Polymorphisms and mutations of *ADAMTS13* in the Japanese population and estimation of the number of patients with Upshaw–Schulman syndrome

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Upshaw–Schulman syndrome (USS), also called hereditary thrombotic thrombocytopenic purpura, is an autosomal recessive disease characterized by thrombocytopenia and microangiopathic hemolytic anemia. USS is associated with hereditary severe deficiency of plasma ADAMTS13 activity; patients with USS have homozygous or compound heterozygous mutations in the *ADAMTS13* gene [1–5]. ADAMTS13 is a plasma metalloprotease that regulates platelet aggregation through the cleavage of von Willebrand factor (VWF) multimers. ADAMTS13-deficient plasma derived from patients with USS contains unusually large VWF multimers, which can induce unwanted hyperaggregation of platelets and microvascular thrombi. In this study, we analyzed the relationship between genetic variation of *ADAMTS13* and plasma ADAMTS13 activity in the Japanese general population. In addition, on the basis of the data obtained via our genetic analysis, we estimated the number of patients with USS in Japan.

The population examined is based on the Suita Study [6], an epidemiologic study consisting of randomly selected Japanese residents of Suita City, which is located in the second largest urban area in Japan. Our study protocol was approved by the ethical review committee of the National Cerebral and Cardiovascular Center, and only subjects who provided written informed consent for genetic analyses were included.

To identify common polymorphisms in the population, we first sequenced all 29 exons and exon–intron boundaries of *ADAMTS13* using 346 consecutive subjects, by means of previously described methods [2]. We identified 25 polymorphisms with allele frequencies of the respective minor allele > 0.01, including two in the promoter region, 10 in the exons, and 13 in the introns. Of these, six were missense single-nucleotide polymorphisms (SNPs): p.T339R (c.1016C > G), p.Q448E (c.1342C > G), p.P475S (c.1423C > T), p.P618A (c.1852C > G), p.S903L (c.2708C > T), and p.G1181R (c.3541G > A). Next, we performed TaqMan genotyping assays (Applied Biosystems, Tokyo, Japan) for the missense SNPs, using 3616 subjects whose plasma ADAMTS13 activities had been measured with the FRETS-VWF73 assay [7]. Allele frequencies for the minor alleles were 0.027 for p.T339R, 0.192 for p.Q448E, 0.050 for p.P475S, 0.027 for p.P618A, 0.048 for p.S903L, and 0.022 for p.G1181R. The observed genotypes did not deviate significantly from Hardy–Weinberg equilibrium. The p.T339R and p.P618A SNPs were in absolute linkage disequilibrium (r^2 = 0.97), whereas the other missense SNPs were not strongly linked (r^2 < 0.11).

The p.Q448E and p.P475S SNPs, but not the other missense SNPs, were significantly associated with plasma ADAMTS13 activity (Fig. 1A). The ADAMTS13 activity (97% ± 25% in men, 111% ± 28% in women, mean ± standard deviation) of p.Q448E heterozygotes (QE) and minor allele homozygotes (EE) was slightly but significantly higher than that of major allele homozygotes (QQ) (91% ± 24% in men, 104% ± 26% in women). In contrast, the ADAMTS13 activity (79% ± 20% in men, 92% ± 24% in women) of p.P475S heterozygotes (PS) and minor allele homozygotes (SS) was significantly lower than that of major allele homozygotes (PP) (94% ± 24% in men, 108% ± 27% in women). The difference in activity was consistent with the observation that the recombinant ADAMTS13-P475S mutant has approximately 70% of the activity of wild-type ADAMTS13 [8]. It is interesting that p.P618A was not associated with plasma ADAMTS13 activity in the present study, whereas the conditioned medium of HEK293 cells expressing the A618 variant showed lower levels of activity (27%) and antigen (14%) than the wild type [9]. PolyPhen-2, a program that predicts damaging missense mutations [10], identified p.T339R and p.P618A as 'possibly damaging' and 'probably damaging', respectively, whereas the other four SNPs were predicted to be 'benign'.

As the *ADAMTS13* locus is near (130–190 kb) the *ABO* locus on chromosome 9q34, we compared the frequencies of the SNPs among ABO blood group genotypes (Fig. 1B). The relative frequencies of p.T339R minor allele homozygotes and

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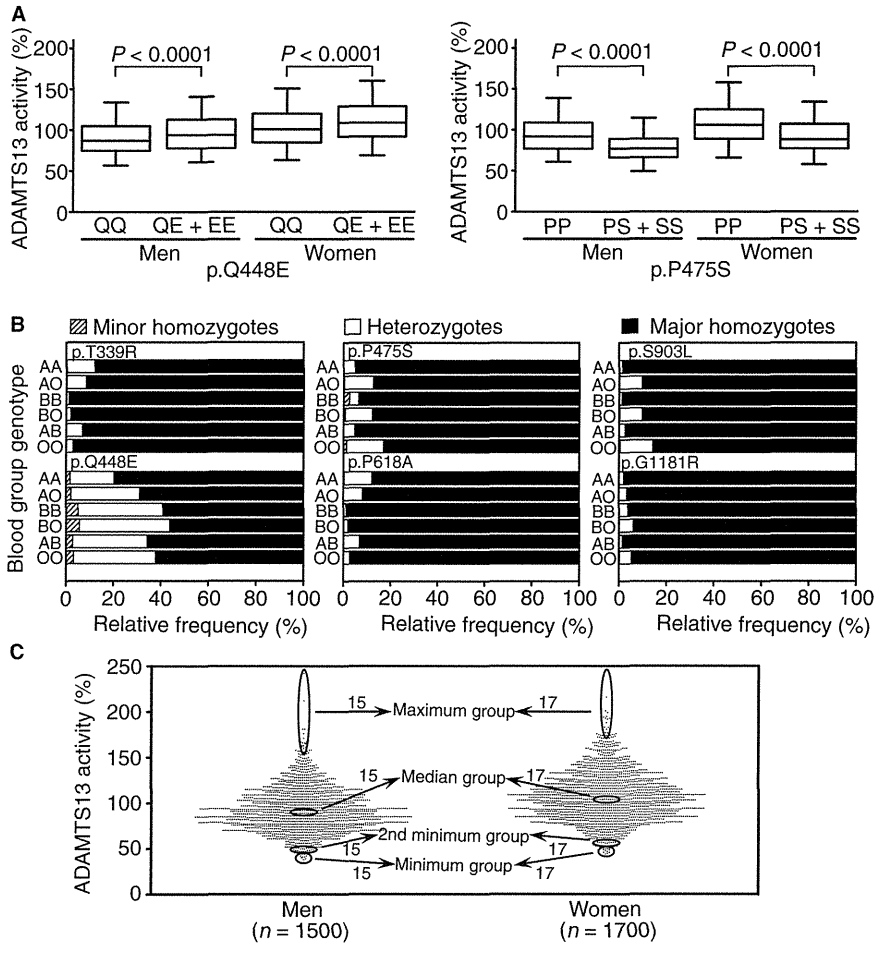

		Min. (n = 32)	2nd min. (n = 32)	Med (n = 32)	Max. (n = 32)
Average activity		47.1%	53.1%	97.6%	183%
Common SNP	p.T339R	2	1	3	0
	p.Q448E	8 (1)	8	8	16
	p.P475S	9 (2)	6	2	1
	p.P618A	2	1	3	0
	p.S903L	2	3	5	3
	p.G1181R	2	4	1	1
Rare mutation		7	3	2	2

Fig. 1. *ADAMTS13* variation in a Japanese general population. (A) Box-and-whisker plot (5th–95th percentiles) of plasma ADAMTS13 activity in each genotype of p.Q448E and p.P475S. *P*, Kruskal–Wallis test. (B) The relative frequency of minor homozygotes, heterozygotes and major homozygotes for each genetic polymorphism. (C) Scatter dot plot of plasma ADAMTS13 activity for men and women. On the basis of these activity measurements, 128 subjects were selected for sequencing of *ADAMTS13*. (D) The numbers of minor allele carriers in each group. One in eight p.Q448E carriers and two in nine p.P475S carriers in the minimum group were homozygotes for the respective minor alleles.

heterozygotes were higher for AA, AO and AB than for BB, BO and OO, suggesting that p.T339R is associated with the blood group A allele. The p.P618A SNP, which is tightly associated with p.T339R, exhibited the same pattern. The p.P475S and p.S903L SNPs tended to be associated with the blood group O allele.

