J Infect Chemother 20 (2014) 128–130

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Case report

Staphylococcal endocarditis as the first manifestation of heritable protein S deficiency in childhood

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ARTICLE INFO

Article history:
Received 30 May 2013
Received in revised form
11 August 2013
Accepted 28 August 2013

Keywords:
Protein S deficiency
Infective endocarditis
Staphylococcus aureus
Vegetation
Thromboembolism

ABSTRACT

A 12-year-old Japanese girl developed infective endocarditis and central nervous system disease. The previously healthy girl showed altered consciousness and abnormal behaviors along with the classical signs of septic emboli. *Staphylococcus aureus* was isolated from peripheral blood, but not, the pleocytotic cerebrospinal fluid. Diagnostic imaging studies revealed a vegetative structure in the morphologically normal heart, and multiple thromboembolisms in the brain and spleen. Low plasma activity of protein S (12%) and thrombophilic family history allowed the genetic study, demonstrating that she carried a heterozygous mutation of *PROS1* (exon 13; 1689C > T, p.R474C). Surgical intervention of the thrombotic fibrous organization and subsequent anticoagulant therapy successfully managed the disease. There are no reports of infective endocarditis in childhood occurring as the first presentation of heritable thrombophilia. Protein S deficiency might be a risk factor for the development or exacerbation of infective endocarditis in children having no pre-existing heart disease.

1. Introduction

Infective endocarditis (IE) is an infection of the mural endocardium caused by various bacteria or fungi. It rarely occurs in children presenting with vegetations, septic emboli, valve damages, and heart failure [1]. The majority of patients have underlying conditions such as rheumatic valvulopathy or congenital heart disease (CHD) [2]. Antibiotic managements controlled rheumatic heart diseases, and appreciably reduced the IE-risk in patients with unrepaired CHD. However, the incidence of pediatric IE is still increasing [2,3]. Advances in the cardiac surgery improved the survival rate of CHD patients, and might increase the number of IE-risk patients harboring bioprosthetic and synthetic devices. Recent reports delineated a continuing shift in the epidemiology of pediatric IE toward a proportion of children without pre-existing heart disease [3,4]. The shift accounts for the growing numbers of preterm infants and children who need indwelling central venous catheters for the management. On the other hand, there is little information about the etiology of IE occurring in previously healthy children who have no structural heart disease.

Protein S (PS) deficiency is one of the genetic traits predisposing persons to hypercoagulability [5]. Homozygous *PROS1* mutation leads to fetal loss or, if any, neonatal purpura fulminans. Adult carriers of the heterozygous mutation are at high risk of stroke, pulmonary embolism, and deep vein thrombosis. PS deficiency can be acquired in vitamin K deficiency, warfarin or sex hormone therapy, pregnancy, liver disease, and infections. PS-deficient patients are prone to thromboembolic events during cardiac surgery. There are no reports of IE as the first manifestation of heritable thrombophilia.

We report herewith a pediatric case of PS-deficient heterozygote, beginning with an intractable IE and systemic thromboembolism in no pre-existing heart disease.

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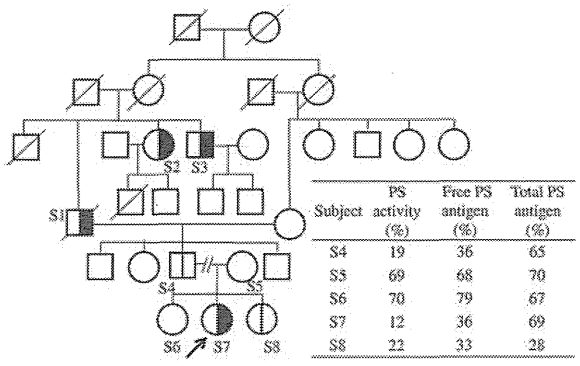
http://dx.doi.org/10.1016/j.jiac.2013.08.002

2. Case report

A 12-year-old Japanese girl was transferred to our hospital for the treatment of IE and central nervous system (CNS) disease of unknown etiology. The patient with no previous illness developed headache and vomiting. The next day (the 2nd day of illness), she showed fever and disturbed consciousness. On the 3rd day of illness, the patient was admitted to our affiliated hospital because CNS infection was suspected. Peripheral blood studies showed a leukocyte count of 16.09 × 10^9/L and C-reactive protein (CRP) of 16.18 mg/dl. Cerebrospinal fluid (CSF) analysis revealed a cell count of 54/μl with 46% polymorphonuclear cells and 54% lymphocytes, glucose of 85 mg/dl, and protein of 32.2 mg/dl. Methicillin-sensitive *Staphylococcus aureus* (MSSA) was isolated from the peripheral blood but not CSF cultures. Electroencephalogram revealed diffuse slow wave. Cefotaxime, panipenem/betamipron and acyclovir were intravenously started for the treatment of meningoencephalitis. Because of the thrombophilic family history and an abnormal structure in the left ventricle assessed by echocardiography, she was referred to our hospital at the 5th day of illness.

On admission, an afebrile well-nourished girl showed a pulse rate of 56 beats/min, a respiratory rate of 22 breaths/min and a blood pressure of 110/59 mmHg. The patient was listless but showed no meningeal signs. The Glasgow Coma Scale was E4V5M6. Auscultations revealed normal heart and respiratory sounds. There was no hepatosplenomegaly. Splinter hemorrhage and non-tender petechiae were found on the toes. Roth spots were determined on the retina. Complete blood cell counts showed a leukocyte count of 10.59 × 10^9/L with 83.5% segmented neutrophils, a hemoglobin concentration of 12.3 g/dl and a platelet count of 185 × 10^9/L. Liver and kidney functions were normal. CRP concentration was 7.45 mg/dl. Serum brain natriuretic peptide levels were elevated [51.3 pg/mL; reference range (rr): 0–18.4]. Coagulation studies revealed normal ranges of fibrinogen 321 mg/dl, prothrombin time (PT%) 78%, and activated partial thromboplastin time 32.5 s (rr: 26–41), and slightly increased levels of fibrinogen degradation products 5.4 μg/ml (rr: <5.0), D-dimer 2.0 μg/ml (rr: <0.5), thrombin-antithrombin complex 3.7 ng/ml (rr: <3.0), and plasmin α2-antiplasmin complex 1.2 μg/ml (rr: <0.8). Plasma anticoagulant activities showed low PS of 12% (rr: 59–128), subnormal protein C (PC) of 66% (rr: 75–131), and normal antithrombin of 103% (rr: 80–120). Protein induced by vitamin K absence/antagonist-II was undetectable. Normal PS antigen levels (69%; rr: 65–135) and free-PS antigen levels (36%; rr: 60–150) favored the diagnosis of type III PS deficiency. Detailed family history disclosed that two siblings of the paternal grandfather were diagnosed as having PS deficiency because of mesenteric artery thrombosis and pulmonary infarction in their fifties. Asymptomatic father and younger sister of the patient had low PS activity (Fig. 1).

Venous blood cultures yielded MSSA. Sterile CSF showed a cell count of 98/μl with 88% polymorphonuclear leukocytes and 12% lymphocytes, glucose of 99 mg/dl, and protein of 21.0 mg/dl. Transesophageal echocardiography revealed a vegetative structure (13 × 5 mm) in the left ventricular outflow tract. Head magnetic resonance imaging showed multiple white matter lesions with high signal intensity of T1-weighted fluid-attenuated inversion recovery and diffusion weighted image (DWI) on the splenium of corpus callosum and frontal and parietal lobes, indicating cerebral venous infarction. These determined the diagnosis of IE and cerebral infarctions associated with PS deficiency. Cefazolin (CEZ) and gentamicin (GM) therapy led to a partial resolution of symptoms but not vegetative mass. Because of the flare of fever and peripheral thrombotic lesions, the intraventricular lesion was removed by cardiac surgery on the 26th day of illness. The pedunculated structure located on the left ventricle wall adjacent to left coronary



Subject	PS activity (%)	Free PS antigen (%)	Total PS antigen (%)
S4	19	36	65
S5	69	68	70
S6	70	79	67
S7	12	36	69
S8	22	33	28

Fig. 1. Family pedigree of the present patient (arrow), and plasma protein S (PS) activity, free PS antigen, and total PS antigen in Subjects 4–8 (table). Subject 1 developed thromboembolisms in the leg at 49 years of age, cerebral infarction at 50 years, and mesenteric arterial thrombosis at 62 years. Subject 2 suffered from mesenteric arterial thrombosis at 59 years of age. Subject 3 had a history of pulmonary infarction. Subjects 4 (42 years old) and 8 (10 years old) had no history of thrombosis. Subjects 2 and 3 had been diagnosed as having PS deficiency. S: Subject.

cusp, containing organizing thrombi with granulation tissue, but not mesenchymal cells or bacterial colonies (Fig. 2). Antimicrobial (CEZ + GM) and anticoagulant therapy (aspirin + warfarin following heparinization) was then started. After the stop of antimicrobial agents at the 50th day of illness, she is alive and well on warfarin therapy. The genetic study demonstrated that the patient was a heterozygous carrier of the reported mutation in exon 13 of *PROS1* (1689C > T, Arg474Cys) [6].

3. Discussion

Associated conditions in IE children without preexisting heart disease include neoplasms, prematurity, connective tissue disorders, and diabetes mellitus [3]. However, inherited thrombophilia

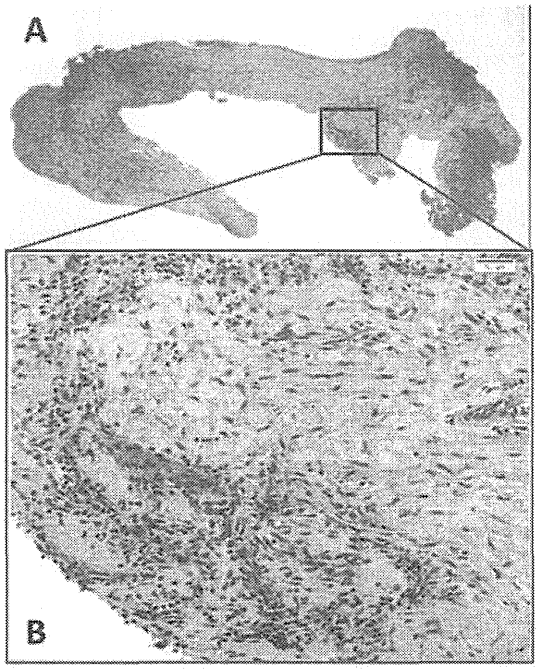


Fig. 2. Histological examination of a pedunculated structure on the left ventricle wall contained organizing thrombi with granulation tissue and fibrous tissue (Hematoxylin and eosin stain, original magnification A: × 40, B: × 400). There are edematous and myxoid changes, but no mesenchymal cells or bacterial colonies were observed.

had not been recognized a risk of IE. Lin et al. [4] characterized the feature of IE in non-CHD children; prevalent infection of *S. aureus* (MRSA and MSSA), vegetations in the right-sided heart, older age, and higher requirement of surgical intervention. Although the present patient later disclosed an episode of deciduous tooth loss two weeks prior to the onset of disease, the contribution remains unknown to the development of staphylococcal IE. The pathological findings of the intracardiac mass might be suggestive for nonbacterial thrombotic endocarditis (NBTE), arising in association with primary antiphospholipid syndrome, myeloproliferative disorders and malignancy [7]. On the other hand, granulation in the lesion, not usually observed in NBTE, indicated the presence of inflammation. Absent mesenchymal cells precluded the diagnosis of myxomatous tumor. The present case fulfilled the clinical criteria of IE, although *S. aureus* was identified from blood culture one time only. Furthermore, cerebral infarction, the most serious extracardiac complication of two different types of endocarditis, did not recur on anticoagulant therapy after the surgical intervention. These observations suggested that the predisposition of hypercoagulability could precipitate the formation of intra-ventricular clot with certain triggers such as occult bacteremia, and resulted in the intractable IE. Even if IE occurred in chance association with PS deficiency, it is not to say exaggerated that thrombophilia was one of the exacerbating factors of IE.

