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血小板血栓形成を制御する遺伝子の同定と
その成果を用いた予防と治療の個別化

分担研究報告書

血小板血栓形成を制御する遺伝子の同定とその成果を用いた予防と治療の個別化に関する研究

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研究要旨

本年度では、血栓形成のセンサー分子として同定したADP受容体$P2Y_{12}$、抑制因子として同定したSemaphorin 3Aに関して、これら分子の作用機構を詳細に解析した。その結果$P2Y_{12}$を介したADPの持続的な刺激が、インテグリン$\alpha_{IIb}\beta_3$の活性化を維持する上で必須であることを明らかにした。またSemaphorin 3AはRacを抑制すると共にPI3K系を抑制することによりインテグリン$\alpha_{IIb}\beta_3$の活性化を抑制することが示唆された。

A．研究目的

現代社会において、メタボリック症候群の増加および人口の高齢化により動脈硬化を基盤とした病的動脈血栓症による死因が本邦および世界における死因の約３割を占めるにいたっており、病的血栓制御法の開発は極めて急務の課題である。

血栓は、血栓形成に関してADPなどの促進因子およびNOなどの抑制因子のバランスにより制御されており、病的状態ではそのバランスが破綻していると考えられる。本研究では、病的動脈血栓形成を制御する遺伝子を同定するとともに、血栓形成に関し促進因子および抑制因子を解析し、予防および治療の新たな戦略を構築することを目的とした。昨年度の実績として、血栓形成のセンサー分子としてADP受容体$P2Y_{12}$、抑制因子としてSHPS-1、Semaphorin 3Aおよびアディポネクチンを同定した。本年度は、上記の成果の中で$P2Y_{12}$およびSemaphorin 3Aの機能に関してさらに詳細な分子機序を解析した。

B．研究方法

$P2Y_{12}$ 欠損血小板は PAR1 での刺激時、一過性の血小板凝集を示すが血小板凝集は直ぐに解離し、活性化 $a_{IIb}b_3$ を認識する PAC-1 抗体の結合は PAR1 刺激において検出されなかった。この成績は、$P2Y_{12}$ が欠損すると PAR1 刺激において $a_{IIb}b_3$ は一過性に活性化するものの、$a_{IIb}b_3$ の活性化を維持できないことを示唆している。この現象より、本研究では $a_{IIb}b_3$ の活性化を維持するには血小板から放出された ADP とその受容体である $P2Y_{12}$ からのシグナルが必須であるとの仮説をたて、この仮説を検証するために、初めに正常血小板をトロンビンにて刺激し放出反応および $a_{IIb}b_3$ 活性化を完了させた後に、$P2Y_{12}$ 阻害剤 AR-C69931MX 1mM などの種々阻害剤を加え PAC1 抗体結合フローサイトメーターにて測定した。また、トロンビンの濃度を一定に保ち、血小板数を減少させ $a_{IIb}b_3$ 活性化を観察した。

Semaphorin 3A に関しては、IgG Fc 部分との融合蛋白（Sema/Fc）を作製し、血小板 $a_{IIb}b_3$ 活性化に対する影響および種々のシグナルへの影響を検討した。PI3 kinase 活性化は AKT のリン酸化にて、Rap1 の活性化は pull-down 法にて検討した。

C．研究成果

血小板をトロンビンで刺激すると、血小板の放出反応が完了しPセレクチンが発現すると共に $a_{IIb}b_3$ が活性化する。この条件では、$a_{IIb}b_3$ の活性化は数時間持続する。しかしながら、興味深いことに血小板が活性化した後に $P2Y_{12}$ 阻害剤 AR-C69931MX 1mM を加えると、P-selectin の発現は変化しないが、$a_{IIb}b_3$ が不活性化されることが今回の検討で明らかになった。この $a_{IIb}b_3$ の不活性化は、$P2Y_{12}$ 阻害剤特異的であり、セロトニン受容体やトロンボキサン A2 受容体、a2-アドレナリン受容体の阻害剤では認められなかった。さらに血小板数を 50,000/ml から 500/ml まで希釈すると、トロンビンで刺激しても $a_{IIb}b_3$ 活性化は観察されないこと、ここに少量の ADP を加えると $a_{IIb}b_3$ が再活性化することが明らかになった。また、$a_{IIb}b_3$ の活性化、不活性化と Rap1 の活性化、不活性化と連関していることが示された。

トロンビン刺激血小板に対する Sema3A/Fc の作用は以下のとおりであった。1)Sema/Fc はトロンビン刺激による血小板内 Ca^{2+} 濃度変化や PKC 活性化には影響しない。2)Sema/Fc はトロンビン刺激による Akt のリン酸化および Rap 活性化を抑制した。一方、convulxin 刺激に対しては、Sema3A/Fc は GPVI 下流のシグナル伝達分子である LAT・SLP-76・PLCg のリン酸化には影響を与えなった。Sema/Fc はトロンビン刺激時と同様に convulxin による Akt リン酸化および Rap 活性化を抑制した。一方、Sema3A/Fc はトロンビン刺激時とは異

なり convulxin による血小板内 Ca^{2+}の増加を抑制した。

以上の成績を総合すると、Semaphorin 3A はアゴニストの種類に関係なく Akt や Rap1 の活性化を抑制することから、その上流の PI3K 経路を抑制していることが示唆された。

