脈の閉塞を抑制しコラーゲン・エピネフリン混合液の静注によるマウスの死亡を減弱させた[20]. この物質は出血量には影響しないことから，血栓を惹起するポリリン酸を迅速に中和する抗血栓物質と考えられた．また，17万個以上の低分子化合物ライブラリーと42個の陽イオン性化合物をスクリーニングすることにより，generation 1.0 cationic PAMAM dendrimerを含め，数種の化合物がポリリン酸阻害剤として同定された[21]. 今後，これらの低分子化合物の抗血栓作用に関する研究が進むと考えられる．

D. ポリリン酸の生合成にかかわる酵素とその遺伝子欠損マウスの解析

酵母ではポリリン酸は乾燥重量の10～20%にまで達し，主に液胞に蓄えられている[6]. 酵母のポリリン酸の量はイノシトールピロリン酸に依存している．最も豊富に含まれているイノシトールピロリン酸は，イノシトールピロリン酸5-IP_7 (5-diphosphoinositol pentakisphosphate) であり，これはinositol hexakisphosphate (IP_6) からIP_6キナーゼの作用により合成される．IP_6キナーゼを欠損している酵母はイノシトールポリリン酸が欠乏し，ポリリン酸量が大きく減少する．哺乳動物にはIP_6キナーゼ遺伝子は3つある．そのうち，IP_6キナーゼ1遺伝子欠損マウスは低体重，インスリン量低下，精子形成欠損を示し，胚性線維芽細胞のIP_7量が30%にまで減少した．この遺伝子欠損マウスのATPとADPに量的な変化はなかったが，ポリリン酸量は明らかに減少していた．血小板機能や血栓能を調べたところ，トロンビン刺激による血小板Pセレクチンの表出とADP刺激によるインテグリンの活性化には差がみられなかったものの，トロンビン刺激による洗浄血小板の凝集能は低下し，ADP刺激した血小板の上清は血漿の凝固時間の延長能を示した．この遺伝子欠損マウスでは，尾の出血時間が延長し，ADP静注による肺塞栓モデルで高い生存率を示した．これらの結果から，IP_6キナーゼは血小板のポリリン酸濃度を制御し，哺乳動物の止血に働いていることが明らかになった[22]. 哺乳動物では，ポリリン酸の生合成に関わる酵素類は明らかではなく，IP_6キナーゼがどのような機構でポリリン酸量を変化させるかは不明である．

むすび

ここで述べたように，ポリリン酸は内因系凝固反応惹起物質であり，トロンビンによるXIとVのフィードバック活性化の促進物質である．また，プレカリクレインを活性化することによりブラジキニンを産生する炎症惹起物質でもある．ポリリン酸は血小板放出反応の結果として凝固系に作用するので，ポリリン酸の血小板由来の凝固促進因子としての機能は，永らく不明であった1次止血と2次止血の間を繋ぐものであり，血栓塞栓症と炎症性疾患の治療に新しい考えを示すものである．

さて，止血反応や血栓形成におけるポリリン酸の役割を考えてみたい．組織因子が露出した部位にフィブリンができる．その部位に接着した血小板は，組織因子を覆って隠してしまう．3次元に成長する血栓内でフィブリン形成が継続的に進むためには，凝固の開始反応が継続する必要があり，血小板から放出されるポリリン酸はXII活性化を惹起して継続的なフィブリンの形成に寄与すると考えられる．フィブリン形成時にポリリン酸がないと機械的に弱いフィブリン線維になり，塞栓のリスクが高くなるとも考えられる．これまで，凝固反応は組織因子-VIIa複合体でだけ始まると記述されてきたが，ポリリン酸-XIIでもトロンビン産生とフィブリン形成が起こるので，生体内の血栓形成時の凝固反応機構をもう一度考え直す必要があるだろう．組織因子-VIIaとポリリン酸-XII

のどちらがフィブリン形成に重要であるかは，直接比べることはできないが，血管床と血管障害機序によって異なるだろうと考えられる．

ポリリン酸は血小板にだけ含まれているのではない．約60個のリン酸からなるポリリン酸がラットbasophilic leukemia（好塩基性白血病）マスト細胞に同定された[23]．このポリリン酸はヒスタミン含有顆粒中ではなく，セロトニン含有顆粒中に存在した．この顆粒はacidocalcisomesに酷似する．マスト細胞由来のポリリン酸はブラジキニン形成能をもつ．ポリリン酸はヒト好塩基球中にも検出された[23]．

DNA複製の研究でノーベル賞を受賞した故Arthur Kornberg博士は，1990年代になってDNA複製から微生物のポリリン酸へ研究を移し，ポリリン酸の研究を進め，亡くなる2カ月前にポリリン酸に関する総説をまとめた[24]．ポリリン酸の凝固や炎症での機能は，いまだdiscovery phaseである．最近，ポリリン酸は細胞外ヒストンによる血小板の活性化を増強し[25]，NF-κB活性化を介した炎症反応を引き出す[26]と報告された．今後，更に多くのポリリン酸の病態生理学的な機能が同定されると考えられる．ポリリン酸は血栓領域だけでなく，多くの分野の医薬品開発の標的物質と考えられるであろう．

文献

1) Ruiz FA, Lea CR, Oldfield E, et al. Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. J Biol Chem. 2004; 279: 44250-7.
2) Smith SA, Mutch NJ, Baskar D, et al. Polyphosphate modulates blood coagulation and fibrinolysis. Proc Natl Acad Sci U S A. 2006; 103: 903-8.
3) Muller F, Mutch NJ, Schenk WA, et al. Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. Cell. 2009; 139: 1143-56.
4) Morrissey JH. Polyphosphate: a link between platelets, coagulation and inflammation. Int J Hematol. 2012; 95: 346-52.
5) Morrissey JH, Choi SH, Smith SA. Polyphosphate: an ancient molecule that links platelets, coagulation, and inflammation. Blood. 2012; 119: 5972-9.
6) Brown MR, Kornberg A. Inorganic polyphosphate in the origin and survival of species. Proc Natl Acad Sci U S A. 2004; 101: 16085-7.
7) Hernandez-Ruiz L, Saez-Benito A, Pujol-Moix N, et al. Platelet inorganic polyphosphate decreases in patients with delta storage pool disease. J Thromb Haemost. 2009; 7: 361-3.
8) Xu J, Zhang X, Pelayo R, et al. Extracellular histones are major mediators of death in sepsis. Nat Med. 2009; 15: 1318-21.
9) Fuchs TA, Brill A, Duerschmied D, et al. Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci U S A. 2010; 107: 15880-5.
10) Caen J, Wu Q. Hageman factor, platelets and polyphosphates: early history and recent connection. J Thromb Haemost. 2010; 8: 1670-4.
11) Mutch NJ, Myles T, Leung LLK, et al. Polyphosphate binds with high affinity to exosite II of thrombin. J Thromb Haemost. 2010; 8: 548-55.
12) Naito K, Fujikawa K. Activation of human blood coagulation factor XI independent of factor XII. Factor XI is activated by thrombin and factor XIa in the presence of negatively charged surfaces. J Biol Chem. 1991; 266: 7353-8.
13) Gailani D, Broze GJ, Jr. Factor XI activation in a revised model of blood coagulation. Science. 1991; 253: 909-12.
14) Choi SH, Smith SA, Morrissey JH. Polyphosphate is a cofactor for the activation of factor XI by thrombin. Blood. 2011; 118: 6963-70.
15) Mutch NJ, Engel R, de Willige SU, et al. Polyphosphate modifies the fibrin network and down-regulates fibrinolysis by attenuating binding of tPA and plasminogen to fibrin. Blood. 2010; 115: 3980-8.
16) Smith SA, Morrissey JH. Polyphosphate enhances fibrin clot structure. Blood. 2008; 112: 2810-6.
17) Montilla M, Hernandez-Ruiz L, Garcia-Cozar FJ, et al. Polyphosphate binds to human von Willebrand factor in vivo and modulates its interac-

tion with glycoprotein Ib. J Thromb Haemost. 2012; 10: 2315-23.
18) Smith SA, Choi SH, Davis-Harrison R, et al. Polyphosphate exerts differential effects on blood clotting, depending on polymer size. Blood. 2010; 116: 4353-9.
19) Muhl L, Galuska SP, Oorni K, et al. High negative charge-to-size ratio in polyphosphates and heparin regulates factor VII-activating protease. FEBS J. 2009; 276: 4828-39.
20) Jain S, Pitoc GA, Holl EK, et al. Nucleic acid scavengers inhibit thrombosis without increasing bleeding. Proc Natl Acad Sci U S A. 2012; 109: 12938-43.
21) Smith SA, Choi SH, Collins JNR, et al. Inhibition of polyphosphate as a novel strategy for preventing thrombosis and inflammation. Blood. 2012; 120: 5103-10.
22) Ghosh S, Shukla D, Suman K, et al. Inositol hexakisphosphate kinase 1 maintains hemostasis in mice by regulating platelet polyphosphate levels. Blood. 2013; 122: 1478-86.
23) Moreno-Sanchez D, Hernandez-Ruiz L, Ruiz FA, et al. Polyphosphate is a novel pro-inflammatory regulator of mast cells and is located in acidocalcisomes. J Biol Chem. 2012; 287: 28435-44.
24) Rao NN, Gomez-Garcia MR, Kornberg A. Inorganic polyphosphate: Essential for growth and survival. Annu Rev Biochem. 2009; 78: 605-47.
25) Semeraro F, Ammollo CT, Morrissey JH, et al. Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. Blood. 2011; 118: 1952-61.
26) Bae JS, Lee W, Rezaie AR. Polyphosphate elicits pro-inflammatory responses that are counteracted by activated protein C in both cellular and animal models. J Thromb Haemost. 2012; 10: 1145-51.

ultrastructural and functional characterization. *J Thromb Haemost* 2010; **8**: 173–84.
13 Mangin P, Yap CL, Nonne C, Sturgeon SA, Goncalves I, Yuan Y, Schoenwaelder SM, Wright CE, Lanza F, Jackson SP. Thrombin overcomes the thrombosis defect associated with platelet GPVI/FcRgamma deficiency. *Blood* 2006; **107**: 4346–53.
14 Kalia N, Auger JM, Atkinson B, Watson SP. Critical role of FcR gamma-chain, LAT, PLCgamma2 and thrombin in arteriolar thrombus formation upon mild, laser-induced endothelial injury *in vivo*. *Microcirculation* 2008; **15**: 325–35.
15 Konstantinides S, Ware J, Marchese P, Mus-Jacobs F, Loskutoff DJ, Ruggeri ZM. Distinct antithrombotic consequences of platelet glycoprotein Ibalpha and VI deficiency in a mouse model of arterial thrombosis. *J Thromb Haemost* 2006; **4**: 2014–21.
16 Massberg S, Gawaz M, Gruner S, Schulte V, Konrad I, Zohlnhofer D, Heinzmann U, Nieswandt B. A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall *in vivo*. *J Exp Med* 2003; **197**: 41–9.
17 Eckly A, Hechler B, Freund M, Zerr M, Cazenave JP, Lanza F, Mangin PH, Gachet C. Mechanisms underlying FeCl(3) -induced arterial thrombosis. *J Thromb Haemost* 2011; **9**: 779–89.
18 Inoue O, Suzuki-Inoue K, McCarty OJ, Moroi M, Ruggeri ZM, Kunicki TJ, Ozaki Y, Watson SP. Laminin stimulates spreading of platelets through integrin alpha6beta1-dependent activation of GPVI. *Blood* 2006; **107**: 1405–12.
19 Brill A. A ride with ferric chloride. *J Thromb Haemost* 2011; **9**: 776–8.
20 Wong LC, Langille BL. Developmental remodeling of the internal elastic lamina of rabbit arteries: effect of blood flow. *Circ Res* 1996; **78**: 799–805.
21 Marsh LE, Lewis SD, Lehman ED, Gardell SJ, Motzel SL, Lynch JJ Jr. Assessment of thrombin inhibitor efficacy in a novel rabbit model of simultaneous arterial and venous thrombosis. *Thromb Haemost* 1998; **79**: 656–62.
22 Pinel C, Wice SM, Hiebert LM. Orally administered heparins prevent arterial thrombosis in a rat model. *Thromb Haemost* 2004; **91**: 919–26.
23 Wang X, Cheng Q, Xu L, Feuerstein GZ, Hsu MY, Smith PL, Seiffert DA, Schumacher WA, Ogletree ML, Gailani D. Effects of factor IX or factor XI deficiency on ferric chloride-induced carotid artery occlusion in mice. *J Thromb Haemost* 2005; **3**: 695–702.
24 Pozgajova M, Sachs UJ, Hein L, Nieswandt B. Reduced thrombus stability in mice lacking the alpha2A-adrenergic receptor. *Blood* 2006; **108**: 510–4.

von Willebrand factor-to-ADAMTS13 ratio increases with age in a Japanese population

K. KOKAME,* T. SAKATA,† Y. KOKUBO‡ and T. MIYATA*
**Department of Molecular Pathogenesis, †Laboratory of Clinical Chemistry* and *‡Department of Preventive Cardiology, National Cerebral and Cardiovascular Center, Suita, Osaka, Japan*

To cite this article: Kokame K, Sakata T, Kokubo Y, Miyata T. von Willebrand factor-to-ADAMTS13 ratio increases with age in a Japanese population. *J Thromb Haemost* 2011; **9**: 1426–8.