The major concern is the location of the vegetative lesion; why did it originate from the out-flow tract of left ventricle, but not, of right one in this patient? There are many reported cases of PS-deficient patients who developed arterial thromboembolism including stroke and mesenteric artery thrombosis. PS-deficient children might have a potential risk of arterial thrombosis [8]. Nevertheless, the association of heterozygous PS deficiency with venous but not arterial thrombosis is proven [9]. Girolami et al. [10] reported a 31-year-old man who developed both left and right atrial thrombosis, as presenting symptom of heritable PS deficiency. Jobic et al. [11] described a 77-year-old woman with unremarkable medical history who had native aortic valve thrombosis associated with PS deficiency. Maldjian et al. [12] reported that a previously healthy 39-year-old woman developed intracardiac thrombus due to PS-deficiency. On the other hand, no pediatric patient with isolated PS deficiency was reported to suffer from intracardiac thrombosis. Paç et al. [13] reported a case of biventricular cardiac thrombi in a 3-year old-girl with PC and PS deficiencies after the treatment of urinary tract infection and pneumonia. Mattiau et al. [14] described a 2-year-old girl with PC deficiency who presented with thrombus in the left ventricle and systemic emboli after the treatment of pneumonia. In these cases, intracardiac thrombi occurred in association with infection, but not limited to either-sided heart. To the best of our knowledge, there is only one case having a large vegetation of IE and PS deficiency [15]. The clinical course of the 81-year-old woman with no pre-existing heart disease was progressive. Taken together, surgical intervention must be inevitable for the treatment of thrombophilic IE patient, irrespective of the location of vegetative lesions.

The most prevalent form of heritable thrombophilia in Japan is PS deficiency, because of the polymorphism of PS-tokushima [16]. However, the mutation carriers are considerably found in the series of deep vein thrombosis [6,16]. The patient's pedigree disclosed the segregation of thrombophilic patient and PS deficiency. Secondary decrease of plasma PS activity in infection makes it difficult to diagnose heterozygous PS deficiency. The management of infection-associated hypercoagulability in such mutant carriers should be individualized according to the types of thrombophilia. Further study is needed to determine whether heritable thrombophilia is a risk factor for the development of IE in previously healthy non-CHD children. However, we should pay more attention to heritable thrombophilia in the management of pediatric IE.

Conflicts of interest

The authors state that they have no conflicts of interest.

Acknowledgments

We thank Prof. Yoshinao Oda and Dr. Kenichi Kohashi and Yuichi Yamada (Department of Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan) and Dr. Hatsue Ueda (Department of Pathology, National Cerebral and Cardiovascular Center, Osaka, Japan) for helpful discussion. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a grant from the Ministry of Health, Labour and Welfare of Japan.

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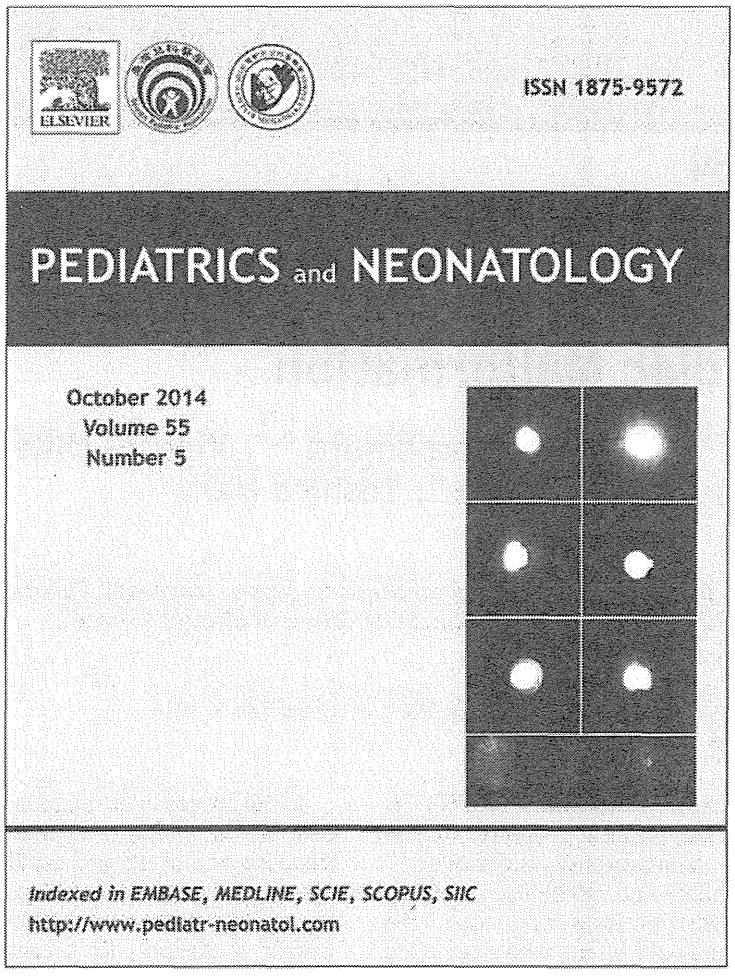

Pediatrics and Neonatology (2014) 55, 412–413

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BRIEF COMMUNICATION

Tachyarrhythmia-induced Cerebral Sinovenous Thrombosis in a Neonate Without Cardiac Malformation

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Received Nov 19, 2013; received in revised form Jan 26, 2014; accepted Feb 5, 2014
Available online 28 May 2014

Neonatal cerebral sinovenous thrombosis (CSVT) is becoming increasingly diagnosed because of greater clinical awareness and improved neuroimaging techniques.[1,2] Despite the substantial mortality and morbidity, the etiology of pediatric CSVT has not been fully understood.[3] We herein report a case of 13-day-old infant who developed intractable paroxysmal supraventricular tachycardia, which was subsequently complicated with CSVT.

A 13-day-old Japanese male was admitted to our affiliated hospital because of poor feeding and not doing well for 2 days. The infant was delivered at term with a birth weight of 3458 g and full APGAR scores. There was no cardiomyopathy or prothrombotic disorder in his relatives. On admission, he presented tachycardia at 300 beats/minute with hypotension. The electrocardiogram (ECG) showed narrow QRS tachycardia with following P wave suggesting the diagnosis of paroxysmal supraventricular tachycardia (Supplementary Figure 1). Cardioversion (0.5 J/kg twice) after the intubation allowed restoration of a sinus rhythm. The patient was then transferred to our hospital for intensive treatment.

Initial physical examination demonstrated an afebrile and pale infant. He had a sinus rhythm but presented cold extremities and prolonged capillary refill time (>3 seconds). Auscultation revealed a gallop rhythm of the heart. Chest X-ray examination demonstrated pulmonary congestion and cardiac dilatation with a cardiothoracic ratio of 61%. The echocardiogram demonstrated an impaired left ventricular function with ejection fraction at the level of 50%. Laboratory examinations are as follows: white blood cell count 10.68×10^9/L; hematocrit 35.7%; platelet count 157×10^9/L; creatinine kinase 342 U/L; creatine kinase-MB 71 U/L; lactate dehydrogenase 601 U/L; C-reactive protein 0.11 mg/dL; troponin-T 0.154 ng/mL; prothrombin time—international normalized ratio 1.62; activated partial thromboplastin time 40.1 seconds; fibrinogen degradation products 63.1 μg/ml; D-dimer 28.5 μg/mL; and brain natriuretic peptide 3956 pg/mL.

The ECG during tachycardia documented the characteristics of atrioventricular reentrant tachycardia at 300 beats/minute. The tachycardia was terminated by the intravenous rapid infusion of ATP three times. The ECG during sinus rhythm showed no delta wave, suggesting the possibility of concealed WPW syndrome. Oral propranolol (1–2 mg/kg/day) and flecainide (3–6 mg/kg/day) succeeded to prevent the recurrence of refractory arrhythmia. The echocardiogram showed a smooth recovery of the left ventricular function to the normal level of 63% 2 days after admission.

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http://dx.doi.org/10.1016/j.pedneo.2014.02.003

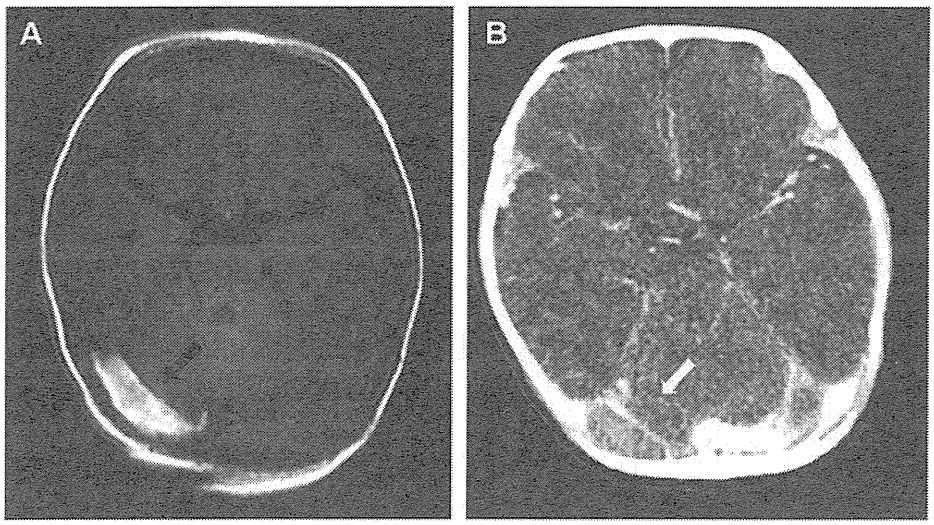

Figure 1 (A) T1-weighted magnetic resonance imaging. (B) Contrast-enhanced computed tomography of the brain after restoration of sinus rhythm. High signal intensity on T1-weighted magnetic resonance imaging (arrow) and flow disturbance on computed tomography (white arrow) confirmed the diagnosis of right transverse sinus thrombosis.

Magnetic resonance imaging (MRI) was performed 9 days after admission, in order to assess the cerebral damage due to cardiogenic shock. CSVT was indicated on MRI findings (Figure 1A). Computed tomography (CT) of the brain determined a right transverse sinus thrombosis (Figure 1B). Continuous infusion of fractionated heparin (10–22 unit/kg/hour, activated partial thromboplastin time 42–50 seconds) was started. Two weeks after the anticoagulant therapy, CT demonstrated recanalization of the transverse sinus. There were no laboratory data indicating inherited or acquired thrombophilic predispositions (on 12 days after admission): normal plasma activity of protein C 69%, protein S 58% and antithrombin 91%, and undetectable titer of anticardiolipin antibody 2 U/mL. The patient received no additional anticoagulant therapy. CSVT did not recur for 6 months after the first event.