D．考案

本年度の検討にて、$a_{IIb}b_3$ の活性化が可逆的であること、ADP-$P2Y_{12}$ のシグナルにより $a_{IIb}b_3$ の活性化が制御されていることが明らかとなった。血小板は活性化時 ADP を放出するが、血小板 1 個あたりの放出 ADP 濃度が低いために、autocrine 作用によっては $a_{IIb}b_3$ 活性化を維持することはできず、$a_{IIb}b_3$ の活性化には paracrine 的にあるまとまった血小板（血栓）からの ADP が必要であることも明らかとなった。また、ADP-$P2Y_{12}$ 系は $a_{IIb}b_3$ の活性化を維持するのに必須の分子であることも明らかになった。このような分子機構を介して、ADP-$P2Y_{12}$ 系は血栓の大きさを規定するセンサー分子として作用すると考えられる。一方、Semaphorin 3A は血小板機能を抑制するが、その抑制は Rac1 の抑制による細胞骨格系の変化の抑制のみならず、PI3K 系を抑制し血小板凝集や $a_{IIb}b_3$ の活性化を抑制することが、本年度の研究で明らかになった。今後は in vivo での抗血栓作用に関してさらに検討する予定である。

E．結論

$P2Y_{12}$ は $a_{IIb}b_3$ の活性化維持に必須の分子であり、この作用を介して血栓形成のセンサー分子として機能していること、一方 semaphorin 3A は PI3K を抑制することにより $a_{IIb}b_3$ の活性化を抑制することが明らかとなった。

F．健康危険情報

なし

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H. 知的財産権の出願・登録状況

なし

別添５

Ⅲ．研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の
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Journal of Thrombosis and Haemostasis, **4**: 2010–2013

ORIGINAL ARTICLE

Plasma protein S activity correlates with protein S genotype but is not sensitive to identify K196E mutant carriers

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See also Okada H, Yamazaki T, Takagi A, Murate T, Yamamoto K, Takamatsu J, Matsushita T, Naoe T, Kunishima S, Hamaguchi M, Saito H, Kojima T. *In vitro* characterization of missense mutations associated with quantitative protein S deficiency. This issue, pp 2003–9.

Summary. *Background:* Protein S (PS) is an anticoagulant protein that functions as a cofactor for activated protein C (APC), and congenital PS deficiency is a well-known risk factor for the development of deep vein thrombosis (DVT). Recently, we and others identified the K196E missense mutation in the second epidermal growth factor-like domain of PS as a genetic risk factor for DVT in the Japanese population. The incidence of this mutation is high in the Japanese population. *Objectives:* In the present study, we investigated the relationship between plasma PS activity and the presence of the K196E mutation. *Patients and methods:* We measured PS activity as a cofactor activity for APC in 1862 Japanese individuals and determined the PS K196E genotype in this population. *Results:* Individuals heterozygous for the mutant E-allele had lower plasma PS activity than wildtype subjects (mean ± SD, 71.9 ± 17.6%, $n = 34$ vs. 87.9 ± 19.8%, $n = 1828$, $P < 0.0001$). However, the PS activity of several heterozygous individuals ($n = 8$) was greater than the population average. In contrast, multiple wildtype subjects ($n = 26$) had PS activity less than 2 SD below the population mean, indicating that other genetic or environmental factors affect PS activity. *Conclusions:* Plasma PS activity itself is not suitable for identifying PS 196E carriers and other methods are required for carrier detection.

Keywords: deep vein thrombosis, missense mutation, protein S.

Introduction

Protein S (PS) is an important regulator of coagulation that serves as a cofactor for activated protein C (APC), the anticoagulant protease that proteolytically degrades activated factor (F) V and FVIII [1]. Individuals with homozygous or compound heterozygous deficiency for PS develop disseminated thrombosis after birth, and heterozygosity for PS deficiency increases the risk of deep vein thrombosis (DVT) [2,3].

Recently, we and others identified that a PS missense mutation prevalent in the Japanese population, which causes Lys196 to be replaced by Glu (K196E mutation, formerly known as PS Tokushima, and referred to as K155E mutation), is a genetic risk factor for the development of DVT [4,5]. This mutation lies within the second epidermal growth factor-like domain of PS, and, *in vitro*, K196E mutant PS has decreased APC cofactor activity and poorly accelerates prothrombinase inactivation [6–8]. This missense mutation was originally identified in Japanese patients with PS deficiency suffering from DVT [9,10]. However, the plasma PS activity in individuals with this mutation remained controversial. In one report, PS activity was decreased in carriers of the K196E mutation with normal PS levels [9]. In contrast, another study found PS activity within the normal range in affected individuals [10].

We identified 66 heterozygotes and no homozygotes for the mutant PS 196E-allele from a population of 3651 individuals [5]. Therefore, the frequency of the mutant E-allele in the Japanese population was about 0.009. Extrapolating from these values, we estimated that approximately one out of every 55 Japanese individuals is heterozygous for the E-allele [11]. Thus, a substantial number of Japanese carry the E-allele for PS and are at increased risk for the development of DVT. Given the relatively high frequency of this mutation and its strong correlation with DVT, it may be advisable to screen individuals for the presence of this mutation so that carriers can avoid additional environmental risk factors associated with DVT. An appropriate screening test is lacking, however, and we hypothesized that plasma PS activity levels may directly correlate with PS genotype. If this were the case, genetic testing

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would not need to be undertaken to determine the PS genotype of a large population.