We then utilized the plasma ADAMTS13 activity data to estimate the frequency of hereditary ADAMTS13 deficiency. In the population, 3200 DNA samples (1500 men and 1700 women) were available, from a quantitative standpoint, for sequencing of *ADAMTS13*. We selected 128 subjects according to their plasma ADAMTS13 activity (Fig. 1C): 32 subjects of the 'minimum' group (average activity, 47.1%), consisting of 15 men and 17 women with the lowest activities in each gender; 32 subjects of the 'second minimum' group (53.1%), consisting of 15 men and 17 women with the second lowest activities; 32 subjects of the 'median' group (97.6%), consisting of 15 men and 17 women with median activities; and 32 subjects of the 'maximum' group (183%), consisting of 15 men and 17 women with the highest activities. Each group corresponds to 1% of the population examined. All DNA samples from the four groups were subjected to *ADAMTS13* sequencing, which

revealed that 70 individuals had at least one of the six missense SNPs described above (Fig. 1D). Of these, only p.P475S showed a significant difference in minor allele frequency among four groups (P = 0.028, chi-square test). In addition, 14 individuals had rare non-synonymous mutations: seven (p.F324L, p.F418L, p.I673F, p.Q773X, p.Y1074AfsX46, p.R1095Q, and p.S1314L) in the 'minimum' group; three (p.I380T, p.Y1074AfsX46, and p.R1274C) in the 'second minimum' group; two (p.Q723K and p.N1321S) in the 'median' group; and two (p.L19F and p.R268Q) in the 'maximum' group. Of these, p.I673F (c.2017A > T) and p.Y1074AfsX46 (c.3220delTACC) had been identified as causative mutations in patients with USS [11,12]. All of the others were newly identified mutations.

To estimate the number of individuals with a hereditary ADAMTS13 deficiency, we generated several hypotheses: (i) as two individuals in each of the 'median' and 'maximum' groups had rare mutations, two of every 32 people should have a mutation that does not cause a functional defect of ADAMTS13; (ii) thus, five (= 7 – 2) individuals in the 'minimum' group and one (= 3 – 2) individual in the 'second minimum' group should be the heterozygotes carrying a mutation with a functional defect; (iii) other than these six (= 5 + 1) individuals in the 'minimum' and 'second minimum' groups, no individual should have any mutations that confer a functional defect. These hypotheses were consistent with a prediction based on PolyPhen-2: the p.S1314L, p.I380T, p.Q723K, p.N1321S, p.L19F and p.R268Q mutations are 'benign', p.I673F and p.R1274C are 'possibly damaging', and the others are 'probably damaging'. According to the hypotheses, we estimated that six of 3200 individuals were heterozygotes for ADAMTS13 deficiency. This estimation suggested that ~ 1 individual in 1.1×10^6 (= 6/3200 × 6/3200 × 1/4) should be a homozygote or a compound heterozygote for ADAMTS13 deficiency. In Japan, which has a population of approximately 1.3×10^8, ~ 110 individuals may have hereditary ADAMTS13 deficiency or USS. If we adjusted our estimate of ADAMTS13 deficiency from 6/3200 to 7/3200 or 5/3200, the number of patients would be 160 or 80, respectively. The validity of these calculation procedures was confirmed by StaGen Co., Ltd (Chiba, Japan), a company specializing in genetics, statistics, and data analysis.

In conclusion, this study demonstrated that, in the Japanese general population, there are six common missense SNPs: p.T339R, p.Q448E, p.P475S, p.P618A, p.S903L, and p.G1181R. Of these, p.Q448E and p.P475S are significantly associated with plasma ADAMTS13 activity. Allele frequencies of these SNPs correlate with ABO blood group. Finally, we estimated the number of patients with USS in Japan, yielding a figure that corresponds to approximately three times the number of patients already diagnosed as having this condition. Because of insufficient sample sizes, we may have underestimated the prevalence of USS. Further studies are needed to obtain more reliable conclusions.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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加齢とプロテインS*

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1．はじめに

プロテインSの血中抗原量や活性は，遺伝因子や環境因子により変動する．遺伝因子としてプロテインSの抗原量や活性に影響を与えるミスセンス変異やプロモータ領域の変異が挙げられる．環境因子として，年齢，性，ホルモン，妊娠，肝疾患，炎症が挙げられる．一方，プロテインSの抗凝固活性はC4BP結合型には見られず，C4BPに結合しない遊離型プロテインS(free protein S；fPS)が抗凝固活性を示す．プロテインS抗原量の測定は，総プロテインS(total protein S；tPS)抗原量およびC4BPに結合していないfPS抗原量が測定可能であり，fPS量がよりプロテインS抗凝固活性を反映すると考えられる．

本稿では，プロテインS抗原量および抗凝固活性の加齢による変動を中心に，これまでヨーロッパおよび本邦で行われた一般住民を対象にした研究の成果を紹介したい．

2．オランダで行われた小規模なプロテインS抗原量の研究[1]

男性225名，女性168名(計393名)の健常人を対象に，tPS量，fPS量，プロテインC抗原量，X因子抗原量の測定が行われた．X因子はプロテインSやプロテインCと同様に，ビタミンK依存性の凝固因子なので，これらに関連して測定された．対象者の年齢は18～60歳以上であった．経口避妊薬を服用する女性は除外した．両プロテインS抗原量ともに，男性が女性より高い値を示したが，特に，50歳までのtPS量とfPS量はともに女性で有意な低値を示した．閉経前の女性はtPS量の低値を示したが，閉経後のtPS量は男性と差は見いだせなかった．fPS量は男性が有意に高く，閉経前の女性が最も低く，閉経後の女性はこれら2群の中間を示した．本研究は対象者総数が393名であり，これをさらに性別と10歳代に分けているので，一つのグループは40名程度と小規模であるものの，tPS量とfPS量の性差と，女性での閉経前後の変化を示した．

3．小児と100歳老齢者のプロテインS抗原量の研究[2,3]

1～16歳までの246名の小児を対象に33種の凝血学的パラメーターを測定した研究が報告されている．この研究では，tPS量とfPS量も測定されている．両プロテインS抗原量は生後6か月で，すでに成人と同程度であった．しかし，生後1日目に測定すると，プロテインS抗原量は極めて低い値(0.3 U/ml程度)を示した[2]．一方，100歳以上の高齢者(合計25名，男性9名，女性16名)のtPS量を測定した報告によると，100歳以上の老齢者のtPS量は18～50歳のグループや51～69歳のグループのtPS量と差が見られなかった．

*：Aging and protein S
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表　西スコットランドで測定された総プロテインS抗原量(tPS)と遊離プロテインS抗原量(fPS)

	人数	25歳未満	25～45歳未満	45歳以上	全体
tPS(%)					
男性	2,106	106(72～168)	109(72～162)	110(73～171)	108(72～164)
女性					
ホルモン* あり	487	84(56～136)	89(56～148)	95(57～157)	87(57～142)
ホルモンなし	1,195	96(65～141)	96(67～152)	99(71～146)	96(68～148)
fPS(%)					
男性	2,106	119(71～177)	115(68～177)	113(67～175)	115(68～176)
女性	1,682	96(51～155)	98(55～156)	100(60～152)	98(54～155)

tPS値(%)およびfPS(%)値は中央値，括弧内は2.5～97.5パーセンタイル値を示す．
女性のtPS量は経口避妊薬服用・ホルモン補充療法(*)で低値を示したので，その有無で分類した．

4．西スコットランドでのプロテインS抗原量の大規模な研究[4]

西スコットランドに在住の心臓，腎臓，肝臓に疾患をもたない3,788名(男性2,106名，女性1,682名，17～68歳)の血中プロテインS抗原量(tPS，fPS)が報告されている．男性の平均年齢は32歳，女性は35歳であった．女性の87%(1,467名)は閉経前であり，31%は経口避妊薬を服用していた．197名の女性は閉経しており，その15%はホルモン補充療法を受けていた．

表に西スコットランドで測定されたtPS量およびfPS量の年齢別の中央値と2.5～97.5%値を男女に別けて示した．両プロテインS抗原量は，ともに男性は女性より高値を示した．即ち，男性のtPS量は女性より平均13.6%高かった($p<0.001$)．男性ではtPS量は年齢により変化しなかったが，fPS量は加齢により少し減少した($p=0.001$)．女性では，tPS量は加齢により増加したが($p<0.001$)，fPS量は変わらなかった．閉経はtPS量に影響しなかった．経口避妊薬により約8%のtPS量の減少を認めた($p<0.001$)．fPS量は加齢により上昇した($p<0.02$)が，調整を行うとその有意差は消失し，最も大きな因子は閉経であった．経口避妊薬はfPS量に影響しなかった．閉経後の女性へのホルモン補充療法はtPS量，fPS量ともに影響を与えなかった．

表には，tPS量およびfPS量の年齢別の中央値と2.5～97.5%値を男女別に示している．全対象者の下方2.5%に当たるtPS量は56～73%であり，経口避妊薬を服用する若い女性が最も低く，男性が最も高かった．全対象者の下方2.5%に当たるfPS量でも，若い女性が51%と最も低く，男性が60%と最も高かった．

5．吹田研究で行ったプロテインS活性の大規模な研究[5]