This is the first report of neonatal CSVT as a complication of paroxysmal supraventricular tachycardia. Perinatal complications, dehydration, sepsis, meningitis, and inherited thrombophilias such as antithrombin, protein C, or protein S deficiency are the major associations with pediatric CSVT.[4,5] A recent case series reported that none of the neonates with CSVT had persistently low activity of protein C, protein S, or antithrombin, and some of them were considered to have acquired prothrombotic states.[4] This patient showed no evidence of prothrombotic disorders, although FD fibrinogen degradation products P and D-dimer had already been elevated on admission. Pediatric venous thrombosis occurs at the highest incidence in neonates. Extremely high brain natriuretic peptide level suggested the presence of cardiac deterioration due to prolonged tachycardia. We speculate that the cardiogenic shock led to the stasis of cerebral sinus venous flow that predisposed the neonate to developing thrombosis.

No randomized clinical trials have been conducted concerning anticoagulation therapy for neonatal CSVT. However, the American College of Chest Physicians recommended initial anticoagulation except in the presence of significant hemorrhage.[6] The favorable response to the short-term anticoagulant therapy in the present case might corroborate that CSVT was not associated with inherited coagulopathy, but the transient circulatory disturbance.

Because the symptoms of CSVT are nonspecific, and often subtle or asymptomatic, the diagnosis is delayed and may be missed altogether. Our patient did not present any neurological symptom at the diagnosis of CSVT. Intensive imaging tests including brain echogram, CT, and MRI, and appropriate anticoagulant therapy should be considered for the neonates having sustained tachyarrhythmia with cardiac failure.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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Rheumatology Advance Access published May 11, 2014

Concise report

doi:10.1093/rheumatology/keu180

Early progression of atherosclerosis in children with chronic infantile neurological cutaneous and articular syndrome

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Abstract

Objective. Chronic inflammation plays a key role in the development of atherosclerosis. Early progression of atherosclerosis has been reported in patients with RA. Cryopyrin-associated periodic syndromes (CAPS) are autosomal dominant autoinflammatory disorders caused by heterozygous *NLRP3* gene mutations. Chronic infantile neurological cutaneous and articular (CINCA) syndrome is the most severe form of CAPS and patients display early onset of rash, fever, uveitis and joint manifestations. However, there has been no previous report on atherosclerosis in patients with CAPS. The objective of this study is to assess the development of atherosclerosis in patients with CINCA syndrome.

Methods. Intima–media thickness (IMT) of the carotid arteries, stiffness parameter β, ankle brachial index (ABI) and pressure wave velocity (PWV) were evaluated by ultrasonography in 3 patients with CINCA syndrome [mean age 9.0 years (S.D. 5.3)] and 19 age-matched healthy controls [9.3 years (S.D. 4.3)].

Results. The levels of carotid IMT, stiffness parameter β and PWV in CINCA syndrome patients were significantly higher than those in healthy controls [0.51 mm (S.D. 0.05) *vs* 0.44 (0.04), $P = 0.0021$; 6.1 (S.D. 1.7) *vs* 3.9 (1.0), $P = 0.0018$; 1203 cm/s (S.D. 328) *vs* 855 (114), $P = 0.017$, respectively].

Conclusion. Patients with CINCA syndrome showed signs of atherosclerosis from their early childhood. The results of this study emphasize the importance of chronic inflammation in the development of atherosclerosis. Further analysis on atherosclerosis in young patients with CINCA syndrome may provide more insights into the pathogenesis of cardiovascular disease.

Key words: ankle–brachial index, atherosclerosis, chronic infantile neurologic cutaneous and articular syndrome, cryopyrin-associated periodic syndromes, intima-media thickness, pulse wave velocity.

Introduction

It is well known that chronic inflammation is a predisposing factor for atherosclerosis. There has been considerable interest regarding the possible causal role of inflammation in the development of atherosclerosis in adult patients with RA, SLE and familial Mediterranean fever (FMF). Patients with SLE, APS or RA have increased mortality rates related to early atherosclerosis. Relative risk of 5 for myocardial infarction, 6–10 for stroke in SLE patients and 3.6 for cardiovascular deaths in RA patients has been reported [1]. Furthermore, the American Heart Association has reported that chronic inflammatory disease is one of the eight high-risk factors for atherosclerosis, even in children [2].

Cryopyrin-associated periodic syndromes (CAPS), including chronic infantile neurological cutaneous and articular (CINCA) syndrome, Muckle–Wells syndrome and familial cold autoinflammatory syndrome, are autosomal dominant autoinflammatory syndromes caused by heterozygous mutations of the *NLR family pyrin domain*

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Submitted 25 November 2013; revised version accepted 11 March 2014.

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containing 3 (*NLRP3*) gene. It has been reported that disease associated *NLRP3* mutation causes IL-1β oversecretion by caspase-1 activation. CINCA syndrome, the most severe form among them, is characterized by chronic systemic inflammation manifested as early onset of rash, fever, uveitis, chronic meningitis and joint symptoms [3]. However, there has been no previous report evaluating atherosclerosis in patients with CAPS.

Several physiological examinations are applied to assess atherosclerosis. Carotid intima–media thickness (cIMT) is known to be an indicator of atherosclerosis for adults and children [4]. In fact, increased cIMT has been shown in children with obesity, hyperlipidaemia and diabetes mellitus [5]. It has been reported that stiffness parameter β is more useful in detecting atherosclerotic changes in earlier stages than cIMT [6]. Also, pulse wave velocity (PWV) and ankle–brachial index (ABI) are simplified parameters of the severity of atherosclerosis and predictors of prognosis in adult patients with cardiovascular disease [7, 8]. The objective of this study is to assess the development and progression of atherosclerosis in young patients with CINCA syndrome by measuring cIMT, stiffness parameter β, PWV and ABI.

Patients and methods

Study population

Three patients (a 5-year-old boy [9], a 7-year-old girl [10] and a 15-year-old boy [11]) with CINCA syndrome and 19 age-matched healthy controls were enrolled in this study. *NLRP3* mutations were observed in all three patients. The parameters of atherosclerosis were investigated in these three patients who were in remission for 1 year after the initiation of canakinumab treatment. The Institutional Review Board of Kyushu University Hospital approved the study and informed consent was obtained from each subject.

Sonographic study

Carotid artery US was performed with an iE33 ultrasound machine (Philips, Amsterdam, The Netherlands) using an 11 MHz probe. Measurements were obtained with subjects in the supine position by experienced sonographers blinded to the subjects' clinical status. Ultrasonographic images of the right and left common carotid arteries (CCAs) of each subject at the lower third cervical region proximally and 1 cm above the carotid bulb distally in the longitudinal plane were obtained. CCA IMT measurements of the distal CCA posterior wall were done manually by the distance measurement system of the sonography device after magnification of the images. Three measurements were made in a non-neighbouring fashion within an ~1 cm segment from both the left and right CCA proximal and distal portions. The IMT was measured during end diastole. Mean IMT was calculated as the average of three consecutive measurements of maximum far wall thickness obtained from the CCA. Measurement of the internal diameter of the CCA was performed for three consecutive heartbeats. Intraobserver variability was 1.7% for IMT and 3.1% for arterial wall diameter measurements. The stiffness parameter β was calculated from this formula [12]: $\beta = [\ln(SBP/DBP)]/(\Delta D/D)$, where SBP is the systolic blood pressure, DBP is the diastolic blood pressure, D is carotid artery diastolic diameter and ΔD is the change in artery diameter during systole.

PWV and ABI

PWV and ABI were measured using a BP-203RPEIII (Omron Colin, Tokyo, Japan). PWV, ABI, the blood pressure of the extremities, ECG and heart sounds were synchronously measured and then automatically recorded. Electrodes were contacted on both wrists and a microphone was attached to the left margin of the sternum. The extremities were then wrapped by cuffs that were connected to a pulse monitor. The volume wave and time difference emitted from the pulse monitor were recorded. The pulse wave was defined as the value obtained by dividing the distance between the two points by the time spent in transferring the pulse. In the current study, the pulse wave was measured in the brachial artery and ankle (baPWV). The ABI was defined as the ratio between the systolic pressure measured in the ankle and that measured in the brachial artery.

Laboratory evaluation

In the morning, after an overnight fast, venous blood was sampled for the measurement of serum concentrations of glucose, total cholesterol, triglycerides and standard CRP.

Statistical analysis

Data are expressed as mean (S.D.). Differences between data were studied using the Student's *t* test. Analytical statistics of data between group comparisons of categorical data parameters were performed by using the chi-square test. Statistical significance was taken as $P < 0.05$. All statistical analyses were performed using JMP8 (SAS Institute, Tokyo, Japan).

Results

Clinical characteristics of the study group are presented in Table 1. Age, sex and triglyceride levels were similar between patients with CINCA syndrome and control subjects ($P = 0.65$, 0.53 and 0.17, respectively). Total cholesterol levels in CINCA syndrome patients were significantly lower than those in healthy controls, although they were within normal ranges in both groups. CRP concentrations in the patient group were significantly higher than in healthy controls [5.76 mm (S.D. 2.05) *vs* 0.08 (0.16), $P < 0.0001$].

All subjects tolerated the sonographic examination well. Sonographic study results and normal values of the parameters for the age of the patients [13, 14] are summarized in Table 2. Carotid artery analysis revealed that the IMT and stiffness parameter β of patients with CINCA syndrome were significantly higher than those of healthy controls [0.51 mm (S.D. 0.05) *vs* 0.44 (0.04), $P = 0.0021$, and 6.1 (S.D. 1.7) *vs* 3.9 (1.0), $P = 0.018$, respectively).

TABLE 1 Clinical and laboratory characteristics of the subjects

	Patient 1	Patient 2	Patient 3	CINCA syndrome ($n = 3$), mean (S.D.)	Controls ($n = 19$), mean (S.D.)	P-value
Gender, male/female	Male	Female	Male	2/1	9/10	0.53
Age, years	5	7	15	9.0 (5.3)	9.3 (4.3)	0.65
BMI, kg/m^2	16.0	15.5	16.8	16.1 (0.6)	17.3 (2.9)	0.51
Systolic blood pressure, mmHg	91	96	128	105 (20)	99 (8)	0.38
Diastolic blood pressure, mmHg	45	50	68	54 (12)	53 (4)	0.73
Total cholesterol, mg/dl	123	122	131	125 (5)	159 (17)	0.0046
Triglycerides, mg/dl	61	79	157	99 (51)	70 (28)	0.17
Glucose, mg/dl	93	85	102	94 (3)	94 (6)	0.95
CRP, mg/dl	0.26	1.62	5.55	2.48 (2.75)	0.08 (0.16)	<0.0001

CINCA syndrome: chronic infantile neurological cutaneous and articular syndrome.

TABLE 2 Ultrasonographic examination, baPWV and ABI in CINCA syndrome patients and control subjects

	Patient 1	Patient 2	Patient 3	CINCA syndrome ($n = 3$), mean (S.D.)	Controls ($n = 19$), mean (S.D.)	P-value
Intima–media thickness, mm (normal value for each age) [13]	0.47 (0.40)	0.5 (0.40)	0.57 (0.50)	0.51 (0.05)	0.44 (0.04)	0.0021
Systolic diameter, mm	5.5	5.8	5.8	5.7 (0.2)	6.2 (0.8)	0.30
Diastolic diameter, mm	4.8	5.2	5.4	5.1 (0.3)	5.3 (1.7)	0.63
Stiffness parameter β (normal value for each age) [14]	4.8 (3.4)	5.7 (3.7)	7.6 (4.5)	6.1 (1.7)	3.9 (1.0)	0.018
Right baPWV, cm/s	1068	920	1566	1185 (338)	850 (114)	0.0025
Left baPWV, cm/s	1053	1022	1587	1221 (318)	859 (114)	0.0014
Averaged baPWV, cm/s (normal value for each age) [15]	1061 (<941)	971 (<919)	1577 (1041)	1203 (328)	855 (114)	0.0017
Right ABI	1.15	0.91	0.98	1.00 (0.10)	1.04 (0.10)	0.67
Left ABI	1.16	0.95	0.92	0.99 (0.10)	1.06 (0.10)	0.48
Averaged ABI (normal value for each age) [15]	1.16 (>1.00)	0.93 (>1.00)	0.95 (>1.00)	0.99 (0.10)	1.05 (0.10)	0.54

CINCA syndrome: chronic infantile neurological cutaneous and articular syndrome; baPWV: brachial artery pulse wave velocity; ABI: ankle–brachial index.