ADAMTS13, a plasma metalloprotease, regulates platelet aggregation through shear stress-dependent specific cleavage of von Willebrand factor (VWF) multimers. Severe deficiency of plasma ADAMTS13 activity results in accumulation of unusually high-molecular-weight VWF multimers in plasma, and can cause a systemic disease, thrombotic thrombocytopenic purpura (TTP) [1–3]. Previously, we developed a simple and quantitative assay for measuring ADAMTS13 activity using FRETS-VWF73, a fluorogenic peptide substrate [4]. In this study, we used the assay to measure plasma ADAMTS13 activity in 3616 individuals from the Japanese general population.

We used plasma samples from the previously published Suita Study [5–7], an epidemiological study consisting of randomly selected Japanese residents of the city of Suita, which is located in the second largest urban area in Japan. Participants between the ages of 30 and 79 years were randomly selected from the municipality population registry and stratified into groups by sex and age in 10-year increments in 1989. They underwent regular health check-ups between September 1989 and March 1994. Subjects have continued to visit the National Cerebral and Cardiovascular Center every 2 years for regular health check-ups. Our study protocol was approved by the ethical review committee, and only subjects who provided written informed consent for genetic analyses were included.

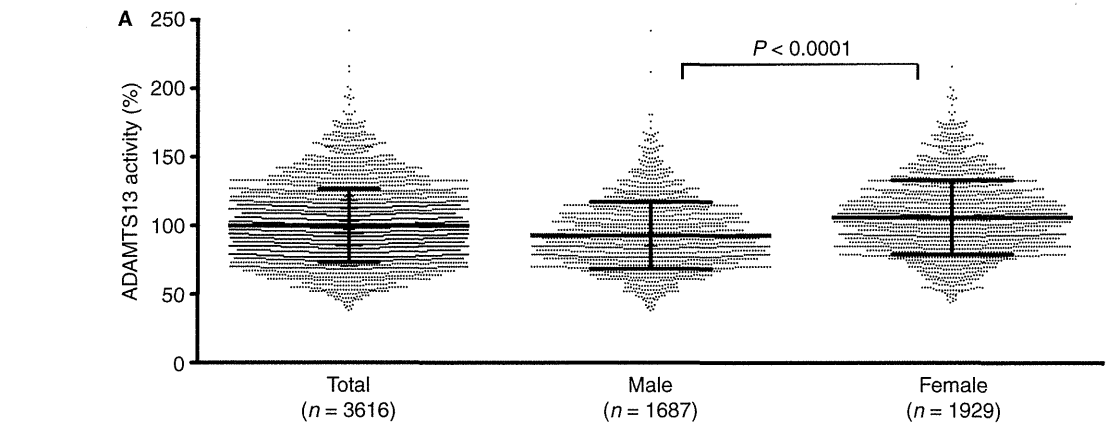
When the mean of all plasma ADAMTS13 activity values was set at 100%, the standard deviation (SD) was 27% (Fig. 1A). The fifth percentile, 25th percentile, median, 75th percentile and 95th percentile were 61%, 81%, 97%, 116% and 148%, respectively. The mean activity of men (93 ± 24%, mean ± SD, n = 1687) was significantly lower ($P < 0.0001$) than that of women (106 ± 27%, n = 1929), consistent with the previous report [4].

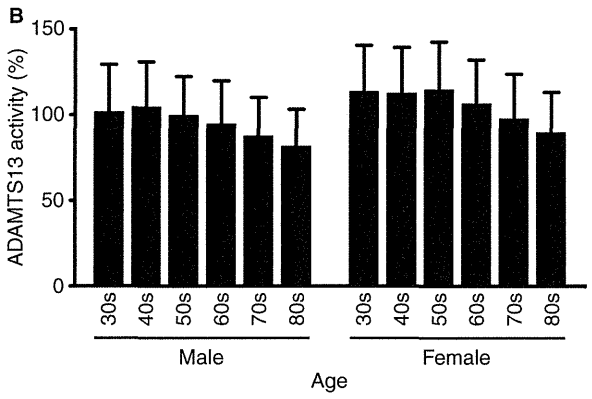
In both men and women, the plasma ADAMTS13 activity tended to decrease with age, especially after age 60 (Fig. 1B). A linear regression model also indicated the decrease with age (regression coefficient of −0.642 and 95% confidence intervals (CI) of −0.740 to −0.544 in men; −0.663 and −0.767 to −0.558 in women). We also measured plasma VWF antigen levels

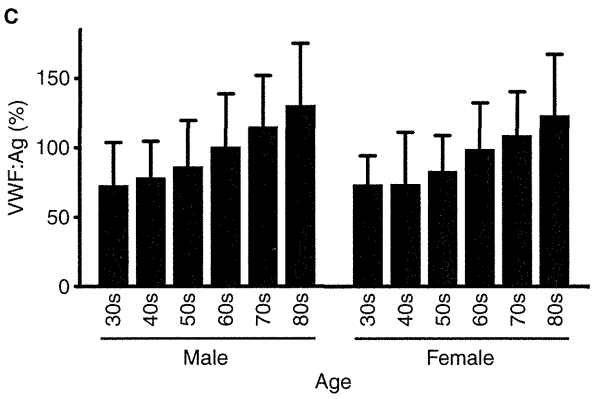
Correspondence: Koichi Kokame, Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan.
Tel.: +81 6 6833 5012; fax: +81 6 6835 1176.
E-mail: kame@ri.ncvc.go.jp

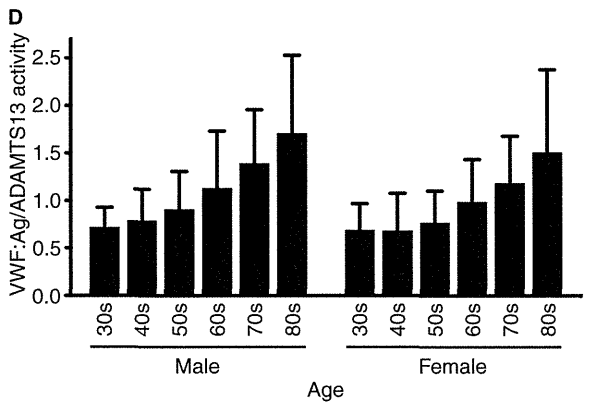
DOI: 10.1111/j.1538-7836.2011.04333.x

Received 30 March 2011, accepted 24 April 2011



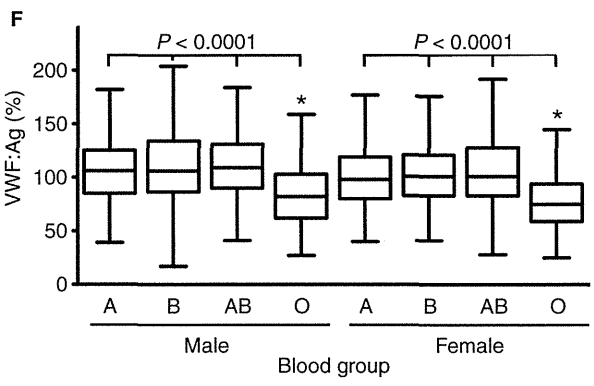






E

		Q1	Q2	Q3	Q4
Male $n = 1687$	ADAMTS13 activity (%)	38–76	76–90	90–109	109–242
	VWF:Ag (%) ± SE	107 ± 11	106 ± 11	108 ± 11	111 ± 11
		$P = 0.153$			
Female $n = 1929$	ADAMTS13 activity (%)	44–86	86–104	104–123	123–216
	VWF:Ag (%) ± SE	103 ± 9	99 ± 10	99 ± 10	101 ± 9
		$P = 0.670$			



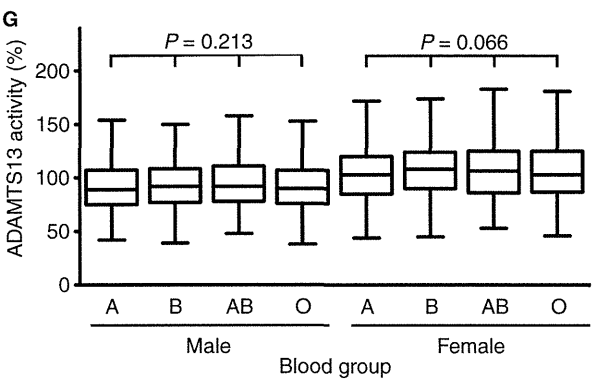


Fig. 1. Plasma ADAMTS13 activity in a Japanese general population. (A) Scatter dot plot of total, male and female plasma ADAMTS13 activity. The mean of all values was set at 100%. Lines indicate the means with SD. *P*, *t*-test. (B, C, D) Age-specific ADAMTS13 activity (B), VWF:Ag (C) and VWF:Ag-to-ADAMTS13 activity ratio (D). The mean of all VWF:Ag values was set at 100%. Error bars indicate SD. (E) Analysis of VWF:Ag by quartiles of plasma ADAMTS13 activity. *P*, ANCOVA. (F, G) Box-and-whisker plot of blood group-specific VWF:Ag (F) and ADAMTS13 activity (G). *P*, Kruskal–Wallis test. *Only blood group O had significantly different VWF:Ag from other blood groups.

(VWF:Ag) of all subjects using an immuno-turbidimetric assay, STA LIATEST VWF (Diagnostica Stago, Parsippany, NJ, USA). In both men and women, VWF:Ag increased with age (Fig. 1C), as reported previously [8]. The linear regression coefficient was 1.37 (95% CI, 1.21–1.52) in men and 1.30 (1.17–1.42) in women. Because of combined effects of the increase in VWF:Ag and the decrease in ADAMTS13 activity, the VWF:Ag-to-ADAMTS13 activity ratio was dramatically increased with age (Fig. 1D). This may partly explain the prothrombotic state of elderly men and women, because the imbalance between VWF and ADAMTS13 may be involved in thrombotic diseases such as acute myocardial infarction [9], advanced liver cirrhosis [10] and coronary artery disease [11]. As the FRETS-VWF73 assay itself was not affected by VWF concentration in plasma samples (0–160 $\mu g\ mL^{-1}$, data not shown), the reduced ADAMTS13 activity in the plasma of elderly subjects was not considered to be due to the assay-dependent artifactual phenomenon. In fact, when age-adjusted VWF:Ag was compared among quartiles of ADAMTS13 activity in the population using SPSS Statistics (IBM, Tokyo, Japan), no significant association between VWF:Ag and ADAMTS13 activity was observed in men (ANCOVA, $P = 0.153$) or in women ($P = 0.670$) (Fig. 1E).

ABO blood group has a significant influence on VWF:Ag; individuals with blood group O have lower VWF:Ag values than those of non-O groups [8]. We genotyped the ABO blood group by TaqMan assay (Applied Biosystems, Tokyo, Japan), which detects two polymorphisms, c.261Gdel and c.526C > G, on *ABO*. As expected, the subjects of blood group O exhibited a significantly lower VWF:Ag (men, 88 ± 43%; women, 80 ± 28%, mean ± SD) than those of the other blood groups (men A, 110 ± 35%; men B, 112 ± 36%; men AB, 115 ± 44%; women A, 103 ± 37%; women B, 105 ± 36%; women AB, 106 ± 31%) (Fig. 1F). In contrast, the plasma ADAMTS13 activity in men (A, 92 ± 24%; B, 94 ± 24%; AB, 96 ± 24%; O, 93 ± 25%) and women (A, 104 ± 26%; B, 109 ± 28; AB, 109 ± 28%; O, 106 ± 28) was not significantly associated with ABO blood group (Fig. 1G). This is consistent with the finding that ADAMTS13 antigen levels are not associated with ABO blood group in 387 male subjects [12]. The results are also consistent with the fact that VWF [13] but not ADAMTS13 [14] contains ABO blood group-related N-linked oligosaccharides.

In conclusion, this study demonstrated that, in the Japanese general population, the plasma ADAMTS13 activity is lower in men than in women, decreases with age, and is not significantly associated with ABO blood group. The VWF:Ag-to-ADAMTS13 activity ratio is increased with age in both men and women, and this increase may be involved in the prothrombotic state of elderly individuals.

Acknowledgements

This work was supported by grants-in-aid from the Ministry of Health, Labour, and Welfare of Japan; from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan.

Disclosure of conflict of interests

The authors state that they have no conflict of interest.