In this study, we examined the relationship between PS activity and the presence of the K196E mutation. The mean PS activity of individuals heterozygous for the K196E mutation was significantly less than that of wildtype individuals. However, there was substantial overlap in PS activity between these populations, and, thus, PS activity is not an appropriate method to differentiate K196E carriers from the general population.

Methods

We previously measured the PS activity in a population of Japanese individuals as part of the Suita Study, and we determined their genotype with respect to the PS K196E mutation [5,12]. The ability of PS to act as a cofactor for PC activation was measured on the basis of the activated partial thromboplastin time assay using Staclot PS (Diagnostica Stago, Asnières, France) [12]. The plasma levels of PS activity were expressed as percentages of the levels obtained from commercially available standard human plasma (Behringwerke, Marburg, Germany). The intra-assay coefficient of variation for PS activity was 6.9% (n = 10). The PS K196E genotype was determined by the TaqMan genotype discrimination method [5], using the primers 5′-ACCACTGTTCCTGTAAAAATGGTTT/5′-TGTGTTTTAATTCTACCATCCTGCT and the probes 5′-VIC-CAAATGAGAAAGATTGTAAAG-MGB (the mutant E-allele)/5′-FAM-CAAATAAGAAAGATTGTAAAG-MGB (the wild-type allele). The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center. PS activity was measured in 2690 population individuals [12] and the genotype was determined in 3651 individuals [5]. The 1862 individuals with both known PS activity and genotype were used for analysis in this study. Plasminogen activity was previously measured using the chromogenic assay method with streptokinase as the activator and the specific substrate S-2251 (Chromogenix AB, Stockholm, Sweden) [13]. Plasminogen activity was determined in 4517 individuals [13], and the plasminogen A620T mutation genotype was determined in 3295 out of 4517 individuals by the TaqMan method using the primers 5′-TGTGGAGGCACCTTGATATCC/5′-TGTCAATTGTCCCCTAAACATACTTC and the probes 5′-VIC-TGTTGACTACTGCCCACT-MGB (the mutant T-allele)/5′-FAM- TGTTGACTGCTGCCCACT-MGB (the wild-type allele). Analysis of variance was used to compare mean values between groups by Student's t-test using JMP v 5.1 software (SAS Institute Inc., Cary, NC, USA).

Results

We measured the PS activity in 1862 individuals of known PS genotype, and we compared the activity of wildtype and heterozygous individuals. Within this population, 1828 subjects harbored the wildtype allele while 34 were heterozygous for the K196E mutation. No individuals were homozygous for the mutant E-allele. Within the total population, the mean ± SD PS activity was 87.6% ± 19.9%.

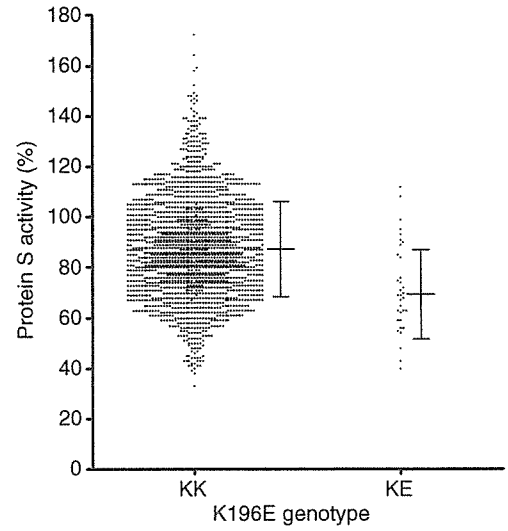

Fig. 1. Protein S (PS) activity in wild-type and K196E heterozygous individuals. Mean ± SD PS activity in heterozygous and wild-type individuals was 71.9% ± 17.6% (n = 34) and 87.9% ± 19.8% (n = 1828) (P < 0.0001), respectively.

Individuals heterozygous for the K196E mutation had reduced plasma PS activity compared to individuals with the KK genotype (mean ± SD, 71.9% ± 17.6%, n = 34 vs. 87.9% ± 19.8%, n = 1828, P < 0.0001) (Fig. 1). However, several heterozygous individuals with the mutant E-allele (n = 8) had measured PS activity greater than the total population average, while 26 wildtype subjects had PS activity at least 2SD less than the population mean (47.8%). Thus, PS activity does not appear to be a useful surrogate marker for PS genotype.

To determine whether an individual's genotype for any coagulation related protein could be determined by measuring the activity of the respective factor, we further examined the genotype and plasma activity of plasminogen in 3295 subjects. We identified 92 individuals heterozygous for the plasminogen A620T mutation, and the plasma plasminogen activity of these individuals was significantly less than wildtype individuals. Furthermore, there was little to no overlap between the measured plasminogen activities of wildtype and heterozygous individuals. Thus, the concept we originally wished to test was validated (Fig. 2).

There are well-documented gender- and age-related differences in PS activity [14], and this was true for our study population as reported [11] (Fig. 3A). When we examined the relationship between PS activity, genotype, and age, we observed decreased PS activity across all ages for individuals with the KE-genotype (Fig. 3B).