吹田研究は，大阪府吹田市の住民を対象として1989年に始まったコホート研究である．住民台帳から30～70歳代まで10歳代ごとに男女別に無作為に抽出し，郵送で健診案内を郵送し参加を呼びかけた．15,200名を抽出した中で，これまでに8,360名が健診受診している．日本の多くのコホートが農漁村の在住者を対象とするのに対し，吹田研究は都市部の住民を対象とする点に特徴がある．筆者らは健診受診された2,690名(男性1,252名，女性1,438名，33～89歳)のプロテインS活性をStaclot protein S(Diagnostica Stago社)を用いて測定した．本研究の特徴は，計画健診により無作為に抽出された30歳代から80歳代までの広い年齢層の対象者のプロテインS活性(抗原量ではなく)を測定した点にあり，国民の3分の2を占める都市部住民の年齢と性別によるプロテインSの抗凝固活性の変化を把握できる．

プロテインS抗凝固活性は，男性が女性より高値を示した(92.6±21.4%，82.9±17.8%，平均値±標準誤差，$p<0.0001$)．特に男性では年齢により活性が低下したが(correlation coefficient，$r=-0.366$)，女性では変化が見られなかった．

プロテインS活性を性別，10歳年令別に区別した値を図に示す．これらの結果から，①男性のプロテインS活性は40歳代の102%をピークに，それ以降は徐々に低下し80歳代では78%にまで低下する，②一方，女性では30歳代・40歳代は79%と低値を示すものの，それ以降は下ることなく60齢代では逆に85%にまで上昇する，③男女

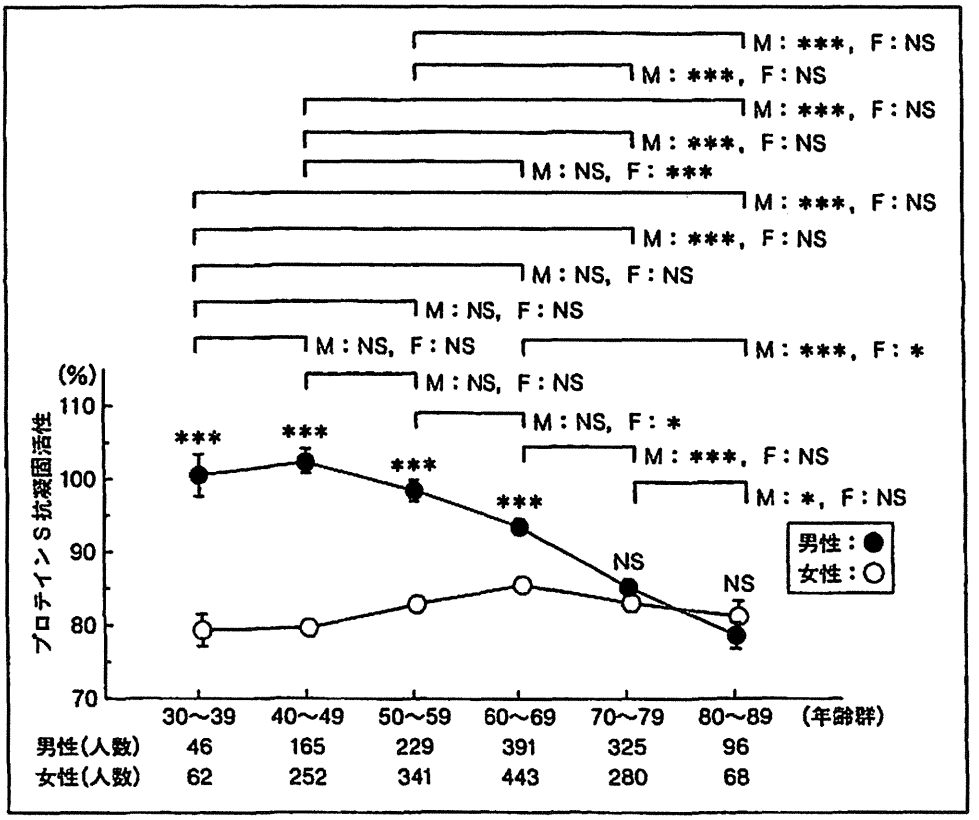

図　日本人2,698名のプロテインS抗凝固活性
男性1,252名，女性1,446名のプロテインS活性を10歳代ごとに平均値+/−標準誤差を示す．年齢別の男女群間の比較はMann-Whitney検定を用いた．男女別年齢群間の比較はSteel-Dwass検定を用いた．
***：$p<0.001$，**：$p<0.01$，*：$p<0.05$，NS：not significant.

で比較すると，30歳代～60歳代までは，男性のプロテインS活性は女性より明らかに高値を示す，④しかし，70歳代・80歳代では男女差がみられない，ことが明らかとなった．

男性のプロテインS活性が女性より高いという結果は，西スコットランドでのプロテインS抗原量の研究とよく一致する．しかし，男性のプロテインS活性は加齢により低下するという結果は，西スコットランドの抗原量の研究と一致しない．これは研究対象者の年齢の違いで説明される．すなわち，吹田研究は80歳代という高齢までの活性を測定しているので，加齢による活性の低下を観察したが，西スコットランドの研究では，25歳未満，25～45歳未満，45歳以上，という大まかな年齢の分け方をしているので，加齢による抗原量の低下を見いだせなかったと考えられる．

6．おわりに

プロテインSの抗原量と活性には性差がみられ，特に若い女性では低下が大きく，プロテインSの検査値を見るときに注意が必要である．男性は，70歳代・80歳代の高齢者でプロテインS活性の低下がみられた．こういった性と年齢によるプロテインS抗原量と活性の変化は，欠乏症の同定の際に，考慮すべきであろう．

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日本人の ADAMTS13

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ADAMTS13 of Japanese people

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Key words: ADAMTS13, VWF, thrombotic thrombocytopenic purpura, Upshaw-Schulman syndrome, polymorphism

1. はじめに

ADAMTS13は，von Willebrand因子（von Willebrand factor：VWF）マルチマーを切断することで血小板凝集を調節する血漿プロテアーゼである．その極端な活性低下は，血液中に通常検出されない異常高分子量のVWFマルチマーを増加させ，血栓性血小板減少性紫斑病（thrombotic thrombocytopenic purpura：TTP）の原因となる[1)-3)]．したがって，TTPの診断にはADAMTS13活性の測定が有効であり，これまでに種々の測定方法が開発されてきた．現在，FRETS-VWF73蛍光定量法[4)]とAct-ELISA発色定量法[5)]がよく普及している．我々は，簡便でハイスループット測定にも適している蛍光定量法を用いて，日本人地域一般住民の血漿ADAMTS13活性を測定した[6)]．本稿では，遺伝子解析の結果[7)]とあわせて，日本人のADAMTS13に関して紹介する．

2. 日本人のADAMTS13活性

国立循環器病研究センター予防健診部で1989年から実施されている都市部一般住民を対象とした「吹田研究」[8)-10)]の一部として，男女合わせて3616人の血漿ADAMTS13活性を測定した[6)]．日本の中で地域差が存在する可能性もあるが，現時点でそれを示すデータは存在しないため，本稿では吹田研究の対象者が日本人一般住民を代表しているとみなして論じる．

全試料のADAMTS13活性平均値を100%とすると，標準偏差（standard deviation：SD）は27%であった（**図1**）．パーセンタイル値はそれぞれ，5パーセンタイル：61%，25パーセンタイル：81%，中央値：97%，75パーセンタイル：116%，95パーセンタイル：148%となり，3616人中の最高値は242%，最低値は38%であった．男性のADAMTS13活性平均値93%（SD：24%，n=1687）は女性の平均値106%（SD：27%，n=1929）に比べて有意に低かった（$P<0.0001$）．男女差が生じる理由は不明である．

男性と女性のいずれにおいても，ADAMTS13活性は60歳台以上で徐々に低下する傾向が見られた（**図2**）．一方，ADAMTS13の基質であるVWFの血中抗原量は加齢によって増加することが知られている[11)]．今回の試料でも，予想通り，高齢者ほどVWF抗原量は増加していた（**図2**）．VWF量の増加とADAMTS13活性の減少

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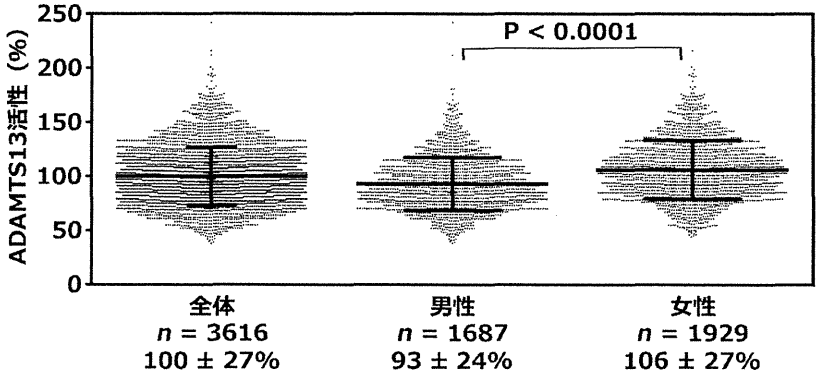