References

1 Sadler JE. von Willebrand factor, ADAMTS13, and thrombotic thrombocytopenic purpura. *Blood* 2008; **112**: 11–8.
2 Moake J. Thrombotic microangiopathies: multimers, metalloprotease, and beyond. *Clin Transl Sci* 2009; **2**: 366–73.
3 Tsai HM. Pathophysiology of thrombotic thrombocytopenic purpura. *Int J Hematol* 2010; **91**: 1–19.
4 Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T. FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. *Br J Haematol* 2005; **129**: 93–100.
5 Sakata T, Mannami T, Baba S, Kokubo Y, Kario K, Okamoto A, Kumeda K, Ohkura N, Katayama Y, Miyata T, Tomoike H, Kato H. Potential of free-form TFPI and PAI-1 to be useful markers of early atherosclerosis in a Japanese general population (the Suita Study): association with the intimal-medial thickness of carotid arteries. *Atherosclerosis* 2004; **176**: 355–60.
6 Kimura R, Kokubo Y, Miyashita K, Otsubo R, Nagatsuka K, Otsuki T, Sakata T, Nagura J, Okayama A, Minematsu K, Naritomi H, Honda S, Sato K, Tomoike H, Miyata T. Polymorphisms in vitamin K-dependent γ-carboxylation-related genes influence interindividual variability in plasma protein C and protein S activities in the general population. *Int J Hematol* 2006; **84**: 387–97.
7 Kokubo Y, Kamide K, Okamura T, Watanabe M, Higashiyama A, Kawanishi K, Okayama A, Kawano Y. Impact of high-normal blood pressure on the risk of cardiovascular disease in a Japanese urban cohort: the Suita study. *Hypertension* 2008; **52**: 652–9.
8 Gill JC, Endres-Brooks J, Bauer PJ, Marks WJ Jr, Montgomery RR. The effect of ABO blood group on the diagnosis of von Willebrand disease. *Blood* 1987; **69**: 1691–5.
9 Matsukawa M, Kaikita K, Soejima K, Fuchigami S, Nakamura Y, Honda T, Tsujita K, Nagayoshi Y, Kojima S, Shimomura H, Sugiyama S, Fujimoto K, Yoshimura M, Nakagaki T, Ogawa H. Serial changes in von Willebrand factor-cleaving protease (ADAMTS13) and prognosis after acute myocardial infarction. *Am J Cardiol* 2007; **100**: 758–63.
10 Uemura M, Fujimura Y, Matsumoto M, Ishizashi H, Kato S, Matsuyama T, Isonishi A, Ishikawa M, Yagita M, Morioka C, Yoshiji H, Tsujimoto T, Kurumatani N, Fukui H. Comprehensive analysis of ADAMTS13 in patients with liver cirrhosis. *Thromb Haemost* 2008; **99**: 1019–29.
11 Miura M, Kaikita K, Matsukawa M, Soejima K, Fuchigami S, Miyazaki Y, Ono T, Uemura T, Tsujita K, Hokimoto S, Sumida H, Sugiyama S, Matsui K, Yamabe H, Ogawa H. Prognostic value of plasma von Willebrand factor-cleaving protease (ADAMTS13) antigen levels in patients with coronary artery disease. *Thromb Haemost* 2010; **103**: 623–9.
12 Chion CKNK, Doggen CJM, Crawley JTB, Lane DA, Rosendaal FR. ADAMTS13 and von Willebrand factor and the risk of myocardial infarction in men. *Blood* 2007; **109**: 1998–2000.
13 Matsui T, Titani K, Mizuochi T. Structures of the asparagine-linked oligosaccharide chains of human von Willebrand factor: occurrence of blood group A, B, and H(O) structures. *J Biol Chem* 1992; **267**: 8723–31.
14 Hiura H, Matsui T, Matsumoto M, Hori Y, Isonishi A, Kato S, Iwamoto T, Mori T, Fujimura Y. Proteolytic fragmentation and sugar chains of plasma ADAMTS13 purified by a conformation-dependent monoclonal antibody. *J Biochem* 2010; **148**: 403–11.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 29, pp. 26158–26165, July 22, 2011

NDRG4 Protein-deficient Mice Exhibit Spatial Learning Deficits and Vulnerabilities to Cerebral Ischemia*[S]

Received for publication, May 2, 2011, and in revised form, May 29, 2011 Published, JBC Papers in Press, June 2, 2011, DOI 10.1074/jbc.M111.256446

Hitomi Yamamoto[‡], Koichi Kokame[‡1], Tomohiko Okuda[‡], Yukako Nakajo[§], Hiroji Yanamoto[§2], and Toshiyuki Miyata[‡]

From the [‡]Department of Molecular Pathogenesis and [§]Laboratory of Neurology and Neurosurgery, National Cerebral and Cardiovascular Center, Suita, Osaka 565-8565, Japan

The N-myc downstream-regulated gene (NDRG) family consists of four related proteins, NDRG1-NDRG4, in mammals. We previously generated NDRG1-deficient mice that were unable to maintain myelin sheaths in peripheral nerves. This condition was consistent with human hereditary motor and sensory neuropathy, Charcot-Marie-Tooth disease type 4D, caused by a nonsense mutation of NDRG1. In contrast, the effects of genetic defects of the other NDRG members remain unknown. In this study, we focused on NDRG4, which is specifically expressed in the brain and heart. *In situ* mRNA hybridization on the brain revealed that NDRG4 was expressed in neurons of various areas. We generated NDRG4-deficient mice that were born normally with the expected Mendelian frequency. Immunochemical analysis demonstrated that the cortex of the NDRG4-deficient mice contained decreased levels of brain-derived neurotrophic factor (BDNF) and normal levels of glial cell line-derived neurotrophic factor, NGF, neurotrophin-3, and TGF-β1. Consistent with BDNF reduction, NDRG4-deficient mice had impaired spatial learning and memory but normal motor function in the Morris water maze test. When temporary focal ischemia of the brain was induced, the sizes of the infarct lesions were larger, and the neurological deficits were more severe in NDRG4-deficient mice compared with the control mice. These findings indicate that NDRG4 contributes to the maintenance of intracerebral BDNF levels within the normal range, which is necessary for the preservation of spatial learning and the resistance to neuronal cell death caused by ischemic stress.

N-myc downstream-regulated gene (NDRG)[3] family members NDRG1-NDRG4 are intracellular proteins, consist of 340–394 amino acid residues, and share 53–65% sequence identity with each other. Furthermore, accumulating evidence implicates their roles in development, cancer metastasis, and the immune system (1–5).

We originally identified RTP (NDRG1) as a homocysteine-responsive gene in human umbilical vein endothelial cells (6), which is also called DRG1, Cap43, Rit42, Ndr1, and PROXY-1. NDRG1 expression is induced by a number of conditions, such as DNA damage, hypoxia, and intracellular calcium ion elevation (4). Overexpression of NDRG1 suppresses the metastatic potency of some types of cancer cells (4) and enhances the degranulation of mast cells in response to various stimuli (7). A nonsense mutation of *NDRG1* causes hereditary motor and sensory neuropathy, Charcot-Marie-Tooth disease type 4D, which presents as distal muscle wasting and atrophy, foot and hand deformities, tendon areflexia, sensory loss, and deafness in afflicted individuals (8). We previously generated NDRG1-deficient mice and revealed the essential role of NDRG1 in the cytoplasm of Schwann cells for the maintenance of myelin sheaths in peripheral nerves (9). A frame shift deletion of *Ndrg1* in Greyhounds also causes polyneuropathy (10).

Similar to NDRG1, the expression of NDRG2 is induced by stress conditions such as hypoxia (1). NDRG2 expression is up-regulated in cortical pyramidal neurons, senile plaques, and the cellular process of dystrophic neurons in the Alzheimer's brain, whereas expression is decreased in the rat frontal cortex after antidepressant treatment and electroconvulsive therapy (11). NDRG2 also plays a role in aldosterone-mediated epithelial sodium channel function (12), dendritic cell differentiation (13), and insulin action (14). NDRG3, on the other hand, may play a role in spermatogenesis because it is found in the outer layers of the seminiferous epithelium (3). Overexpression of NDRG3 contributes to the angiogenesis of tumors via up-regulation of chemokines (3).

In contrast to other NDRG members, NDRG4 expression is detected specifically in the brain and heart (15). In the embryonic mouse heart, NDRG4 expression is down-regulated under severe ventricular hypoplasia caused by Tbx2 misexpression, implying that NDRG4 is involved in cell growth and proliferation (16). However, information on the physiological function of NDRG4 is lacking. In the mouse brain, NDRG4 is identified in the neuronal cytoplasm of the cerebrum and cerebellum (17). Down-regulation of NDRG4 in PC12 cells results in extending shorter neurites in response to NGF (18). Considering that NDRG4 expression is induced by treatment with homocysteine in rat aortic smooth muscle cells (19), we speculated that

* This work was supported by grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan; from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

[1] To whom correspondence may be addressed: 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Fax: 81-6-6835-1176; E-mail: kame@ri.ncvc.go.jp.

[2] To whom correspondence may be addressed: 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Fax: 81-6-6835-6894; E-mail: hyanamot@res.ncvc.go.jp.

[3] The abbreviations used are: NDRG, N-myc downstream-regulated gene; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; NT-3, neurotrophin-3; GFAP, glial fibrillary acidic protein; MCAO, middle cerebral artery occlusion; rCBF, regional cerebral blood flow.

NDRG4 is stress-related and has a cell-protective role in neurological disorders and cerebrovascular disease. This was supported by our finding that NDRG4 mRNA expression is decreased in the brain of patients with Alzheimer's disease (15).

Neurotrophins such as brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), NGF, neurotrophin-3 (NT-3), and TGF-β1 are essential for the survival and homeostatic maintenance of central neurons (20). BDNF, especially, is a potent modulator of synaptic connectivity in the central nervous system, influencing synaptic structure and function. The reduced levels of BDNF in the entorhinal cortex or forebrain are associated with poor memory (21, 22). BDNF also has neuroprotective action in models of ischemia. Increased BDNF levels in the brain for an appropriate period prior to the ischemic insult increases the resistance of the brain against lethal stresses caused by severe ischemia (23, 24). In contrast, a deficiency in endogenous BDNF renders the brain more susceptible to ischemic injury (25) and more suppressive to infarct tolerance by the preconditioning of spreading depression (26).

In this study, we generated NDRG4-deficient mice to reveal the roles of NDRG4 in the brain. As a result, we found that under the condition of NDRG4 deficiency, mice showed impaired phenotypes in spatial learning and neuroprotection with decreased levels of BDNF.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-NDRG1 rabbit antiserum was raised against recombinant glutathione S-transferase-fusion protein of human NDRG1 (27). Anti-NDRG2, anti-NDRG3, and anti-NDRG4 rabbit antisera were raised against the synthetic peptides Q^{351}SSESGTLPSGPPGH^{365} for mouse NDRG2 (17), F^{343}SRSVTSNQSDGTQE^{357} for mouse NDRG3 (17), and C-N^{214}RPGTVPNAKTLR^{226}-$CONH_2$ for mouse NDRG4, respectively, which were conjugated with keyhole limpet hemocyanin. Polyclonal antibodies in the antisera were purified by antigen-immobilized affinity column chromatography. Anti-NeuN and anti-glial fibrillary acidic protein (GFAP) were purchased from Millipore and Dako, respectively.

Construction of the Targeting Vector—We previously isolated and characterized genomic clones carrying *NDRG4* (15). The NDRG4-B and NDRG4-B^{var} isoforms are the alternative splicing products, whereas the NDRG4-H isoform is produced by the alternative promoter usage. The initiating Met codons for NDRG4-B/B^{var} and NDRG4-H exist in exons 5 and 3, respectively. Exon 6 is common to all isoforms. The loxP-flanked pST-neoB cassette (28) was inserted within exon 6. The ~11-kb sequence was inserted into the diphtheria toxin A fragment cassette vector (29), and the DNA was linearized by SalI digestion for electroporation.

Generation of NDRG4-deficient Mice—R1 mouse embryonic stem cells (30) were electroporated with the targeting vector and selected in medium containing G418. Targeted clones were identified by Southern blotting using the Gene Images Random-prime system (GE Healthcare) with 5′- and 3′-external probes. These cells were injected into blastocysts to obtain chimeras, which were crossed with wild-type C57BL/6 mice (Japan SLC) for germ line transmission of the disrupted *Ndrg4* allele. The genotypes of the offspring were examined by PCR analysis of DNA isolated from ear biopsy using three primers; P1 (CATCTCTCCAAGAGCCAGAGTGT), P2 (AAGATGCAGCCACACTTACGATT), and P3 (AACAGTAACAGCTTCCCACATC). Heterozygous mice with the disrupted *Ndrg4* allele were backcrossed with wild-type C57BL/6 mice. The mouse experiments were approved by the Animal Care and Use Committee of the National Cerebral and Cardiovascular Center in Japan, and were performed in accordance with the institutional and national guidelines and regulations.

Western Blotting Analysis—Protein expression was analyzed by Western blotting as described previously (31). Briefly, organs perfused with PBS (10 mM sodium phosphate, 150 mM NaCl (pH 7.4)) were homogenized in SDS sample buffer (10 mM Tris-HCl, 2% SDS, 50 mM DTT, 2 mM EDTA, 0.02% bromphenol blue, 6% glycerol (pH 6.8)), boiled for 7 min, and subjected to SDS-PAGE. Proteins in the gels were transferred to an immunoblot PVDF membrane (Bio-Rad). Following a blocking step with 5% skim milk, the membrane was incubated with anti-NDRG4, anti-NDRG1, anti-NDRG2, or anti-NDRG3 and probed with HRP-labeled goat anti-rabbit IgG (Kirkegaard and Perry Laboratories). The membrane was developed using Immobilon Western chemiluminescent HRP substrate (Millipore), and chemiluminescence was detected by a LAS-3000 image analyzer (GE Healthcare).

In Situ mRNA Hybridization—Digoxigenin-labeled riboprobes were prepared for nucleotide positions 1269–1777 (NDRG4-a) and 1811–2343 (NDRG4-b) of mouse NDRG4 (NM_145602). The paraffin-embedded brain sections (6 μm thick) were dewaxed, rehydrated, and treated with proteinase K (8 μg/ml) for 30 min at 37 °C. The sections were acetylated by 0.25% acetic anhydride and hybridized with the riboprobes (300 ng/ml) for 16 h at 60 °C. Following treatment with RNase A (50 μg/ml) for 30 min at 37 °C and 0.5% blocking reagent (Roche), the sections were incubated with anti-DIG alkaline phosphate conjugate (Roche). Colorimetric reactions were performed with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate solution (Sigma), and then the sections were counterstained with Kernechtrot stain solution (Mutoh). Because NDRG4-a and NDRG4-b riboprobes exhibited quite similar performance, only the data from NDRG4-a riboprobes are shown.