Discussion

DVT is a multi-factorial disease caused by the interaction of environmental and genetic factors. In Caucasian populations,

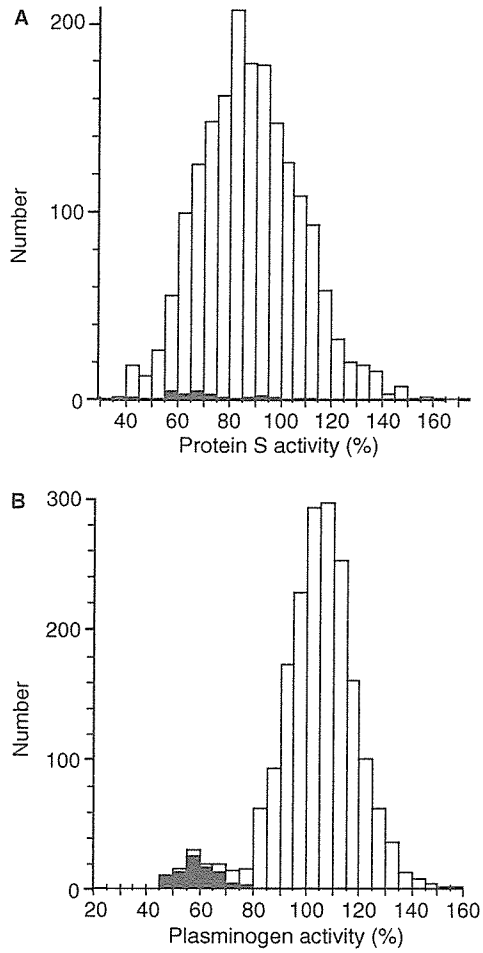

Fig. 2. Histogram representation of protein S (PS) (A) and plasminogen (B) activity in wildtype and heterozygous individuals. PS activity was measured in 1862 individuals, and plasminogen activity was measured in 3295 individuals. Activity was divided into groups by 5% increments, and mutation carriers are shown in closed bars.

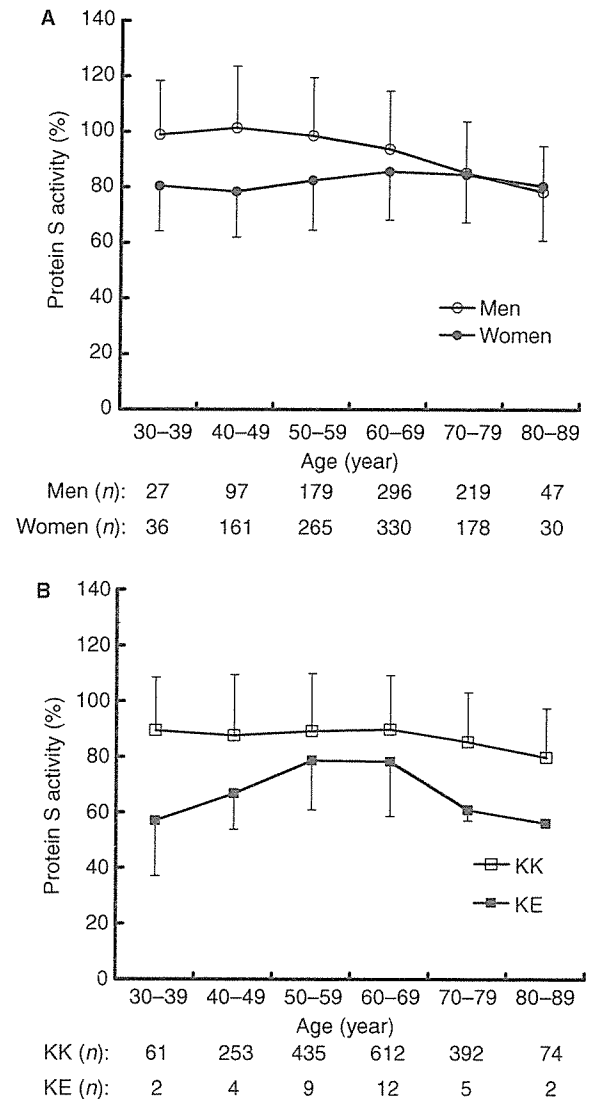

Fig. 3. Protein S (PS) activity divided in sex, age, and genotype. Open circles and closed circles in (A) show the mean PS activity in men and women, respectively. Open squares and closed squares in (B) show the mean PS activity in wild-type (KK-genotype) and heterozygote (KE-genotype). Error bars represent SD.

the FV Leiden (FVL) mutation, R506Q mutation in FV, is an important risk factor for the development of DVT. FVL carriers can be readily identified using the APC resistance test [15]. A FVL carrier will exhibit a prolonged clotting time in an activated thromboplastin time assay following the addition of APC. The incidence of this particular mutation varies in different ethnic populations [16,17] and is not observed in the Japanese [18]. In contrast, the PS K196E mutation present in the Japanese population is a genetic risk factor for DVT [4,5]. Therefore, a plasma assay for detecting PS 196E carriers should be developed. To understand the relation of the PS activity with the K196E mutation, we examined the PS activity and the K196E genotype in the Japanese population enrolled in the Suita Study.