図1　日本人一般住民の血漿 ADAMTS13 活性
全試料の平均値を 100％とした．バーとエラーバーはそれぞれ平均値と SD を表す．男性の ADAMTS13 活性は女性よりも有意に低い．

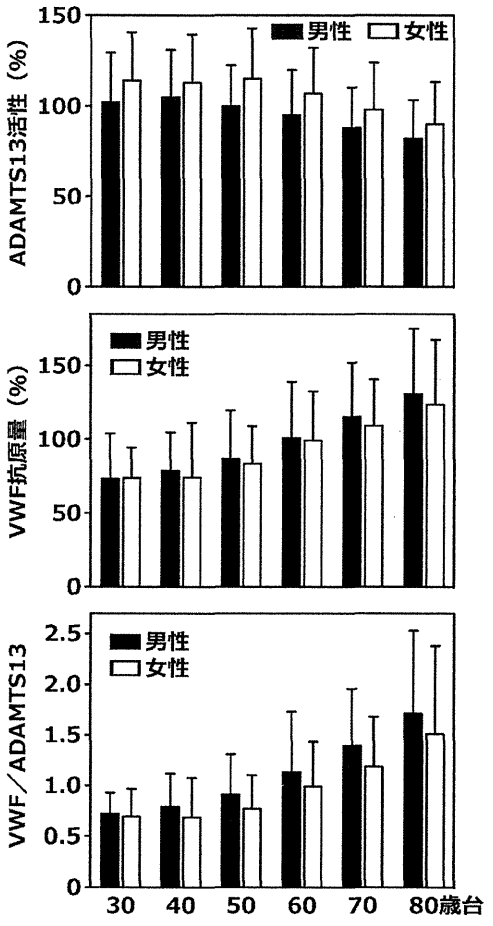

図2　ADAMTS13 活性および VWF 抗原量と年齢
男女とも，加齢に伴って ADAMTS13 活性は減少し，VWF 抗原量は増加する．したがって，VWF 抗原量／ADAMTS13 活性の比は著しく増加する．

は，いずれも血小板凝集を促進する方向に働くため，両者の比，すなわち VWF 量／ADAMTS13 活性は血栓傾向の指標となる．そこで，VWF/ADAMTS13 比を年齢層別にプロットすると，加齢に伴う増加傾向が顕著に現れた（**図2**）．これは高齢者における血栓傾向を部分的に説明できるデータかもしれない．なお，FRETS-VWF73 を用いて測定する ADAMTS13 活性は，試料中の VWF 量の影響を受けないので，加齢による ADAMTS13 活性の低下は，加齢による VWF 量の増加が測定系に与えた影響ではない．実際，年齢による差を補正して統計解析したところ，ADAMTS13 活性と VWF 量に有意な相関は見られなかった（データ省略）．

ABO 血液型の O 型の人では，他の型に比べて血中 VWF 量が低い[11]．本研究の対象者でも，男女いずれにおいても，O 型は他の型に比べて有意に低い VWF 量を示した（**図3**）．一方，ADAMTS13 活性と血液型に有意な相関は見られなかった（**図3**）．この結果は，血中 ADAMTS13 抗原量と血液型は相関しないという，387 人の男性で解析したイギリスの報告と一致する[12]．VWF 分子には血中からのクリアランスに関与する血液型糖鎖が結合している[13]．一方，ADAMTS13 分子には血液型糖鎖が結合していない[14]ため，クリアランスに血液型間の違いが生じないのであろう．

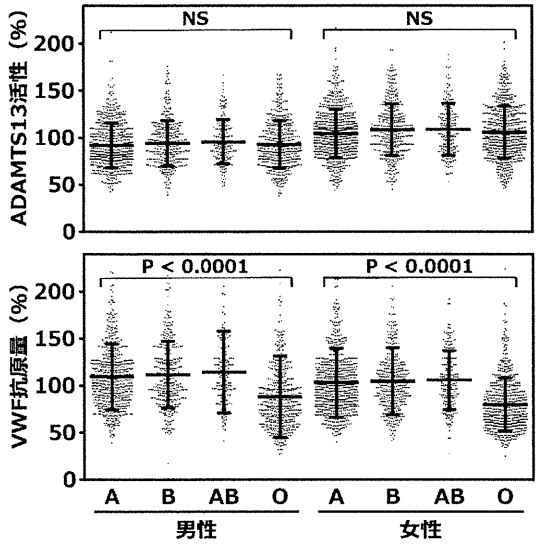

図3　ADAMTS13活性およびVWF抗原量と血液型
ADAMTS13活性とABO血液型に有意な相関は見られない．VWF抗原量はO型で有意に低い．

3. 日本人のADAMTS13遺伝子多型

TTP症例の多くは二次性あるいは特発性であるが，常染色体劣性遺伝を示す遺伝性（家族性）の症例も稀に見つかる．遺伝性TTPはUpshaw－Schulman症候群（Upshaw-Schulman syndrome：USS）とも呼ばれ，その実体は先天性ADAMTS13欠損症であり，患者はADAMTS13遺伝子異常のホモ接合体あるいは複合ヘテロ接合体である[1) 15)-19)]．我々は，奈良県立医科大学の藤村吉博教授と共同で日本人USS患者の遺伝子解析を行ってきたが，その過程で，USSの原因変異とは考えにくい，比較的高頻度に見られる塩基変異，すなわち遺伝子多型をしばしば同定した．

そこで，日本人が保有するADAMTS13遺伝子多型を調べ上げるために，吹田研究対象者から連続抽出した346人のADAMTS13遺伝子の全エキソン（29個）および近傍のイントロンの塩基配列を解析した[7)]．その結果，マイナーアレル頻度（minor allele frequency：MAF）が0.01を超える多型が25個同定され，すべて1塩基多型（single nucleotide polymorphism：SNP）であった．その内訳は，プロモータ領域に2個，エキソンに10個，イントロンに13個である．エキソンに同定された10個のSNPのうち，6個はアミノ酸残基の置換を伴うミスセンス多型で，N末端側から順に，T339R（c.1016C＞G），Q448E（c.1342C＞G），P475S（c.1423C＞T），P618A（c.1852C＞G），S903L（c.2708C＞T），G1181R（c.3541G＞A）であった（**表1**）．

次に，それぞれのMAFを求めるため，ADAMTS13活性を測定した3616検体でTaqMan法によるジェノタイピングを行った．Q448Eが最も高頻度でMAFは0.192，続いて，P475Sが0.050，S903Lが0.048，T339RとP618Aが0.027，G1181Rが0.022であった．T339RとP618Aはほぼ完全に連鎖不平衡を示し（$r^2 = 0.97$），他のミスセンスSNPは互いに強い相関を示さなかった（$r^2 < 0.11$）．

表1　日本人のADAMTS13遺伝子ミスセンス多型と血漿ADAMTS13活性

多型	遺伝型	男性			女性		
		n	活性±SD（%）	*P*	*n*	活性±SD（%）	*P*
T339R	TT	1589	93 ± 24	0.236	1808	106 ± 27	0.116
	TR+RR	87	90 ± 23		107	103 ± 28	
Q448E	QQ	1099	91 ± 24	＜0.0001	1226	104 ± 26	＜0.0001
	QE+EE	578	97 ± 25		687	111 ± 28	
P475S	PP	1535	94 ± 24	＜0.0001	1734	108 ± 27	＜0.0001
	PS+SS	152	79 ± 20		195	92 ± 24	
P618A	PP	1466	93 ± 25	0.488	1713	107 ± 27	0.120
	PA+AA	79	91 ± 23		96	103 ± 29	
S903L	SS	1504	93 ± 25	0.125	1750	106 ± 27	0.283
	SL+LL	174	90 ± 23		166	108 ± 25	
G1181R	GG	1612	93 ± 24	0.863	1834	106 ± 27	0.981
	GR+RR	72	94 ± 27		82	106 ± 29	

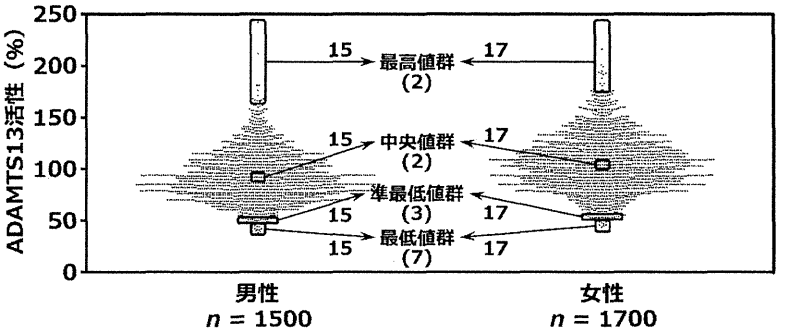

図4　先天性 ADAMTS13 欠損症者数の推定
ADAMTS13 活性を指標にして 3200 人から 128 人（32 人×4 群）を抽出し，ADAMTS13 遺伝子の塩基配列を解析した．括弧内の数字は，各群に同定された稀な非同義変異のアレル数．

