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The role of epidermal growth factor-like domain-related abnormalities, protein S Tokushima, and anti-protein S autoantibodies in pregnancy loss

Yoshihiro Sato [a,b], Toshitaka Sugi [a,*], Rie Sakai [a], Hiroyuki Kuma [c]

[a] Laboratory for Recurrent Pregnancy Loss, Sugi Women's Clinic, Kanagawa, Japan
[b] Yoshihiro Women's Clinic, Tokyo, Japan
[c] Faculty of Pharmaceutical Sciences, Nagasaki International University, Nagasaki, Japan

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ABSTRACT

Background: Protein S (PS) deficiency and autoantibodies that bind to PS (anti-PS) have been described in patients with adverse pregnancy outcomes, including pregnancy loss. PS Tokushima is a congenital abnormality of the second epidermal growth factor (EGF)-like domain, and anti-PS has been reported to recognize EGF-like domains.
Objectives: We evaluated the role of PS Tokushima and anti-PS in patients with pregnancy loss.
Methods: Patients with recurrent early pregnancy loss (n = 324; group A), those with one or more mid-to-late pregnancy loss (n = 196; group B), and infertile women having no pregnancy loss (n = 650; group C) were screened for PS type II deficiency and anti-PS. Patients who were diagnosed with PS type II deficiency underwent genetic analysis for the detection of PS Tokushima.
Results: The incidence of patients with PS Tokushima was 1.85 %, 5.10 %, and 1.23 % in groups A, B, and C, respectively. The incidence of patients with PS Tokushima was significantly higher in group B ($p = 0.0027$) than in group C. The incidence of patients with anti-PS was 20.1 %, 23.0 %, and 19.2 % in groups A, B, and C, respectively. The incidence of patients with anti-PS was significantly higher in groups A ($p = 0.0229$), B ($p = 0.0071$), and C ($p = 0.0288$) than in previously reported healthy nonpregnant women (7.1 %, 4/56).
Conclusions: Our data suggest that PS Tokushima is associated with mid-to-late pregnancy loss, while anti-PS are associated with recurrent early pregnancy loss, mid-to-late pregnancy loss, and infertility.

1. Introduction

Protein S (PS) is a vitamin K-dependent glycoprotein that functions as a cofactor to activated protein C (APC), which is an anticoagulant serine protease, and is an important regulator of blood coagulation (Rezende et al., 2004). Mature PS has a modular structure consisting of a γ-carboxyglutamic acid domain, a thrombin-sensitive region, four epidermal growth factor (EGF)-like domains (EGF1-4), and a sex hormone-binding globulin-like domain containing two laminin G-like--domain-like repeats.

Recently, many studies have suggested an association between adverse pregnancy outcomes and PS deficiency (Rey et al., 2003; Robertson et al., 2006; Paidas et al., 2005; Hojo et al., 2008; Ebina et al., 2015). Rey et al. performed a meta-analysis and reported that PS deficiency was associated with recurrent pregnancy loss and fetal loss after 22 weeks (Rey et al., 2003). Robertson et al. reported in a systematic review that PS deficiency was associated with late fetal loss (Robertson et al., 2006).

PS Tokushima (p.Lys196Glu), a genetic mutation and one of the molecular defects identified in EGF2 of PS (Yamazaki et al., 1993a; Hayashi et al., 1994), is found in 1.6 %–1.8 % of healthy Japanese individuals, whereas it is found in 5 %–10 % of Japanese patients with venous thromboembolism (VTE) (Kinoshita et al., 2005; Miyata et al., 2009; Yamazaki et al., 1993b; Kimura et al., 2006). This mutation was responsible for PS type II deficiency, namely, PS qualitative deficiency, which is characterized by decreased PS activity with normal PS antigen levels (Hayashi et al., 1994; Tsuda et al., 2002; Gandrille et al., 2000).

Some studies have described autoantibodies that bind to PS (anti-PS) in patients with adverse pregnancy outcomes (Marozio et al., 2011; Sato et al., 2018). It was reported that 20 out of 100 patients with recurrent

* Corresponding author at: Laboratory for Recurrent Pregnancy Loss, Sugi Women's Clinic, 2-12-1-7F, Shinyokohama, Kohoku, Yokohama, Kanagawa, 222-0033, Japan.
E-mail address: sugi@sugi-wc.jp (T. Sugi).

