma sample. The intra-assay CV was determined by measuring the absorbance of three KE plasma samples five times in a single run. The inter-assay CV was determined by measuring the absorbance of three KE plasma samples in five separate runs.

To examine the linearity of dilution, we serially diluted FL-PS-E from 0 to 2.5 μg ml^{-1} in TBS alone, or a 20-fold diluted PS-deficient plasma sample contained in the STA Staclot Protein S reagent kit (Roche Diagnostics, Mannheim, Germany), or 20-fold diluted KK plasma. In the analytical recovery experiments, we assayed two different 20-fold diluted KE plasma samples in duplicate after the addition of three different amounts of FL-PS-E (25, 50, 100 ng). Serial twofold dilutions from 0 to 2.5 μg mL^{-1} of FL-PS-E in 20-fold diluted KK plasma were used for a standard curve. The recovery was calculated using the formula: (detected concentration/expected concentration) × 100.

Results and Discussion

We screened supernatants of hybridoma cells from 1,672 wells by preferential binding to a recombinant mutant PS EGF-like domain, EGF-PS-E, compared to its wild-type counterpart, EGF-PS-K. We obtained three monoclonal antibodies, 4B1, 15C8 and 16E3, all of which bound to mutant EGF-PS-E but not to wild-type EGF-PS-K.

We compared the binding of the three monoclonal antibodies to wild-type and mutant forms of PS EGF domains and full-length PS. All three antibodies bound to the mutant EGF domain EGF-PS-E (Fig 1A) as well as the full-length mutant FL-PS-E (Fig 1B). Among the antibodies, 15C8 bound most strongly to mutant FL-PS-E (Fig 1B) and was thus used for the subsequent experiments. The Western blot analysis showed that an anti-His tag antibody detected both His-tagged wild-type FL-PS-K and mutant FL-PS-E (Fig 2A), whereas the 15C8 antibody specifically detected FL-PS-E (Fig 2B).

For widespread clinical laboratory use to identify PS K196E carriers, we developed a sandwich ELISA system for detecting the PS K196E mutant in 20-fold diluted plasma using a PS polyclonal antibody as the capture antibody and the PS K196E mutation-specific monoclonal antibody 15C8 as the detection antibody. In the 133 human plasma samples examined in the present study, all 11 PS K196E heterozygote (KE) samples showed distinctly higher absorbance compared to the 122 wild-type (KK) samples (absorbance range: KK, −0.01–0.07; KE, 0.30–1.00) (Fig 3). In addition, the KE plasma samples obtained from a warfarin-treated individual and a pregnant individual also showed higher absorbance compared to the wild-type samples (absorbance: warfarin, 0.30; pregnancy, 0.37).

The intra-assay CVs and inter-assay CVs obtained from three plasma samples ranged from 1.4% to 3.1% and from 8.1% to 14.7%, respectively. All were within a generally acceptable range (<10% for the intra-assay CV and <15% for the inter-assay CV) [25, 26]. We therefore concluded that the PS K196E ELISA system can discriminate KE samples from KK samples.

To examine the linearity of the PS K196E concentration in the ELISA system, we made three different standard curves obtained from a series of the FL-PS-E dilution in TBS alone, 20-fold diluted PS-deficient plasma, and 20-fold diluted KK plasma. All gave good linearity (Fig 4, R^2 = 0.99–1.00). The standard curves prepared from dilution with TBS alone and with the PS-deficient plasma were similar (Fig 4). The FL-PS-E standards diluted with wild-type KK plasma showed lower absorbance values than those with TBS alone or with PS-deficient plasma

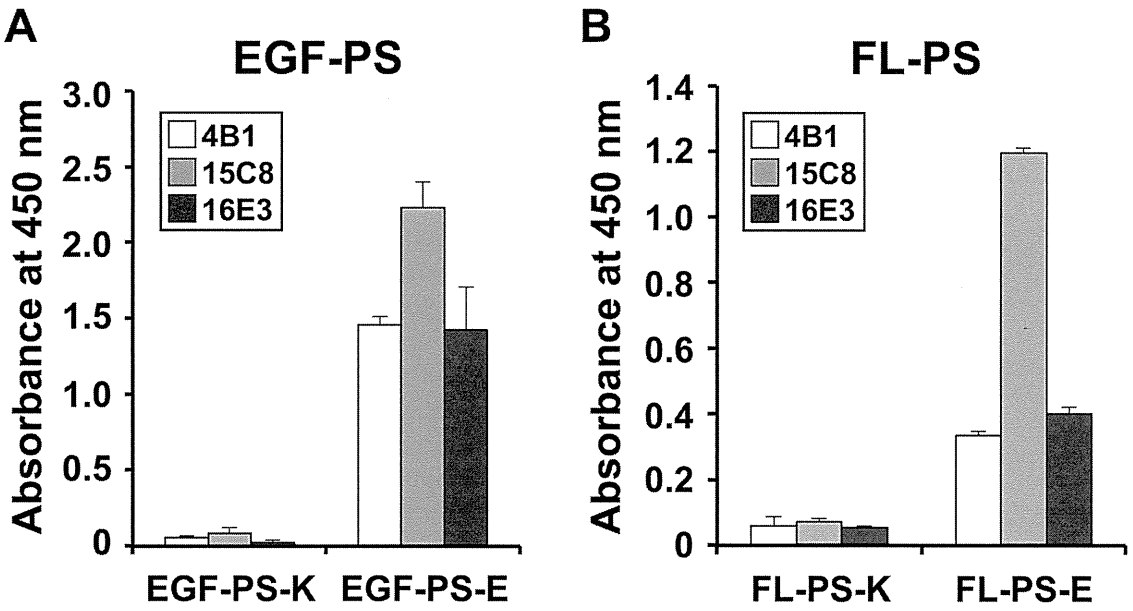

Fig 1. Binding of PS K196E mutation-specific monoclonal antibodies to recombinant PS proteins. The 96-well plates were coated with EGF-PS-K or EGF-PS-E (A) or FL-PS-K or FL-PS-E (B). The plates were then incubated with each of three monoclonal antibodies, 4B1, 15C8, and 16E3. HRP-labeled anti-mouse IgG antibody was used for the second antibody. Bound HRP was developed with TMB substrate, and the absorbance was measured at 450 nm. Error bars: SD (n = 3).

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(Fig 4). Wild-type PS-K and mutant PS-E may compete for binding to a PS polyclonal antibody immobilized on the microplate wells, resulting in the low absorbance values in the FL-PS-E standards diluted with wild-type KK plasma.

We next performed recovery experiments. The recoveries of three different amounts of FL-PS-E (25, 50, and 100 ng) in two different KE plasma samples ranged from 95% to 106% (recovery of FL-PS-E: 25 ng, 95%: 50 ng, 100%: 100 ng, 106%) when the standard curve obtained from the FL-PS-E standards diluted with KK plasma was used. When the standard curves were obtained from the FL-PS-E standards diluted with PS-deficient solution such as TBS or PS-deficient plasma, the recovery was low (22%–76%, 25%–67%, respectively).

The competition between wild-type PS-K and mutant PS-E for binding to the immobilized PS polyclonal antibody raises the limitation of the ELISA system in terms of quantification. That is, the concentration of PS-K in each KE plasma sample will affect the absorbance value in the ELISA system. Currently, we cannot overcome this problem.

Ethnic heterogeneity of genetic mutations in the human genome has been reported, and it is important to consider the ethnic variability of genes for properly assessing the risk of thrombosis and VTE in specific populations [27]. For example, carrier testing for factor V Leiden mutation is now one of the most frequently ordered molecular genetic tests in Caucasian populations [28]. However, this testing is not useful for East Asian populations because these populations do not have the factor V Leiden mutation [11,12]. On the other hand, PS K196E is a genetic mutation found in about 1.8% of the Japanese population [14,16,17], and the prevalence of the mutation in Japanese VTE patients is 5%–10% [13,21,22]. Given the frequency of the PS K196E mutation and its association with VTE, screening for PS K196E carriers will be valuable, especially for high-risk VTE populations such as patients with cardiovascular diseases or cancer. In addition to the clinical application, our PS K196E mutation-specific ELISA system would also be useful in research seeking to identify the association of this mutation with thrombosis-related diseases.