Immunohistochemistry—Serial sections for *in situ* mRNA hybridization were deparaffinized, rehydrated, and boiled by microwave irradiation in 10 mM citrate buffer (pH 6.0). After incubation with 0.3% hydrogen peroxide, the sections were blocked, incubated with anti-NeuN, and stained using Histofine MOUSESTAIN Kit (Nichirei) and diaminobenzidine according to the manufacturer's instructions. Alternate sections were blocked with Protein Block Serum-Free (Dako) and the Avidin/Biotin blocking kit (Vector Laboratories), and then incubated with anti-GFAP. Following incubation with biotin-conjugated goat anti-rabbit Ig (Dako), the sections were treated with HRP-conjugated streptavidin (Nichirei) and stained with diaminobenzidine. The sections were counterstained with Mayer's hematoxylin (Mutoh).

Measurement of Neurotrophin Levels—Protein levels of BDNF, GDNF, NGF, NT-3, and TGF-β1 were measured as

described (32). The PBS-perfused cerebral cortex described above was excised from each mouse (7–18 weeks old) and homogenized. The protein levels were measured using a two-site sandwich ELISA, Emax Immunoassay System (Promega). The protein concentration in each sample was measured using a BCA protein assay kit (Thermo Scientific).

Morris Water Maze (MWM) Test—We conducted the MWM test (33) using modifications as described previously (32). In a 64 × 91 cm-sized pool of opaque water (from a non-toxic agent), a 10 × 10 cm-square-shaped platform was hidden at a fixed position 2 cm under the surface of the water. The temperature of the water was kept at 24–25 °C during the procedure. Each mouse (6–8 weeks old) performed four trials per day, over five consecutive days, without any prior or subsequent training. We defined a successful escape, *i.e.* standing on the platform, as a stop for more than 1 s with all limbs on the platform. The cut-off time in a trial was set at 300 s. Mice that failed to reach the platform in 300 s were removed from the water, and the time needed to escape to the platform (escape latency) became 300 s. In each trial, the escape latency, the total path length needed to navigate to the platform, and the maximum swimming speed were analyzed using a video-tracking system, Smart (Panlab).

Middle Cerebral Artery Occlusion (MCAO) Model—Temporary focal ischemia was induced using the three-vessel occlusion technique as described previously (34). Briefly, under halothane-inhalation anesthesia, the left middle cerebral artery of each mouse (8–19 weeks old) was cauterized at the lateral border of the olfactory tract, and bilateral common carotid arteries were clip-occluded for 15 min followed by reperfusion. After opening the skull and subsequent cauterization of the MCA, the wound for the surgical MCA obstruction was closed within 3 min to avoid hypothermic neuroprotection against reperfusion injury (35). The rectal temperature was regulated so that it stayed within the physiological range (36.5–37.5 °C) using a temperature controller (NS-TC10, Neuroscience) during the operation. The heart rate and mean blood pressure were monitored via the tail artery using indirect blood pressure meter BP-98AW (Softron).

Regional Cerebral Blood Flow (rCBF) in the Penumbra-like Peripheral Area—The rCBF was monitored using the laser-Doppler blood flowmetry meter TBF-LN1 (Unique Medical) (34). The measurement area was set in the penumbra-like peripheral area of the ischemic region at 2 mm caudal and 1 mm dorsal to the crossover point of the left middle cerebral artery and the lateral surface of the olfactory tract. The rCBF was measured just before (control), during, and after MCAO.

Cerebral Function—Twenty-four hours or 7 days after MCAO, neurological deficits were examined according to a published scoring scale, with some modifications (35). Balance in the body trunk while being lifted by the tail was graded according to the following criteria: 0, no deficit (no twisting of the body); 1, mild deficit (asymmetric twisting tendency of the body); and 2, severe deficit (repeated asymmetric twisting of the body). Motor function of the extremities while being lifted by the tail was graded as follows: 0, no deficit (symmetrical movement of the forelimbs); 1, mild deficit (intermittent asymmetrical flexion of the forelimbs); and 2, severe deficit (continuous asymmetrical flexion of the forelimbs). The neurological deficit score (from 0 to 4) comprises the sum of the grades of the balance in body trunk and motor function of extremities.

Measurement of Infarcted Volume—Mice were perfused transcardially with heparinized PBS at 24 h or 7 days after MCAO to wash out any blood components from the brain tissue, which visualizes intraluminal blood coagulation or thrombosis formation, if any. The brain was removed and cut from the frontal tip into 1-mm-thick slices. Viable tissue was stained red with 2% 2,3,5-triphenyltetrazolium chloride followed by fixation with 4% paraformaldehyde in PBS. The infarct and total hemispheric areas of each slice were measured by tracing the borders in a computer-assisted image-analysis system, WinROOF (Mitani). To assess the total infarct volume after MCAO, an edema index was calculated by dividing the total volume of the hemisphere ipsilateral to the MCAO by the volume of the contralateral hemisphere. The infarcted volume was adjusted by dividing the volume by the edema index. The value of edema index at 7 days after MCAO could be considered 1.00, as found in our previous study (35).

Statistical Analysis—Data are presented as the means ± S.D. We used unpaired Student's *t* tests for comparisons within each parameter. Probability values of < 0.05 were considered statistically significant.

RESULTS

Localization of NDRG4 in Mouse Brain—We performed *in situ* mRNA hybridization to investigate the cellular localization of NDRG4 in the adult mouse brain. NDRG4 mRNA was widely distributed in various parts of brain (Fig. 1, *A* and *B*), including the olfactory bulb, olfactory tuberculum, cerebral cortex, striatum, hippocampus, dentate gyrus, thalamus, hypothalamus, mesencephalon, cerebellum, pons, and medulla oblongata (supplemental Fig. S1). To identify the cell types that were positive for the NDRG4 riboprobe, we analyzed serial sections by *in situ* mRNA hybridization for NDRG4 (Fig. 1, *C* and *D*) in combination with immunostaining for NeuN, a marker of neurons (*E* and *F*) or GFAP, a marker of astrocytes (*G* and *H*). NDRG4 expression was mainly observed in NeuN-positive cells but not in GFAP-positive cells, indicating that NDRG4 was specifically expressed in neurons. This was consistent with our previous finding that NDRG4 protein was expressed in neurons of the cerebral cortex and Purkinje cells of the cerebellum (17).

Generation of NDRG4-deficient Mice—To elucidate the effects of NDRG4 deficiency in neuronal cells, we generated NDRG4 knockout mice using gene targeting strategies (Fig. 2*A*). The *Ndrg4* gene covers all NDRG4 protein isoforms, NDRG4-B, NDRG4-B^{var}, and NDRG4-H (15). The genomic DNA fragment encompassing exon 6, which is the most upstream common coding region of NDRG4 isoforms, was used to construct the targeting vector. The genotype was confirmed by genomic PCR analysis (Fig. 2*B*). The F1 mice with one *Ndrg4*-disrupted allele (*Ndrg4*$^{+/-}$) were backcrossed with wild-type C57BL/6 mice (*Ndrg4*$^{+/+}$). *Ndrg4*$^{+/-}$ mice were then crossed to generate the NDRG4-deficient mice (*Ndrg4*$^{-/-}$). *Ndrg4*$^{-/-}$ mice were born normally with the expected Mendelian distribution. The numbers of *Ndrg4*$^{+/+}$, *Ndrg4*$^{+/-}$, and *Ndrg4*$^{-/-}$ live births were 56, 115, and 51, respectively (p =

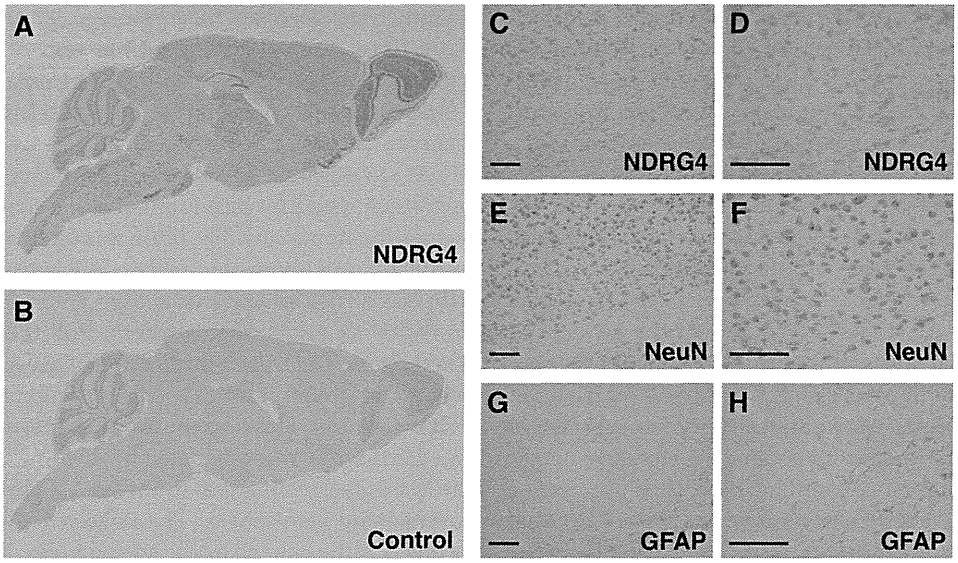


FIGURE 1. **Localization of NDRG4 in the mouse brain.** Sagittal sections were prepared from 8-week-old wild-type mice and subjected to *in situ* mRNA hybridization of NDRG4 (*A* and *B*). Digoxigenin-labeled antisense riboprobes for NDRG4 (*A*) but not sense riboprobes (*B*, negative control) produced positive signals (*blue*). The nuclei were counterstained in *red*. *C–H*, serial coronal sections of wild-type mouse brain were subjected to *in situ* mRNA hybridization of NDRG4 (*C* and *D*, *blue* signals) and immunostaining of NeuN (*E* and *F*, *brown* signals) or GFAP (*G* and *H*, *brown* signals). The nuclei were counterstained in *red* (*C* and *D*) and *blue* (*E–H*). *D*, *F*, and *H* are higher-magnification images of *C*, *E*, and *G*, respectively. *Scale bars* = 100 μm.

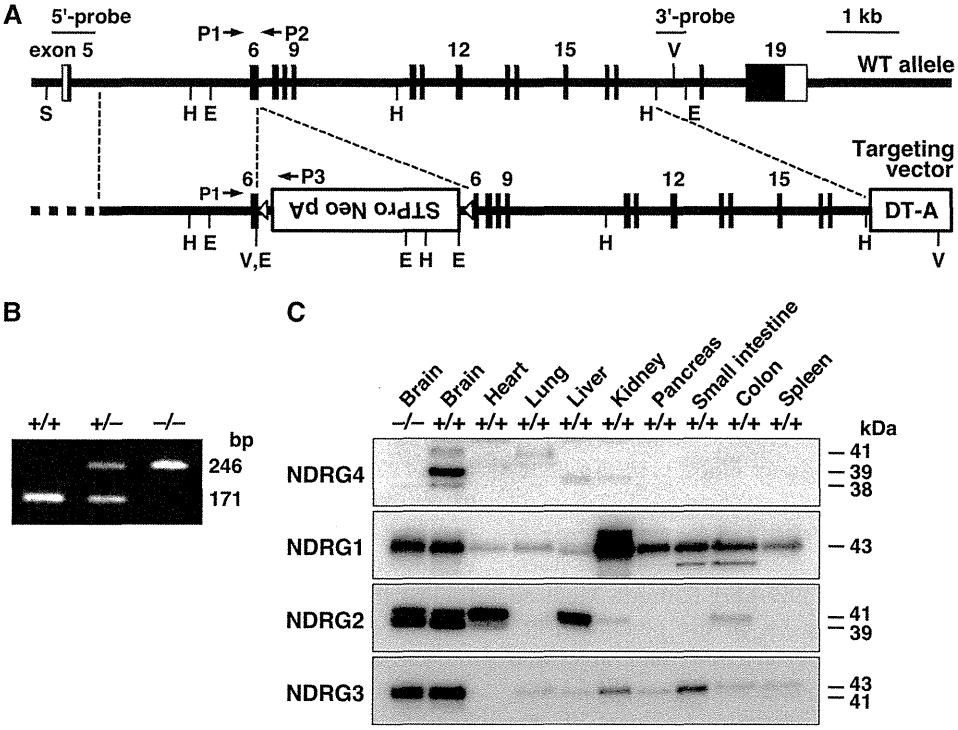


FIGURE 2. **Generation of NDRG4-deficient mice and expression pattern of NDRG family proteins in mouse organs.** *A*, targeting strategy for the *Ndrg4* gene knockout. *Solid boxes* represent open reading frames of *Ndrg4*. The loxP-flanked (*open triangles*) pSTneoB cassette with a polyadenylation signal (*STPro Neo pA*) was inserted into exon 6, and the diphtheria toxin A fragment cassette (*DT-A*) was included at the 3′ end of the vector. The 5′- and 3′-external probes used for Southern blotting selection of ES clones are shown by *bars*. The PCR primers (*P1*, *P2*, and *P3*) for genotyping are shown by *arrows*. *S*, SalI; *H*, HindIII; *E*, EcoRI; *V*, EcoRV. *B*, genotyping of wild-type (*Ndrg4*$^{+/+}$), heterozygous NDRG4-deficient (*Ndrg4*$^{+/-}$), and homozygous NDRG4-deficient (*Ndrg4*$^{-/-}$) mice. PCR amplification of the *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ alleles resulted in products of 171 and 246 bp, respectively. *C*, expression patterns of NDRG family proteins in mouse organs. Equal protein amount of organ homogenates from 17-week-old *Ndrg4*$^{-/-}$ and *Ndrg4*$^{+/+}$ mice were subjected to Western blotting analysis using each antibody. Anti-NDRG4 detected NDRG4-B (38 kDa), NDRG4-B^{var} (39 kDa), and NDRG4-H (41 kDa) in the *Ndrg4*$^{+/+}$ brain but not in the *Ndrg4*$^{-/-}$ brain. The expression of NDRG1, NDRG2, and NDRG3 was not affected by the lack of NDRG4 in the brain.