4. ADAMTS13 遺伝子多型と活性

1 つの対象者集団で ADAMTS13 活性と遺伝子多型の両データを得ることができたので，活性と多型の相関を調べた（**表 1**）．活性を低下させる多型として知られる P475S[16] の保有者は，今回の解析でも，男女とも非保有者に比べて約 15％の活性低下を示した．これは，組換え ADAMTS13-P475S 変異体が正常型の約 70％の活性を示すという結果[20] とよく一致する．また，Q448E 保有者では，男女とも 6～7％の活性上昇が見られた．これは，多検体を解析することによって今回初めて得られた知見である．P475S の活性低下と Q448E の活性上昇は軽微なレベルなので，これらを保有していることで TTP 発症リスクが影響を受ける可能性は低いと思われる．これら 2 個以外の 4 個のミスセンス多型は，血漿 ADAMTS13 活性と有意な相関を示さなかった（**表 1**）．

5. 先天性 ADAMTS13 欠損症者数の推定

日本に USS 患者は何人いるのか．USS のように，症状が類似した疾患が多く，しかも程々に稀な遺伝性疾患の場合，発症頻度や患者数を明らかにすることは意外に難しい．浸透率（遺伝子異常を持っている場合に発症する率）が不明なので，先天性 ADAMTS13 欠損者数＝USS 患者数とは言い切れないが，今回の吹田研究のデータから先天性 ADAMTS13 欠損者数の推定を試みた．

ADAMTS13 活性を測定した全検体のうち，DNA が利用可能な 3200 人（男性 1500 人，女性 1700 人）を母集団とし，その中から 4 群を抽出した（**図 4**）．まず，男女それぞれ ADAMTS13 活性が最も低い 15 人あるいは 17 人を合わせた 32 人を最低値群（母集団の 1％に相当）とした．さらに，男女それぞれ最低値群の直上に位置する 15 人と 17 人の計 32 人を準最低値群として，中央値を中心とした男性 15 人と女性 17 人の計 32 人を中央値群として，活性が最も高かった男性 15 人と女性 17 人の計 32 人を最高値群として抽出した．各群の活性平均値は，最低値群 47.1％，準最低値群 53.1％，中央値群 97.6％，最高値群 183％であった．これら 128 人（32 人×4 群）の ADAMTS13 遺伝子をシーケンシング解析した結果，70 人がミスセンス多型の少なくとも 1 個を保有しており，P475S のみ，有意に群間の差異を示した（$P = 0.028$）．そして，興味深いことに，128 人のうち 14 人が，稀な非同義変異アレルをヘテロ接合体として保有していた．その内訳は，最低値群に 7 人（F324L，F418L，I673F，Q773X，Y1074AfsX46，R1095Q，S1314L），準最低値群に 3 人（I380T，Y1074AfsX46，R1274C），中央値群に 2 人（Q723K，N1321S），最高値群に 2 人（L19F，R268Q）で，うち I673F と Y1074AfsX46 は，USS 原因変異として我々がすでに報告したものと同じであった[19) 21)]．この 2 変異以外はすべて未報告の変異であった．

この結果から先天性ADAMTS13欠損症者の頻度を推定するために，以下の前提条件を仮定した．1）中央値群32人および最高値群32人にもそれぞれ2人の非同義変異アレル保有者が存在したことから，ADAMTS13活性域に関係なく32人に2人の割合で，活性に無害の変異アレルの保有者（ヘテロ接合体）が存在する．2）最低値群32人のうち5人（7人－2人）および準最低値群32人のうち1人（3人－2人）は活性を低下させる変異アレルを保有している．3）その6人（5人＋1人）以外に，活性を低下させる変異アレルの保有者は母集団（3200人）に存在しない．

これらの前提条件が正しいとすれば，日本の一般住民3200人のうち6人の割合で，ADAMTS13遺伝子異常アレルのヘテロ接合体が存在することになる．したがって，劣性遺伝形式を示す先天性ADAMTS13欠損症者，すなわち異常アレルのホモ接合体あるいは複合ヘテロ接合体の頻度は，約110万人に1人（＝6/3200×6/3200×1/4）と推定される．これは，日本に約110人の先天性ADAMTS13欠損症者が存在することを示す．しかしながら，このような頻度を正確に見積もるには，3200人という母集団は小さいかもしれない．例えば，この計算のもととなったヘテロ接合体の存在頻度6/3200が5/3200あるいは7/3200であれば，日本の先天性ADAMTS13欠損症者数は80人ないし160人となる．このような推定がどのくらい正しいか分からないが，これまでに日本で同定されたUSS患者数が40人強であることを考えると，実態から大きくかけ離れていないのではないかと思われる．

6. おわりに

本稿では，TTP患者ではなく，一般住民（全員が健常者とは限らない）を対象にして，ADAMTS13活性と遺伝子多型について紹介した．典型的なTTP症例ではADAMTS13活性の極端な低下（5%未満あるいは10%未満）が危機的症状の要因となる．一方，中程度に低下したADAMTS13活性（例えば10～30%）は，敗血症や肝疾患など種々の疾患の病態や重症度に対して何らかの影響を与えている可能性がある[22) 23)]．また，ADAMTS13の活性や抗原量が播種性血管内凝固症候群や慢性肝炎などTTP以外の疾患の発症や再発，予後予測などのマーカーになる可能性もある[24) 25)]．それらを考える上で，一般住民のADAMTS13に関するデータが役立つことを期待する．なお，本稿で紹介した吹田研究の成果は，対象としてご協力いただいた市民の皆様や，国立循環器病研究センター・分子病態部・宮田敏行部長，予防健診部・小久保喜弘医長をはじめとして，多くの方々との共同作業で得られたものであり，ここに感謝の意を表します．

Disclosure of Conflict of Interests

The authors indicated no potential conflict of interest.

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More on: racial differences in venous thromboembolism

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We read with great interest the review article by Zakai and McClure [1] regarding racial differences in venous thromboembolism (VTE). While racial differences in the incidence of VTE have been reported, the race-specific genetic risk factors for VTE have remained obscure until recently. Factor V Leiden mutation and prothrombin G20210A mutation are commonly found in populations of European origin as modest genetic risk factors for VTE [1], and Asians have neither mutation. Although the incidence of VTE in African-Americans is more than 5-fold greater than that in Asian-ancestry populations, and European and Hispanic populations are at intermediate risk of VTE, Asian populations may have race-specific mutations for VTE. Recently, we identified a protein S K196E mutation as a genetic risk factor for VTE in a Japanese population [2–4]. As Zakai and McClure have described a high prevalence of protein C and protein S deficiency in Japanese, Taiwanese and Thai populations with VTE, the protein S K196E mutation can account for the high prevalence of protein S deficiency in Japanese subjects. An *in vitro* study showed that the recombinant protein S with the K196E mutation lost activated protein C-dependent anticoagulant activity [5].

So far, three independent case–control studies, all performed in Japan, have reached the same conclusion – that the protein S K196E mutation is a risk factor for VTE, with odds ratios between 3.74 and 8.56 [2–4]. The prevalence of this mutation has been examined in four cohorts, and 77 heterozygous carriers have been identified in 4319 Japanese subjects [2–4,6], indicating a mutant allele frequency of 0.0089. None were homozygotes. The total population of Japan is now 127 million. Therefore, approximately 10 000 Japanese may be homozygotes. Three VTE patients homozygous for protein S K196E mutation have been identified with a prevalence of one homozygote out of approximately 85 VTE patients [2,3]. One homozygote showed 78% total protein S antigen, 94% free protein S antigen, and 35% protein S anticoagulant activity [2]. Another homozygote showed 39% protein S anticoagulant activity but no data on antigen levels. The third homozygote

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did not have any antigen or activity data [3]. The protein S K196E mutation can be found in VTE patients with congenital protein C deficiency, facilitating the development of VTE [4,7], and is frequently present in VTE patients with pregnancy [8]. The genotype-phenotype study of the Japanese general population showed that the individuals heterozygous for the mutant E-allele had 16% lower protein S anticoagulant activity than wild-type subjects [9].

The protein S K196E mutation seems to be race specific, because so far this mutation has not been identified in a Caucasian population. We believe that the protein S K196E mutation should be present in other east Asian populations, including the Chinese and Koreans; however, so far there are no reports on those populations. Some genetic mutations specific to eastern Asian populations, in particular the plasminogen A610 T mutation with an allele frequency of 0.020 in Japanese subjects and the ADAMTS13 P475S mutation with an allele frequency of 0.050 in Japanese subjects, have been reported in other east Asian populations [10]. These mutations are low-frequency variations, with allele frequencies between 0.05% and 0.5% [11]. Factor V Leiden mutation and prothrombin G20210A mutation in Caucasian populations are also regarded as low-frequency variations. Thus, as previously described [11], the low-frequency genetic variations would be important for specific phenotypes such as VTE, and the protein S K196E mutation is a race-specific genetic risk factor for VTE. Recent genomewide genetic analysis in Asian populations revealed the southern migration route to eastern Asia [12]. The low-frequency genetic variation should have occurred recently and should be fixed within a specific population. This accumulating body of evidence strongly suggests that genetic studies should be carried out in each ethnic population and that studies of common variations as well as low-frequency variations would be valuable.