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pregnancy loss were seropositive for anti-PS, which recognized EGF1-4 in PS (Sato et al., 2018).

PS Tokushima and anti-PS are common in the EGF-like domain-related abnormalities in PS. In this study, we investigated the association among PS Tokushima, anti-PS, and recurrent early and mid-to-late pregnancy loss.

2. Materials and methods

2.1. Patients

From May 2018 to February 2020, plasmas from 324 patients with recurrent early pregnancy loss (group A), 196 patients with one or more mid-to-late pregnancy loss (group B) and 650 infertile women having no pregnancy loss (group C) who were referred to Sugi Women's Clinic were screened for PS-specific activity and anti-PS by immunoblotting. Patients who were diagnosed with PS type II deficiency by low PS-specific activity underwent genetic analysis for the detection of PS Tokushima.

Patients with recurrent early pregnancy loss (group A) had three or more pregnancy losses before 10 weeks gestation. Those with mid-to-late pregnancy loss (group B) had one or more pregnancy losses after 10 weeks gestation. Infertile women (group C) had no pregnancy loss. They were patients with repeated implantation failure defined as having three or more embryo transfers with good-quality embryos. We did not enroll patients with repeated implantation failure into the group A nor the group B. Ectopic pregnancy and/or elective abortion were excluded. The main biological and clinical characteristics of the three groups of patients are given in Table 1. No patient was pregnant and taken any treatment when plasma was obtained.

Blood samples were collected in non-activating plastic tubes containing 3.2 % trisodium citrate (9:1 v/v). After centrifugation, plasma aliquots were immediately stored at −60 °C until use. Informed consent was obtained from all subjects. The study protocol was approved by the local ethics review committee.

2.2. Protein S-specific activity

The total PS activity and antigen levels were measured by the chromogenic method and latex agglutination method, respectively, using commercial kits (Shino-Test, Tokyo, Japan). The total PS activity to total PS antigen level ratio was defined as PS-specific activity. Based on data on healthy individuals from a previous study, the cutoff value to diagnose PS type II deficiency by PS-specific activity was 0.78 (mean - 3.0 standard deviation) (Tsuda et al., 2012; Noguchi et al., 2019).

2.3. Gene analyses

Genomic DNA was prepared from peripheral blood leukocytes, and the exon and exon-intron boundary regions were amplified using polymerase chain reaction (PCR). PS Tokushima samples were analyzed by PCR-restriction fragment length polymorphism with a mutagenic primer (5′−CCATCCTGCTCTTACCTTTACAATCTGACT-3′) and a normal primer (5′-CTCCTGAAAAGTTCTCTGCA-3′). The amplified fragment from the mutant allele was digested by Hinf I, and two fragments (404 and 30 bp) were detected. In contrast, the normal allele yielded only a 434-bp fragment after enzyme digestion (Yamazaki et al., 1993b).

2.4. SDS-PAGE and immunoblotting for Anti-PS

SDS-PAGE was performed using 10 % polyacrylamide gel. PS (Enzyme Research Laboratories, IN, USA) was prepared in non-reducing forms. PS (5 μL of 10 μg/mL solution) was applied to each lane. Transfer to polyvinylidene difluoride (PVDF) membrane was done for 20 min at 0.1 amps. Membranes were blocked for 1.5 h with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) at pH 7.3. Incubation with patient plasma (1/100) was done for 2 h followed by three washes with 0.05 % Tween 20/TBS. The membrane was exposed to horseradish peroxidase-conjugated polyclonal antibodies to human IgG or IgM for 1 h followed by washing as above. Immunobands were developed using 3,3′,5,5′-tetramethylbenzidine.

In our immunoblot, we evaluated the intensity of immunobands visually compared with negative controls. A plasma was considered positive if anti-PS IgG and/or IgM was positive.

2.5. Statistical analysis

Differences between the two groups were analyzed for statistical significance ($P < 0.05$) by the Fisher's exact test.

3. Results

As shown in Table 2, the incidence of patients who were diagnosed with PS type II deficiency was 2.47 % (8/324), 5.61 % (11/196), and 1.54 % (10/650) in groups A, B, and C, respectively. The incidence of patients with PS type II deficiency was significantly higher in patients with one or more mid-to-late pregnancy loss ($p = 0.0030$, odds ratios [OR]: 3.81, 95 % confidence interval [CI]: 1.59–9.10) than in infertile women having no pregnancy loss.