0.77, chi-square test). Both male and female *Ndrg4*$^{-/-}$ mice were fertile.

NDRGs Expression in Mouse Organs—To examine the expression patterns of NDRG family proteins in *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice, we performed a Western blotting analysis of their organs as adults. NDRG4 was specifically expressed in the brain, and little or no signal was detected in other tissues (Fig. 2*C*). The three isoforms, NDRG4-B (38 kDa), NDRG4-B^{var} (39

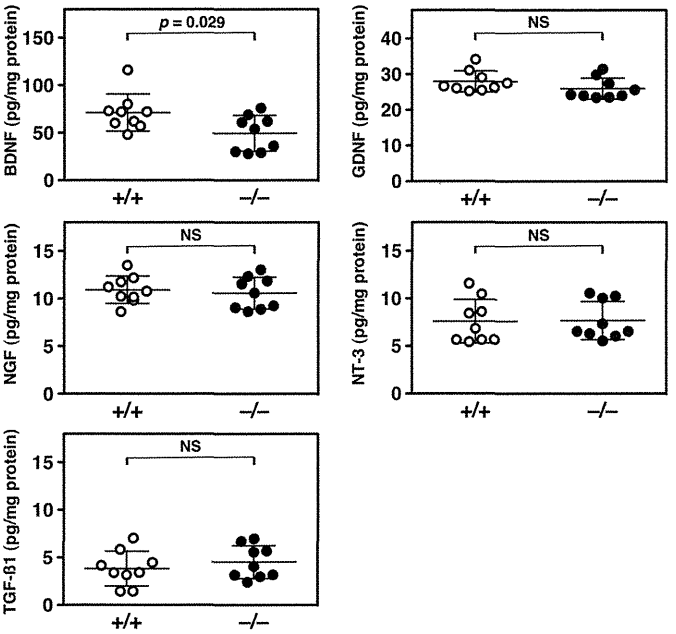


FIGURE 3. **Neurotrophins in the mouse cortex.** The protein levels of BDNF, GDNF, NGF, NT-3, and TGF-β1 in the cortex isolated from the *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice were measured by ELISA. Data are expressed as the mean ± S.D. ($n = 9$). *NS*, $p > 0.05$.

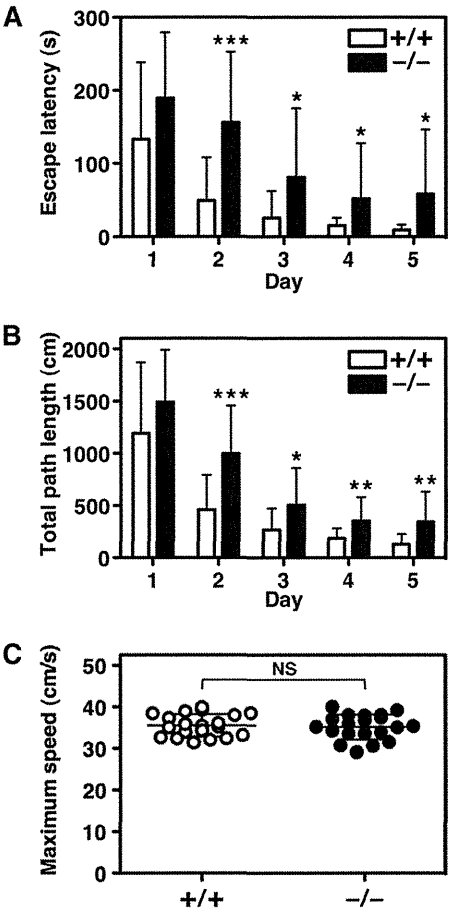


FIGURE 4. **MWM test.** Spatial learning and memory function of the *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice were tested in the MWM task. The escape latency (*A*), total path length (*B*), and maximum swimming speed (*C*) to the hidden platform on trials over five consecutive days are shown. The *Ndrg4*$^{-/-}$ mice exhibited inferior performance in escape latency and total path length in the MWM task as compared with *Ndrg4*$^{+/+}$ mice. However, the maximum swimming speed was equivalent between groups. Data are mean with error bars of S.D. ($n = 20$ in each experimental group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *NS*, $p > 0.05$.

kDa), and NDRG4-H (41 kDa), were detectable in the brain of *Ndrg4*$^{+/+}$ mice, whereas they were absent in the *Ndrg4*$^{-/-}$ brain. As described previously (15), NDRG1 was ubiquitously expressed in all tested organs. NDRG2 was mainly expressed in the brain, heart, and liver, with weaker expression in the kidney and colon. NDRG3 was observed in the brain, kidney, and small intestine. The expression levels of NDRG1, NDRG2, and NDRG3 in the brain were not affected by a lack of NDRG4, suggesting that there were no compensatory up-regulation mechanisms of gene expression.

Neurotrophin Levels—To investigate whether NDRG4 deficiency impacts brain function, we measured the protein levels of major neurotrophins in the brain (Fig. 3). The quantification of BDNF in the cortex homogenates revealed a significant decrease of BDNF in *Ndrg4*$^{-/-}$ (49.4 ± 18.8 pg/mg protein, $n = 9$) compared with *Ndrg4*$^{+/+}$ (71.1 ± 19.5 pg/mg protein, $n = 9$) mice. In contrast, the levels of GDNF, NGF, NT-3, and TGF-β1 in the cortex were not significantly different between the *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice (GDNF, 28.0 ± 3.0 *versus* 26.0 ± 3.0 pg/mg protein; NGF, 10.9 ± 1.4 *versus* 10.6 ± 1.7 pg/mg protein; NT-3, 7.6 ± 2.3 *versus* 7.7 ± 2.0 pg/mg protein; TGF-β1, 3.8 ± 1.8 *versus* 4.5 ± 1.7 pg/mg protein; $n = 9$). Therefore, we expected that abnormal regulation of BDNF protein levels may be involved in the development of the phenotypes of *Ndrg4*$^{-/-}$ mice.

Spatial Learning Ability—To confirm whether the lack of NDRG4 affects the ability of spatial learning and memory, we analyzed the performance of the mice in the MWM task. We found that escape latency to the hidden platform was significantly longer after the first trial for *Ndrg4*$^{-/-}$ mice compared with *Ndrg4*$^{+/+}$ mice (Fig. 4*A*). The total path length needed to navigate to the platform was also significantly longer in *Ndrg4*$^{-/-}$ than in *Ndrg4*$^{+/+}$ mice after the first trial (Fig. 4*B*). In contrast, there were no significant differences in the maximum swimming speed between *Ndrg4*$^{+/+}$ (35.5 ± 2.7 cm/s) and *Ndrg4*$^{-/-}$ (35.2 ± 3.0 cm/s) mice, indicating that *Ndrg4*$^{-/-}$ mice have normal sensorimotor function (Fig. 4*C*). These results indicated that poor performance of *Ndrg4*$^{-/-}$ mice in the MWM test was caused by the attenuation of spatial learning ability accompanied with BDNF reduction.

Neuronal Damage after Focal Ischemia—To elucidate whether NDRG4 is involved in the neuroprotective actions of BDNF, we explored the effect of NDRG4 deficiency on the development of neuronal damage after MCAO. We first performed transcardiac perfusion of PBS 24 h or 7 days after ischemia and confirmed that there was no thrombus formation except for the coagulated point in the proximal part of the middle cerebral artery in both *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice by visual inspection. A 2,3,5-triphenyltetrazolium chloride staining assay for viable cells at 24 h after a 15 min of MCAO demonstrated larger infarct lesion sizes in *Ndrg4*$^{-/-}$ mice compared with in *Ndrg4*$^{+/+}$ mice (Fig. 5, *A* and *B*). There were no differences in the edema index between the groups (1.07 ± 0.04 in *Ndrg4*$^{+/+}$ and 1.06 ± 0.03 in *Ndrg4*$^{-/-}$, $n = 10$). Corrobo-

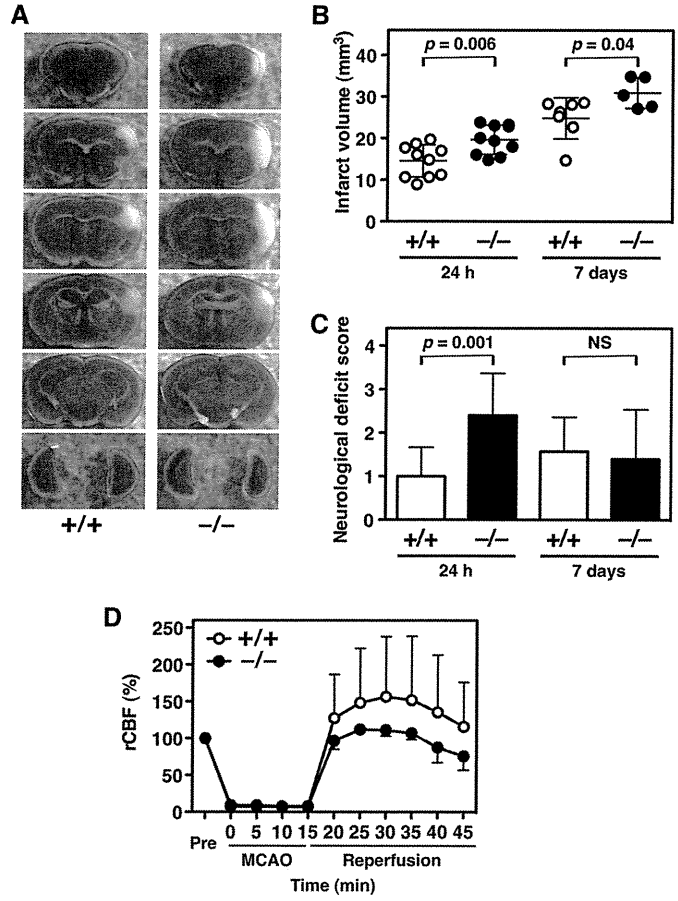


FIGURE 5. **Induction of temporary focal ischemia.** *A*, representative images of six corresponding coronal sections from *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mouse brains at 24 h after MCAO. The 1-mm-thick slices were stained with 2,3,5-triphenyltetrazolium chloride. The sizes of infarcted region (*white area*) were larger in the brain slices of *Ndrg4*$^{-/-}$ mice compared with *Ndrg4*$^{+/+}$ mice. *B*, quantification of infarct volumes (mm^3) at 24 h and 7 days after MCAO. *Ndrg4*$^{-/-}$ mice (●) had larger infarct volumes than *Ndrg4*$^{+/+}$ mice (○). Data are mean ± S.D. (n = 10). *C*, neurological deficit scored at 24 h and 7 days after MCAO. *Ndrg4*$^{-/-}$ mice had a severe neurological deficit score compared with *Ndrg4*$^{+/+}$ mice. Data are mean ± S.D. (n = 10). *D*, rCBF in the penumbra-like peripheral area of the ischemic legion. The rCBF was measured by laser-Doppler blood flowmetry system. The rCBFs were expressed as percentages of their preischemic normal values. During MCAO, rCBF was reduced to an equivalent level, and reperfusion was achieved in both groups, although the rCBF values of *Ndrg4*$^{-/-}$ mice were lower compared with *Ndrg4*$^{+/+}$ mice. Data are mean with error bars of S.D. in *Ndrg4*$^{+/+}$ (○, n = 7) and *Ndrg4*$^{-/-}$ mice (●, n = 5). The differences in rCBF between *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice at each point were not significant.

rating the histological results, MCAO-treated *Ndrg4*$^{-/-}$ mice showed more severe neurological deficits compared with *Ndrg4*$^{+/+}$ mice in the cerebral function scoring test (Fig. 5*C*). At 7 days after the 15-min MCAO, the infarction volumes were significantly larger in *Ndrg4*$^{-/-}$ mice compared with *Ndrg4*$^{+/+}$ mice, as seen in the acute phase study (Fig. 5*B*). However, differences in neurological deficit scores between *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice decreased at the end of the observation period (Fig. 5*C*).