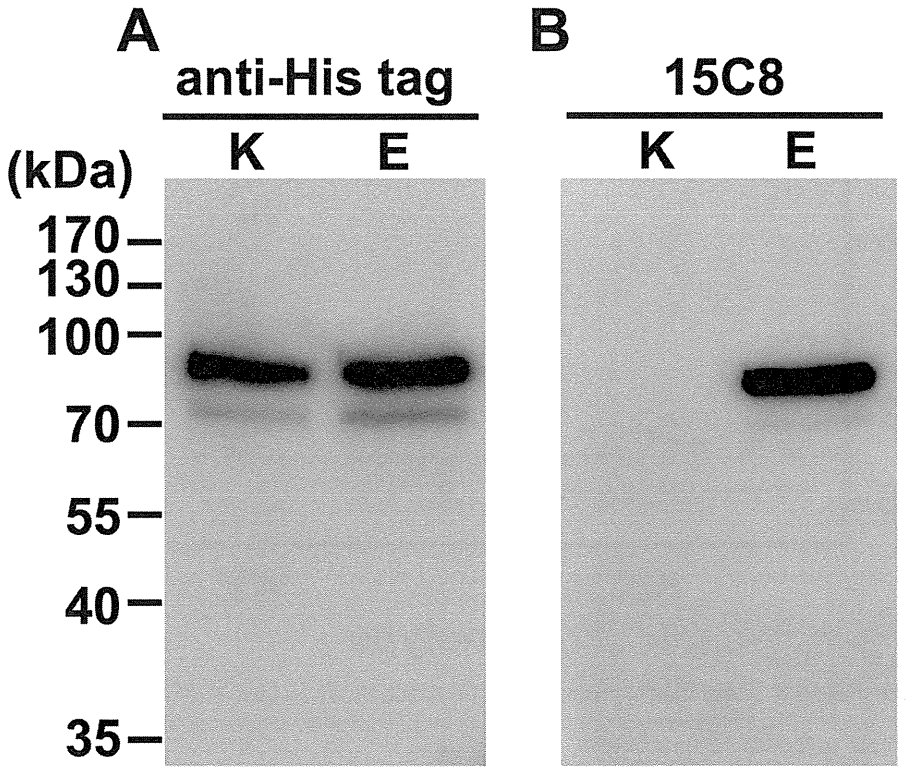

Fig 2. Western blot analysis of the recombinant PS proteins using the PS K196E mutation-specific monoclonal antibody. FL-PS-K and FL-PS-E were separated by SDS-PAGE under reducing conditions, transferred to a membrane, and incubated with the anti-His tag antibody (A) or the PS K196E mutation-specific monoclonal antibody 15C8 (B), and then with HRP-labeled anti-mouse IgG. The proteins were visualized using a chemiluminescent substrate. The molecular weights are shown on the left.

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PS type II deficiency is characterized by a reduced anticoagulant activity within normal limits of antigen levels, and it could be expected to show decreased PS-specific activity that can be obtained from the anticoagulant activity divided by the antigen level. An assay system for measuring PS-specific activity was previously developed for identifying the PS K196E mutation [29]. In that study, low PS-specific activity of < 0.69 was observed in individuals with the PS K196E mutation. Unfortunately, this method also identified individuals with low PS-specific activity who had the PS C247F mutation and warfarin-treated patients [29]. Thus, an assay system for PS-specific activity is not specific for identifying individuals with the PS K196E mutation. Our newly developed ELISA system specifically detected the PS K196E mutant and thus will be useful for the identification of PS K196E carriers in clinical settings.

A potential limitation of our mutation-specific ELISA system is that it cannot distinguish the homozygotes from the heterozygotes. Plasma PS antigen levels are expected to be strongly correlated with the absorbance values in the PS K196E ELISA system in homozygotes and heterozygotes with differing slopes. However, the present measurements using 11 KE plasma samples did not show a good correlation between the total PS antigen levels and the absorbance values in the PS K196E ELISA system (data not shown). The reasons for this might be the limited sample size and the heterogeneity of the patients' background. We should examine the correlation using a much larger sample size in the near future. The discrimination of homozygotes from heterozygotes cannot be achieved using the PS K196E ELISA system at this time.

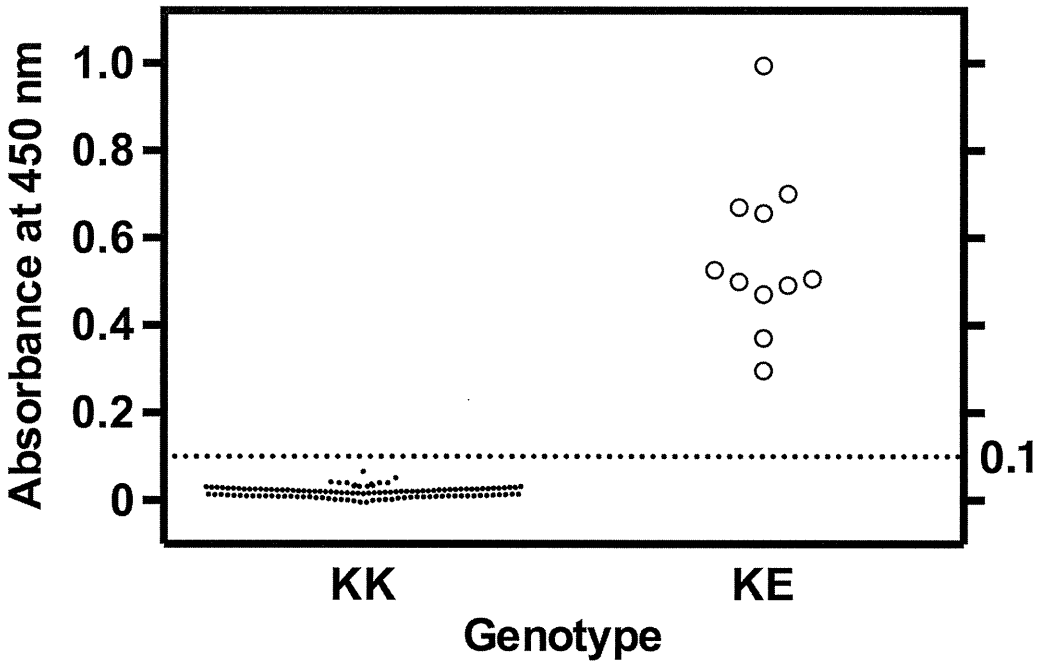

Fig 3. Detection of PS K196E mutant in plasma by the PS K196E ELISA system. Plasma samples diluted 20-fold using TBS were measured by the PS K196E ELISA system. The absorbance ranges of 122 KK and 11 KE plasma samples were –0.01–0.07 and 0.30–1.00, respectively. All data points represent the mean of three wells performed in triplicate.

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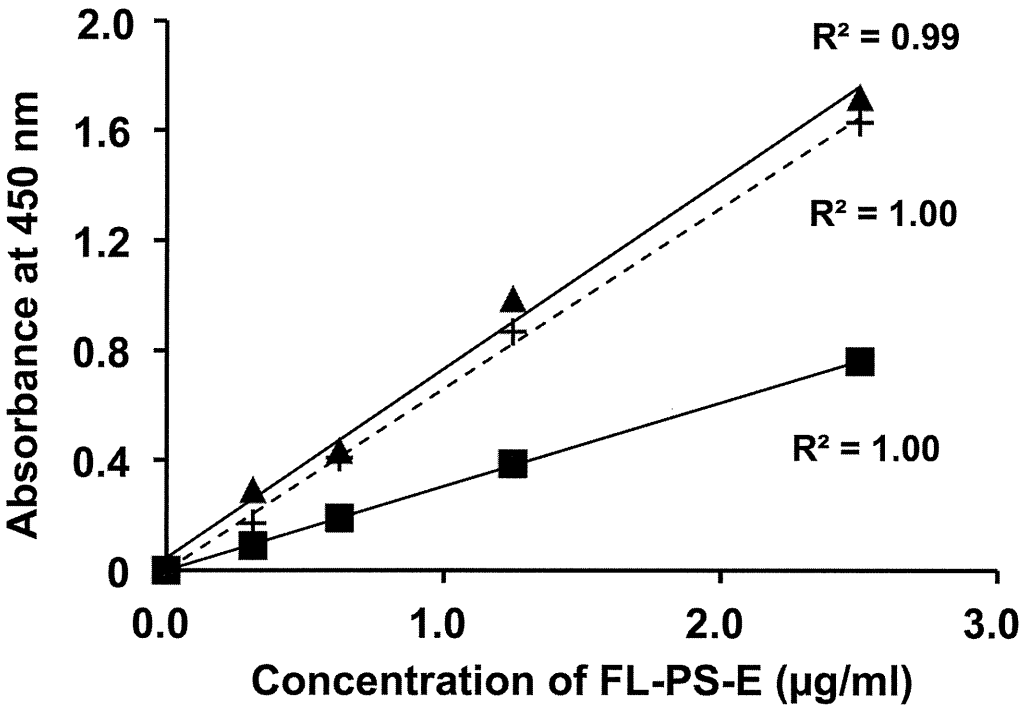

Fig 4. Dilution plots of FL-PS-E in the PS K196E ELISA system. FL-PS-E was serially diluted from 0 µg mL^{-1} to 2.5 µg mL^{-1} in TBS (▲), 20-fold diluted PS-deficient plasma (+), or a 20-fold diluted KK plasma sample (■).

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Conclusions

We developed a novel ELISA system for detecting a PS K196E mutant in plasma. This system will be a useful tool for identifying PS K196E carriers who are at high risk for VTE in the clinical environment.

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Author Contributions

Conceived and designed the experiments: TM. Performed the experiments: KM MA. Analyzed the data: KM MA KK TM. Contributed reagents/materials/analysis tools: KM MA KK AS EM. Wrote the paper: KM MA KK TM.