The plasma PS activity in individuals with the PS K196E mutation remained controversial [6,9,10]. In one report, four members in a family who carried this mutation showed the PS activity with 37%, 72%, 101%, and 77%, respectively [10]. In a second family in this report, two members carried this mutation with the PS activity with 87% and 92%. On the basis of these results, the authors suggested this mutation as a phenotypically neutral polymorphism. In contrast, another study identified the same mutation correlated with low PS activity [6,9]. In this study, the authors identified this mutation in three patients with DVT. In addition, four individuals who did not show history of thrombosis were carriers of this mutation. All of these carriers showed low PS activity (mean ± SD, 43.1% ± 9.1%). Thus, so far, the relationship between the plasma PS activity and K196E mutation has not been settled. To address this issue, we have measured the PS activity and determined the genotype in the general Japanese population. As the results, we found that individuals heterozygous for the PS K196E mutation had reduced plasma PS activity compared to wildtype subjects, but this difference was relatively small and did not sufficiently differentiate between the two genotypes. In contrast, plasma

plasminogen activity was an effective test for segregating wildtype individuals and those heterozygous for the plasminogen A620T mutation. Thus, plasma PS activity is influenced by environmental factors to a greater extent than plasminogen activity.

The environmental factors such as age, sex hormone, and inflammation, are known to influence the PS activity [19]. As shown in Fig. 3, gender- and age-related differences in PS activity were observed in the general Japanese population. In addition, plasma PS activity might be influenced by other genetic factors. Genome scan for plasma free PS levels indicated a quantitative trait locus on human chromosome 1q [20]. This region contains *C4BPA* and *C4BPB* genes that are differentially regulated by acute phase cytokines [21]. PS can bind to the β-chain of C4 binding protein and not to the α-chain. The resulting alterations in the synthesis of C4 binding protein isoforms may affect the equilibrium between bound and free PS. Alternative means must be developed for the identification of PS K196E carriers to reduce the risk of DVT in affected individuals.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Review Article

Genetic Risk Factors for Deep Vein Thrombosis among Japanese: Importance of Protein S K196E Mutation

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Abstract

There is mounting evidence that mutations associated with a given disease arise with different frequencies among ethnic groups, thus ethnicity-specific studies are needed to identify causative mutations and properly assess risk. In particular, ethnic differences in the genetic background of thrombophilia have been reported. We recently conducted a large-scale analysis of the plasma activities of proteins C, S, antithrombin, and plasminogen within the Japanese general population. We found age- and sex-related differences and estimated the prevalence of deficiencies of protein C (0.13%), antithrombin (0.15%), protein S (1.12%), and plasminogen (4.29%). We also evaluated the genetic contribution to deep vein thrombosis and found that protein S mutation K196E is a genetic risk factor in the Japanese population. We estimated allele frequency to be 0.009, suggesting that 1 of 12,000 Japanese may be homozygous for the E allele, thus possibly as many as 10,000 individuals. Accordingly, a substantial proportion of the Japanese population carries the protein S E allele and is at risk of developing deep vein thrombosis. Given the frequency of this mutation and its strong correlation with deep vein thrombosis, it may be valuable to conduct a large-scale screening for this allele and advise concerned persons to avoid environmental risk factors known to be associated with deep vein thrombosis.

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Key words: Protein S; Deep vein thrombosis; Genetic risk; General population

1. Two Natural Anticoagulant Pathways: Protein C System and Protease Inhibitor System

Regulation of coagulation is achieved by a finely tuned balance between procoagulant and anticoagulant potencies. Generation of the multifunctional protease thrombin is a key event resulting from activation of the blood coagulation system. To regulate thrombin formation in plasma, 2 anticoagulant systems act in synergy. The first is known as the protein C anticoagulant pathway, the second as the heparan sulfate–dependent protease inhibitor system [1,2]. The protein C system controls 2 critical reactions: activation of factor X and activation of prothrombin. In this system, the thrombin-thrombomodulin complex activates protein C bound to its endothelial cell receptor, which is constitutively expressed. Resulting activated protein C (APC) has a relatively long half-life in circulation (approximately 20 minutes) and proteolytically inactivates activated factors V (FVa) and VIII (FVIIIa). Protein S accelerates inactivation of FVa and FVIIIa by APC. In the protease inhibitor system, antithrombin and tissue factor pathway inhibitor neutralize key coagulation proteases, in particular activated factors VII, IX, and X, in addition to thrombin. Inactivation of these proteases is heparan sulfate–dependent and occurs on the endothelium, lowering the potency of coagulant activity. Thus 2 systems involving a total of 6 proteins mainly control coagulation. Genetic or acquired deficiencies of any of these proteins may lead to vein thrombosis. Deficiency in protein C, protein S, or antithrombin is a major risk factor for vein thrombosis among white people [2,3]. Lack of data concerning the prevalence of these deficiencies in the general population of other ethnic groups renders it hazardous to extrapolate risk factors for vein thrombosis.

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2. Ethnic Differences in Genetics of Thrombophilia

There is growing evidence that within different ethnic groups, mutations associated with disease arise with different frequencies, thus ethnicity-specific studies are needed to identify causative mutations and to properly assess risk [4,5]. It is now well documented that there are ethnic differences in the genetic background of thrombophilia.

Factor V Leiden is an established genetic risk factor predominantly found in white populations [3,6]. This factor historically was found in the plasma of patients with deep vein thrombosis [7]. When plasma showed reduced anticoagulant response to the addition of APC, the phenotype was called APC resistance. Genetic study of APC resistance revealed a single nucleotide mutation in the gene of coagulation factor V: G-to-A missense mutation at position 1691 of the transcript resulting in replacement of Arg506 with Gln [8]. Arg506 is a target site for APC-catalyzed inactivation of FVa. Therefore a simple explanation for the mechanisms of APC resistance is that the Arg506-to-Gln change endorses resistance against proteolysis by APC, leading to impaired down-regulation of FVa [1,3,6].