Physiological measures of *Ndrg4*$^{+/+}$ and *Ndrg4*$^{-/-}$ mice, heart rate (24 h, 477 ± 100 *versus* 464 ± 95 beat/min, n = 10; 7 days, 497 ± 57 *versus* 483 ± 131 beat/min, n = 7 and 5, respectively) and mean blood pressure (24 h, 46 ± 8 *versus* 55 ± 13 mm Hg, n = 10; 7 days, 68 ± 10 *versus* 63 ± 25 mm Hg, n = 7 and 5, respectively) were not significantly different during ischemic treatment. These results indicate that NDRG4 is essential for the acquisition of normal resistance to the acute and chronic phase of cerebral ischemia through the retention of BDNF levels.

rCBF—We monitored the rCBF in the penumbra-like peripheral area of the ischemic legion to exclude the possibility that the larger infarct in the *Ndrg4*$^{-/-}$ brain was due to a decrease of rCBF during MCAO. Using laser-Doppler blood flowmetry, we found that rCBF values during the 15-min MCAO were equivalently reduced in *Ndrg4*$^{+/+}$ (8.4 ± 3.2%) and *Ndrg4*$^{-/-}$ (7.1 ± 2.0%) mice (Fig. 5*D*). After MCAO, perfusion was observed in both groups as expected, but the rCBF values were relatively lower in *Ndrg4*$^{-/-}$ than in *Ndrg4*$^{+/+}$ mice, which indicates that the sensitivity to ischemic stress is increased under a deficiency of NDRG4.

DISCUSSION

In this study, we revealed that NDRG4 was involved in the retaining of BDNF levels in the cortex. We also revealed that NDRG4-deficient mice showed cognitive deficits and impaired cerebral infarction tolerance. Although these phenomena in the brain seem to be physiologically distinct from each other, the abilities in learning/memory and neuroprotection are both appropriate indicators of biological activities involving BDNF (20).

BDNF participates in synaptic plasticity and memory processing in the adult brain (22). Indeed, mice that lack BDNF in their forebrain fail to learn the MWM task (21), whereas an increase in BDNF levels in the brain improves spatial learning and memory (22, 26). These observations are consistent with our findings that *Ndrg4*$^{-/-}$ mice have lower amounts of cortex BDNF than *Ndrg4*$^{+/+}$ and impaired spatial learning and memory function. Because BDNF also increases the survivability of neurons against ischemia, decreased levels of BDNF in the *Ndrg4*$^{-/-}$ cortex can explain the enlarged lesion sizes that appeared after the stress induced by temporary focal ischemia (23–26). BDNF-mediated production of prostacyclin (36) may be associated with the neuronal vulnerabilities of the *Ndrg4*$^{-/-}$ mice because prostacyclin has a potent neuroprotective effect against focal cerebral ischemia (37). Despite the decreased levels of BDNF in the cortex of *Ndrg4*$^{-/-}$ mice, the neurological deficits were recovered at 7 days after ischemia. It needs further investigations to clarify the mechanisms of neurologic recovery in *Ndrg4*$^{-/-}$ mice. Some signaling pathways mediated by BDNF receptors such as tropomyosin-related kinase B might be up-regulated by a sustained decrease of BDNF in *Ndrg4*$^{-/-}$ mice.

The expression of NDRG4 is decreased in the brains of patients with Alzheimer's disease (15), and BDNF expression is also decreased in the cortex of Alzheimer's patients (22), observations consistent with our current finding of decreased levels of BDNF in the *Ndrg4*$^{-/-}$ mouse brain. Therefore, it is likely that NDRG4 exists upstream of the BDNF production. A decrease of NDRG4 may cause neuronal vulnerability via an associated reduction of BDNF levels and thus may be a potential contributor or a risk factor in the pathogenesis of Alzheimer's disease.

Although the molecular mechanisms by which NDRG4 influences cerebral BDNF levels are unknown, NDRG4-mediated signaling pathways may play an essential role in BDNF synthesis and secretion. BDNF secretion is dependent on the activation of voltage-gated Na^+ channels and the subsequent of Ca^{2+} influx through voltage-gated N-type Ca^{2+} channels (38). In addition, BDNF release is involved in caffeine/ryanodine-sensitive Ca^{2+} release from intracellular stores. These findings support the idea that NDRG4 might regulate BDNF secretion via Ca^{2+} mobilization.

In contrast to the dysfunctional effects of NDRG4 deficiency on the central nervous system, NDRG1 deficiency results in peripheral nervous system defects. Although a brain magnetic resonance imaging study demonstrated subcortical white matter abnormalities in sibling patients with Charcot-Marie-Tooth disease type 4D (39), the lack of NDRG1 exhibited no adverse effects on higher brain functions (9) and on brain anatomy (17), suggesting that other NDRG members may compensate for the NDRG1 deficiency in the central nervous system. Similarly, the mild phenotypes of the *Ndrg4*$^{-/-}$ mice may be due to the compensatory action of the other NDRG members. Further analysis using double-knockout mice such as *Ndrg1*$^{-/-}$*Ndrg4*$^{-/-}$ may reveal the overlapping roles of the NDRG members.

Although the NDRG4 mRNA is abundantly expressed in the human brain and heart (15, 16), Western blotting analysis in the present study could only detect the NDRG4 protein isoforms in the brain but not in the heart of the wild-type mice. This was probably due to the extremely low levels of NDRG4 protein in the heart. This unexpected finding may be caused by the low translational efficiency or the instability of NDRG4 mRNA in the heart. However, recent reports implicate biological roles of NDRG4 in the heart. The knockdown of NDRG4 during embryonic development in zebrafish results in phenotypes such as a hypoplastic heart with pericardial edema, a dilated atrium, looping defects, reduced circulation, and a slower heart rate with weaker contraction (40). Severe ventricular hypoplasia down-regulates NDRG4 expression in the mouse embryonic heart (16). These reports indicate that NDRG4 is necessary for the normal regulation of myocardial proliferation and cardiac growth during early cardiogenesis. In addition, human chromosome 16q21 near *NDRG4* was identified as the locus that influences QT interval duration (41, 42). Although we currently do not find any histological and functional abnormalities for the *Ndrg4*$^{-/-}$ heart, more detailed studies may reveal the roles of NDRG4 on cardiac function.

In conclusion, we found that NDRG4 has an essential role in retaining normal spatial learning and memory, in protecting cerebral neurons against severe ischemic stress, and in maintaining BDNF levels in the brain within the normal range. Although the mechanisms by which NDRG4 influences intracerebral BDNF levels are yet unidentified, the decreased level of cortical BDNF may induce impairments in the central nervous system of *Ndrg4*$^{-/-}$ mice. Further investigation of *Ndrg4*$^{-/-}$ mice, including brain vasculature characterization and neurogenesis, may provide insight into effective therapies for some central nervous system diseases, including Alzheimer's disease and ischemic stroke.

Acknowledgment—We thank Dr. Yuka Eura for experimental materials.

REFERENCES

1. Yao, L., Zhang, J., and Liu, X. (2008) *Acta Biochim. Biophys. Sin.* **40,** 625–635
2. Schilling, S. H., Hjelmeland, A. B., Radiloff, D. R., Liu, I. M., Wakeman, T. P., Fielhauer, J. R., Foster, E. H., Lathia, J. D., Rich, J. N., Wang, X. F., and Datto, M. B. (2009) *J. Biol. Chem.* **284,** 25160–25169
3. Wang, W., Li, Y., Li, Y., Hong, A., Wang, J., Lin, B., and Li, R. (2009) *Int. J. Cancer* **124,** 521–530
4. Kitowska, A., and Pawelczyk, T. (2010) *Acta Biochim. Pol.* **57,** 15–21
5. Melotte, V., Qu, X., Ongenaert, M., van Criekinge, W., de Bruïne, A. P., Baldwin, H. S., and van Engeland, M. (2010) *FASEB J.* **24,** 4153–4166
6. Kokame, K., Kato, H., and Miyata, T. (1996) *J. Biol. Chem.* **271,** 29659–29665
7. Taketomi, Y., Sunaga, K., Tanaka, S., Nakamura, M., Arata, S., Okuda, T., Moon, T. C., Chang, H. W., Sugimoto, Y., Kokame, K., Miyata, T., Murakami, M., and Kudo, I. (2007) *J. Immunol.* **178,** 7042–7053
8. Kalaydjieva, L., Gresham, D., Gooding, R., Heather, L., Baas, F., de Jonge, R., Blechschmidt, K., Angelicheva, D., Chandler, D., Worsley, P., Rosenthal, A., King, R. H., and Thomas, P. K. (2000) *Am. J. Hum. Genet.* **67,** 47–58
9. Okuda, T., Higashi, Y., Kokame, K., Tanaka, C., Kondoh, H., and Miyata, T. (2004) *Mol. Cell. Biol.* **24,** 3949–3956
10. Drögemüller, C., Becker, D., Kessler, B., Kemter, E., Tetens, J., Jurina, K., Jäderlund, K. H., Flagstad, A., Perloski, M., Lindblad-Toh, K., and Matiasek, K. (2010) *PLoS ONE* **5,** e11258
11. Takahashi, K., Yamada, M., Ohata, H., Momose, K., Higuchi, T., Honda, K., and Yamada, M. (2005) *Int. J. Neuropsychopharmacol.* **8,** 381–389
12. Boulkroun, S., Fay, M., Zennaro, M. C., Escoubet, B., Jaisser, F., Blot-Chabaud, M., Farman, N., and Courtois-Coutry, N. (2002) *J. Biol. Chem.* **277,** 31506–31515
13. Choi, S. C., Kim, K. D., Kim, J. T., Kim, J. W., Yoon, D. Y., Choe, Y. K., Chang, Y. S., Paik, S. G., and Lim, J. S. (2003) *FEBS Lett.* **553,** 413–418
14. Burchfield, J. G., Lennard, A. J., Narasimhan, S., Hughes, W. E., Wasinger, V. C., Corthals, G. L., Okuda, T., Kondoh, H., Biden, T. J., and Schmitz-Peiffer, C. (2004) *J. Biol. Chem.* **279,** 18623–18632
15. Zhou, R. H., Kokame, K., Tsukamoto, Y., Yutani, C., Kato, H., and Miyata, T. (2001) *Genomics* **73,** 86–97
16. Dupays, L., Kotecha, S., Angst, B., and Mohun, T. J. (2009) *Dev. Biol.* **333,** 121–131
17. Okuda, T., Kokame, K., and Miyata, T. (2008) *J. Histochem. Cytochem.* **56,** 175–182
18. Ohki, T., Hongo, S., Nakada, N., Maeda, A., and Takeda, M. (2002) *Brain Res. Dev. Brain Res.* **135,** 55–63
19. Nishimoto, S., Tawara, J., Toyoda, H., Kitamura, K., and Komurasaki, T. (2003) *Eur. J. Biochem.* **270,** 2521–2531
20. Mattson, M. P. (2008) *Ann. N.Y. Acad. Sci.* **1144,** 97–112
21. Gorski, J. A., Balogh, S. A., Wehner, J. M., and Jones, K. R. (2003) *Neuroscience* **121,** 341–354
22. Nagahara, A. H., Merrill, D. A., Coppola, G., Tsukada, S., Schroeder, B. E., Shaked, G. M., Wang, L., Blesch, A., Kim, A., Conner, J. M., Rockenstein, E., Chao, M. V., Koo, E. H., Geschwind, D., Masliah, E., Chiba, A. A., and Tuszynski, M. H. (2009) *Nat. Med.* **15,** 331–337
23. Schäbitz, W. R., Schwab, S., Spranger, M., and Hacke, W. (1997) *J. Cereb. Blood Flow Metab.* **17,** 500–506
24. Yanamoto, H., Nagata, I., Sakata, M., Zhang, Z., Tohnai, N., Sakai, H., and Kikuchi, H. (2000) *Brain Res.* **859,** 240–248
25. Endres, M., Fan, G., Hirt, L., Fujii, M., Matsushita, K., Liu, X., Jaenisch, R., and Moskowitz, M. A. (2000) *J. Cereb. Blood Flow. Metab.* **20,** 139–144
26. Yanamoto, H., Xue, J. H., Miyamoto, S., Nagata, I., Nakano, Y., Murao, K., and Kikuchi, H. (2004) *Brain Res.* **1019,** 178–188
27. Agarwala, K. L., Kokame, K., Kato, H., and Miyata, T. (2000) *Biochem. Biophys. Res. Commun.* **272,** 641–647
28. Takagi, T., Moribe, H., Kondoh, H., and Higashi, Y. (1998) *Development*