The averaged baPWV of the patients was significantly higher than that of controls [1203 cm/s (S.D. 328) *vs* 855 (114), $P = 0.017$) (Table 2). There was no significant difference in ABI between the two groups, although the values of two patients were lower than the normal range [15].

Discussion

In the present study we found that patients with CINCA syndrome develop atherosclerosis from early childhood. There have been many previous studies describing atherosclerosis associated with inflammatory diseases such as RA, SLE and FMF [1]. However, this is the first report showing the youngest group of patients who developed atherosclerosis associated with inflammatory disorders.

It has been shown that inflammation plays an important role in the development of atherosclerosis. The presence of macrophages and activated lymphocytes within the plaques supports the nature of an immune system-mediated inflammatory disorder of atherosclerosis. It has been shown that higher disease activity representing higher inflammatory burden is associated with increased cardiovascular events in patients with RA and SLE [16]. It may be induced by elevated inflammatory cytokines, which can cause the development of endothelial dysfunction in atherosclerotic processes. In addition, changes in lipid metabolism and a wide variety of immune and inflammatory alterations that directly affect the endothelium, vascular smooth muscle cells and inflammatory cellular components of the atherosclerotic plaque may also play important roles in the development and progression of atherosclerosis in patients. CINCA syndrome is the most severe form of CAPS, and patients display severe systemic inflammation from the neonatal period [3].

Therefore it is reasonable to assume that the progression of atherosclerosis from childhood in three patients with CINCA syndrome is closely related to chronic systemic inflammation. It was reported that the incidence of atherosclerosis could be reduced by aggressive disease-modifying therapies in patients with RA and SLE [16]. In patients with CINCA syndrome, we can investigate the association between inflammation and atherosclerosis without any effect of classical risk factors such as obesity, smoking, hyperlipidaemia or diabetes. This may provide a novel clue to clarify the role of inflammation in the development of atherosclerosis.

In patients with FMF and SLE, age and disease duration were reported to be associated with the severity of atherosclerosis [17]. In the present study we found that the oldest patient (patient 3) with the longest disease duration had the most advanced atherosclerosis, which is in line with this report. Early diagnosis and effective treatment for chronic inflammation in these patients have been emphasized in preventing cardiovascular disease because a negative correlation between the duration of anti-inflammatory treatment and IMT has been observed in SLE patients [18].

Interestingly, improvements in PWV and cIMT [19] were reported in patients with RA after sufficient infliximab treatment. In patients with CINCA syndrome, canakinumab was reported to induce rapid and sustained remission of symptoms [20]. It is possible that a significant improvement in atherosclerosis will be observed in our patients with CINCA syndrome after canakinumab treatment in the near future.

However, there are some limitations in the present study. First, our study contains only a small number of patients because of the extremely rare incidence of this disease. Second, the parameters investigated in this study are considerably variable with the age of the subjects. It is also possible that the values of the parameters change because of the measurement equipment. Multicentre and long-term follow-up analysis with standardized procedures and tools on a larger number of the patients are necessary to provide more precise information on the pathogenesis of atherosclerosis.

Conclusion

Patients with CINCA syndrome developed atherosclerosis from early childhood. Atherosclerosis in CINCA syndrome patients may be a prototype of cardiovascular disease predominantly induced by chronic inflammation.

Rheumatology key messages

- Patients with CINCA syndrome develop atherosclerosis from early childhood.
- This report shows the youngest group of patients who developed atherosclerosis associated with inflammatory disorders.
- Early treatment with anti-IL-1β antibody might be beneficial in preventing atherosclerosis in CINCA syndrome.

Acknowledgements

We thank Junji Kishimoto (Digital Medicine Initiative, Kyushu University Hospital, Fukuoka, Japan) for the contribution of statistical analysis.

Funding: This work was supported by a grant for Research on Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan to T.H. and a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (24791072) to K.Y.

Disclosure statement: The authors have declared no conflicts of interest.

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THROMBOSIS AND HEMOSTASIS

Novel FV mutation (W1920R, FV_{Nara}) associated with serious deep vein thrombosis and more potent APC resistance relative to FV_{Leiden}

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Key Points

- FV_{Nara} (W1920R), associated with serious deep vein thrombosis, is more resistant to APC relative to FV_{Leiden} (R506Q).
- This mechanism results from significant decreases in FVa susceptibility to APC and FV cofactor activity for APC.

Factor V (FV) appears to be pivotal in both procoagulant and anticoagulant mechanisms. A novel homozygote (FV_{Nara}), a novel mechanism of thrombosis associated with Trp1920→Arg (W1920R), was found in a Japanese boy and was associated with serious deep vein thrombosis despite a low level of plasma FV activity (10 IU/dL). Activated partial thromboplastin time–based clotting assays and thrombin generation assays showed that FV_{Nara} was resistant to activated protein C (APC). Reduced susceptibility of FVa_{Nara} to APC-catalyzed inactivation and impaired APC cofactor activity of FV_{Nara} on APC-catalyzed FVIIIa inactivation contributed to the APC resistance (APCR). Mixtures of FV-deficient plasma and recombinant FV-W1920R confirmed that the mutation governed the APCR of FV_{Nara}. APC-catalyzed inactivation of FVa-W1920R was significantly weakened, by ~11- and ~4.5-fold, compared with that of FV–wild-type (WT) and FV_{Leiden} (R506Q), respectively, through markedly delayed cleavage at Arg506 and little cleavage at Arg306, consistent with the significantly impaired APC-catalyzed inactivation. The rate of APC-catalyzed FVIIIa inactivation with FV-W1920R was similar to that without FV, suggesting a loss of APC cofactor activity. FV-W1920R bound to phospholipids, similar to FV-WT. In conclusion, relative to FV_{Leiden}, the more potent APCR of FV_{Nara} resulted from significant loss of FVa susceptibility to APC and APC cofactor activity, mediated by possible failure of interaction with APC and/or protein S. (*Blood.* 2014;123(15):2420-2428)

Introduction

Factor V (FV) contributes to opposing mechanisms in the regulation of coagulation.[1,2] The procoagulant action of FV is associated with cofactor activity for FXa in the prothrombinase complex, which catalyzes the conversion of prothrombin to thrombin on a phospholipid (PL) surface.[3-5] FV is converted to FVa by proteolytic cleavage by thrombin. Development of a hypercoagulant state is controlled by downregulation by activated protein C (APC) with protein S (PS). Hence, FVa is rapidly inactivated by proteolytic cleavage of the heavy chain (HCh) at Arg306, Arg506, and Arg679.[6,7] Cleavage at Arg506 is essential for the exposure of other cleavage sites but is not directly required for the decrease in activity. Cleavage at Arg306 results in near-complete loss of FVa activity. Nevertheless, any defect of 1 or more cleavage reactions significantly affects the processes of APC-induced inactivation.[8,9] The alternative function of FV is as an anticoagulant cofactor of APC in FVIIIa inactivation.[1] FVIIIa functions as a cofactor in the tenase complex and is responsible for PL-dependent FXa generation by FIXa.[10-12] In the process of APC-induced FVIIIa inactivation, FV acts as an anticoagulant cofactor of APC with PS, resulting in acceleration of FVIIIa inactivation through cleavage at Arg336.[13,14] This anticoagulant activity of FV is mediated by a product of proteolysis by APC before cleavage by thrombin. Cleavage at Arg506 of FV attached to the B domain is essential to the anticoagulant activity of FV, whereas cleavage at Arg306 appears to contribute less to this mechanism.[15,16] Any molecular defect of these cleavage reactions confers APC resistance (APCR).[1]

A point mutation of the *F5* gene, Arg506→Gln (R506Q; FV_{Leiden}), is the major cause of APCR[2] and is detected in ~20% of Caucasians with deep venous thrombosis (DVT).[17,18] The loss of the APC cleavage site at Arg506 in FV_{Leiden} results in a loss of APC-induced FVa inactivation and impairment of FV cofactor activity of APC in FVIIIa inactivation. Rare FV point mutations Arg306→Thr (R306T; $FV_{Cambridge}$)[19,20] and Arg306→Gly (R306G; $FV_{Hong\ Kong}$)[20,21] affect the APC cleavage site at Arg306 and are associated with mild APCR.[22] No FV mutations linked to APCR have been identified in Japanese populations, however. We describe the findings in a Japanese boy with severe DVT in the paradoxical presence of FV deficiency with FV activity (FV:C) 10 IU/dL. We have identified a novel mechanism of thrombosis associated with a Trp1920→Arg (W1920R) mutation in the *F5* gene (FV_{Nara}). The defect resulted in APCR more potent than that seen with FV_{Leiden}.

Submitted October 1, 2013; accepted January 28, 2014. Prepublished online as *Blood* First Edition paper, February 12, 2014; DOI 10.1182/blood-2013-10-530089.

K.N. and K.S. contributed equally to this study.

Presented in abstract form at the 53rd annual meeting of the American Society of Hematology, San Diego, CA, December 12, 2011.

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Materials and methods

Blood samples were obtained after informed consent following local ethical guidelines. DNA direct sequencing and the expression of recombinant protein were approved by the Medical Research Ethics Committee of Tokyo Medical University. This study was conducted in accordance with the Declaration of Helsinki.

Reagents

The pMT2/FV mammalian expression plasmid containing the full-length *F5* cDNA was provided by Dr Kaufman (University of Michigan, Ann Arbor, MI). The EZ1 DNA Blood Kit, QIAquick Gel Extraction Kit, and QIAfilter Plasmid Kit (Qiagen, Dusseldorf, Germany) and the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) were purchased. Recombinant FVIII was a generous gift from Bayer Corporation, Japan. A monoclonal antibody (mAb)C5,[23] recognizing the C-terminus of the FVIII A1 domain, was provided by Dr Carol Fulcher (Scripps Research Institute, La Jolla, CA). FV, FIXa, FX, FXa, α-thrombin, APC, PS, mAbAHV-5146 against the FV HCh (Hematologic Technologies, Essex Junction, VT), lipidated TF (Innovin; Dade Behring, Marburg, Germany), and fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland) were purchased commercially. FV-deficient plasmas (George King. Overland Park, KS), PT, and activated partial thromboplastin time (aPTT) reagent (Instrumentation Laboratory, Bedford, MA; Sysmex, Kobe, Japan) were purchased. PL vesicles containing phosphatidylserine/phosphatidylcholine/phosphatidylethanolamine, 10%/60%/30%, were prepared using *N*-octylglucoside.[24]

DNA direct sequencing

Genomic DNA was extracted from leukocytes, using the BioRobot EZ1 workstation. PCR assays were performed with *Taq* DNA polymerase (TaKaRa-Bio, Otsu, Japan). PCR products were electrophoresed on agarose gels and purified by gel extraction. Purified PCR products from genomic DNA and *F5* plasmids were confirmed by sequencing using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and analyzed with a 3730 DNA analyzer. All sequences were compared with wild-type (WT) *F5* sequences (GenBank number Z99572).