The factor V Leiden mutation has a high prevalence, between 2% and 15%, in the general white population [9]. The prevalence is as high as 60% in selected patients with vein thrombosis [9]. Overall, the factor V Leiden mutation is the most common genetic risk factor for vein thrombosis in white populations, yet mutation is absent in other populations, including Japanese [10].

Another genetic polymorphism, prothrombin G20210A, has been identified as a genetic risk factor for vein thrombosis in whites [11]. Carriers of the 20210A allele have an increased plasma level of prothrombin, which may be a risk factor for vein thrombosis [11]. This polymorphism has extreme difference in prevalence among various ethnic groups and is absent in the Japanese population [12].

3. The Suita Study: A Japanese General Population

The National Cardiovascular Center conducted the Suita Study for the purpose of identifying the most common risk factors or characteristics that contribute to cardiovascular disease in the Japanese population. A large group of participants without overt symptoms of cardiovascular disease who had not had a heart attack or stroke were observed over a long period [13,14]. The study was based on a random sampling of 14,200 Japanese residents of Suita, a city near Osaka and part of the second-largest urban area of Japan. The 14,200 residents, between 30 and 89 years of age, were arbitrarily selected from the city population registry and were stratified by sex and decennial boundary. Letters were sent to the selected residents asking them whether they would be willing to participate in this study, which was started in 1989 on a cohort basis. By February 1997, 52.7% of the selected subjects (n = 7347) had paid an initial visit to the National Cardiovascular Center. After February 1997, participants visited the National Cardiovascular Center every 2 years for regular health checkups. In addition to routine blood examinations (total cholesterol, high-density lipoprotein cholesterol, triglycerides, glucose, glycosylated hemoglobin [HbA1c], systolic, and diastolic blood pressure), a number of thrombosis-related parameters were measured, including antithrombin, anticoagulant proteins C and S, and fibrinolytic protein plasminogen [15-17]. Examination of these thrombosis-related parameters provides invaluable information concerning thrombosis and hemostasis, and it is reasonable to believe that results obtained in the Suita Study are representative of the Japanese general population.

Overall, 12 thrombosis-related parameters were examined in the Japanese population. Results concerning antithrombin, proteins C and S, tissue factor pathway inhibitor, and fibrinolytic protein plasminogen have been published [15-18]. We describe and summarize these results in view of plasma activity level and introduce the genetics of thrombosis.

4. Plasma Activity of Antithrombin, Proteins C and S, and Plasminogen in the Japanese General Population: Age- and Sex-Related Differences and Prevalence of Deficiency

It has been reported that deficiency of proteins C and S or antithrombin may affect 1.1% to 3% of patients with vein thrombosis and as many as 5% to 9% of patients with recurrent disease and juvenile patients [19,20]. However, in a study measuring the prevalence of protein C deficiency in the general population of Scotland, investigators found a prevalence of no more than 0.2% [21], and in a study in the Midwest of the United States, investigators found a prevalence of 0.3% to 0.5% [22]. The prevalence of antithrombin deficiency was reported to be 0.16% in the general population of Scotland [23]. Thus large studies have been performed in the United States and Europe. Only small-scale investigations have been conducted in the Asian population [24], and prevalence was not assessed.

The first report [25] linking plasminogen deficiency to thrombosis was followed by a number of publications on plasminogen deficiency. Subsequent studies, however, challenged the link between plasminogen deficiency and thrombosis [26,27]. Among 1192 consecutive patients with a history of venous and/or arterial thrombosis, plasminogen deficiency was not found to be a risk factor for thrombosis [27]. In a large cohort study performed in Scotland, investigators also found no such link. Twenty-eight persons with plasminogen deficiency were identified among 9611 donors, giving a prevalence of 0.29% [26]. This prevalence was not significantly different from the prevalence (0.54%) calculated from studies of thrombotic cohorts in the literature, suggesting that plasminogen deficiency can be excluded as a risk factor for thrombosis. Intrinsic limitations in these studies, however, prevented complete exclusion of plasminogen deficiency as a risk factor. For example, comparison of the frequencies among populations in relation to geographic distance has not been carefully examined.

We measured plasma levels of antithrombin, protein C, and plasminogen in 4517 persons from the Japanese general population. Antithrombin activity was measured through its heparin cofactor activity with S-2238 as a chromogenic substrate. Protein C level was measured after activation of protein C activator (Protac) with S-2366 as substrate. Plasminogen was measured with S-2251 as a chromogenic substrate

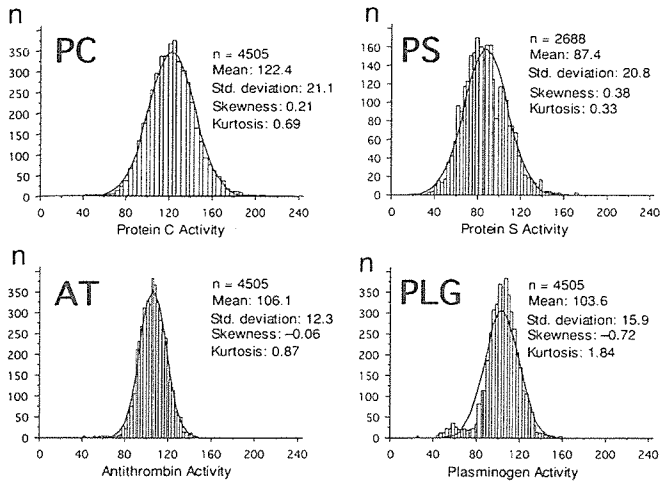

Figure 1. Distribution of plasma activity for protein C (PC), protein S (PS), antithrombin (AT), and plasminogen (PLG) in the Japanese general population. Protein C, antithrombin, and plasminogen activities were measured in 4517 subjects. Protein S activity was measured in 2690 subjects. Mean value, standard deviation, skewness, and kurtosis coefficients of each protein are shown.

after activation by streptokinase. We also used a Staclot protein S kit (Diagnostica Stago, Asniéres, France) to measure protein S cofactor efficiency in 2690 individuals in relation to the effect of APC on activated partial thromboplastin time.