The incidence of patients with PS Tokushima was 1.85 % (6/324), 5.10 % (10/196), and 1.23 % (8/650) in groups A, B, and C, respectively. The incidence of patients with PS Tokushima was significantly higher in patients with one or more mid-to-late pregnancy loss ($p = 0.0027$, OR: 4.31, 95 % CI: 1.68–11.09) than in infertile women having no pregnancy loss.

As shown in Table 2, the incidence of patients with anti-PS was 20.1 % (65/324), 23.0 % (45/196), and 19.2 % (125/650) in groups A, B, and C, respectively. The incidence of patients with anti-PS was significantly higher in groups A ($p = 0.0229$, OR: 3.26, 95 % CI: 1.14–9.35), B ($p = 0.0071$, OR: 3.87, 95 % CI: 1.33–11.30), and C ($p = 0.0288$, OR: 3.10, 95 % CI: 1.10–8.72) than in our previously reported healthy nonpregnant

Table 1
Characteristics of the three groups of women in this study.

	Recurrent early pregnancy loss (Group A)	Mid-to-late pregnancy loss (Group B)	Infertile women having no pregnancy loss (Group C)
No. of patients	324	196	650
Age, mean (range)	36.7 (25–47)	34.4 (24–46)	38.5 (25–53)
No. of pregnancy losses, mean (range)			
Before 10th week of pregnancy	3.3 (3–11)	1.5 (1–5)	0
Beyond 10th week of pregnancy	0	1.1 (1–4)	0

Table 2
Positive rates for PS type II deficiency, PS Tokushima, and anti-PS autoantibodies (anti-PS).

	Recurrent early pregnancy loss (n = 324; group A)	Mid-to-late pregnancy loss (n = 196; group B)	Infertile women having no pregnancy loss (n = 650; group C)
PS type II deficiency	8/324 (2.47 %)	11/196 (5.61 %)	10/650 (1.54 %)
PS Tokushima	6/324 (1.85 %)	10/196 (5.10 %)	8/650 (1.23 %)
Anti-PS	65/324(20.1 %)	45/196(23.0 %)	125/650(19.2 %)

PS; protein S.

women (7.1 %, 4/56) (Sato et al., 2018). Anti-PS were associated with recurrent early pregnancy loss, mid-to-late pregnancy loss, and infertility.

Table 3 shows the positivity rates for anti-PS in patients with or without PS Tokushima. In group A, the incidence of anti-PS was 0.0 % (0/6) and 20.4 % (65/318) in PS Tokushima and non-PS Tokushima, respectively. In group B, the incidence of anti-PS was 10.0 % (1/10) and 23.7 % (44/186) in PS Tokushima and non-PS Tokushima, respectively. In group C, the incidence of anti-PS was 25.0 % (2/8) and 19.2 % (123/642) in PS Tokushima and non-PS Tokushima, respectively. In all groups, there was no association between anti-PS and PS Tokushima (group A: $p = 0.6040$, group B: $p = 0.4593$, group C: $p = 0.6539$).

Table 3
Positive rates for anti-PS autoantibodies (anti-PS) in patients with or without PS Tokushima.

	Recurrent early pregnancy loss (n = 324; group A)	Mid-to-late pregnancy loss (n = 196; group B)	Infertile women having no pregnancy loss (n = 650; group C)
PS Tokushima (+)	0/6 (0.0 %)	1/10 (10.0 %)	2/8 (25.0 %)
PS Tokushima (−)	65/318 (20.4 %)	44/186 (23.7 %)	123/642 (19.2 %)

PS; protein S.

4. Discussion

To the best of our knowledge, this is the first study to suggest an association between PS Tokushima and mid-to-late pregnancy loss and between anti-PS and repeated implantation failure as well as pregnancy loss.

In a previous report, genetic analysis of 304 healthy subjects (168 men aged 22–74 years and 136 women aged 23–74 years) revealed PS Tokushima in 5 subjects (1.6 %, 5/304) (Kinoshita et al., 2005). In this study, PS Tokushima was detected in 5.10 % of the patients with mid-to-late pregnancy loss, which is up to 4-fold higher than that detected in infertile women having no pregnancy loss ($p = 0.0027$, OR: 4.31, 95 % CI: 1.68–11.09) and up to 3-fold higher than that detected in previously reported healthy controls ($p = 0.0329$, OR: 3.22, 95 % CI: 1.08–9.55) (Kinoshita et al., 2005).