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Atypical haemolytic uraemic syndrome in a Japanese patient with *DGKE* genetic mutations

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Dear Sirs,

Atypical haemolytic uraemic syndrome (aHUS) is characterised by thrombosis in the microvasculature and is caused by dysregulation of the complement alternative pathway via mutations or autoantibodies. Recessive mutations in the diacylglycerol kinase ε gene (*DGKE*) were recently identified in aHUS patients under two years old (1, 2) as well as in patients with membranoproliferative glomerulonephritis, membranoproliferative-like glomerular microangiopathy, or thrombotic microangiopathy (TMA) (3, 4). A clinical feature of patients with homozygous or compound heterozygous *DGKE* mutations is initial acute kidney injury, typically in children less than one year old. The aHUS caused by *DGKE* mutations is independent of complement dysregulation (5) and the exact mechanism is not known. Loss of DGKE expression in endothelial cells showed a proinflammatory and prothrombotic phenotype, with increased expressions of ICAM-1 and tissue factor (6).

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To obtain additional clinical information for aHUS patients with *DGKE* mutations, we performed a genetic analysis of *DGKE* in Japanese patients with an aHUS onset in the first two years of life. Japan's Nara Medical University has functioned as a TMA referral centre since 1988 (7), and has collected 1,122 Japanese TMA patients until the end of 2013. The database includes 77 patients with aHUS, which is defined by acute renal failure, thrombocytopaenia, and microangiopathic haemolytic anaemia, with no severe ADAMTS13 activity deficiency or Shiga toxin-producing *Escherichia coli* infection. Patients with organ or haematopoietic stem cell transplantation were excluded from aHUS. Some of genetic analyses of aHUS have been previously reported (8, 9). From the database, we selected 14 aHUS patients with a disease onset in the first two years of life. Direct sequencing of the polymerase amplification reaction products was performed using the 3730xl DNA Analyzer (Applied Biosystems Japan, Tokyo, Japan) (8). The study protocol was approved by the Ethical Committee of the National Cerebral and Cardiovascular Center, the Hiroshima Prefectural Hospital, and Nara Medical University, and written informed consents for genetic analysis were obtained.

Among the 14 selected patients, we identified one patient who had a splice site mutation c.1213–2A>G derived from his father and a frameshift mutation c.71delT encoded with p.Leu24Cysfs*145 derived from his mother (▶Figure 1). Both mutations are likely deleterious for the DGKE function and pathogenic loss-of-function mutations. Neither genetic mutation was found in the NCBI database. A DNA sequence analysis in six complement-related genes (*CFH*, *C3*, *MCP*, *CFI*, *CFB* and *THBD*) showed that the patient had the missense polymorphisms *CFH*: p.Val62Ile, p.Glu936Asp, *CFB*: p.Leu9His, p.Arg32Gln, and *THBD*: p.Ala473Val, but did not have mutations predisposing for aHUS. The patient did not have autoantibodies against complement factor H.

Another aHUS patient who presented aHUS at eight months of age had a missense mutation, p.Ile195Met, in the *DGKE* gene. *In silico* analyses for functional prediction of the missense mutation suggested that the p.Ile195Met mutation in *DGKE* was "tolerated" by the SIFT algorithm (10) and "benign" by the Polyphen-2 algorithm (11). To date, all of the aHUS patients with *DGKE* mutations are in the homozygous or compound heterozygous state, and parents with heterozygous *DGKE* mutation had no clinical abnormalities (5). We thus did not regard a *DGKE* p.Ile195Met mutation as a disease-causing mutation. The remaining 12 patients did not carry the nonsynonymous *DGKE* mutations.

We previously reported the clinical phenotypes of the former patient with compound heterozygous *DGKE* mutations (12). He was a male baby who developed plasmapheresis-resistant aHUS at four months of age and showed extremely severe hypertension. His C3 level was undetectable (< 20 mg/dl), and he had high lactate dehydrogenase and creatinine levels. He received repeated plasma infusions and nine sessions of plasmapheresis. However, no treatment was effective for his haemolysis and renal failure. His severe hypertension did not initially respond to fluid removal by haemodiafiltration and was also refractory to treatment with a large intravenous dose of nicardipine chloride, per-oral enarapril, and losartan. Finally, treatment with the complement C5 blockage drug eculizumab every three weeks for 17 months resulted in the control of severe hypertension and the cessation of peritoneal dialysis (12). After the administration of eculizumab, the platelet counts and C3 level increased and the lactate dehydrogenase levels decreased.

The number of aHUS patients with *DGKE* mutations who were treated with eculizumab at the acute phase and as maintenance therapy is limited. At the acute phase, one patient showed a negative

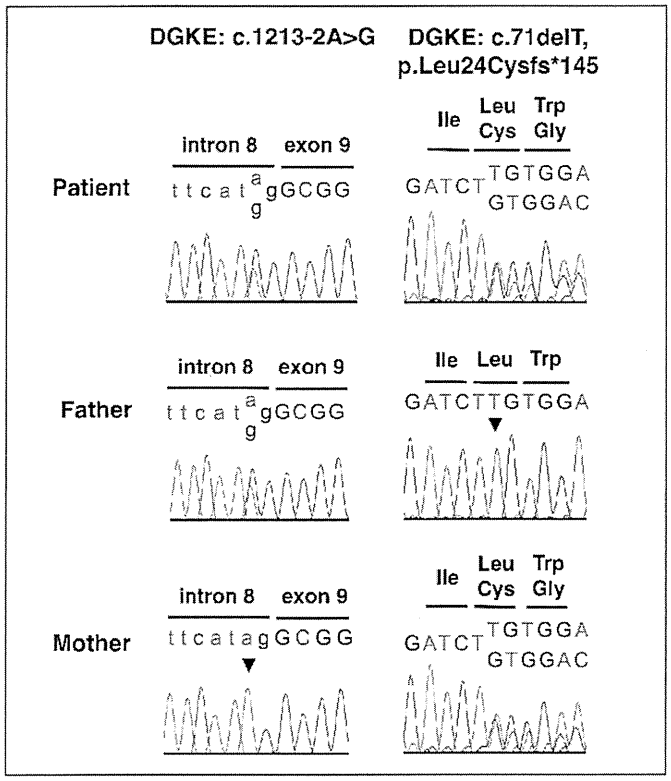

Figure 1: Chromatograms of two mutations in the *DGKE* gene in an affected patient and his parents. Small letters and capital letters are intron and exon, respectively. The A of the ATG translation initiation start site was designated as position +1, and the initial Met was denoted as +1. The c.1213–2A>G mutation disturbs the invariant splicing consensus sequence AG at the end of the intron. The c71delT mutation produces the frameshift, leading to the stop codon downstream at 145 amino acid residues.

response and another showed a positive response (1, 2). Lemaire et al. reported seven aHUS patients with *DGKE* mutations treated with eculizumab (1). None of the patients showed an abnormality in the complement system. In that study, one patient with *DGKE* mutations had aHUS recurrences even after eculizumab treatment. The genetic study of a Spanish aHUS registry reported an aHUS patient with concurrent *DGKE* and *C3* mutations who was treated with eculizumab (2). After presenting with aHUS at eight months of age, she had several aHUS recurrences, and biweekly plasma infusions were effective in normalising blood parameters; subsequent eculizumab treatment resolved the infection-associated edemas that were typical in this patient. Sanchez Chinchilla et al. suggested that the association of *DGKE* mutations concomitant with a *C3* gene mutation in this particular patient possibly contributed to more severe disease with chronic activation of TMA and a positive response to eculizumab treatment.

We report here a patient with *DGKE* mutations who presented plasmapheresis-resistant aHUS and severe hypertension in the first year of life. We did not identify mutations predisposing for aHUS in 6 complement genes in the patient, however he was successfully treated with eculizumab. The treatment strategy for aHUS patients with *DGKE* mutations is not yet settled (1, 2, 5). Further studies are needed to identify the appropriate therapeutic strategies for aHUS patients with *DGKE* mutations.

Conflicts of interest

T. Miyata has received lecturing fees from Bayer, Daiichi Sankyo, Boehringer Ingelheim, Shino-Test, Kyowa Kirin and Bristol-Myers. T. Ohta has received lecturing fees from Asahikasei Pharma, Pfaizer, Alexion Pharma, Daiichi Sankyo, Kyowa Kirin and Kyorin. Y. Fujimura is a recipient of research grant from Alexion Pharmaceuticals. None of the other authors declares a conflict of interest.

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Regular Article

THROMBOSIS AND HEMOSTASIS

Exacerbated venous thromboembolism in mice carrying a protein S K196E mutation

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Key Points

- A protein S-K196E mutation reduced its activated protein C cofactor activity in recombinant murine protein S-K196E and in K196E mutant mice.
- Mice carrying a protein S-K196E mutation or heterozygous protein S deficiency were more vulnerable to venous thrombosis than wild-type mice.