Expression of recombinant FV

The mutations were introduced independently into a pMT2/FV plasmid by site-directed mutagenesis.[25] The WT and mutant plasmids used in transfection experiments were purified. Vectors expressing recombinant proteins were transfected into HEK293 cells, using the lipofection method. After 60 hours, the culture media and cells were harvested. Conditioned media (CM) were collected, centrifuged to remove cell debris, and stored at −80°C. FV antigen (FV:Ag) levels in CM and cell lysates were measured by enzyme-linked immunosorbent assay (ELISA; Affinity Biologicals). FV:C in CM was measured in PT-based clotting assays, using the ACL9000 coagulation analyzer (Instrumentation Laboratory). The specific activity of FV was calculated as the ratio of FV:C to the concentration of FV:Ag, both of which were measured in CM. The proteins were harvested in serum-free medium and concentrated by filtration (cutoff ~100 kDa).

APCR assay

aPTT-based assays. The APC-resistance kit, which is not approved and not commercially available in Japan, was provided by Instrumentation Laboratory for research use. This assay was performed using ACL9000 with predilution of sample plasmas in FV-deficient plasma. The APC sensitivity ratios (APCsrs) were expressed as ratios of aPTT clotting times in the presence of APC divided by clotting times in its absence. This assay reflects the effect of APC on inactivation of both FVa and FVIIIa; hence, a low level of APCsr indicates a defect in the inactivation of FVa and/or FVIIIa and, consequently, reflects APCR.

Thrombin generation-based assays. Calibrated automated thrombin generation assay (Thrombinoscope) was performed as previously reported.[26] Platelet-poor plasma (PPP) or platelet-rich plasma (PRP) was preincubated for 10 minutes with TF (5 pM), APC (8 or 40 nM), and PL (0 or 10 μM), respectively. PRP was adjusted to 15×10^4 platelets/μL. Measurements were commenced after the addition of $CaCl_2$ and fluorogenic substrate (final concentration [f.c.] 16.7 mM and 417 μM, respectively). Fluorescent signals were monitored continuously in a Fluoroskan microplate reader (Thermo Fisher Scientific, Franklin, MA). For data analyses, the parameters (lag time, peak thrombin, time to peak, and endogenous thrombin potential [ETP]) were derived. The APCsrs were expressed as ratios of the parameter in the absence (or presence) of APC, divided by the ratio in its presence (or absence).

FXa generation-based assays. Normal or patient's plasma was mixed with FV-deficient plasma in various proportions and assayed using the FXa generation assay (COATEST SP-FVIII, Chromogenix, Milan, Italy), with minor modifications.[27] The test specifically quantifies FVIIIa:C in 16-fold diluted plasma by measuring intrinsic FXa generation mediated by excess exogenous FIXa and FX with PL. The simultaneous addition of APC (40 nM) with cofactors PS and FV in plasma inhibits intrinsic FXa generation by inactivating FVIIIa. The APCsrs were expressed as ratios of the amount of generated FXa in the absence of APC divided by that in its presence. A low level of APCsr indicates a defect in FVIIIa inactivation and, consequently, reflects APCR.

Prothrombinase assay

FV (2 nM) was activated by thrombin (20 nM) for 1 minute, followed by the addition of hirudin. The reactants were mixed with prothrombin (1.4 μM), PL, and 5-dimethylamino-naphthalene-1-sulfonylarginine-*N*-(3-ethyl-1,5-pentanediyl)-amide (30 μM), followed by initiation by the addition of FXa (10 pM). Aliquots were removed to assess the initial rates of product formation, and the reactions were quenched with EDTA (f.c. 50 mM). Rates of thrombin generation were determined at absorbance 405 nm (Abs_{405}) after the addition of S-2238 (f.c. 0.46 mM). Thrombin generation was quantified from a standard curve prepared using known amounts of thrombin.

FV–PL binding

Binding of FV to immobilized PL was examined in ELISAs.[28] α-phosphatidyl-*L*-serine (5 μg/mL) in methanol was added to microtiter wells and air-dried. The wells were blocked by the addition of gelatin solution (5 mg/mL), and serial dilutions of FV were added and incubated at 37°C for 2 hours. Bound FV was quantified by the addition of anti-FV mAbAHV-5146 (2.5 μg/mL) and goat anti-mouse peroxidase-linked antibody, followed by measuring at Abs_{492}. The amount of nonspecific immunoglobulin G (IgG) binding without FV was <3% of the total signal. Specific binding was estimated by subtracting the amount of nonspecific binding.

APC-catalyzed inactivation of FVa

FV (8 nM) was incubated with thrombin (100 nM) for 5 minutes at 37°C, and reaction was terminated by the addition of hirudin (25 U/mL). Samples containing the generated FVa (2 nM) were incubated with APC (25 pM), and PS (30 nM) with PL (20 μM), for the indicated times. Aliquots were obtained from the mixtures and diluted ~30-fold. Residual FV:C was measured in aPTT-based clotting assays. The presence of thrombin and hirudin in the diluted samples had little effect in these assays.

APC cofactor activity of FV

The APC cofactor activity of FV variants was measured in a FVIIIa degradation assay,[29] with minor modifications. FVIII (10 nM) and PL (20 μM) were activated by thrombin (5 nM) for 30 seconds, and the reaction was terminated by the addition of hirudin (2.5 U/mL). The generated FVIIIa was then incubated with APC (0.5 nM) and PS (5 nM) with various concentrations of FV variants for 20 minutes. The reactants were diluted 9-fold before incubation with FIXa (2 nM) and FX (200 nM) for 1 minute. Generated FXa was measured in a chromogenic assay with S-2222 at Abs_{405}. Relative FVIII:C was calculated from the amounts of generated FXa.

Table 1. FV levels and APCR in plasmas of the patient and family members

Case	FV:C (IU/dL)	FV:Ag (IU/dL)	*F5* mutation	aPTT-based APCR assay		
				aPTT, seconds		
				Minus APC	Plus APC	APCsr (plus/minus APC)
FV_{Nara} family members						
Patient	10.0	40.0	W1920R homozygote	79.7	131	1.64
Father	74.1	74.0	W1920R heterozygote	43.3	98.0	2.26
Mother	87.6	75.0	W1920R heterozygote	39.0	104	2.67
Brother	109	77.0	WT	32.2	98.4	3.06
Sister-1	113	101	WT	32.6	107	3.28
Sister-2	132	115	WT	32.2	99.8	3.10
FV_{Leiden} patient						
Patient 1	56.5	96.0	R506Q heterozygote	35.2	57.2	1.63
Patient 2	65.2	61.0	R506Q heterozygote	36.8	61.2	1.66
FV-deficient patient						
Patient 1	50.0	44.0		39.2	124	3.16
Patient 2	54.0	53.0		38.4	118	3.07
Patient 3	55.0	47.0		43.4	124	2.85
Healthy controls						
Male (n = 17)				32.6 ± 0.70	108.4 ± 4.50	3.32 ± 0.11
Female (n = 15)				33.5 ± 1.47	108.6 ± 3.64	3.24 ± 0.08

All data were measured at least 3 times, and the average values are shown. For the levels of healthy controls, the average value ± standard deviation is shown.

Western blotting

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed using 8% gels, followed by western blotting.[30] Protein bands were probed using the indicated mAbs, followed by the addition of goat anti-mouse peroxidase-linked antibody.[30] Signals were detected using enhanced chemiluminescence, and densitometric scans were quantified using Image J 1.34.

Results

Patient's profile

A 13-year-old boy was admitted for massive DVT in association with swelling of the lower extremities. There was no personal or family history of thrombosis. Laboratory findings demonstrated prolonged PT and aPTT (18.9/67.6 seconds; control, 12.2/30.2 seconds). FV:C and FV:Ag were 10 and 40 IU/dL, respectively, indicating a cross-reactive material–reduced reaction. Anti-FV inhibitor was not detected. Other procoagulant and anticoagulant factors, including fibrinolytic factors and antiphospholipid syndrome-associated factors, were within normal range. Free tissue factor pathway inhibitor was 20.2 ng/mL (normal, 15-35 ng/mL).[31] His parents and 3 siblings had normal levels of FV:C and FV:Ag (Table 1). He was treated with warfarin to maintain prothrombin time-international normalized ratio 2.5 to 3.0. Nevertheless, a fresh thrombus developed in his left external iliac-vein, and the right inferior vena cava was completely occluded. After heparinization and urokinase therapy, the patient was treated with higher doses of warfarin to maintain prothrombin time-international normalized ratio 4.0 to 5.0, and he has since been free of recurrent DVT.

Gene analysis

Direct sequencing identified a W1920R homozygous mutation of exon 20 of *F5* in the patient (Figure 1). His parents heterozygously carried this mutation, but it was undetected in his siblings. Neither the FV_{Leiden} mutation (R506Q) nor FV-HR2 haplotype (H1299R and D2194G) were found in the patient or his family members. The W1920R mutation was not detected in 100 alleles from Japanese control subjects using direct sequencing, and the novel *F5* missense mutation that we identified was designated FV_{Nara}.

APCR in FV_{Nara} plasma

We investigated whether the FV_{Nara} was resistant to APC. aPTT-based APCR assays, reflecting APC inactivation of FVa and FVIIIa, were performed. The APCsr obtained in patient's plasma was similar to those of FV_{Leiden} patients and significantly lower than those in healthy control patients (Table 1). APCsrs in the parents were intermediate, and those in the siblings were equal to the levels of healthy controls. APCsrs of 3 mild FV-deficient patients with FV:C ~50 IU/dL were similar to those of control. APCsrs of inherited FV-deficient patients with FV:C ~10 IU/dL were not measurable, however, because the clotting times after the addition of APC were markedly prolonged (data not shown). These results demonstrated that the FV_{Nara} mutation conferred APCR.

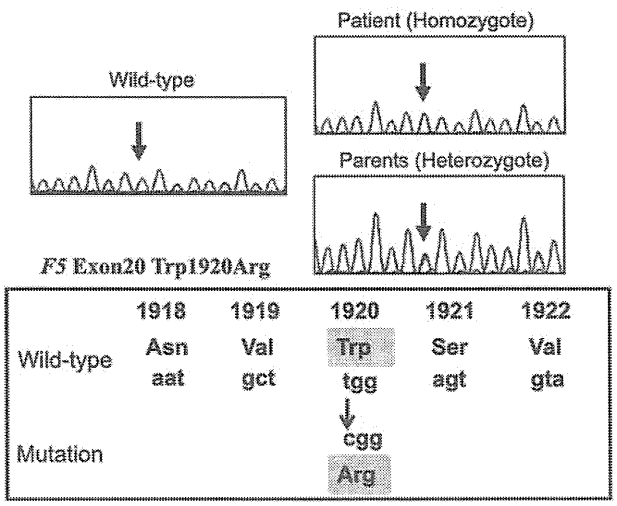

Figure 1. DNA direct sequencing of exon 20 of *F5* gene from the patient, his parents, normal WT. The mutation (T–C) is present at codon 1920, resulting in a Trp1920→Arg substitution in the FV protein (FV_{Nara}).