125, 21–31
29. Yagi, T., Nada, S., Watanabe, N., Tamemoto, H., Kohmura, N., Ikawa, Y., and Aizawa, S. (1993) *Anal. Biochem.* **214,** 77–86
30. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90,** 8424–8428
31. Kokame, K., Agarwala, K. L., Kato, H., and Miyata, T. (2000) *J. Biol. Chem.* **275,** 32846–32853
32. Nakajo, Y., Miyamoto, S., Nakano, Y., Xue, J. H., Hori, T., and Yanamoto, H. (2008) *Brain Res.* **1241,** 103–109
33. Morris, R. (1984) *J. Neurosci. Methods* **11,** 47–60
34. Yanamoto, H., Nagata, I., Niitsu, Y., Xue, J. H., Zhang, Z., and Kikuchi, H. (2003) *Exp. Neurol.* **182,** 261–274
35. Yanamoto, H., Nagata, I., Niitsu, Y., Zhang, Z., Xue, J. H., Sakai, N., and Kikuchi, H. (2001) *Stroke* **32,** 232–239
36. Santhanam, A. V., Smith, L. A., and Katusic, Z. S. (2010) *Stroke* **41,** 350–356
37. Lin, H., Lin, T. N., Cheung, W. M., Nian, G. M., Tseng, P. H., Chen, S. F., Chen, J. J., Shyue, S. K., Liou, J. Y., Wu, C. W., and Wu, K. K. (2002) *Circulation* **105,** 1962–1969
38. Kolarow, R., Brigadski, T., and Lessmann, V. (2007) *J. Neurosci.* **27,** 10350–10364
39. Echaniz-Laguna, A., Degos, B., Bonnet, C., Latour, P., Hamadouche, T., Lévy, N., and Leheup, B. (2007) *Neuromuscul. Disord.* **17,** 163–168
40. Qu, X., Jia, H., Garrity, D. M., Tompkins, K., Batts, L., Appel, B., Zhong, T. P., and Baldwin, H. S. (2008) *Dev. Biol.* **317,** 486–496
41. Newton-Cheh, C., Eijgelsheim, M., Rice, K. M., de Bakker, P. I., Yin, X., Estrada, K., Bis, J. C., Marciante, K., Rivadeneira, F., Noseworthy, P. A., Sotoodehnia, N., Smith, N. L., Rotter, J. I., Kors, J. A., Witteman, J. C., Hofman, A., Heckbert, S. R., O'Donnell, C. J., Uitterlinden, A. G., Psaty, B. M., Lumley, T., Larson, M. G., and Stricker, B. H. (2009) *Nat. Genet.* **41,** 399–406
42. Pfeufer, A., Sanna, S., Arking, D. E., Müller, M., Gateva, V., Fuchsberger, C., Ehret, G. B., Orrú, M., Pattaro, C., Köttgen, A., Perz, S., Usala, G., Barbalic, M., Li, M., Pütz, B., Scuteri, A., Prineas, R. J., Sinner, M. F., Gieger, C., Najjar, S. S., Kao, W. H., Mühleisen, T. W., Dei, M., Happle, C., Möhlenkamp, S., Crisponi, L., Erbel, R., Jöckel, K. H., Naitza, S., Steinbeck, G., Marroni, F., Hicks, A. A., Lakatta, E., Müller-Myhsok, B., Pramstaller, P. P., Wichmann, H. E., Schlessinger, D., Boerwinkle, E., Meitinger, T., Uda, M., Coresh, J., Kääb, S., Abecasis, G. R., and Chakravarti, A. (2009) *Nat. Genet.* **41,** 407–414

Int J Hematol (2011) 94:150–155
DOI 10.1007/s12185-011-0902-z

ORIGINAL ARTICLE

Genetic analysis of patients with deep vein thrombosis during pregnancy and postpartum

Reiko Neki · Tomio Fujita · Koichi Kokame · Isao Nakanishi · Masako Waguri · Yuzo Imayoshi · Noriyuki Suehara · Tomoaki Ikeda · Toshiyuki Miyata

Received: 30 April 2011 / Revised: 13 July 2011 / Accepted: 13 July 2011 / Published online: 3 August 2011

Abstract Deep vein thrombosis (DVT) is a serious pregnancy-related complication. Recent studies indicate that the genetic background for DVT differs with ethnicity. In our study, we enrolled 18 consecutive Japanese patients who had developed DVT during pregnancy and postpartum. We performed a genetic analysis of three candidate genes for DVT, protein S, protein C and antithrombin, in these patients. We found that four patients had missense mutations in the protein S gene, including the K196E mutation in two patients, the L446P mutation in one patient, and the D79Y and T630I mutations in one patient, as well as one patient with the C147Y mutation in the protein C gene. All five patients with genetic mutations had DVT in their first two trimesters. Nine of the patients without genetic mutations developed DVT in the first two trimesters, and four in the postpartum period. Thus, genetic mutations in the protein S gene were predominant in pregnant Japanese DVT women, and DVT in pregnant women with genetic mutations occurred more frequently at the early stage of pregnancy than postpartum. Considering the rapid decrease in protein S activity during pregnancy, we may need to assess thrombophilia in women before pregnancy.

Keywords Deep vein thrombosis · Protein S · Thrombophilia · Pregnancy

1 Introduction

Venous thromboembolism is the leading cause of maternal deaths in Western countries [1]. The incidence of pregnancy-related venous thromboembolism was 13 per 10,000 deliveries [2]. A 30-year population-based study reported that the unadjusted incidence of deep vein thrombosis (DVT) was 151.8 per 100,000 woman-years [3]. Most studies have found that the risk for thrombosis were 3–12 times higher in postpartum than during pregnancy [3, 4]. One study, however, reported twice as many events antenatally as postpartum [5]. Most of these studies involved patients in Western countries. A study in Japan showed that pulmonary thromboembolism occurred in 0.02% of total births, and the mortality rate was 2.5 per 100,000 deliveries [6]. Women with pregnancy-related thrombosis tend to have inherited thrombophilia, thus the prevention of DVT during pregnancy and postpartum is important for pregnant women. Therefore, the identification of inherited or acquired thrombophilia in pregnant women is urgently needed for the prevention of pregnancy-related thrombosis.

R. Neki (✉) · T. Ikeda
Department of Perinatology and Gynecology, National Cerebral and Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan
e-mail: rneki@hsp.ncvc.go.jp

T. Fujita · I. Nakanishi · M. Waguri
Department of Maternal Medicine, Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Osaka, Japan

K. Kokame · T. Miyata
Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, Suita, Osaka, Japan

Y. Imayoshi
Department of Clinical Medicine, Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Osaka, Japan

N. Suehara
Department of Obstetrics, Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Osaka, Japan

In Caucasian populations, two thrombotic mutations, the factor V Leiden mutation and the prothrombin G20210A mutation, for venous thromboembolism are widely distributed, with 30–60% of women with pregnancy-related thrombosis having these mutations [7, 8]. Both mutations are well-established risk factors for venous thromboembolism during pregnancy and postpartum in Caucasian populations. Several prophylactic therapies for pregnant women, such as heparin administration in the perinatal period, are recommended based on the type of thrombophilia and history of thrombosis [9]. However, these two genetic mutations are not found in the Japanese population [10, 11]. Thus, Caucasians and Japanese have clear genetic differences for thrombosis [12].

Deficiencies in protein S, protein C, and antithrombin are well-known risk factors for DVT [13]. The frequency of protein C deficiency and antithrombin deficiency in the general Japanese population was estimated to be 0.13 and 0.15%, respectively, and was comparable to the Caucasian population [12, 14–16]. The frequency of protein S deficiency in Japanese, however, seemed to be higher than that in Caucasians [17, 18], although the assays for plasma protein S levels differed among the studies. Actually, the frequency of protein S deficiency in 2,690 individuals randomly selected from the general Japanese population was estimated to be 1.12%, higher than reported in Caucasian populations (0.03–0.13%) [17, 18]. In a study of Japanese patients with venous thromboembolism, the frequency of inherited protein S deficiency was higher than that in Caucasian patients [19, 20]. It was recently reported that 17% of Japanese patients with venous thromboembolism had genetic mutations in the protein S gene [20]; this was much higher than in selected Caucasian patients with thromboembolism (1.4–8.6%) [13]. Furthermore, we and others reported the significant association with a missense mutation, K196E, in the protein S gene and venous thromboembolism in Japanese populations [19, 21, 22]. The carriers of this mutation showed low protein S activity [19, 23]. The prevalence of this mutant allele in the general Japanese population was about 0.009, suggesting that a substantial proportion of the Japanese population carried the protein S E-allele and was at risk of developing DVT [12, 19, 21, 22, 24]. This mutation seems to be ethnically specific, because it has not so far been identified in Caucasians.

It is well recognized that plasma levels of protein S activity and antigen are significantly reduced during pregnancy [25] and in oral contraceptive users [26]. The activities of protein S, protein C, and antithrombin can be affected at the acute stage of thrombotic events or after antithrombotic therapies. Therefore, the plasma assay may have an intrinsic limitation for the diagnosis of thrombophilia, and alternative ways to diagnose thrombophilia are expected. Genetic analysis might fulfill this requirement if it is applicable.

In this study, we performed DNA analysis for the genes of protein S, protein C, and antithrombin in patients with DVT during pregnancy and postpartum. We measured their plasma activities of protein S, protein C, and antithrombin. Based on these analyses, we described the clinical characteristics of the DVT events in patients with genetic mutation.

2 Materials and methods

2.1 Study patients

In this study, 18 consecutive patients with DVT during pregnancy and postpartum were enrolled from two tertiary perinatal centers: the National Cerebral and Cardiovascular Center and the Osaka Medical Center and Research Institute for Maternal and Child Health. Both centers are located in the Osaka Prefecture, which has the third-largest population in Japan. Postpartum was defined as the first 3 months after delivery. DVT was diagnosed by ultrasonography, venography, or magnetic resonance imaging angiography. We enrolled only patients with symptomatic DVT. Each patient's age, body mass index, gestational weeks of DVT onset, complications of pregnancy, delivery mode, and other information were reviewed.

The protocol of this study was approved by the Ethics Review Committee of the National Cerebral and Cardiovascular Center and by that of the Osaka Medical Center and Research Institute for Maternal and Child Health. Only those who had given written informed consent for genetic analyses were included.

2.2 Activity measurements of protein S, protein C, antithrombin, and antiphospholipid syndrome screening

The plasma samples were obtained after at least 3 months' postpartum and at least 3 months without the use of warfarin. Samples were subjected to a thrombophilia screening, including prothrombin time, activated partial prothrombin time, and activities of protein S, protein C, and antithrombin. Protein S activity was measured as cofactor activity for activated protein C on the basis of the activated partial thromboplastin time assay using Staclot protein S (Diagnostica Stago, Asnieres, France) [18]. Protein C amidolytic activity was measured using S-2366 as a chromogenic substrate and Protac derived from *Agkistrodon contortrix* venom as the activator [16]. Antithrombin activity was measured as a heparin cofactor activity using chromogenic substrate S-2238 (Chromogenix AB, Stockholm, Sweden) [16, 27]. Samples were also subjected to an antiphospholipid syndrome screening of

lupus anticoagulant, anticardiolipin antibody, and anti-β2-glycoprotein-I antibody [28].

2.3 DNA sequencing of protein S, protein C, and antithrombin genes

We sequenced the entire coding region of protein S, protein C, and antithrombin genes in 18 patients with DVT. The method of direct sequencing using the 96-capillary 3730xl DNA Analyzer (Applied Biosystems Japan, Tokyo, Japan) has been described previously [20, 29]. We have adopted the numbering standards of the Nomenclature Working Group, wherein the A of the ATG of the initiator Met codon is denoted as nucleotide +1, and the initial Met residue is denoted as amino acid +1 [30].

3 Results

3.1 DVT history of enrolled patients

We enrolled 18 Japanese symptomatic DVT patients in this study, and only one patient had previous DVT event. All patients were negative for the antiphospholipid syndrome. Thirteen patients were primiparous and five were multiparous. One patient without genetic mutation had a history of miscarriage. One patient without genetic mutation had a history of first trimester artificial abortion that was also complicated with DVT at the time. As an additional risk factor, two out of 13 DVT patients without genetic mutation showed hyperemesis, but all five patients with genetic mutation did not show hyperemesis. Other risk factors such as bed rest, preeclampsia, multiple pregnancy, and preterm labor were not observed in all 18 patients. One patient without genetic mutation had the travelers' thrombosis in the first trimester. One patient without genetic mutation showed paradoxical embolism after DVT postpartum.

3.2 Identification of genetic mutation in DVT patients

We sequenced the coding regions of the protein S, protein C, and antithrombin genes in the 18 DVT patients and identified missense mutations in the protein S gene in four cases, and in the protein C gene in one case, but not in the antithrombin gene (Table 1). Two patients, cases 1 and 2, had the K196E mutation in the protein S gene; this is the most popular thrombophilic mutation in the Japanese population [19, 21, 24]. These two patients had protein S anticoagulant activity above 50% (Table 1). Case 3 had a missense mutation, L446P, in the protein S gene. Case 4 had two missense mutations, D79Y and T630I, in the protein S gene with very low anticoagulant activity of 4%, with family history of DVT in her father. The protein S anticoagulant activities during pregnancy in cases 2, 3, and 4 were decreased to 25, <20, and <1%, respectively. Case 5 had the C147Y mutation in the protein C gene with 45% amidolytic activity. Her protein C activity did not change during pregnancy (Table 1). None of the 18 patients with DVT had nonsynonymous mutations in the antithrombin gene. All patients were not obese with body mass index between 18 and 24. Case 1, 2, and 3 had term vaginal delivery; however, case 4 and 5 had cesarean section due to other obstetric indication.

3.3 Onset of DVT in patients with genetic mutation

Table 2 shows the onset of the DVT events in patients with or without genetic mutation. DVT was found in all five patients with genetic mutations in their first and second trimesters, but not in postpartum. In 13 patients without genetic mutations, DVT events occurred in postpartum for four patients and in the first and second trimesters for nine patients. Two out of four patients without genetic mutation underwent cesarean section. Thus, DVT in pregnant patients with genetic mutation tended to occur in the first and second trimesters and not postpartum.

4 Discussion

Although the relationship between DVT and genetic mutations in protein S, protein C, and antithrombin genes is well established, the clinical courses of DVT patients with genetic mutation among Japanese women during pregnancy and postpartum have not been well characterized. Recent genetic analysis of inherited thrombophilia revealed ethnic differences in DVT between Caucasians and Asians [19, 21], suggesting that the study of venous thromboembolism within individual ethnic populations is highly valuable [12]. It has been established that Caucasians have factor V Leiden mutation and prothrombin G20210A mutation as genetic risk factors for DVT, whereas Japanese do not carry them [10, 11]. However, Japanese have the K196E mutation in the protein S gene as a genetic risk for DVT [19, 21, 22]. The study of DVT in a Japanese population without factor V Leiden mutation or prothrombin G20210A mutation may reveal different clinical characteristics and give rise to hitherto unrecognized issues. In particular, sub-group analyses, such as DVT during pregnancy and postpartum, would be valuable. In the present study, we enrolled 18 pregnant Japanese women with DVT and found that five out of 18 patients (28% patients) had genetic mutations in the protein S or protein C gene. None carried mutations in the antithrombin gene.