The results of our study are consistent with the results of previous reports. A meta-analysis showed an association between PS deficiency and nonrecurrent fetal loss after 22 weeks of gestation (OR: 7.39, 95 % CI: 1.28–42.63) (Rey et al., 2003). A systematic review showed that PS deficiency was significantly associated with late pregnancy loss (OR: 20.09; 95 % CI: 3.70–109.15) (Robertson et al., 2006).

In this study, no association was observed between PS Tokushima and recurrent early pregnancy loss. This result is also consistent with a previous report in which 335 patients with recurrent early pregnancy loss were investigated (Matsukawa et al., 2017).

The frequency of anti-PS was significantly higher in patients with recurrent early pregnancy loss, mid-to-late pregnancy loss, and infertility than in previously reported healthy controls (Sato et al., 2018). There was no association between PS Tokushima and anti-PS, suggesting that these may have different pathogenicities.

PS Tokushima was responsible for PS type II deficiency, which causes thrombophilia (Hayashi et al., 1994; Tsuda et al., 2002; Gandrille et al., 2000), although the inhibition of PS activity by anti-PS depended on the antigenic binding sites of anti-PS. The recognition of EGF1-2 by anti-PS may inhibit PS activity, causing thrombophilia and leading to pregnancy loss, similar to PS Tokushima (Sato et al., 2018).

An interesting hypothesis of the pathogenicity of anti-PS is that the recognition of EGF-like domains in PS and EGF family proteins by anti-PS may inhibit uterine and placental angiogenesis that is crucial for the survival of the embryo and fetus, leading to pregnancy loss (Sato et al., 2018). PS has been known as a natural anticoagulant protein, and it has been suggested to have an important role in multiple biological processes, including coagulation, apoptosis, angiogenesis/vasculogenesis, and cancer progression (Suleiman et al., 2013). PS was observed to accumulate around damaged placental trophoblastic cells in early and late pregnancy, which indicated a possibility that PS could protect or restore damaged villi and had physiological effects on the placenta (Matsumoto et al., 2008).

Autoantibodies, which recognize EGF-like domains in PS, might be related to the inhibition of angiogenesis and clearance of apoptotic cells during normal pregnancy (Sato et al., 2018).

In this study, it was found that anti-PS were associated with not only pregnancy loss but also repeated implantation failure. However, the negative influence of thrombophilia is unclear at the stage of implantation. In this study, PS Tokushima, a type of thrombophilia, was not associated with infertility.

EGF family proteins play an important role in implantation. In particular, heparin-binding EGF-like growth factor (HB-EGF) is crucial for normal implantation and is involved in blastocyst adhesion and development (Raab et al., 1996; Leach et al., 2004).

From these findings, it is reasonable to believe that the recognition of EGF-like domains in PS and EGF family proteins by anti-PS is associated with repeated implantation failure.

This study had two limitations. First, only PS type II-deficient patients that were identified by a low PS-specific activity were tested for genetic analysis of PS Tokushima. However, it was reported that the PS-specific activity had a sensitivity of 100.0 % for diagnosing PS Tokushima (Noguchi et al., 2019); therefore, it is highly probable that patients who were negative for PS type II deficiency did not have PS Tokushima. Second, there were no healthy controls in this study. Further studies performing the genetic analysis of PS Tokushima for all patients and screening healthy controls are necessary to obtain more accurate results.

In summary, our data suggested that PS Tokushima is associated with mid-to-late pregnancy loss, while anti-PS is associated with recurrent early pregnancy loss, mid-to-late pregnancy loss, and repeated implantation failure. Although PS Tokushima and anti-PS are common in the EGF-like domain-related abnormaities in PS, no direct association was established. PS Tokushima and anti-PS may have different pathogenecities.

Addendum

Study concept and design, Y. Sato, T. Sugi, and H. Kuma; acquisition of data, Y. Sato, T. Sugi, and R. Sakai; analysis and interpretation of data, Y. Sato, T. Sugi, and H. Kuma; patient inclusion and management, Y. Sato and T. Sugi; drafting of the manuscript, Y. Sato, T. Sugi, and H. Kuma; study supervisor, T. Sugi.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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