Protein S (PS) acts as an anticoagulant cofactor for activated protein C in regulation of blood coagulation. The K196E mutation in PS is a race-specific genetic risk factor for venous thromboembolism with a prevalence of ~2% within the Japanese population. To evaluate the thrombosis risk of the PS-K196E mutation, we generated PS-K196E knockin mice and heterozygous PS-deficient mice. We analyzed their thrombotic states, comparing with mice carrying the factor V Leiden mutation (FV-R504Q), a race-specific genetic risk for venous thrombosis in whites. PS-K196E mice grew normally but had decreased activated protein C cofactor activity in plasma. Purified recombinant murine PS-K196E showed the same decreased activated protein C cofactor activity. A deep vein thrombosis model of electrolytic inferior vena cava injury and pulmonary embolism models induced by infusion of tissue factor or polyphosphates revealed that PS-K196E mice, heterozygous PS-deficient mice, and FV-R504Q mice were much more susceptible to venous thrombosis compared with wild-type mice. Transient middle cerebral artery ischemia-reperfusion injury model studies demonstrated that both PS-K196E mice and heterozygous PS-deficient mice had cerebral infarction similar to wild-type mice, consistent with human observations. Our in vitro and in vivo results support a causal relationship between the PS-K196E mutation and venous thrombosis and indicate that PS-K196E mice can provide an in vivo evaluation system to help uncovering racial differences in thrombotic diseases. (*Blood.* 2015;126(19):2247-2253)

Introduction

Protein S (PS) is a vitamin K–dependent plasma glycoprotein that is mainly synthesized in liver and endothelial cells. PS acts as an anticoagulant cofactor for activated protein C (APC) in the proteolytic inactivation of factor Va (FVa) and factor VIIIa (FVIIIa) and negatively regulates blood coagulation.[1,2] In human plasma, ~60% of PS forms a complex with C4b binding protein (C4BP), and the formation of this complex abolishes the APC cofactor activity of PS. PS also acts as a cofactor for tissue factor pathway inhibitor during the inhibition of factor Xa (FXa) in human plasma, but this cofactor activity has not been detected in mouse plasma.[3]

PS deficiency is a risk factor for developing venous thromboembolism (VTE).[4] Homozygous or compound heterozygous PS deficiency is an extremely rare disorder that causes purpura fulminans in affected newborns.[5] Heterozygous PS deficiency is milder but firmly associated with an increased risk of VTE.[4] The risk of thrombosis in individuals with PS deficiency is increased with other genetic or acquired factors predisposing to thrombosis.

A rare variant in the PS gene, *PROS1*, that is present in 1.8% of Japanese, which causes a Lys196 to Glu substitution in PS (PS-K196E, rs 121918474, c.586A>G, also known as PS Tokushima,[6] PS-K155E in the mature protein numbering[7]) is a genetic risk factor for VTE in the Japanese population (odds ratio = 3.7-8.6).[8-11] The K196E mutation in PS is located in the second epidermal growth factor (EGF)-like domain.[2,7,9,11-14] We estimate that ~1 out of every 12 000 Japanese individuals is homozygous for the 196E allele, representing a total of as many as 10 000 individuals.[13] Thus, a substantial number of Japanese carry the mutant PS 196E allele, as a heterozygote or homozygote, and a likely risk of development of VTE. The phenotype-genotype analysis in individuals with PS-K196E mutation showed that heterozygotes had 16% lower PS anticoagulant activity than wild-type individuals, although the anticoagulant activities between heterozygotes and wild-types are substantially overlapped.[12] PS-K196E mutation appears to be a genetic risk for deep vein thrombosis (DVT) during pregnancy,[15] and PS-K196E mutation concomitant with other predisposing mutations for thrombosis was found in patients with VTE.[16] This mutation, however, did not increase the risk for adverse pregnancy outcomes defined by 2 or more miscarriages, fetal growth restriction, and/or intrauterine fetal death.[17] PS-K196E mutation seems to be Japanese specific, because the mutation has not been observed in the white, Chinese, or Korean populations.[11,18,19]

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There is an Inside *Blood* Commentary on this article in this issue.

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Racial differences in the genetic background of venous thrombosis have been well documented.[20] As an example, the factor V (FV) Leiden mutation, R506Q, that is a VTE risk factor is predominantly found in white populations.[21] This mutation renders FVa resistant to the inactivation by APC, resulting in a hypercoagulable state.[22,23] A mouse model carrying the homologous murine FV Leiden mutation (R504Q) showed enhanced venous thrombosis, consistent with an increased incidence of VTE in human FV Leiden carriers.[24,25] Thus, FV Leiden mice provide a valuable in vivo model of thrombosis-associated diseases in whites and have been studied under various stimulations or pathophysiologic conditions.[25-27]

Recent guidelines for establishing pathogenic causality of rare genetic variants emphasize that the strongest evidence for causality comes from disruption of the candidate gene in a model organism (eg, a mouse) that recapitulates the pathology in humans.[20] Hence, we generated a mouse colony carrying the PS-K196E mutation. Based on PS-knockout (PS-KO) studies,[3,28] homozygous PS deficiency in mice leads to embryonic lethality with a fulminant coagulopathy and hemorrhages. Heterozygous PS-deficient mice had reduced plasma PS antigen levels and APC cofactor activity, and they survived to adulthood but showed severe thrombosis after induction of pulmonary embolism (PE) by injection of tissue factor.

In the present study, we generated PS-K196E knockin mice and analyzed their phenotypes, comparing with heterozygous PS-deficient mice and FV-R504Q knock-in mice. Our data show that the PS-K196E mutation in mice increases the risk of thrombotic diseases, proving the pathogenic causality of this mutation beyond doubt. The PS K196E mice may be valuable for future pathogenic studies related to venous thrombosis.

Materials and methods

Generation of PS-K196E knockin mice and PS-KO mice, genetic analysis, and RNA analysis

The targeting vector was introduced into 129/SvEvTac-derived embryonic stem cells by electroporation.[29-32] The male chimeras were bred to wild-type C57BL/6J females and F1 offspring with the PS-K196E mutation or PS deficiency were backcrossed to C57BL/6J mice (Japan SLC, Hamamatsu, Japan) for 10 generations. The detailed materials and methods are described in the supplemental Data (available on the *Blood* Web site). All animal procedures were approved by the Animal Care and Use Committee of the National Cerebral and Cardiovascular Center and performed in accordance with institutional and national guidelines and regulations.

Plasma APC cofactor activity and PS antigen assays

Blood was collected from anesthetized mice via the retro-orbital plexus into tubes containing a 0.1 volume of 3.8% sodium citrate. Plasma was prepared from blood by centrifugation at 1000*g* for 10 minutes.

Plasma APC cofactor activity was measured by an activated partial thromboplastin time (APTT)-based clotting assay using a recombinant mouse APC.[33] Plasma samples (40 μL) were pretreated with 450 nM recombinant mouse APC or Owren-Koller buffer (10 μL) for 1 min at 37°C. STA-Cephascreen reagent (50 μL; Diagnostica Stago, Asnières-sur-Seine, France) was added and incubated for 3 min at 37°C. The clotting time was recorded using a coagulometer (STart4; Diagnostica Stago) after adding 25 mM $CaCl_2$ (50 μL). The prolongation of clotting time by the addition of APC to wild-type plasma was considered as 100% APC cofactor activity.

Plasma PS antigens were measured by enzyme-linked immunosorbent assay using antibodies against human PS. Plasma samples in 0.5% bovine serum albumin were applied to rabbit anti–human PS-coated (Dako, Glostrup, Denmark) microwell plates for 2 hours at room temperature. Bound PS was detected by incubation with peroxidase-conjugated rabbit anti-human PS prepared using Peroxidase Labeling Kit-NH_2 (Dojindo, Kumamoto, Japan) for 1 hour. Bound antibody was detected using SureBlue Reserve TMB Microwell Peroxidase Substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) as absorbance change at 450 nm. The PS level measured in wild-type mouse plasma was arbitrarily defined as 100%.

APC cofactor activity analysis of recombinant mouse PS proteins in vitro

Recombinant mouse wild-type PS and PS-K196E mutant proteins were made as described.[33] SDS-PAGE analysis under reducing conditions revealed that the recombinant proteins were ~95% pure (data not shown). APC cofactor activity of recombinant PS was determined by modified APTT and FXa 1-stage clotting assays as described previously.[33] In a modified APTT assay, 10 μL normal mouse pooled plasma (BioreclamationIVT, Baltimore, MD), 5 μL human fibrinogen (2 mg/mL; Enzyme Research Laboratories, South Bend, IN), 25 μL recombinant mouse APC (36 nM), 25 μL recombinant mouse PS (0, 3.38, 6.75, 13.5, and 27 nM), and 25 μL APTT-XL (Thermo Fisher Scientific, Waltham, MA) were mixed and incubated for 3 minutes at 37°C. The clotting time was recorded after adding 25 μL $CaCl_2$ (25 mM). In an FXa 1-stage clotting assay, 10 μL normal mouse pooled plasma, 25 μL of a mixture (4:1) of phospholipid vesicles (25 μM, 80% 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine/20% 1,2-dioleoyl-sn-glycero-3-phosphatidylserine; Avanti Polar Lipids, Alabaster, AL) plus human fibrinogen (2 mg/mL), 25 μL recombinant mouse APC (53 nM), 25 μL recombinant mouse PS (0, 0.63, 1.25, 2.5, 5, and 10 nM), and 25 μL mouse FXa (Haematologic Technologies, Essex Junction, VT) were mixed and incubated for 3 minutes at 37°C. The clotting time was recorded after adding 25 μL $CaCl_2$ (25 mM). Each assay was performed in triplicate. The APC cofactor activity of recombinant mouse PS was evaluated based on the slope of a linear increase in clotting time with the dosage of recombinant mouse PS.