Figure 1 shows distributions of proteins C and S activities as well as antithrombin and plasminogen levels in the Japanese general population. It appears that SDs for proteins C and S activity are larger than for antithrombin and plasminogen activity. Plasminogen activity is characterized by a large peak centered at 104% but also includes a small and broad peak corresponding to 60% activity. A smaller peak corresponds to plasminogen deficiency.

Figure 2 shows the age (32-89 years) and sex distribution for protein C and S activity as well as antithrombin and plasminogen levels. Analysis of activity through decennial regrouping showed that activity of all proteins was significantly reduced in men older than 50 years. A decrease in protein C and S activity was particularly noticeable. In contrast, protein C activity significantly increased in women older than 40 years. A sex-related difference also was observed for men 30 to 39 years and 40 to 49 years of age, who had higher proteins C and S activity than women in the same age groups.

Table 1 shows the mean ± SD for plasma levels of proteins C and S in 10-year age groups of men and women. In the 30- to 39-year and 40- to 49-year age groups, protein S activity was 22% and 23% lower in women than in men, respectively. In the 50- to 59-year age group, protein S activity also was 16% lower in women than in men. The reduced protein S activity in women may lead to misjudgment of protein S deficiency in women. Thus data obtained from a large general population are needed for unambiguous identification of protein S deficiency.

Sex- and age-related variation also was found with respect to antithrombin and plasminogen levels, but the differences were smaller (Figure 2). It is generally believed that thrombotic tendency in elderly persons is due to low anticoagulant activity. According to the results of our study, this finding would be true for Japanese men but not for women.

By measuring plasma activity, we estimated the prevalence of deficiency for each factor, as shown in Table 2. For protein C and antithrombin, we also calculated the ratio of antithrombin to protein C activity (AT/PC ratio). Using as the criterion an AT/PC ratio higher than 3 SD (1.27) associated with protein C activity lower than 3 SD (59.3%), we identified 6 of 4517 individuals as potentially having heterozygous protein C deficiency, implying a prevalence of 0.13%. Using the same approach and criterion, we identified 7 individuals as potentially having antithrombin deficiency, implying a prevalence of 0.15% [16]. Still using the same criterion, we identified 14 of 1252 men and 23 of 1438 women as potentially having protein S deficiency. Thus prevalence of protein S deficiency was estimated to be 1.12% in men and 1.60% in women. However, prevalence of protein S deficiency in women might have been overestimated because of interference with hormonal state. Hence we believe that 1.12% is likely to represent the true prevalence of protein S deficiency in the Japanese general population [17]. With respect to plasminogen, identification of deficiency was straightforward because of the small but distinctive peak at around 60% plasminogen activity, corresponding to individuals with plasminogen deficiency (Figure 1). To unambiguously differentiate plasminogen deficiency from normal plasminogen level, we used mean –2 SD of the calculated ratio of antithrombin to plasminogen activity (0.69) [15]. Accordingly, the prevalence of plasminogen deficiency in the Japanese population would be 4.29%.

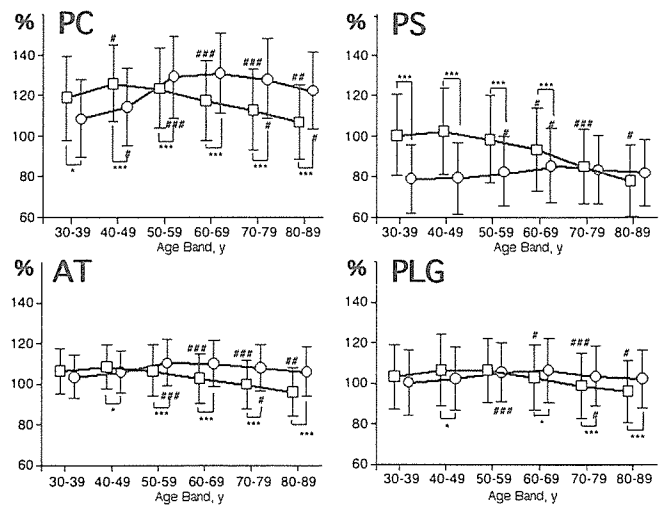

Figure 2. Sex- and age-related differences in plasma activity of protein C (PC), protein S (PS), antithrombin (AT), and plasminogen (PG) in the Japanese general population. Protein C, antithrombin, and plasminogen activity was measured in 2090 men and 2427 women. Protein S activity was measured in 1252 men and 1438 women. Activity was analyzed in 10-year age groups. Results show mean value. Error bars indicate standard deviation for each age group; squares, activity in men; circles, activity in women. #$P < .05$, ##$P < .001$, ###$P < .0001$ compared with those in the preceding age group (same sex). *$P < .05$, **$P < .001$, ***$P < .0001$ sex difference within same age group.