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This proof-of-principle study illustrates that NGS enables rapid genetic diagnosis of a PFD in a single test. In this example, we were able to restrict SNV mapping to a subgroup of 57 genes implicated in secretion, so that it was feasible to use Sanger sequencing to determine whether each potential SNV was a true-positive or a false-positive call. Although successful in HPS, restricting analysis to a subgroup of genes is likely to reduce the diagnostic yield of NGS for other PFDs where it is less easy to select a candidate gene list. Strategies such as whole-exome sequencing may circumvent this difficulty by increasing the overall sensitivity of NGS for pathogenic SNVs. However, increasing the number of mapped genes is also expected to yield significantly larger numbers of irrelevant SNVs and false-positive calls. Alternative NGS strategies require further evaluation in other PFDs to determine the optimum diagnostic and cost-effective approach.

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The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. The 216 candidate PFD genes.
Data S2. The accessible internet-based resources used in this work.

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RGS2 deficiency in mice does not affect platelet thrombus formation at sites of vascular injury

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RGS2 is a member of the regulator of G-protein signaling (RGS) protein family that plays a key role in the regulation of G-protein-coupled receptors. RGS2 inhibits Gq-mediated and Gi-mediated signaling by activating the intrinsic GTPase of the Gα subunit [1,2]. RGS2 also interacts with Gs and adenylyl cyclase, and suppresses Gs signaling independently of GTPase-activating protein activity [2,3]. In platelets, the activation of the Gq-coupled or Gi-coupled receptors by thrombin, thromboxane A_2 and ADP stimulates platelet aggregation, whereas the activation of the Gs-coupled prostacyclin receptor inhibits aggregation [4,5]. Recently, active roles of RGS proteins in regulating platelet G-protein-coupled receptors have been

reported [6,7]. Mice carrying a G186S mutation in the α subunit of Gi2 that impairs RGS–Gi2 interactions showed increased platelet aggregation in vitro and enhanced platelet thrombus formation after vascular injury *in vivo* [6]. These results indicate that platelet RGS proteins are essential for regulating thrombogenesis at the sites of vascular injury. A G23D mutation in the human *RGS2* gene was reported to increase the proportion of RGS2 isoforms that strongly interact with adenylyl cyclase. Platelets from carriers of this mutation showed decreased Gs activity after stimulation of the prostacyclin receptor, suggesting that RGS2 is a negative regulator of platelet Gs signaling [7]. In this study, we analyzed platelet thrombus formation in RGS2-deficient mice to fully characterize the physiologic importance of RGS2 in platelets.

RGS2-deficient ($Rgs2^{-/-}$) mice on the C57BL/6J genetic background [8,9] were provided by M. E. Medelsohn (Tufts University School of Medicine). Blood samples were collected from male mice aged 8–12 weeks. All animal procedures were approved by the Animal Care and Use Committees of the National Cerebral and Cardiovascular Center. We confirmed the presence of RGS2 transcripts in platelets of wild-type C57BL/6J ($Rgs2^{+/+}$) mice by RT-PCR (data not shown), as previously reported in rat platelets [10]. No RGS2 transcripts were detected in platelets of $Rgs2^{-/-}$ mice. Platelet counts were (810 ± 77) × 10^9 L^{-1} (mean ± standard deviation [SD], n = 20) for $Rgs2^{+/+}$ mice and (781 ± 84) × 10^9 L^{-1} (n = 20) for $Rgs2^{-/-}$ mice, with no significant difference ($P > 0.05$ by Student's t-test) between the genotypes.

We studied platelet Gs activity by using whole-blood aggregation-inhibition analysis. Platelet aggregation in whole blood was measured by a screen filtration pressure method with a WBA-Neo aggregometer (ISK, Tokyo, Japan) [11,12]. Four different concentrations (100, 200, 400 and 800 μg L^{-1}) of the Gs agonist prostaglandin E_1 (PGE_1) were added to whole blood 1 min before aggregation was initiated with 4 mg L^{-1} collagen. The 50% inhibitory concentrations (IC_{50}) of PGE_1 calculated from the dose–response curve were 308 ± 34 μg L^{-1} (mean ± SD, n = 8) for $Rgs2^{+/+}$ mice and 330 ± 75 μg L^{-1} (n = 8) for $Rgs2^{-/-}$ mice, with no significant difference between the genotypes. We also examined the inhibitory responses to PGE_1 on collagen-induced platelet aggregation in platelet-rich plasma, as previously reported in humans [7]. Aggregation was monitored by light transmission with an MCM Hema Tracer 704 aggregometer (Tokyo Koden, Tokyo, Japan), as described previously [13]. Again, the responses were comparable in $Rgs2^{+/+}$ mice (IC_{50} = 6.46 ± 2.77 μg L^{-1}, mean ± SD, n = 4) and $Rgs2^{-/-}$ mice (IC_{50} = 8.72 ± 2.69 μg L^{-1}, n = 4), suggesting normal platelet Gs activity in $Rgs2^{-/-}$ mice.

To clarify the consequences of RGS2 deficiency in platelet thrombus formation under physiologic blood flow, we performed a whole-blood perfusion assay on a collagen-coated surface, as described previously [13,14]. Under both low (200 s^{-1}) and high (1500 s^{-1}) shear rate conditions, we did not detect any difference in either rate or extent of thrombus accumulation between $Rgs2^{+/+}$ and $Rgs2^{-/-}$ mice (Fig. 1A).

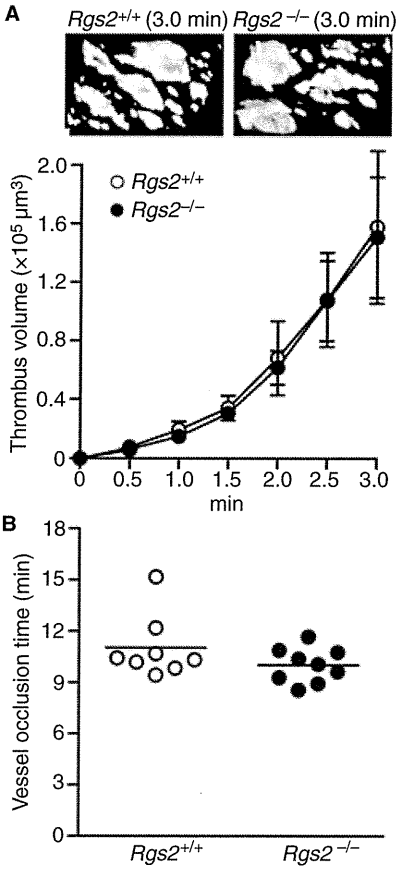

Fig. 1. Platelet thrombogenesis in RGS2-deficient mice. (A) In vitro thrombogenesis on a collagen surface under flow. Whole blood from $Rgs2^{+/+}$ or $Rgs2^{-/-}$ mice containing mepacrine-labeled platelets was perfused at a wall shear rate of 1500 s^{-1}. The cumulative thrombus volume, analyzed with a multidimensional imaging system, was measured every 0.5 min up to 3 min. Data are the mean ± standard error of the mean of 12 mice for each genotype. No significant differences were observed between $Rgs2^{+/+}$ and $Rgs2^{-/-}$ mice by two-way repeated measures analysis of variance. The typical images of thrombi at 3 min after perfusion are shown in the upper panels. The cumulative thrombus volume at a shear rate of 200 s^{-1} was also similar between the genotypes (n = 11). (B) *In vivo* thrombogenesis in ferric chloride-injured mesenteric arterioles. Endogenous platelets of live mice were fluorescently labeled with a DyLight488-conjugated rat IgG against mouse glycoprotein Ibβ, and observed in mesenteric arterioles after injury with 10% ferric chloride. The vessel occlusion time is shown. Student's t-test showed no significant differences between the genotypes. Time to first thrombus formation also did not differ between genotypes. The symbols represent data from a single mouse. The bars represent the mean values of groups.

These results suggest that RGS2 does not substantially contribute to the regulation of platelet thrombogenesis under flow. Similar results were obtained *in vivo* in thrombosis experiments using intravital microscopy. We applied 10% ferric chloride topically to the mesenteric arterioles of mice aged 3–5 weeks. Platelet thrombus formation in the arterioles was monitored with a fluorescence microscope and a CCD camera system, as described previously [14]. The diameters and shear rates of the studied arterioles were 123.2 ± 17.7 μm and 1291 ± 163 s^{-1} (mean ± SD, n = 8) for $Rgs2^{+/+}$ mice, and 118.3 ± 13.0 μm and 1299 ± 222 s^{-1} (n = 9) for $Rgs2^{-/-}$

mice, with no significant difference between genotypes. Both the time to first thrombus formation (> 30 μm) and the vessel occlusion time following injury were comparable between *Rgs2*$^{+/+}$ and *Rgs2*$^{-/-}$ mice, indicating that RGS2 does not play a significant role in the regulation of thrombogenesis at the site of arteriolar injury (Fig. 1B).