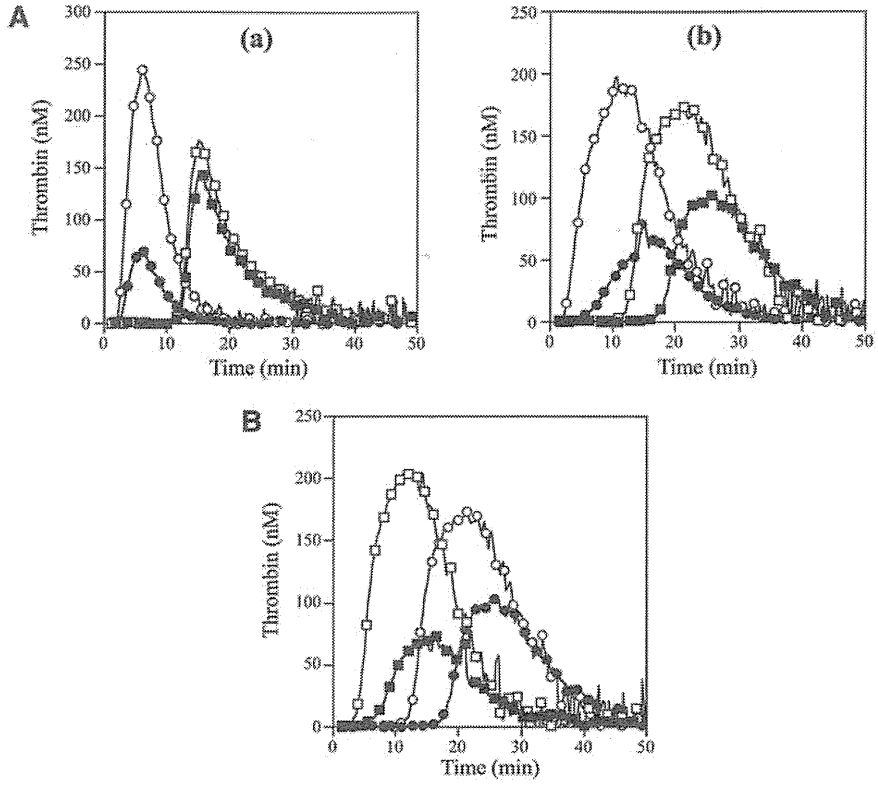

Figure 2. Thrombin generation in the FV_{Nara} patient's plasma. (A) Effects of the addition of APC: thrombin generation after extrinsic activation (TF; 5 pM) of PPP (a) or PRP (b) in normal individuals (circle symbols) and PPP (a) or PRP (b) in FV_{Nara} patient (square symbols) in the absence (open symbols) or presence (closed symbols) of APC was measured as described in "Materials and methods." With PPP, APC (8 nM) was added with PL vesicles (10 μM), whereas with PRP, APC (40 nM) was added without PL. A representative thrombogram is shown. (B) Effects of the addition of native FV: thrombin generation was measured after extrinsic activation (TF; 5 pM) of the patient's PRP with the addition of native FV (circles, 0 IU/dL; squares, 10 IU/dL) in the absence (open symbols) or presence (closed symbols) of APC (40 nM). All experiments were performed at least 3 separate times, and a representative thrombogram is shown.

APCR in the FV_{Nara} patient was examined using PPP in thrombin generation assay initiated by low concentrations of TF with APC. The time-related parameters (lag time and time to peak) and peak thrombin obtained with FV_{Nara} PPP were prolonged and decreased, respectively, compared with control (Figure 2Aa and Table 2). The addition of APC showed that the peak thrombin and ETP with FV_{Nara} were less moderated than those with control, although the time-related readings were unaffected. The APCsrs (minus APC/plus APC) with FV_{Nara} (1.22 and 1.25) were significantly lower than those with control (3.48 and 3.70), supportive of the APCR with FV_{Nara}. Platelet FV also participates in the clotting function of FV; to evaluate the role of platelet FV in these mechanisms, therefore, experiments were repeated using PRP. Similar to PPP, the addition of APC showed that APCsrs (plus APC/minus APC) in time-related parameters in FV_{Nara} were lower than those in control, and the APCsrs (minus APC/plus APC) with FV_{Nara} were also lower than those with control (Figure 2Ab). These findings provided further evidence of APCR with FV_{Nara}. Similar experiments in PPP using 40 nM APC (equal amount in PRP) showed that the thrombin generation of FV_{Nara}, as well as normal plasma, was little detected (data not shown), again confirming that the platelet FV was particularly resistant to APC-mediated inactivation.

To further assess the contribution of plasma FV in the APCR of FV_{Nara}, normal FV was added to the patient's PRP before measuring thrombin generation with APC (Figure 2B). In the presence of normal FV (10 IU/dL) corresponding to the level in patient's plasma, the time-related parameters were moderately shortened, and the peak thrombin and ETP were slightly increased. Thus, the presence of native FV improved reactivity to APC in FV_{Nara} PRP, suggesting that the APCR of FV_{Nara} might be caused by defective patient's plasma FV. Furthermore, even small amounts of plasma FV appeared to influence APC-induced deceleration of blood coagulation.

APC cofactor activity of FV_{Nara}

APCR resulting from a *F5* mutation or mutations is caused by a reduced sensitivity of FVa to APC-catalyzed inactivation[7,32] and/or reduced FV cofactor activity in APC-catalyzed FVIIIa inactivation,[33] but these components are difficult to distinguish. FXa generation

Table 2. Parameters obtained from thrombin generation on the PPP and PRP with FV_{Nara}

	Lag time (APC minus/plus, minutes [APCsr])*	Time to peak (APC minus/plus, minutes [APCsr])*	Peak thrombin (APC minus/plus, nM [APCsr])†	ETP (APC minus/plus, nM × minutes [APCsr])†
PPP				
Control	2.50/2.62 (1.05)	6.12/5.62 (0.92)	242/70 (3.48)	1648/446 (3.70)
FV_{Nara}	12.3/12.5 (1.02)	15.3/15.3 (1.00)	176/144 (1.22)	1711/1373 (1.25)
PRP				
Control	3.01/6.55 (2.18)	11.5/15.7 (1.36)	191/71 (2.69)	2860/968 (2.95)
FV_{Nara}	12.8/18.2 (1.42)	21.2/26.2 (1.23)	169/103 (1.64)	2811/1620 (1.74)
+ FV (10 IU/dL)	2.86/6.45 (2.25)	11.5/16.2 (1.42)	201/70 (2.87)	2988/930 (3.21)

Values were calculated from the parameter data obtained in Figure 2.
*Values were expressed as plus APC divided by minus APC.
†Values were expressed as minus APC divided by plus APC.

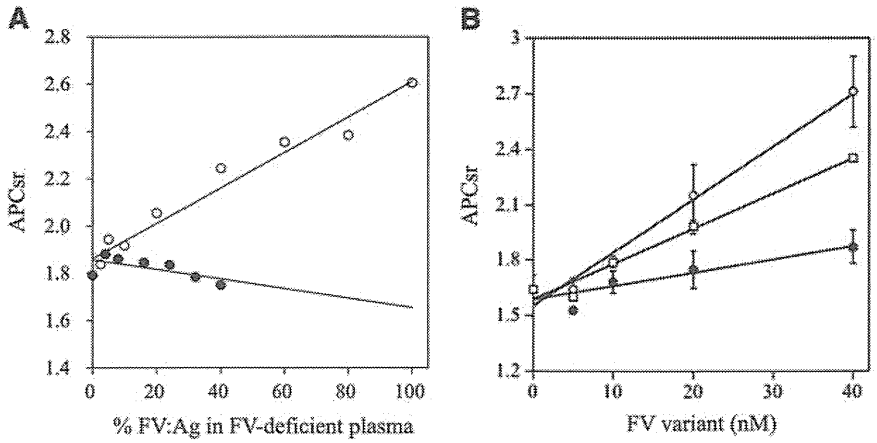

Figure 3. APCR in FXa generation assays. (A) Effects of FV levels on the APCsr in the FV_{Nara} plasma. Normal plasma (open circles) and the patient's plasma (closed circles) were mixed with FV-deficient plasma in various proportions, and FXa generation was measured with COATEST SP FVIII after the simultaneous addition of APC (40 nM) as described in "Materials and methods." The APCsrs were expressed as ratios of the amount of generated FXa in the absence of APC divided by that in its presence. All experiments were performed at least 3 separate times, and the average values are shown. (B) APCR of FV-deficient plasma mixed with recombinant FV-W1920R. Various concentrations of FV (WT, open circles; W1920R, closed circles; R506Q, open squares) were mixed with diluted FV-deficient plasma, and FXa generation was measured with COATEST SP FVIII after the simultaneous addition of APC (40 nM), as described in "Materials and methods." The APCsrs were expressed as ratios of the amount of generated FXa in the absence of APC divided by that in its presence. All experiments were performed at least 3 separate times, and the average and/or standard deviation values are shown.

assays, reflecting intrinsic tenase activity, were used to examine the APC cofactor activity of FV.[27] We therefore determined APCsrs in samples after the addition of APC to probe APCR resulting from defective FV cofactor activity. Normal or FV_{Nara} plasma was mixed with FV-deficient plasma in proportions from 2.5% to 100%. Because FV_{Nara}:Ag was 40 IU/dL, the concentration of FV_{Nara} in these assays varied between 2.5% and 40%. APCsrs increased linearly in proportion to the level of normal FV, and clear differences were demonstrated between 0% and 100% normal plasma (1.8 and 2.6, respectively), similar to an earlier report.[27] APCsrs were independent of the concentration of FV_{Nara}, however, and remained relatively constant or modestly decreased (Figure 3A). The slopes obtained with normal and FV_{Nara} plasmas (ΔAPCsr; 0.72 and −0.18/IU FV, respectively) were significantly different ($P < .01$), indicating that FV_{Nara} possessed little APC cofactor activity.

APCR of FV-W1920R mutant

The measurements of APCR of FV_{Nara} could have been affected by quantitative or qualitative abnormalities of individual plasma components other than FV. To confirm FV specificity, recombinant FV-WT and 2 FV mutant proteins (W1920R and R506Q) were prepared. The levels of FV:Ag and specific activity of the W1920R mutant expressed in CM were similar to the data observed with patient's plasma, at 50% and 45% of WT, respectively. Corresponding levels in CM of R506Q were 125% and 86% of WT, respectively.

APCR assays were repeated using mixtures of FV-deficient plasmas and recombinant FV variants. FXa generation assays were devised in which FV-deficient plasma and FV were diluted 16-fold before mixing with APC; this facilitated measurements at lower levels of FV:Ag (0.3-2.5 nM). These concentrations were comparable to diluted plasma containing physiological levels of FV:Ag (5-40 nM;

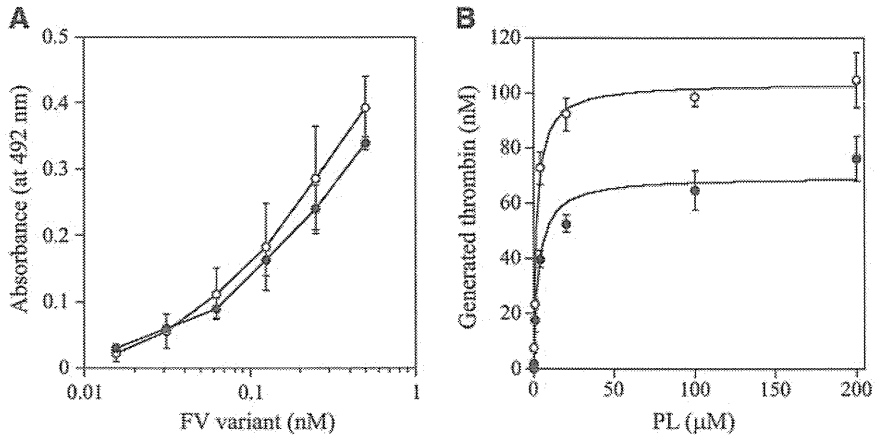

Figure 4. FV-W1920R affecting the association with PL. (A) FV-PL binding. α-Phosphatidyl-L-serine (5 μg/mL) was added to microtiter wells and air-dried. After blocking, serial dilutions of FV were added to the immobilized PL. Bound FV-WT (open circles) and FV-W1920R (closed circles) was quantified by the addition of AHV-5146, as described in "Materials and methods." The average values and standard deviations are shown. (B) The effects of PL on prothrombinase activity; FV (WT, open circles; W1920R, closed circles; 2 nM) was activated by thrombin (20 nM) for 1 minute, followed by the addition of hirudin. The reactants were incubated with prothrombin (1.4 μM) and various amounts of PL, followed by initiation by the addition of FXa (10 pM). Aliquots were removed, and the reactions were quenched by the addition of EDTA. Rates of thrombin generation were determined at Abs_{405} after the addition of S-2238. Thrombin generation was quantified by extrapolation from a standard curve prepared using known amounts of thrombin. The plotted data were fitted using the Michaelis-Menten equation. All experiments were performed at least 3 separate times, and the average values are shown.