Table 1.
Age- and Sex-Related Distribution of Protein C and S Levels in General Population

	Protein C, %				Protein S, %			
	Men		Women		Men		Women	
Age group, y	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD
30-39	66	118.9 ± 20.9	88	108.8 ± 19.2	46	100.7 ± 20.0	62	79.1 ± 17.0
40-49	262	126.0 ± 19.0	388	114.4 ± 19.3	165	102.6 ± 21.6	252	79.4 ± 17.7
50-59	373	123.8 ± 19.7	593	129.4 ± 20.2	231	98.5 ± 21.5	338	82.8 ± 17.1
60-69	660	117.8 ± 19.6	745	131.3 ± 19.8	390	93.6 ± 20.5	442	85.6 ± 18.5
70-79	555	113.1 ± 20.1	491	128.2 ± 19.7	324	85.1 ± 18.6	278	83.6 ± 17.2
80-89	167	107.1 ± 18.7	117	122.7 ± 18.9	96	78.3 ± 17.8	66	82.0 ± 16.6

Prevalence of deficiency estimated in the Japanese general population was then compared with the prevalence reported for the white general population (Table 2). The prevalence values for antithrombin and protein C deficiencies were quite similar, both conditions affecting 1 of 500 to 700 individuals. Differences were nevertheless noticeable with respect to the other factor measured. Plasminogen deficiency in particular has a high prevalence among Japanese but not among whites. Protein S deficiency may also have a higher prevalence among Japanese, even if caution is exercised about such a conclusion, because the assay used and the criteria used to define deficiency differ between studies.

We measured plasminogen, antithrombin, and protein C activity in 108 patients with deep vein thrombosis to estimate prevalence of deficiency (Table 3). Comparison of prevalence in the general population with that in the deep vein thrombosis group revealed that antithrombin and protein C deficiencies were genetic risk factors associated with deep vein thrombosis in the Japanese population (odds ratios, 38 and 52, respectively) [16]. In contrast, there was no evidence of a link between plasminogen deficiency and risk of deep vein thrombosis [15].

5. Genetic Changes in Thrombosis-Related Proteins in the Japanese Population

Factor V Leiden and prothrombin G20210A are genetic risk factors for deep vein thrombosis in white populations, but mutations have not been found in the Japanese population. No other genetic variations have been formally identified as genetic risk factors. Nevertheless, 5 genetic changes in thrombosis-related genes that may have an effect on the occurrence of deep vein thrombosis are known to be present in the Japanese population.

Table 2.
Comparison of Prevalence of Deficiencies of Proteins C and S, Antithrombin, and Plasminogen between Japanese and Westerners*

Deficiency	Population	General Population, %	DVT Group, %
Protein C	Japanese	0.13	6.5
	Westerners	0.15-0.33	3.2
Protein S	Japanese	1.12	ND
	Westerners	0.03-0.13	1.3-2.2
Antithrombin	Japanese	0.15	5.6
	Westerners	0.17	1.1
Plasminogen	Japanese	4.29	2.8
	Westerners	0.3-0.5	ND

*Data from the literature [15-17,19,21,23,53]. Prevalence in the Japanese population was estimated from the plasma activity of each factor. DVT indicates deep vein thrombosis; ND, not determined.

A missense mutation causing an Ala to Thr change at position 620 (A620T) of mature plasminogen has been identified in a Japanese patient with recurrent deep vein thrombosis [25,28]. The mutation was formerly called A601T and was referred to as plasminogen Tochigi, but the numbering standards adopted by the Nomenclature Working Group recommend that the A of the ATG of the initiator Met codon be denoted nucleotide +1 and that the initial Met residue amino acid be denoted +1 [29], causing us to rename several of the mutants we characterized. A patient with the A620T mutation exhibited decreased plasminogen activity, but antigen level was within normal limits [30]. In the mini-plasmin crystal structure, the mutation is located just before the active His residue (Ala55 in the chymotrypsin numbering system) [31]. Small-scale studies have shown that allele frequency for the plasminogen Tochigi mutation is between 0.011 and 0.021 [32,33]. The mutation has been found with an allele frequency of approximately 0.015 in the Chinese Han population and with a frequency of 0.016 in the Korean population [33] but has not been found in white populations [32].

ADAMTS13 is a von Willebrand factor (VWF)-cleaving protease [34]. Defects in the ADAMTS13 gene cause thrombotic thrombocytopenic purpura, a disease characterized by thrombocytopenia and microangiopathic hemolytic anemia with variable degrees of renal failure, neurological dysfunction, and fever. A missense mutation causing replacement of Pro475 by Ser (P475S) in the Cys-rich domain of ADAMTS13 was identified with an allele frequency of approximately 0.05 in the Japanese population [35]. Results of in vitro studies indicated the mutation has low VWF-cleaving activity. Homozygotes for this mutation retain ADAMTS13 activity and thus do not have the thrombotic thrombocytopenic purpura phenotype. Although polymorphism is found in Chinese populations at a lower frequency, it has not been identified in white populations [36-38].

Protein S is an important regulator of coagulation, and a missense mutation causing Lys196 to be replaced by Glu (K196E mutation, formerly known as protein S Tokushima and referred to as K155E mutation) within the second epidermal growth factor–like domain of protein S has been identified in Japanese patients with deep vein thrombosis [39,40]. As in the A620T mutation in plasminogen, protein S activity was decreased in carriers of the K196E mutation, but