DVT model

A mouse DVT model of electrolytic inferior vena cava (IVC) injury was performed according to the method of Diaz et al, with minor modifications.[34,35] Mice were anesthetized with 2.5% 2,2,2-tribromoethanol and kept around 37°C using a heating pad (Bio Research Center, Nagoya, Japan). The IVC was exposed through a midline laparotomy, and all side branches between the renal and iliac veins were ligated with a 7-0 polypropylene suture. An anode of 27G stainless-steel needle electrode (NE-115B; Nihon Koden, Tokyo, Japan) was inserted into the caudal IVC and attached to the anterior wall. A cathode was inserted subcutaneously to complete a circuit. A direct current of 200 μA was applied for 10 minutes using an electric stimulator (SEN-3041; Nihon Koden) with an isolator unit (SS-203J; Nihon Koden). After current load, the needle was gently removed from the IVC and the abdomen was closed by polyglycolic acid suture and cyanoacrylate glue. At 2 days postsurgery, blood was collected and platelet counts were determined using an automatic cell counter (KX-21NV; Sysmex, Kobe, Japan). Thrombi formed in the IVC were removed, weighed in wet condition, and photographed using a digital microscope (VHX-1000; Keyence, Osaka, Japan). Plasma thrombin-antithrombin complex (TAT) and interleukin-6 (IL-6) levels were measured using an Enzygnost TAT micro kit (Siemens AG, Munich, Germany) and a BD OptEIA mouse IL-6 ELISA kit (BD Biosciences, San Jose, CA), respectively.

PE models

A recombinant human tissue factor (TF) reagent (Dade Innovin) containing phospholipids and calcium was purchased from Siemens AG. A high-molecular-weight polyphosphate (HMW polyP) was prepared as reported by Smith et al.[36] Sodium metaphosphate (Sigma-Aldrich Japan, Tokyo, Japan) was washed twice with purified water and solubilized in 250 mM LiCl. The soluble HMW polyP (40-1200 phosphate units long) was precipitated by adding 2.5 volumes of acetone and dissolved in purified water. The concentrations of HMW polyP were determined as phosphate monomer using BIOMOL Green reagent (Enzo Life Sciences, Farmingdale, NY) after complete hydrolysis in 1 N HCl.

Mice were anesthetized with 2.5% 2,2,2-tribromoethanol, and 15 μL/g body weight of the TF reagent (1/30 dilution) or HMW polyP (1.67 g/L) was infused into the IVC.[37] The dose of TF and HMW polyP was chosen such that ~20% of

wild-type mice survived after the infusion. Survival time was recorded until 20 minutes after the infusion, while defining death as respiratory arrest that persisted for at least 2 minutes. Two minutes after the respiratory arrest (but while the heart was still beating) or at the completion of the 20-minute observation period, mice were perfused with 0.5 mL 1% Evans blue via right ventricle. Lungs were excised, photographed, and scored for Evans blue perfusion defects using a scoring scale (from 0 for no occlusion to 4 for complete occlusion). The scores were evaluated by 2 individuals anonymously.

Transient middle cerebral artery (MCA) occlusion model

Cerebral ischemia-reperfusion injury was induced using the 3-vessel occlusion technique as described previously.[38,39] In brief, the left common carotid artery was isolated and occluded by a vascular clip (1-vessel occlusion [1-VO]) under halothane inhalation-anesthesia. A skin incision was made at the midpoint between the left orbit and the external auditory canal. After the removal of the zygomatic bone and downward retraction of the mandibular bone, a craniectomy was made using an electric drill. The distal M1 portion of left MCA, peripheral to the perforating arteries of the basal ganglia, was permanently occluded (2-vessel occlusion [2-VO]) by electrocauterization and cut at the lateral edge of the olfactory tract. Finally, the right common carotid artery was occluded (3-vessel occlusion [3-VO]) using a vascular clip. After 15-minute focal ischemia induced by the 3-VO, 2 clips at the common carotid arteries were removed to establish reperfusion through the collateral arteries (the source of the vascular reserve) distal to the 1-VO in the cortex. During the operation, blood pressure was monitored by an indirect blood pressure meter (BP-98A; Softron, Tokyo, Japan) and rectal temperature was regulated within the range of 36.5 to 37.5°C by a temperature controller (NS-TC10; Neuroscience, Tokyo, Japan). After 24 hours, neurologic deficits were assessed using a scoring scale (from 0 to 4) as described previously,[38,39] and the brains were excised and stained with 2,3,5-triphenyl tetrazolium chloride. The infarcted and the total hemispheric areas of each section were measured using a computer-assisted image-analysis system (WinROOF; Mitani, Tokyo, Japan).[31,39] The infarct volume was adjusted for edema by dividing the volume by the edema index (left hemisphere volume/right hemisphere volume).

Statistical analysis

Statistical significance was assessed by the 1-way analysis of variance followed by the post hoc Bonferroni's multiple comparison test. Data for nonnormal and nonparametric distributions were assessed by the Kruskal-Wallis test followed by the post hoc Dunn's multiple comparison test. Survival rates were analyzed by the Mantel-Cox log-rank test. Differences were considered to be significant at $P < .05$.

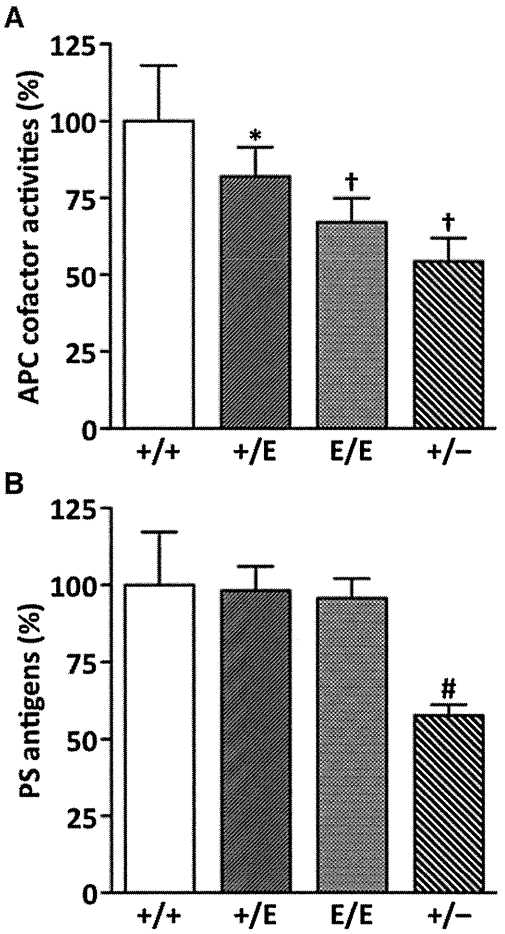

Figure 1. Plasma APC anticoagulant cofactor activity and PS antigen levels. (A) APC cofactor activities. Data are mean ± standard deviation (SD) of $Pros1^{+/+}$ (n = 10), $Pros1^{+/E}$ (n = 10), $Pros1^{E/E}$ (n = 10), and $Pros1^{+/-}$ (n = 8) mice. (B) PS antigens. Data are mean ± SD of 5 mice for each genotype. $^{*}P < .05$ in comparison with $Pros1^{+/+}$ mice. $^{\dagger}P < .001$ in comparison with $Pros1^{+/+}$ mice, and $P < .05$ in comparison with $Pros1^{+/E}$ mice. $^{\#}P < .001$ in comparison with $Pros1^{+/+}$, $Pros1^{+/E}$, and $Pros1^{E/E}$ mice. The levels measured in $Pros1^{+/+}$ mice were defined as 100%.

Results

Generation of PS-K196E and PS-KO mice

To generate PS-K196E mice, we introduced the K196E mutation into the endogenous *Pros1* gene by homologous recombination (supplemental Figure 1A). We confirmed the expected structure of the targeted locus by polymerase chain reaction (data not shown) and Southern blotting (supplemental Figure 1B). *Pros1* messenger RNA was detected in PS-K196E mice with normal sizes (~3.5 kb) and amounts by the northern blot analysis of liver total RNA (supplemental Figure 1C). Expression of the mutant messenger RNA in PS-K196E mice was verified by the appearance of 238-bp and 171-bp fragments in reverse-transcription polymerase chain reaction products after digestion with *NruI* (supplemental Figure 1D) and by direct sequencing of the products. To generate PS-KO mice, we disrupted the *Pros1* gene in a similar manner using a targeting vector that eliminated exon 3 (supplemental Figure 2).

Genotyping of 299 offspring by intercrosses in heterozygous PS-K196E ($Pros1^{+/E}$) mice showed the expected 1:2:1 Mendelian distribution of $Pros1^{+/+}$ (82/299, 27.4%), $Pros1^{+/E}$ (149/299, 49.8%), and $Pros1^{E/E}$ (68/299, 22.7%). $Pros1^{E/E}$ mice were viable and fertile. In contrast, no $Pros1^{-/-}$ pups were obtained in a total of 316 pups from $Pros1^{+/-}$ intercrosses, as previously reported.[3,28] $Pros1^{+/+}$ and $Pros1^{+/-}$ mice were born in the 1:2 distribution (109/316 [34.5%] and 207/316 [65.5%], respectively) by $Pros1^{+/-}$ mice intercrosses, and $Pros1^{+/-}$ mice grew normally. Thus, although PS is essential for embryonic development in mice, the homozygous PS-K196E mutation did not cause embryonic lethality. In addition, $Pros1^{E/-}$ mice were viable when $Pros1^{E/E}$ mice were bred with $Pros1^{+/-}$ mice.