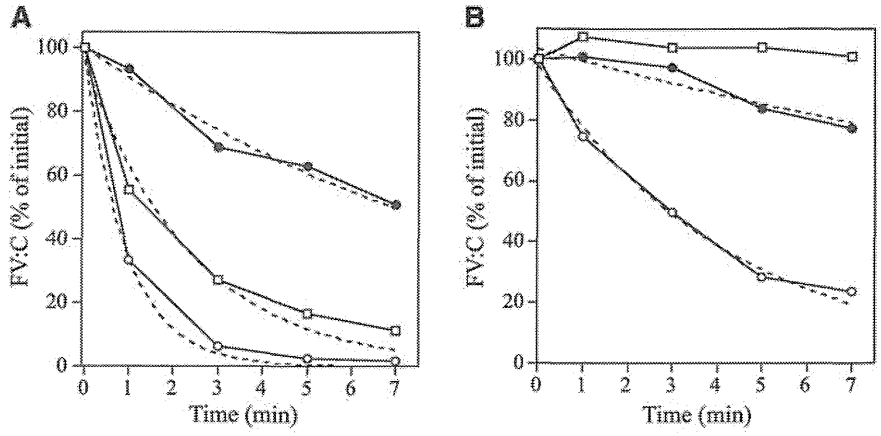

Figure 5. APC-mediated inactivation of FVa-W1920R mutant. FV variants (8 nM) were incubated with thrombin (100 nM) for 5 minutes, and the reaction was terminated by the addition of hirudin (25 U/mL). FVa variant samples (2 nM) were reacted with APC (25 pM) and PL (20 μM) in the presence (A) or absence (B) of PS (30 nM). After dilution, FVa activity was measured in an aPTT-based 1-stage clotting assay. The symbols used are as follows: open circles, WT; closed circles, W1920R; open squares, R506Q. Initial activities of FVa variants were regarded as 100%. The plotted data were fitted in an equation of single exponential decay. All experiments were performed at least 3 separate times, and the average values are shown.

15%-120% of normal FV:Ag). In mixtures with WT, APCsrs increased dose-dependently (ΔAPCsr; 2.85×10^{-2}/nM FV; Figure 3B). The APCsr in W1920R was lower by ~4-fold (0.71×10^{-2}/nM FV) than WT, in keeping with the impairment of APC cofactor activity in W1920R. With R506Q, the APCsr was greater by ~2.7-fold (1.91×10^{-2}/nM FV) than that with W1920R. These findings demonstrated that APCR in FV_{Nara} plasma resulted from W1920R, and the APC cofactor activity of W1920R appeared to be more defective than that of R506Q.

The C1 and/or C2 domains of FV(a) bind to PL membranes,[34,35] which governs the susceptibility of FVa for APC-catalyzed inactivation and the APC cofactor activity of FV. W1920 is in proximity to the PL-binding region or regions,[36] and we speculated that the APCR of FV-W1920R might be a result of significant disturbances in PL binding. The PL binding of W1920R was maintained at ~90% that of WT in ELISA, however (Figure 4A). The influence of PL on prothrombinase activity with W1920R was also investigated. Thrombin generation with W1920R was ~70% the level of that with WT, again supporting the cross-reactive material–reduced type on FV_{Nara}. However, the affinity of PL for W1920R was not significantly different compared with WT (K_m: 3.25 ± 0.77/2.22 ± 0.24 μM, respectively; Figure 4B). Thrombin and PL-dependent FXa activation of W1920R showed a similar reaction to the activation of WT (data not shown). These findings indicated that W1920R–PL interactions were not disturbed.

APC-catalyzed inactivation of FVa-W1920R

APC-catalyzed inactivation of FVa-W1920R, compared with the inactivation of WT and R506Q (FV_{Leiden}), were examined in 1-stage clotting assays. FVa-WT:C was very rapidly decreased after the addition of APC and PS and declined to ~2% of the initial level at 5 minutes (Figure 5A). FVa-R506Q:C was reduced to ~20% of the initial level at 5 minutes. Surprisingly, FVa-W1920R:C decreased very slowly and persisted at ~60% of the initial level. The inactivation rate of W1920R was ~11- and ~4.5-fold lower than the rates of WT and R506Q, respectively (Table 3), indicating significantly defective APC-induced inactivation of W1920R. Without PS, the inactivation rate of FVa-WT:C was significantly decreased, with ~20% of the rate obtained with PS (Figure 5B), whereas inactivation of FVa-R506Q:C was not observed. The inactivation rate of FVa-W1920R:C appeared to be ~40% less than that with PS, but this was ~6-fold lower than that of WT, supporting the theory that W1920R as a cofactor for PS contributed less to the mechanisms of APCR than WT and R506Q.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of APC cleavage was designed to investigate the mechanism or mechanisms contributing to the defective APC-catalyzed inactivation of FVa-W1920R (Figure 6). Using FVa-WT, the band of residues 1 to 506 rapidly appeared within 20 seconds after the addition of APC, followed sequentially by the appearances of the 307 to 506 and 307 to 709 bands, consistent with rapid, consecutive cleavage at Arg506 and Arg306. Cleavage of FVa-R506Q at Gln506 was not evident during a 5-minute reaction. The appearance of the 307 to 709 band was evident at similar velocity to that in WT, but the total ratio of Arg306 cleavage in R506Q was reduced. The appearance of the 1 to 506 band in W1920R was markedly delayed compared with WT, however, and cleavage at Arg306 was not detected. These results suggested that the loss of APC-catalyzed inactivation of FVa-W1920R was a result of a significant delay in cleavage at Arg506 and little cleavage at Arg306.

Cofactor FV-W1920R on APC-catalyzed FVIIIa inactivation

Properties of FV-W1920R as a cofactor for APC were examined in a FVIIIa degradation assay.[29] FV-WT significantly enhanced APC-catalyzed FVIIIa inactivation with a ~3-fold inactivation rate compared with that in its absence, again confirming the APC cofactor activity of FV (Figure 7Aa and Table 4). FV-R506Q moderately diminished APC-catalyzed inactivation with the ~50% inactivation rate of WT. However, the rate with W1920R was similar to that in its absence, emphasizing earlier findings that APC cofactor activity was markedly depleted with W1920R. With regard to the effects of varying amounts of FV on APC cofactor activity, FV-WT enhanced the APC-catalyzed inactivation dose-dependently (50% reduction, 0.25 nM) (Figure 7Ab). Inactivation with R506Q was also enhanced, but the 50% reduction of 0.55 nM was greater than that with WT. However, even at 1 nM of W1920R, little enhancement of this mechanism was demonstrated, indicating that impairment of APC cofactor activity with W1920R was more pronounced than with R506Q.

Table 3. Kinetic parameters determined on APC-catalyzed inactivation of recombinant FVa variants

	PS, $minutes^{-1}$ (-fold)	
FVa variants	Plus	Minus
WT	1.07 ± 0.06 (1)	0.230 ± 0.016 (1)
W1920R	0.100 ± 0.001 (0.09)	0.038 ± 0.008 (0.16)
R506Q	0.417 ± 0.061 (0.39)	Not determined

Values were calculated by nonlinear least squares regression from the data shown in Figure 5A-B, using the single exponential decay.

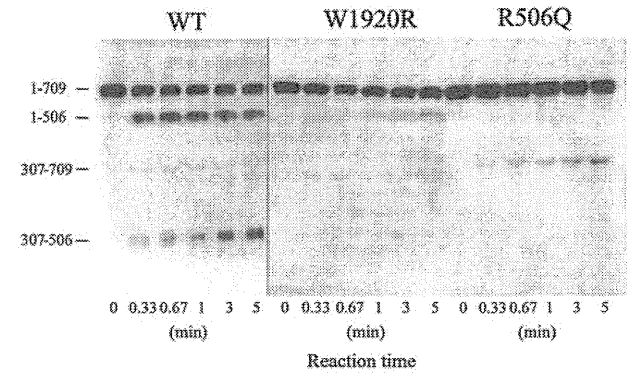

Figure 6. APC-catalyzed proteolytic cleavage of the HCh of FVa-W1920R. FV variants (8 nM) were incubated with thrombin (100 nM) for 5 minutes, and the reaction was terminated by the addition of hirudin (25 U/mL). FVa variant samples (0.5 nM) were incubated with APC (1 nM) and PS (30 nM) in the presence of PL (20 μM) for the indicated times. Samples were analyzed on 8% gels, followed by western blotting using an anti-FV HCh mAb 5146 IgG, as described in "Materials and methods." A vertical line is inserted to indicate a repositioned gel lane.

APC-catalyzed FVIIIa inactivation is regulated by cleavage at Arg336 in A1.[37] Figure 7B illustrates time-dependent cleavage at Arg336 analyzed by western blotting using mAbC5. This mAb recognizes the residues 337 to 372, and the disappearance of this band represents cleavage at Arg336.[38] With FV-WT, intact A1 gradually decreased time-dependently (Figure 7Ba). With FV-W1920R, however, cleavage at Arg336 was not observed during a 10-minute reaction time, and the rate of cleavage with FV-R506Q appeared to be ~40% of that of FV-WT (Figure 7Bb and Table 4), consistent with the results obtained in FXa generation assays. These data confirmed that APC cofactor activity was partially or completely impaired with R506Q and W1920R, respectively.

Discussion

We described a novel FV-W1920R missense mutation associated with APCR in a Japanese boy with serious DVT. The APCR of FV_{Nara} was greater than that of FV_{Leiden} and involved a novel mechanism related to a significant inhibition of cleavage at both sites because of the possible failure of direct and/or indirect interactions with APC and/or PS.

FV-W1920 is a highly conserved residue among other mammalians. According to the crystal structure of APC-inactivated bovine FVa,[36] bovine-W1907 (corresponding to human-W1920) is located on the internal structure in the C1 domain, which is involved in 3 spike-like structures of β-strand as PL-binding sites. W1907 forms hydrogen bonding with W1891 (W1904) and L2013 (L2026), which is located at the first and second spike of PL-binding, respectively. W1920R may have an effect on the PL binding at the C1 interface and may be an unstable molecule by modification (eg, misfolding). However, because a functional assay such as prothrombinase activity, FXa activation, and a PL-binding assay did not indicate significant differences between WT and W1920R, this molecule would be not significantly disturbed structurally; thereby, the reason for secretion defect is unclear.