The question of when DVT events occur in pregnant women with genetic mutations has been debated. Studies of

Table 1 Nonsynonymous mutations identified in protein S and protein C genes in patients ($n = 18$) with DVT during pregnancy and postpartum

Patient	cDNA[a]	Region	Amino acid change	Protein S[b] or protein C[c] activity (%)	Protein S[b] or protein C[c] activity, during pregnancy (%)	Age	Gravida	Parity	Body mass index	Family history	Other complications of pregnancy	Onset of DVT (weeks of gestation)	Delivery mode	Recurrence of DVT	Complication of PTE (weeks of gestation)
Protein S gene															
Case 1	c.586	Exon 6	K196E	57[b]	n.d.	30	1	1	18.6	None	None	27	TVD	None	27
Case 2	c.586	Exon 6	K196E	68[b]	25[b]	27	0	0	20.3	None	None	10	TVD	None	None
Case 3	c.1337	Exon 12	L446P	13[b,d]	<20[b]	30	0	0	18.8	None	None	27	TVD	None	None
Case 4	c.235	Exon 3	D79Y	4[b]	<1[b]	35	0	0	22.5	Father	None	6	C/S	None	None
	c.1889	Exon 15	T630I												
Protein C gene															
Case 5	c.440	Exon 6	C147Y	45[c]	57[c]	28	0	0	24.2	None	None	20	C/S	None	None

TVD term vaginal delivery, *C/S* cesarean section, *PTE* pulmonary thromboembolism

[a] Position from A of initial ATG in cDNA

[b] Protein S anticoagulant activity

[c] Protein C amidolytic activity

[d] Protein S activity was obtained under warfarin treatment

pregnant Caucasian women have reported a 3- to 12-times higher risk of thrombosis postpartum than during pregnancy [3, 4]. On the other hand, a large retrospective study found that events were twice as likely during pregnancy as postpartum [5]. In our new study, we found that Japanese patients with genetic mutations manifested DVT events in their first two trimesters (Table 2). In particular, pregnant Japanese patients with genetic mutation had no DVT events postpartum. Although this trend went against previous findings [3, 4], it was consistent with the results that there were twice as many DVT events during pregnancy as postpartum [5]. DVT onset at the early stage of pregnancy in patients with genetic mutation might be reasonable, since genetic mutation accelerates DVT onset, and patients with mutation might have DVT events in their early stage of pregnancy.

In the present study, we enrolled 18 pregnant Japanese women with DVT and found that four out of 18 patients (22% patients) had genetic mutations in the protein S gene. A previous study on thrombophilia activity screening in Japanese patients with DVT reported a high prevalence of protein S deficiency [31], and this was later confirmed by genetic analysis [19]. Taken together with these previous findings, our study reinforced the theory that protein S deficiency is an important risk factor for DVT in Japanese. This observation was in stark contrast to the case in Caucasians, in whom factor V Leiden and prothrombin G20210A mutations are involved in almost 50% of all DVT cases in pregnant women [8]. It is well known that the level of protein S activity was decreased immediately after pregnancy [25]. Therefore, predisposed thrombophilia should be considered in the care of patients with pregnancy-related complications, and antithrombotic prophylactic therapy might be applicable for those patients. Also, it might be good for women of child-bearing years to know their own thrombophilic nature.

A previous study reported on DNA sequence analyses of the protein S, protein C, and antithrombin genes in 173 Japanese DVT patients [20]. In this study, 55 patients (accounting for 32% of total patients) had nonsynonymous mutations in one of three genes. Among the three genes, mutations in the protein S gene were predominant, being found in 29 patients (17% of the total). Among various nonsynonymous mutations in the protein S gene, the K196E mutation was most prevalent. It was found in one out of 55–70 Japanese individuals, from analyses of general Japanese populations [19, 21, 22, 24]. In our study, we sequenced three genes in 18 patients with pregnancy-related thrombosis and identified missense mutations in five patients (accounting for 28% of the patients). Among five patients, four (22% of the total) had missense mutations in the protein S gene, which reconfirmed the predominance of inherited protein S deficiency in Japanese patients with

Table 2 Onset of DVT according to trimester of pregnancy and postpartum, and according to delivery mode

	Onset of DVT				Delivery mode			Complication of PTE
	First trimester	Second trimester	Third trimester	Postpartum period	Artificial abortion	Term vaginal delivery	Term cesarean section	
Patients with genetic mutation								
Protein S ($n = 4$)	2	2	0	0	0	3	1	1
Protein C ($n = 1$)	0	1	0	0	0	0	1	0
Total ($n = 5$)	2	3	0	0	0	3	2	1[a]
Patients without genetic mutation								
Total ($n = 13$)	4	5	0	4[c]	1[d]	8	4	1[b]

PTE pulmonary thromboembolism

[a] PTE events with genetic mutation occurred during the second trimester

[b] PTE events in the patients without genetic mutation occurred postpartum after cesarean section

[c] Two out of 4 patients without genetic mutation underwent cesarean section

[d] First trimester

DVT. Two of these patients had K196E mutation. Thus, K196E mutation in the protein S gene would be a genetic risk for not only DVT in general, but also for pregnancy-related DVT.

There are limitations to the present study. This was a small-scale retrospective study with 18 patients. We performed genetic analysis in those patients and identified five patients with genetic mutation. To understand the DVT risk in pregnant Japanese patients with inherited or acquired thrombophilia, we will have to recruit patients consecutively and perform thrombophilic screening, including genetic analysis, in the future evaluation.

In conclusion, we identified inherited thrombophilia in pregnant Japanese women with DVT and found protein S deficiency to be a predominant cause of thrombophilia. By DNA sequence analysis, we found two patients with a K196E mutation in the protein S gene that is prevalent in the Japanese population. Since pregnant women showed reduced protein S levels, a diagnosis of protein S deficiency based on its activity has an intrinsic limitation. Since the onset of DVT tends to occur at an early stage during pregnancy, the genetic analysis might be an alternative diagnostic tool.

Acknowledgments We thank Dr. Toshiyuki Sakata for the activity assay, Ms. Junko Ishikawa, Ms. Kanako Sakashita, Ms. Yukiko Sato, and Dr. Shigenori Honda for DNA sequencing, and Dr. Yoshihiro J. Ono for critical reading of the manuscript. This study was supported by a Grant-in-Aid from the Ministry of Health, Labor, and Welfare of Japan; by the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan.

References

1. Marik PE, Plante LA. Venous thromboembolic disease and pregnancy. N Engl J Med. 2008;359:2025–33.
2. Lindqvist P, Dahlback B, Marsal K. Thrombotic risk during pregnancy: a population study. Obstet Gynecol. 1999;94:595–9.
3. Heit JA, Kobbervig CE, James AH, Petterson TM, Bailey KR, Melton LJ III. Trends in the incidence of venous thromboembolism during pregnancy or postpartum: a 30-year population-based study. Ann Intern Med. 2005;143:697–706.
4. Pomp ER, Lenselink AM, Rosendaal FR, Doggen CJ. Pregnancy, the postpartum period and prothrombotic defects: risk of venous thrombosis in the MEGA study. J Thromb Haemost. 2008;6:632–7.
5. McColl MD, Ramsay JE, Tait RC, Walker ID, McCall F, Conkie JA, et al. Risk factors for pregnancy associated venous thromboembolism. Thromb Haemost. 1997;78:1183–8.
6. Kobayashi T, Nakabayashi M, Ishikawa M, Adachi T, Kobashi G, Maeda M, et al. Pulmonary thromboembolism in obstetrics and gynecology increased by 6.5-fold over the past decade in Japan. Circ J. 2008;72:753–6.
7. Kupferminc MJ, Eldor A, Steinman N, Many A, Bar-Am A, Jaffa A, et al. Increased frequency of genetic thrombophilia in women with complications of pregnancy. N Engl J Med. 1999;340:9–13.
8. Gerhardt A, Scharf RE, Beckmann MW, Struve S, Bender HG, Pillny M, et al. Prothrombin and factor V mutations in women with a history of thrombosis during pregnancy and the puerperium. N Engl J Med. 2000;342:374–80.
9. Duhl AJ, Paidas MJ, Ural SH, Branch W, Casele H, Cox-Gill J et al. Antithrombotic therapy and pregnancy: consensus report and recommendations for prevention and treatment of venous thromboembolism and adverse pregnancy outcomes. Am J Obstet Gynecol. 2007;197:457 e1–21.
10. Fujimura H, Kambayashi J, Monden M, Kato H, Miyata T. Coagulation factor V Leiden mutation may have a racial background. Thromb Haemost. 1995;74:1381–2.
11. Miyata T, Kawasaki T, Fujimura H, Uchida K, Tsushima M, Kato H. The prothrombin gene G20210A mutation is not found among Japanese patients with deep vein thrombosis and healthy individuals. Blood Coagul Fibrinolysis. 1998;9:451–2.

12. Miyata T, Kimura R, Kokubo Y, Sakata T. Genetic risk factors for deep vein thrombosis among Japanese: importance of protein S K196E mutation. Int J Hematol. 2006;83:217–23.
13. De Stefano V, Finazzi G, Mannucci PM. Inherited thrombophilia: pathogenesis, clinical syndromes, and management. Blood. 1996; 87:3531–44.
14. Tait RC, Walker ID, Perry DJ, Islam SI, Daly ME, McCall F, et al. Prevalence of antithrombin deficiency in the healthy population. Br J Haematol. 1994;87:106–12.
15. Tait RC, Walker ID, Reitsma PH, Islam SI, McCall F, Poort SR, et al. Prevalence of protein C deficiency in the healthy population. Thromb Haemost. 1995;73:87–93.
16. Sakata T, Okamoto A, Mannami T, Matsuo H, Miyata T. Protein C and antithrombin deficiency are important risk factors for deep vein thrombosis in Japanese. J Thromb Haemost. 2004;2:528–30.
17. Dykes AC, Walker ID, McMahon AD, Islam SI, Tait RC. A study of protein S antigen levels in 3788 healthy volunteers: influence of age, sex and hormone use, and estimate for prevalence of deficiency state. Br J Haematol. 2001;113:636–41.
18. Sakata T, Okamoto A, Mannami T, Tomoike H, Miyata T. Prevalence of protein S deficiency in the Japanese general population: the Suita Study. J Thromb Haemost. 2004;2: 1012–3.
19. Kinoshita S, Iida H, Inoue S, Watanabe K, Kurihara M, Wada Y, et al. Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. Clin Biochem. 2005;38:908–15.
20. Miyata T, Sato Y, Ishikawa J, Okada H, Takeshita S, Sakata T, et al. Prevalence of genetic mutations in protein S, protein C and antithrombin genes in Japanese patients with deep vein thrombosis. Thromb Res. 2009;124:14–8.
21. Kimura R, Honda S, Kawasaki T, Tsuji H, Madoiwa S, Sakata Y, et al. Protein S-K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients. Blood. 2006;107:1737–8.
22. Ikejiri M, Wada H, Sakamoto Y, Ito N, Nishioka J, Nakatani K, et al. The association of protein S Tokushima-K196E with a risk of deep vein thrombosis. Int J Hematol. 2010;92:302–5.
23. Kimura R, Sakata T, Kokubo Y, Okamoto A, Okayama A, Tomoike H, et al. Plasma protein S activity correlates with protein S genotype but is not sensitive to identify K196E mutant carriers. J Thromb Haemost. 2006;4:2010–3.
24. Yamazaki T, Sugiura I, Matsushita T, Kojima T, Kagami K, Takamatsu J, et al. A phenotypically neutral dimorphism of protein S: the substitution of Lys155 by Glu in the second EGF domain predicted by an A to G base exchange in the gene. Thromb Res. 1993;70:395–403.
25. Comp PC, Thurnau GR, Welsh J, Esmon CT. Functional and immunologic protein S levels are decreased during pregnancy. Blood. 1986;68:881–5.
26. Granata A, Sobbrio GA, D'Arrigo F, Barillari M, De Luca P, Egitto M, et al. Changes in the plasma levels of proteins C and S in young women on low-dose oestrogen oral contraceptives. Clin Exp Obstet Gynecol. 1991;18:9–12.
27. Mitsuguro M, Sakata T, Okamoto A, Kameda S, Kokubo Y, Tsutsumi Y, et al. Usefulness of antithrombin deficiency phenotypes for risk assessment of venous thromboembolism: type I deficiency as a strong risk factor for venous thromboembolism. Int J Hematol. 2010;92:468–73.
28. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost. 2006;4:295–306.
29. Kimura R, Kokubo Y, Miyashita K, Otsubo R, Nagatsuka K, Otsuki T, et al. Polymorphisms in vitamin K-dependent gamma-carboxylation-related genes influence interindividual variability in plasma protein C and protein S activities in the general population. Int J Hematol. 2006;84:387–97.
30. Antonarakis SE. Recommendations for a nomenclature system for human gene mutations. Nomenclature Working Group. Hum Mutat. 1998;11:1–3.
31. Tsuda H, Hattori S, Tanabe S, Iida H, Nakahara M, Nishioka S, et al. Screening for aetiology of thrombophilia: a high prevalence of protein S abnormality. Ann Clin Biochem. 1999;36:423–32.