Plasma APC cofactor activities and PS antigens in PS-K196E and PS-KO mice

We measured plasma APC cofactor activity by an APTT-based clotting assay in the absence and presence of recombinant mouse APC as previously reported.[33] The APC cofactor activity determined from the prolongation of clotting time by APC was significantly reduced in $Pros1^{+/E}$, $Pros1^{E/E}$, and $Pros1^{+/-}$ mice compared with $Pros1^{+/+}$ mice (Figure 1A). Similar to human $Pros1^{+/E}$ subjects, $Pros1^{+/E}$ mice had ~18% lower activity (82.0% ± 9.5%, mean ± SD) than $Pros1^{+/+}$ mice (100% ± 18.0%). More severe reductions in the activity were observed in $Pros1^{E/E}$ (67.1% ± 7.8%) and $Pros1^{+/-}$ (54.3% ± 8.3%) mice. Basal APTT values in the absence of added APC were not different

among the mouse groups (*Pros1*$^{+/+}$, 23.3 ± 0.6 s; *Pros1*$^{+/E}$, 23.3 ± 0.6 s; *Pros1*$^{E/E}$, 23.3 ± 0.6 s; *Pros1*$^{+/-}$, 23.3 ± 0.6 s).

The plasma PS antigen levels were normal in *Pros1*$^{+/E}$ mice (98.2% ± 7.9%) and *Pros1*$^{E/E}$ (95.7% ± 6.4%) mice, indicating that PS-K196E mutant was normally secreted into blood but had reduced APC anticoagulant cofactor activity (Figure 1B). In contrast, the PS antigen levels were reduced in *Pros1*$^{+/-}$ mice (57.7% ± 3.5%), corresponding to around 50% reduction in the APC cofactor activity.

In vitro APC cofactor activity of recombinant mouse PS-K196E mutant

To characterize further the effect of K196E mutation on the anticoagulant activity of PS, we prepared recombinant mouse wild-type PS and the PS-K196E mutant and compared their APC cofactor activities using 2 different in vitro assays. In a modified APTT assay, PS-K196E showed 60% APC cofactor activity compared with wild-type PS (Figure 2A). In an FXa 1-stage clotting assay, PS-K196E similarly showed 49% APC cofactor activity compared with wild-type PS (Figure 2B). Thus, the purified PS-K196E mutant had 49% to 60% of normal PS APC cofactor activity.

Exacerbation of VTE in PS-K196E mice

To investigate the effects of PS-K196E mutation on VTE, we performed DVT and PE model experiments. In the DVT model experiment, we applied the electrolytic IVC injury to *Pros1*$^{+/+}$, *Pros1*$^{+/E}$, *Pros1*$^{E/E}$, *Pros1*$^{+/-}$, and homozygous FV-R504Q (*Fv*$^{Q/Q}$) mice. In this model, thrombus formation under continuous blood flow is induced by endothelial activation at the site of anodal electrolysis and thrombi grow to a maximum size at 2 days after the injury.[34] In this study, we applied milder electrolytic stimulation (200 μA, 10 minutes) than that of the original method (250 μA, 15 minutes) to adequately assess prothrombotic states in mice. We measured thrombus weight in IVC at 2 days after the injury and found enhanced thrombus formation in *Pros1*$^{E/E}$, *Pros1*$^{+/-}$, and *Fv*$^{Q/Q}$ mice compared with *Pros1*$^{+/+}$ mice (Figure 3A). Accompanying the increase in thrombus weight, peripheral platelet counts were decreased, and plasma TAT and IL-6 levels were increased in *Pros1*$^{E/E}$, *Pros1*$^{+/-}$, and *Fv*$^{Q/Q}$ mice compared with *Pros1*$^{+/+}$ mice (Figure 3B-D). In *Pros1*$^{+/E}$ mice, thrombus weight, plasma TAT, and IL-6 levels were modestly, although not significantly, increased and platelet counts were significantly decreased compared with *Pros1*$^{+/+}$ mice. Platelet counts, plasma TAT, and IL-6 levels in nontreated mice were not different among the mouse groups. IL-6 has been shown to play a key role in promoting inflammation in mouse DVT models.[40,41] Thus, these results suggest that PS-K196E mutation in mice promotes venous thrombus formation accompanied by enhancement of coagulation and inflammatory responses.

In the PE model experiments, we infused recombinant human TF or HMW polyP into mouse IVC. PolyP is a linear polymer of inorganic phosphates that is ubiquitous from bacteria to humans. PolyP acts as a natural negatively charged surface that activates the intrinsic pathway of blood coagulation.[36] After the induction of PE in mice by TF or HMW polyP, we evaluated the 20-minute survival (Figure 4A-B, respectively). Using a scoring scale from no occlusion (score 0) to complete occlusion (score 4) (Figure 4C), we determined the degree of lung vascular occlusion by perfusion with Evans blue (Figure 4D-E). The survival was significantly reduced in *Pros1*$^{+/E}$, *Pros1*$^{E/E}$, *Pros1*$^{+/-}$, and *Fv*$^{Q/Q}$ mice compared with *Pros1*$^{+/+}$ mice in both TF-induced and HMW polyP-induced PE models. The lung perfusion defect score was inversely correlated with survival and significantly increased in *Pros1*$^{E/E}$, *Pros1*$^{+/-}$, and *Fv*$^{Q/Q}$ mice after TF-induced PE, and in *Pros1*$^{+/E}$, *Pros1*$^{E/E}$, *Pros1*$^{+/-}$, and *Fv*$^{Q/Q}$ mice after HMW polyP-induced PE.

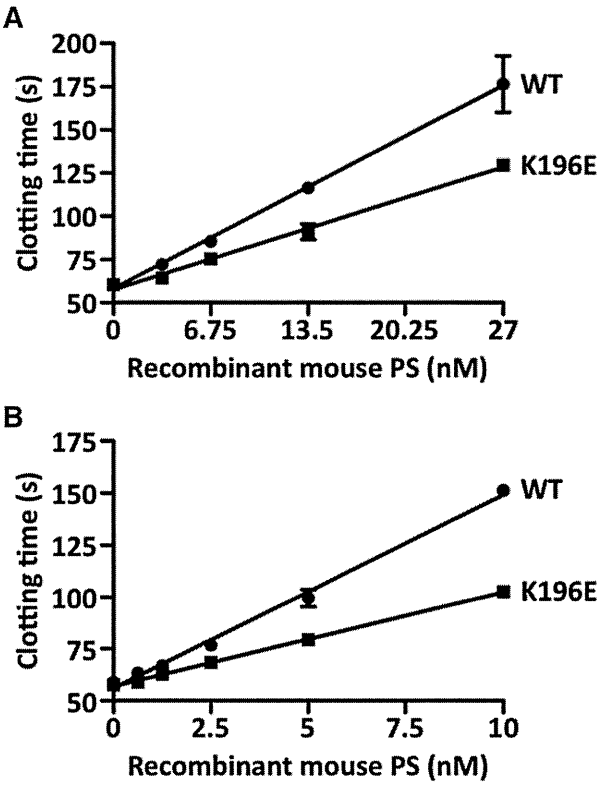

Figure 2. APC anticoagulant cofactor activity of recombinant mouse PS. (A) Modified APTT assay. Increasing concentrations of recombinant mouse wild-type (WT) PS (●) or PS-K196E (■) were added to normal mouse pooled plasma, and a modified APTT clotting assay was performed in the presence of recombinant mouse APC. (B) FXa 1-stage clotting assay. Increasing concentrations of recombinant mouse WT PS (●) or PS-K196E (■) were added to normal mouse pooled plasma, and an FXa 1-stage clotting assay was performed in the presence of recombinant mouse APC. Each assay was performed in triplicate, and data are shown as mean ± SD.

These results suggest that the PS-K196E mutation in mice increases lung vascular occlusion and mortality after induction of PE.

No exacerbation of ischemic stroke in PS-K196E mice

To examine the effects of PS-K196E mutation on arterial ischemic diseases, transient MCA ischemia-reperfusion injury was applied in mice using the 3-VO technique. We previously observed that both *Fv*$^{Q/+}$ and *Fv*$^{Q/Q}$ mice showed increased infarct volumes compared with wild-type mice 24 hours after the ischemia-reperfusion injury induced by the same technique.[42] However, infarct volumes 24 hours after ischemia in *Pros1*$^{+/E}$, *Pros1*$^{E/E}$, and *Pros1*$^{+/-}$ mice were not different from those in *Pros1*$^{+/+}$ mice (Figure 5). The edema index and the neurologic deficit score were also not different among the mouse groups (data not shown). These results suggest that PS-K196E mutation in mice does not cause aggravation of ischemic stroke, unlike FV Leiden mutation.