These results suggest that RGS2 deficiency has no impact on hemostasis *in vivo*. Platelets are thought to express at least 10 different RGS proteins; therefore, other RGS proteins are potentially important.

Addendum

F. Banno: designed research, performed experiments, analyzed and interpreted data, wrote the paper, and approved the final version to be published; T. Nojiri: performed experiments, analyzed data, and approved the final version to be published; S. Matsumoto: performed experiments, analyzed data, and approved the final version to be published; K. Kamide: interpreted data and approved the final version to be published; T. Miyata: designed research, interpreted data, wrote the paper, and approved the final version to be published.

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Disclosure of Conflict of Interests

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Derlin-1 Deficiency Is Embryonic Lethal, Derlin-3 Deficiency Appears Normal, and Herp Deficiency Is Intolerant to Glucose Load and Ischemia in Mice

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Abstract

Accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) causes a cellular condition called ER stress. To overcome ER stress, unfolded proteins are eliminated by an ER-associated degradation (ERAD) system. To explore the physiological requirements for ERAD-related membrane proteins in mammals, we generated Derlin-1–, Derlin-3–, and Herp-deficient mice by gene targeting. Complete loss of Derlin-1 caused embryonic lethality at around E7–E8 (early somite stages). In contrast, Derlin-3– and Herp-deficient mice were born alive with the expected Mendelian frequency, and were superficially indistinguishable from wild-type mice. However, in the Derlin-3– and Herp-deficient mouse organs, the expression levels of ERAD-related proteins were affected under both normal and ER stress conditions; specific effects differed among the organs. Degradation of ERAD substrates was reduced in the Herp-deficient liver, and Herp-deficient mice exhibited impaired glucose tolerance and vulnerability to brain ischemic injury, both of which are known to be implicated in ER stress. Our findings indicate that ERAD or uncharacterized functions involving Derlin-1 are essential in early embryonic development. Derlin-3– and Herp-deficient mice may become useful model animals for investigations of the physiological contribution of ERAD under stressful or pathological conditions.

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Introduction

Unfolded or misfolded proteins accumulated in the endoplasmic reticulum (ER) can induce ER stress, which leads to defects in protein homeostasis. To overcome ER stress, the cells operate several survival systems, one of which is a disposal mechanism called ER-associated degradation (ERAD). In ERAD, misfolded proteins are recognized in the ER and retrotranslocated to the cytosol to be degraded by the ubiquitin-proteasome system [1,2,3,4]. Multiple proteins participate in the process; in particular, ER-membrane proteins including HRD1, Derlin, and Herp work together in functional complexes termed ERAD complexes.

HRD1 is a transmembrane E3 ubiquitin ligase that mediates ubiquitination of ERAD substrates. Yeast Hrd1p/Der3p is essential for degradation of HMG-CoA reductase and mutated carboxypeptidase Y (CPY*) [5,6]. A protein complex consisting of Hrd1p, Hrd3p, Der1p, and Usa1p deals with ER-luminal misfolded proteins; Hrd1p is the central membrane component of this complex [7]. Mammalian HRD1 also functions in ERAD, forming a complex with other proteins [8,9,10].

Mammalian Derlin family proteins, Derlin-1, Derlin-2, and Derlin-3, are orthologs of yeast Der1p, which was identified as a gene that complemented a yeast mutant that cannot degrade CPY* [11]. All Derlin family members contain transmembrane domains and are embedded in the ER membrane [12,13]. In human, Derlin-1 and Derlin-2 mRNAs were expressed ubiquitously, and expression of Derlin-3 mRNA was relatively restricted to several tissues including placenta, pancreas, spleen, and small intestine [13]. Previous reports have delineated their functional roles in ERAD. Derlin-1 is the prime candidate for the ERAD retrotranslocation [12,14]: anti-Derlin-1 antibody inhibited retrotranslocation of ERAD substrates in an *in vitro* retrotranslocation assay [15], and overexpression of a dominant-negative Derlin-1 mutant inhibits ERAD [12]. Overexpression of Derlin-2 or Derlin-3 accelerated ERAD of misfolded glycoproteins, and knockdown of either gene inhibited the degradation [13].

Herp was first reported as an ER stress–responsive protein expressed in cultured human vascular endothelial cells [16]. Herp is a representative target gene of the unfolded protein response (UPR); the induction of Herp mRNA expression following ER stress is mediated via two *cis*-acting ER stress–response elements, ERSE and ERSE-II [17]. Although Herp may play a broad range of biological roles [18,19,20,21,22], recent reports focus on its function in ERAD. Degradation of several ERAD substrates, including connexin 43 [20], CD3δ [9,23], κLC [24], polycystin-2 [25], and α1-antitrypsin null Hong Kong (NHK) [10] is dependent

on Herp. Usa1p, the yeast ortholog of Herp, may be the scaffold protein of the functional complex containing Hrd1p [26]; it is also involved in the degradation of Hrd1p itself [27].

Although growing evidence suggests that ERAD-complex components play important roles in cellular functions, little data exist regarding their systemic significance in mammals. HRD1-deficient mice die *in utero* around embryonic day 13.5, largely due to impairment of definitive erythropoiesis [28]. Derlin-2–deficient mice exhibit perinatal lethality and represented only 4% of mice at weaning [29]. To understand the physiological roles of the Derlin family members and Herp, we generated three mouse knockouts.

Results and Discussion

Offspring from the Cross of Heterozygous Mice

To generate Derlin-1–, Derlin-3–, and Herp-deficient mice, we constructed targeting vectors for disruption of the mouse *Derl1*, *Derl3*, and *Herpud1* genes (Figure 1). All the F1 heterozygous mice, $Derl1^{+/-}$, $Derl3^{+/-}$, and $Herpud1^{+/-}$, were viable and grew with no apparent phenotypic abnormalities under normal breeding conditions. Next, the heterozygous mice were crossed to generate homozygous mutants, and the genotypes of newborn offspring were determined by genomic PCR. From these matings, $Derl3^{-/-}$ and $Herpud1^{-/-}$ newborn mice were born in the expected Mendelian ratios, whereas $Derl1^{-/-}$ mice were not detected, indicating that the complete loss of Derlin-1 severely affected normal embryogenesis (Table 1).

Table 1. Genotype of offspring from the cross of heterozygous mice.

Cross	Genotype			P^a
	+/+	+/−	−/−	
$Derl1^{+/-} \times Derl1^{+/-}$	76	157	0	1.3×10^{-17}
$Derl2^{+/-} \times Derl2^{+/-}$	90	156	0	7.2×10^{-19}
$Derl3^{+/-} \times Derl3^{+/-}$	56	101	39	0.21
$Herpud1^{+/-} \times Herpud1^{+/-}$	76	156	71	0.81

[a]Chi-square test.

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Early Embryonic Death of $Derl1^{-/-}$

To identify the embryonic stages at which $Derl1^{-/-}$ mice die, we analyzed the embryos in uteri of pregnant $Derl1^{+/-}$ female mice that had been crossed with $Derl1^{+/-}$ male mice (Table 2). We collected embryos and determined their genotypes by PCR. In the $Derl1^{+/-}$ crosses, only three (2.6%) $Derl1^{-/-}$ embryos were detected at E10.5, whereas 23 $Derl1^{+/+}$ and 60 $Derl1^{+/-}$ embryos were present. At E8.5, however, the numbers of embryos, 13 ($Derl1^{+/+}$), 28 ($Derl1^{+/-}$), and 11 ($Derl1^{-/-}$), were not significantly different from the expected Mendelian frequency. This suggested that most $Derl1^{-/-}$ embryos are resorbed between E8.5 and E10.5. Indeed, by E10.5, the $Derl1^{+/-}$ crosses resulted in a high frequency (26.5%) of the embryonic resorption compared with 4.5% and 1.9% resorption in wild-type C57BL/6 and ICR, respectively (Table 2). Genotypes of the resorbed embryos were not determined, to avoid miscounting caused by commingling of maternal tissues with embryos. If resorbed embryos were assumed to be $Derl1^{-/-}$, the numbers of embryos [23 ($Derl1^{+/+}$), 60 ($Derl1^{+/-}$), and 34 ($Derl1^{-/-}$)] conformed to the expected Mendelian frequency. The appearances of embryos collected at E8.5 are shown in Figure 2. In contrast to the normal development of $Derl1^{+/+}$ and $Derl1^{+/-}$ embryos, all 11 $Derl1^{-/-}$ embryos showed obvious delay in their development; they appeared as wild-type up to E7.5. Western blotting analysis of the embryos at E7.5 demonstrated the presence of Derlin-1 in $Derl1^{+/+}$ and $Derl1^{+/-}$ and the absence in $Derl1^{-/-}$ embryos (Figure S1). In conclusion, the $Derl1^{-/-}$ mice were embryonic lethal at E7–E8.