Our assays for APC-catalyzed FV cleavage demonstrated little cleavage of W1920R at Arg306, and cleavage at Arg506 was markedly delayed. As discussed earlier, the Arg506 cleavage is considered to be essential for direct and FVIII-related anticoagulant properties of FV,[15,16,29] whereas the Arg306 cleavage is associated with FVa inactivation. Mutations at Arg306, including $FV_{Cambridge}$, contribute to mild APCR, and a similar mutation $FV_{Hong\ Kong}$, appears not to affect the APC response.[19-21] We speculate, therefore, that the impairment of APC-catalyzed cleavage at Arg506 in FV_{Nara} resulted in persistent levels of FVIIIa:C and FVa:C and was the

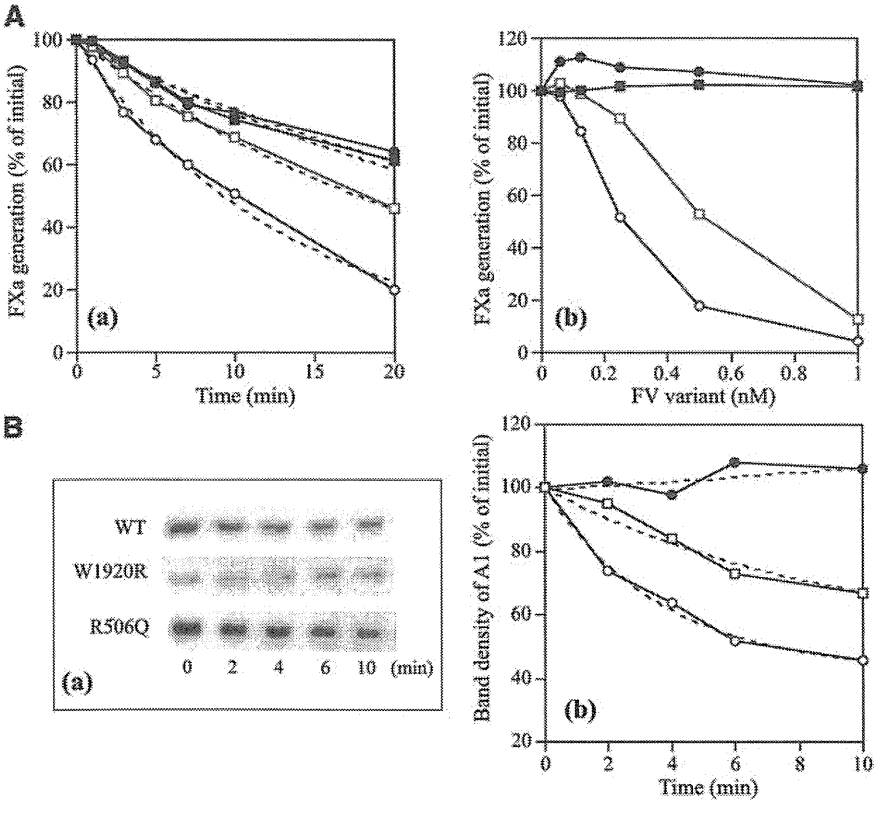

Figure 7. APC cofactor activity of FV-W1920R assessed by the degradation of FVIIIa. (A) FVIIIa inactivation. FVIII (10 nM) with PL (20 μM) was activated by thrombin (5 nM), followed by the addition of hirudin (2.5 U/mL). Generated FVIIIa was incubated either with mixtures of APC (0.5 nM), PS (5 nM), and FV variants (0.5 nM) for the indicated times (a) or with various concentrations of FV variants for 20 minutes (b). FXa generation was initiated by the addition of FIXa (2 nM) and FX (200 nM) for 1 minute. The symbols used are as follows: open circles, WT; closed circles, W1920R; open squares, R506Q; closed squares, no FV. Values of FXa generation at the initial time (a) or in the absence of FV variants (b) were regarded as 100%. The data in (a) were fitted on an equation of single exponential decay (dashed lines). All experiments were performed at least 3 separate times, and the average values are shown. (B) A1 cleavage at Arg336; FVIII (10 nM) with PL (20 μM) was activated by thrombin (5 nM) for 30 second, followed by the addition of hirudin (2.5 U/mL). Generated FVIIIa was incubated with mixtures of APC (0.5 nM), PS (5 nM), and FV variants (0.5 nM) for the indicated times. Samples were analyzed on 8% gels, followed by western blotting using an anti-A1 C5 IgG, as described in "Materials and methods" (a). Band densities of intact $A1^{1-372}$ observed from panel a were measured by quantitative densitometry. Density before the addition of APC was regarded as 100% (b). The plotted data were fitted in an equation of single exponential decay (dashed lines). The symbols used are as follows: open circles, WT; closed circles, W1920R; open squares, R506Q. All experiments were performed at least 3 separate times, and the average values are shown.

Table 4. Kinetic parameters determined on FVIIIa inactivation by APC and PS in the presence of recombinant FV variants

FV variants	Rate constant: *k*, $minutes^{-1}$ (-fold)	
	FVIIIa inactivation	Cleavage at Arg336
WT	0.742 ± 0.034 (1)	0.274 ± 0.034 (1)
W1920R	0.239 ± 0.021 (0.32)	Not determined
R506Q	0.390 ± 0.012 (0.53)	0.110 ± 0.076 (0.40)
None	0.246 ± 0.019 (0.33)	Not determined

Values were calculated by nonlinear least squares regression from the data shown in Figure 7A-B, using the single exponential decay.

most influential defect in the APCR mechanism. The complete loss of cleavage at Arg306 in FV_{Nara} also contributed to the stability of FVa:C. These findings provide potential new insights into the physiological role or roles of FV in (anti)coagulation systems.

The recurrence of DVT in FV_{Nara} was evident despite low levels of plasma FV:C. This observation appears to be unique in FV mutations reported with APCR. The precise reason or reasons for thrombotic symptoms in these circumstances remain to be clarified but could be explained by some laboratory features. First, moderately low levels of plasma FV:C (>5%) promote thrombin generation near to normal plasma and could facilitate significant effects on APCR. The thrombin-related procoagulant capacity of lower levels of FV:C (<2~3%) is very restricted, however, and the anticoagulant function of FV might be negligible. Second, platelet FV:C might be more important than plasma FV:C in physiological procoagulant activity.[39] Platelet FV:C in FV_{Nara} was ~20% (data not shown), and the peak level of thrombin formation in PRP was comparable to that of normal PRP (~90% of normal). The peak thrombin after the addition of APC was increased (145% of normal) (Figure 2Ab). Third, other hemostasis-related mechanisms may have a potential effect on the thrombotic diathesis. Other laboratory findings were within normal range in our patient, however, and we demonstrated directly that FV-W1920R conferred APCR.

The APCR mechanisms in FV_{Nara} appeared to be distinct from other APCR-associated FV mutations. With the exception of $FV_{Liverpool}$ (I359T),[40] all FV mutations have been identified in the HCh and constitute point mutations at cleavage sites that lead to the impairment of FV(a) inactivation. Delayed cleavage at Arg306 was described in $FV_{Liverpool}$, resulting in impaired inactivation of FVa and in clinically severe DVT. This mutation led to the creation of a glycosylation consensus sequence at Asn357 that is slightly remote from the Arg306 site. Cleavage at Arg506 in FVa and APC cofactor activity for FVIIIa inactivation was normal. However, W1920R is significantly remote from the APC-cleavage sites and from moderated cleavage at both Arg306 and Arg506. Although W1920 in FV is close to the PL-binding site,[36] the binding ability of W1920R to PL was almost equivalent to that of FV-WT, indicating that W1920R in FV_{Nara} might affect the direct association (binding-site) or indirect association (conformational change) with APC. In addition, W1920R was little cleaved by APC at Arg306, depending on PS, which might supporting a defective interaction with PS. Our conclusion that FV_{Nara} was resistant to APC inactivation through an impaired interaction with this protease might be further supported by observations that FVa-W1920R, when assembled into prothrombinase, was protected from FXa-catalyzed APC inactivation and that the inhibitory effect of FXa on APC-catalyzed inactivation of FVa-W1920R was observed, similar to WT (data not shown).

FV_{Leiden} is known to be a major cause of hereditary thrombotic diseases among Caucasians.[2,41] Studies of the ethnic distribution of FV_{Leiden} indicated that the mutation was not found in Asians.[17,18] A different FV mutation (E666D) causing APCR coupled with DVT has been reported in China,[42] but there are no reports of FV-associated APCR in Japan. The parents of the current propositus were heterozygous for the W1920R mutation, but they have not developed any thrombotic symptoms to date, although aPTT-based assays demonstrated mild APCR. It is possible that similar to FV_{Leiden}, the heterozygote FV_{Nara} may have a potential risk for thrombosis compounded by other prothrombotic factors.

Acknowledgments

We thank Dr Koji Yada, Dr Hiroaki Minami, and Dr Shoko Furukawa for the clinical support.

This work was partly supported by grants from Ministry of Education, Culture, Sports, Science and Technology (MEXT) Grants-in-Aid for Scientific Research (KAKENHI) (21591370 and 24591558 to K.N.) and an unrestricted research grant from Baxter (to K.F.).

Authorship

Contribution: K.N. and K.A. designed of research; K.N. and K.S. wrote the manuscript; K.N., K.S., K.O., and T.M. performed experiments; K.N., K.S., and K.O. analyzed the data; and K.F. and M.S. supervised the study, interpreted data, and edited the manuscript.

Conflict-of-interest disclosure: K.S. is an endowed assistant professor funded by Baxter; has given lectures at educational symposiums organized by Baxter, Bayer, and Novo Nordisk; has received payment for lectures from Baxter, Bayer, and Novo Nordisk; and has received the Award from Baxter Coagulation Research Fund in Japan. K.F. and K.A. are professors of additional post in Molecular Genetics of Coagulation Disorder without additional salary. K.A. is a board member of the Factor Eight Inhibitor Bypass Activity Post Marketing Surveillance Study Board in Japan organized by Baxter; has received payment for lectures from Baxter, Bayer, Biogen Idec, Kaketsuken, Novo Nordisk, and Pfizer; has received payment for consultancy meetings with Baxter, Bayer, CSL Behring, Kaketsuken, Novo Nordisk, and Pfizer; and has received unrestricted grants supporting research from Pfizer. K.F. is an investigator of Hemophilia Research Study Update organized by Baxter, a board member of the Advate Safety Board in Japan organized by Baxter, and a board member of the Benefix Post Marketing Surveillance Study Board in Japan, organized by Pfizer; has received payment for consultancy meetings with Baxter, Pfizer, Biogen Idec, Bayer, CSL Behring, Kaketsuken, SRL, Mitsubishi Chemical Medience, and Novo Nordisk; has received unrestricted grants supporting research from Baxter, Pfizer, Bayer, Kaketsuken, Japan Blood Products Organization, and CSL Behring; and has received payment for lectures from Baxter, Bayer, Pfizer, Novo Nordisk, CSL Behring, Roche Diagnostics, Fujirebio Inc, and Sekisui Medical. M.S. is a board member of the Feiba and an Advate Safety Board in Japan, organized by Baxter, and a board member of the Benefix Post Marketing Surveillance Study Board in Japan, organized by Pfizer; has received payment for consultancy meetings with Baxter, Pfizer, Biogen Idec, Bayer, CSL Behring, Kaketsuken, Chugai Therapeutic Company, and Novo Nordisk; has received unrestricted grants supporting research from Baxter, Pfizer, Bayer, Kaketsuken, Novo Nordisk, Chugai

Pharmaceutical Company, and CSL Behring; and has received payment for lectures from Baxter, Bayer, Novo Nordisk, and Pfizer. The remaining authors declare no competing financial interests.

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