Discussion

To assess the pathogenic causality of the PS K196E mutation for venous thrombosis, we established colonies of PS-K196E knockin mice and PS-KO mice.[43] Plasma from PS-K196E homozygous mice had 67% of normal APC cofactor activity, similar to purified recombinant murine PS-K196E that had 49% to 60% APC cofactor activity. Mouse C4BP does not contain the PS-binding subunit, the β-chain, which is

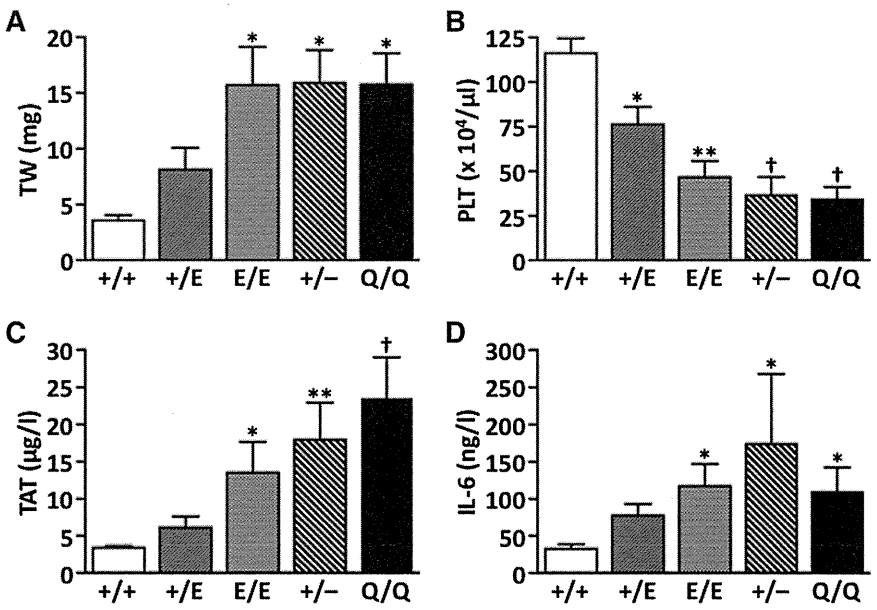

Figure 3. DVT model of electrolytic IVC injury. (A) Thrombus weight (TW) in IVC. (B) Platelet counts (PLT) in peripheral blood in mice with IVC injury. Platelet counts of nontreated control mice were not different among the groups (*Pros1*$^{+/+}$, 118.4 ± 4.5 × 10^4/μL; *Pros1*$^{+/E}$, 131.9 ± 13.1 × 10^4/μL; *Pros1*$^{E/E}$, 110.5 ± 18.7 × 10^4/μL; *Pros1*$^{+/-}$, 103.0 ± 14.5 × 10^4/μL; *FV*$^{Q/Q}$, 111.9 ± 17.4 × 10^4/μL; mean ± standard error of the mean [SEM] of 3 mice). (C) TAT in plasma. TAT of nontreated control mice was not different among the groups (*Pros1*$^{+/+}$, 3.2 ± 0.5 μg/L; *Pros1*$^{+/E}$, 3.0 ± 0.3 μg/L; *Pros1*$^{E/E}$, 2.7 ± 0.5 μg/L; *Pros1*$^{+/-}$, 3.3 ± 0.8 μg/L; *FV*$^{Q/Q}$, 3.5 ± 0.9 μg/L; mean ± SEM of 3 mice). (D) Interleukin-6 (IL-6) in plasma. IL-6 was not detected in nontreated mice of all genotypes (n = 3). Data are mean ± SEM of 12 mice for *Pros1*$^{+/+}$, *Pros1*$^{+/E}$, *Pros1*$^{E/E}$, *Pros1*$^{+/-}$, and *FV*$^{Q/Q}$ mice. *$P < .05$ in comparison with *Pros1*$^{+/+}$ mice. **$P < .001$ in comparison with *Pros1*$^{+/+}$ mice. †$P < .001$ in comparison with *Pros1*$^{+/+}$ mice, and $P < .05$ in comparison with *Pros1*$^{+/E}$ mice.

a pseudogene in mice,[44] so interpretation of APC cofactor activity is uncomplicated by C4BP considerations. The susceptibility of PS-K196E mice to venous thrombosis was determined in multiple models, including (1) an electrolytic IVC model of venous thrombosis that produces a nonocclusive and consistent IVC thrombus in the presence of constant blood flow, (2) TF-initiated pulmonary thrombosis, and (3) HMW-polyP–initiated pulmonary thrombosis. All thrombotic biomarkers or parameters in these thrombosis injury models, including mortality, showed that the PS-K196E mutation caused increased venous thrombosis, generally very similar to PS heterozygosity and to the murine FV Leiden mutation. Heterozygosity for this PS mutation gave a milder thrombotic phenotype than PS mutant homozygosity. These results unambiguously demonstrate a causal link between the PS-K196E mutation and thrombophilia, strongly supporting the PS-K196E mutation as a human genetic risk factor for VTE.[8-11]

The PS lysine residue 196 is located in the second EGF-like domain, and it is highly conserved in PS from human, chimpanzee, rhesus monkey, mouse, rat, opossum, cattle, dog, pig, chicken, and *Xenopus*, indicating its importance for the PS function. Based on modeling of the tertiary structure of the second EGF-like domain of PS, Lys-196 is on the surface of the molecule,[2] where it likely interacts with APC. Consistent with this interpretation for the molecular defect of PS-K196E,

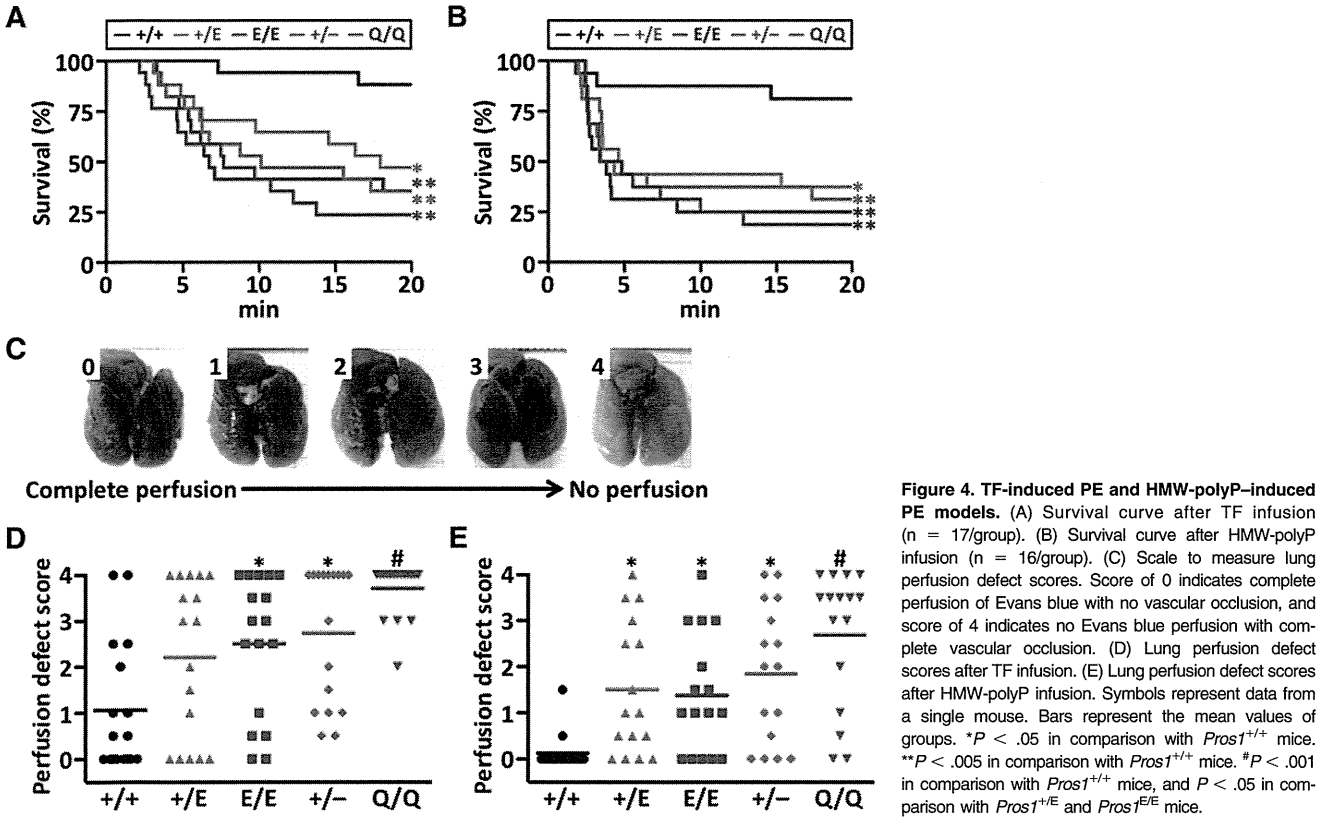

Figure 4. TF-induced PE and HMW-polyP–induced PE models. (A) Survival curve after TF infusion (n = 17/group). (B) Survival curve after HMW-polyP infusion (n = 16/group). (C) Scale to measure lung perfusion defect scores. Score of 0 indicates complete perfusion of Evans blue with no vascular occlusion, and score of 4 indicates no Evans blue perfusion with complete vascular occlusion. (D) Lung perfusion defect scores after TF infusion. (E) Lung perfusion defect scores after HMW-polyP infusion. Symbols represent data from a single mouse. Bars represent the mean values of groups. *$P < .05$ in comparison with *Pros1*$^{+/+}$ mice. **$P < .005$ in comparison with *Pros1*$^{+/+}$ mice. #$P < .001$ in comparison with *Pros1*$^{+/+}$ mice, and $P < .05$ in comparison with *Pros1*$^{+/E}$ and *Pros1*$^{E/E}$ mice.