In contrast, $Derl3^{-/-}$ and $Herpud1^{-/-}$ mice were born and grew normally. These findings indicated that the requirement for each Derlin in embryogenesis was different: Derlin-1 was essential in the early developmental stages (the beginning of the organogen-

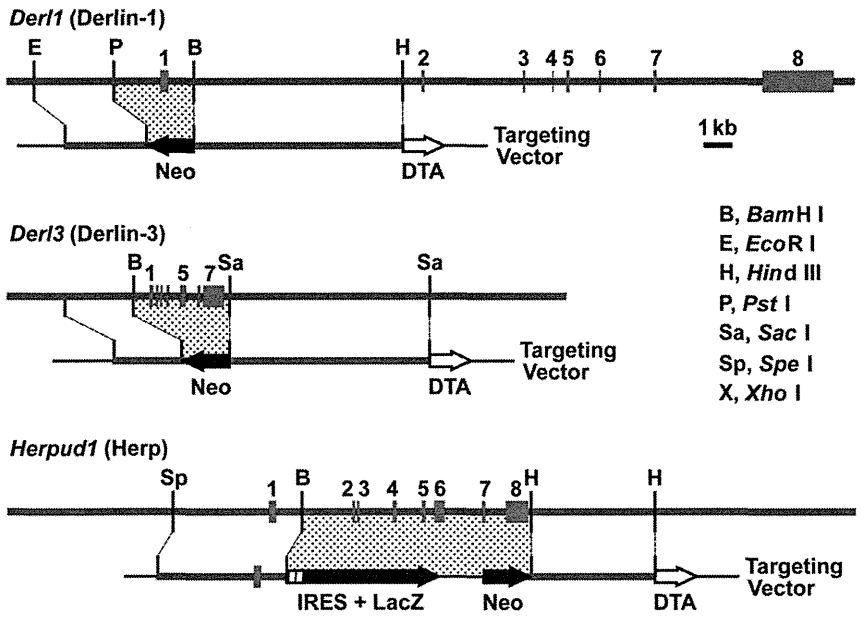

Figure 1. Targeted disruption of Derlin-1, Derlin-3, and Herp. Structures of the mouse *Derl1*, *Derl3*, and *Herpud1* loci, and the targeting vectors for disrupting each gene. Neo; neomycin resistance gene, IRES; internal ribosome entry site, DTA; diphtheria toxin A chain.

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Table 2. Genotype of embryos from the cross of *Derl1*+/− heterozygous mice.

Cross	Embryonic age	Number of embryos	Number of each genotype (%)				P^a	P^b
			+/+	+/−	−/−	Resorbed		
Derl1$^{+/-}$×*Derl1*$^{+/-}$	E10.5	117	23 (19.7)	60 (51.3)	3 (2.6)	31 (26.5)	1.2×10^{-5}	0.34
	E8.5	55	13 (23.6)	28 (50.9)	11 (20.0)	3 (5.5)	0.79	0.97
B6[c]×B6	E10.5	67	64 (95.5)	–	–	3 (4.5)	–	–
ICR[d]×ICR	E10.5	155	152 (98.1)	–	–	3 (1.9)	–	–

The heterozygous mice were mated and the embryos in uteri were genotyped. Resorbed and rudimentary embryos were not genotyped.
[a]Chi-square test of +/+, +/−, and −/−, not including resorbed embryos.
[b]Chi-square test using +/+, +/−, and −/−, counting resorbed embryos as −/−.
[c]C57BL/6JJcl wild-type mice.
[d]Jcl:ICR wild-type mice.
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esis), deletion of Derlin-2 results in perinatal death [29], and Derlin-3 was not essential in development. The loss of Derlin-3 may be compensated by Derlin-1 or Derlin-2. Our findings also indicated that Derlin-1, Derlin-3, and Herp are not essential for cell survival, because even embryonically lethal *Derl1*$^{-/-}$ survived until at least E7.5. This is consistent with the fact that Der1p, Dfm1p (Der1p homolog), and Usa1p are not essential for yeast survival.

Effects of Derlin-3 and Herp Deficiency in Adult Mouse Organs

Although both *Derl3*$^{-/-}$ and *Herpud1*$^{-/-}$ mice were born and grew normally, deficiency of Derlin-3 or Herp may affect functionally related proteins. To investigate the effect of the knockouts on the expression levels of ERAD-related proteins, we prepared tissue homogenates and total RNA from the liver, pancreas, and kidneys of control wild type (WT), *Derl3*$^{-/-}$, and *Herpud1*$^{-/-}$ male mice that had been injected intraperitoneally 12 h before dissection with either PBS (control) or the protein N-glycosylation inhibitor tunicamycin (Tm), in order to induce pharmacological ER stress. The samples were subjected to Western blotting (Figures 3 and S2) and RT-PCR analyses (Figure 4).

In the WT liver, protein levels of Derlin-3 and Herp were quite low under normal conditions (Figure 3, left panels, C), but dramatically elevated 12 h after Tm treatment (left panels, Tm). HRD1, GRP78 (an ER-resident molecular chaperone), and VIMP (VCP-interacting membrane protein) were also increased by Tm treatment in WT mice. Similarly, in the *Derl3*$^{-/-}$ and *Herpud1*$^{-/-}$ liver, the levels of HRD1, GRP78, and VIMP were increased by Tm treatment. Although the Herp level was also increased by Tm treatment in the *Derl3*$^{-/-}$ liver, its levels were reduced by approximately 30% as compared with those in the Tm-treated WT (Figure S2), suggesting some physical or functional interactions between Derlin-3 and Herp. In the *Herpud1*$^{-/-}$ liver, regardless of Tm treatment, HRD1 level was higher than in WT. This may be partly explained by the fact that degradation of yeast Hrd1p is dependent on Usa1p (Herp ortholog) [27]. The levels of Derlin-1, Derlin-2, and AAA-ATPase p97/VCP were little affected by Derlin-3 or Herp deficiencies in the liver.

In the pancreas (Figure 3, middle panels), the levels of Derlin-1 and Derlin-2 were remarkably decreased by deficiency of Derlin-3 but not Herp. In the *Herpud1*$^{-/-}$ pancreas, the level of VIMP was lower than in WT and *Derl3*$^{-/-}$ pancreas, whereas the levels of Derlin-3, HRD1, and GRP78 were higher. In all three mouse strains, Tm had very little effect on expression levels of ERAD-related proteins in the pancreas, in contrast to the situation in the liver and kidneys. Although Derlin-3 was almost absent in the liver (left panels) and kidneys (right panels) under normal conditions, Derlin-3 could be clearly detected in the pancreas even without Tm treatment (middle panels). In the kidneys (right panels), responses to Tm treatment were broadly similar to those in the liver (left panels), but Tm-dependent upregulation of Derlin-3, GRP78, and VIMP in the kidneys was moderate compared to the liver. Thus, these findings indicated that the responses to pharmacologically induced ER stress vary among different organs.

The differences among organs in the effects of Tm and genetic deficiencies were observed not only at the protein level but also the mRNA level: the splicing of XBP1 mRNA, an indicator of the activation of ER stress sensor IRE1 [30], was induced by Tm treatment in the liver and kidneys, but not in the pancreas, of all the three mouse strains (Figures 3 and 4). The mRNA levels of other ERAD-related proteins were also investigated by RT-PCR (Figure 4): some of them (e.g., Derlin-3 and Herp mRNAs) exhibited dynamic changes similar to those observed in their protein levels, in all three organs; others (e.g., Derlin-1, Derlin-2, and HRD1 mRNAs) exhibited changes distinct from those observed in their protein levels. The decrease in the Derlin-1 and Derlin-2 protein levels in the *Derl3*$^{-/-}$ pancreas was likely

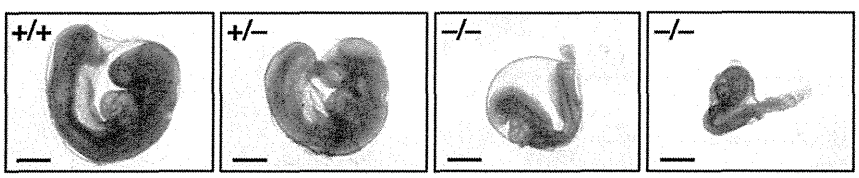

Figure 2. Stereomicroscopic appearance of Derlin-1-deficient mouse embryos. *Derl1*$^{-/-}$ embryos at E8.5 exhibited developmental delay and resorption, respectively, whereas *Derl1*$^{+/+}$ and *Derl1*$^{+/-}$ embryos were normal. Bars, 50 μm.
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