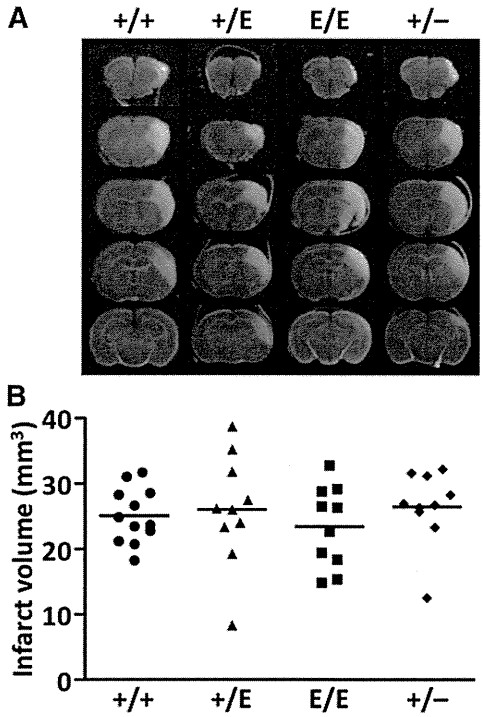

Figure 5. Transient MCA occlusion model using the 3-VO technique. (A) Representative images of coronal sections of *Pros1*$^{+/+}$, *Pros1*$^{+/E}$, *Pros1*$^{E/E}$, and *Pros1*$^{+/-}$ mouse brains. (B) Infarct volumes. No significant differences ($P > .05$) were observed among the groups. Symbols represent data from a single mouse. Bars represent the mean values of groups.

monoclonal antibodies were made that recognize the PS-196E epitope,[46] indicating that residue 196 is likely exposed on the PS surface.

Currently, the extent to which the PS-K196E mutation or PS deficiency are risk factors for arterial occlusive diseases is not clear. To explore the arterial occlusive risk of these PS genetic variations, we used a cerebral focal ischemia-reperfusion model. Because of some limitations of the nylon-thread–induced ischemia/reperfusion MCA occlusion brain injury methodology,[46] we employed the 3-VO technique for rodent ischemic stroke.[38,39] This method consists of temporary occlusion of both common carotid arteries in conjunction with permanent unilateral occlusion of an MCA. The method does not use any foreign materials to occlude the lumen of the vessels and consistently produces focal ischemia, regional cerebral blood flow that is >10% but <20%, and adequate reperfusion in the cortex, achieving good reproducibility for the homogeneous development of cortical infarction.[39] When this method was used to evaluate the susceptibility of PS-K196E mice and PS-KO mice to cerebral ischemia-reperfusion injury, we found that these PS-modified mice did not show any increase in brain infarct volume compared with wild-type control mice. PS protects neurons from ischemic injury in mouse stroke models.[47] Therefore, mice with decreased APC cofactor activity might have been expected to show an aggravation of ischemic stroke, but it was not the case, indicating that the PS-K196E mutation and heterozygosity for wild-type PS do not compromise endogenous PS neuroprotective mechanisms. Because the neuroprotective effects of PS are mediated through its sex-hormone–binding globulin-like region,[48] we speculate that the PS-K196E mutant with the intact sex-hormone–binding globulin-like region retains its neuroprotective actions. Moreover, the neuroprotective effects of pharmacologic APC are based on APC's cell-signaling actions, not on its anticoagulant actions, so loss of APC anticoagulant cofactor activity might not compromise endogenous neuroprotection.[49]

Five rare genetic race-specific variants linked to VTE risk include PS-K196E in Japanese, FV Leiden and prothrombin II ntG20210A in whites, and R189W-protein C and del-Lys193-protein C in Chinese.[11] Whether these mutations have been deleterious or advantageous during evolution is unclear. Each mutation, whether a gain of function or a loss of function, causes increased thrombin generation that may prevent bleeding and achieve hemostasis or increase thrombosis risk. Even today, as in ancient history, bleeding is the leading cause of maternal death linked to childbirth in the absence of modern medical care.[50] Thus, the PS-K196E, like the other 4 VTE-linked mutations, on balance may have benefited the Japanese population historically by reducing maternal death, although it increased VTE risk. If so, this PS-K196E Japanese mutation has been advantageous since its first occurrence after divergence of the Japanese population from other populations and during its subsequent adaptive evolution.

In summary, the murine PS-K196E mutation similarly reduces its APC anticoagulant cofactor activity in plasma and in purified systems, and PS-K196E mice and heterozygous PS deficiency are more vulnerable to venous thrombosis than wild-type mice, proving pathogenic causality for the K196E mutation. Thus, PS-K196E mice may provide a novel murine resource for studies of thrombosis in vivo that may assist defining race-dependent, PS-dependent pathophysiological mechanisms for thrombosis in humans.

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Authorship

Contribution: F.B. designed research, performed most of the experiments, analyzed and interpreted data, and wrote the paper; T.K. performed the MCA ischemia-reperfusion model and analyzed data; J.A.F. performed the recombinant mouse PS experiments and analyzed data; H.Y. established the MCA ischemia-reperfusion model using the 3-vessel occlusion technique and interpreted data; Y.T. analyzed data of the PE model experiments; K.K. constructed the targeting vector for generating the PS-K196E and PS-KO mice; J.H.G. made recombinant murine APC and PS, interpreted data, and wrote the paper; and T.M. designed research, interpreted data, and wrote the paper.

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

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Exacerbated venous thromboembolism in mice carrying a protein S K196E mutation

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Full Length Article

Risks of thromboembolism associated with hormonal contraceptives related to body mass index and aging in Japanese women

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ABSTRACT

Objective: The aim of this study is to estimate the risk of thromboembolism related to body mass index (BMI) and aging among users of hormonal contraceptives in Japan.
Methods: A case–control study of the risk of obesity and a descriptive study of the risk of age were conducted. We used the Pharmaceuticals and Medical Devices Agency database, and extracted thromboembolic events of combined oral contraceptive (COC) products. Control data were from the National Health and Nutrition Survey in Japan. Denominator of descriptive study was from IMS Health, JPM.
Results: A total of 306 thromboembolic events and 6423 controls were analyzed. The odds ratios (95% confidence interval) of the obesity groups (BMI ≥ 25) were 2.32 (1.71–3.15) for venous thromboembolism (VTE), 1.16 (0.62–2.18) for arterial embolism and thrombosis (ATE), and 1.83 (1.38–2.43) for overall thromboembolic events compared with the standard group (BMI of 18.5–24.9) as a reference. The estimated incidence rates of VTE, ATE and overall thromboembolic events per 10,000 person-years in users of therapeutic remedies for dysmenorrhea (35 μg ethinylestradiol combined with norethisterone, 20 μg ethinylestradiol combined with drospirenone and dienogest) among women aged 10–59 years from 2009 to 2013 were 2.38 (2.08–2.74), 0.63 (0.48–0.82), and 3.17 (2.81–3.57), respectively. This tendency was not seen for dienogest.
Conclusions: The risk of VTE in the obesity group among COC users was more than 2 times higher than in the standard group. The incidence rates of VTE in Japanese users of all remedies for dysmenorrhea except dienogest were as high as in people in Western countries.

1. Introduction

Venous thromboembolism (VTE) associated with combined oral contraceptives (COCs; estrogen combined with progestin) was reported for the first time by Jordan WM in 1961 [1]. Since then, the increased risks of VTE associated with current COC use were confirmed [2–11]. The risk of VTE in women using COCs is attributed to changes in hemostasis [12–16].

VTE is an unavoidable adverse event of COCs. However, the VTE risk of COCs in Japanese women is not clear yet, although Adachi et al. reported 29 cases of thromboembolism associated with COC use in Japanese women between 1992 and 2001 throughout Japan [17]. Therapeutic use of estrogen and progestin (low-dose estrogen progestin; LEP) or progestin-only products for dysmenorrhea have been approved for the health insurance coverage since 2008 in Japan, and the associated thromboembolism is thought to increase with the recent increase in the quantity of those prescriptions. The constituents of LEP are the same as COCs.

It is important to identify high-risk persons in order to prescribe COCs safely. Obesity and aging are pointed out as risk factors in Western studies [2,3,9,11,18–25], however, there are few such studies in Japan. The Ministry of Health, Labor and Welfare (MHLW) has made it obligatory for pharmaceutical companies, hospitals, physicians, pharmacists, and others to report the adverse events with medicines. The Pharmaceuticals and Medical Devices Agency (PMDA) accepted these reports from companies, hospitals, physicians and others after April 2004, and established the Japanese Adverse Drug Event Report Database (PMDA database). For encouraging the utilization of adverse event information, it became available as a CSV format after April 2012. Then, we could extract the adverse events including thromboembolism from this

Abbreviations: VTE, venous thromboembolism; ATE, arterial embolism and thrombosis; COCs, combined oral contraceptives; PMDA, Pharmaceuticals and Medical Devices Agency; LEP, low-dose estrogen progestin; MHLW, Ministry of Health, Labor and Welfare; EE, ethinylestradiol; DVT, deep vein thrombosis; PE, pulmonary embolism.

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