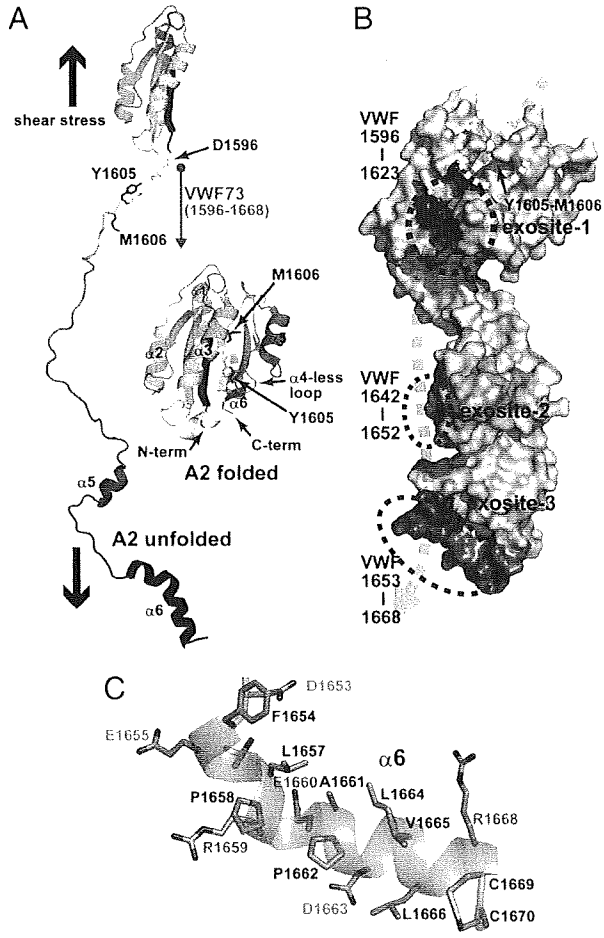

Fig. 4. ADAMTS13-VWF interactions. (*A*) Folded and unfolded structures of the VWF A2 domain. The VWF A2 domain adopts a Rossman fold with a central 6-stranded β-sheet surrounded by 5 α-helices (shown as "A2 folded") (28). The scissile peptide bond (Tyr-1605-Met-1606) is buried within the protein core under static conditions. The C-terminal region (residues 1,596–1,668, corresponding to VWF73) (31) of the A2 domain must be unfolded to expose the scissile bond and the exosite-binding regions under shear-stress conditions (shown as A2 unfolded). (*B*) ADAMTS13-MDTCS-VWF binding model. The molecular surface of the ADAMTS13-MDTCS model is shown in gray and the bound zinc ion is shown in yellow. Residues that mediate VWF binding are depicted as in Fig. 3*C*, and the exosites and the catalytic cleft are indicated by red and yellow dotted ellipsoids, respectively. The dotted green line represents a VWF molecule (residues 1,596–1,668) bound to ADAMTS-MDTCS. (*C*) Close-up view of the α6 helix and surrounding residues in the VWF A2 domain. Hydrophobic residues are indicated with red letters. Systematic charge-to-alanine substitutions revealed that the D1653A and D1663A mutations (cyan) reduced the substrate cleavage, the E1655A mutation (orange) slightly increased cleavage, and the R1659A, E1660A, and R1668A mutations (gray) had no significant effect (34).

ment suggests that these exosites bind collaboratively to multiple discontinuous regions of VWF.

A recent crystallographic study revealed that the Tyr-1605-Met-1606 scissile bond of VWF is buried within the core of the globular A2 domain under static conditions (Fig. 4*A*, A2 folded) (28). When VWF is subjected to fluid shear stress in circulation or denaturants in vitro, the A2 domain unfolds and adopts a partially extended conformation that makes its scissile peptide bond accessible for cleavage by ADAMTS13 (7, 29, 30) (Fig. 4*A*). We previously identified VWF73 (residues 1,596–1,668) as a minimum specific substrate for ADAMTS13 and suggested that a segment (residues 1,660–1,668) of VWF73 contains essential residues for recognition by ADAMTS13 (31). VWF73 is more than 200 Å long at its maximum extension, which is almost twice the distance between the catalytic site and the distal exosite-3 in the current ADAMTS13-MDTCS model. NMR spectroscopy has indicated that VWF73 adopts an unfolded structure (5). Therefore, the ADAMTS-MDTCS appears to be able to accommodate, by an induced-fit mechanism, a partially unfolded VWF73 segment along the extended molecular surface encompassing at least 3 critical exosites (Fig. 4*B*). Exosite-3 forms a cluster of hydrophobic residues rimmed by basic residues (Fig. 3*E*). Both the surface properties and the size of exosite-3 imply that exosite-3 binds to VWF, such that the VWF segment (residues 1,653–1,668) forms an amphipathic α-helix (α6 as in the crystal structure, Fig. 4*C*) and makes contact with ADAMTS13 by facing its hydrophobic residues toward exosite-3. Autoantibodies that inactivate ADAMTS13 are the most frequent cause of acquired TTP. These TTP patients possess antibodies directed against ADAMTS13 residues 657–666 (32) that exactly coincide with the β9-β10 loop, a part of exosite-3.

The present structure suggests a linear correspondence between the ADAMTS13 domains and their interaction sites in the A2 domain of VWF, consistent with previous systematic mutagenesis studies and kinetic analysis by Gao et al. (33). These authors suggested that the S domain contains an exosite that primarily determines catalytic efficiency by interacting with α6 of the VWF A2 domain (33). They identified 3 other VWF segments that interact with the MD, T1, and C domains of ADAMTS13 (17). Our structural and functional data are in good agreement with these observations, suggesting that the catalytic cleft plus exosite-1, exosite-2, and exosite-3 make cooperative, modular contacts with 3 discrete segments of the VWF A2 domain, the residues flanking the cleavage site (P9-P18′, residues 1,596–1,623), residues 1,642–1,652 and the α6 (residues 1,653–1,668) of the A2 domain, respectively (Fig. 4*B*). The model is also consistent with the previous observation that decreasing the length of peptides derived from the C terminus of the VWF A2 domain caused a progressive decrease in their potency as ADAMTS13 inhibitors (34). The elongated structure of the stiff, rod-like T1 module and its nonessential interactions with VWF (17) suggest that its primary role is to position the exosites spatially. The mobility of the domains (Fig. S3 and *SI Text*) suggests that a spectrum of ADAMTS13 conformations exist, with different spatial alignments of the exosites, increasing the possibility of ADAMTS13 interacting with partially unfolded VWF molecules, which also present a wide spectrum of conformations under shear-stress conditions in the circulation. The M domains of ADAMTS4 and ADAMTS5 do not retain specific catalytic activity. The inclusion of the proximal C-terminal domains enhances their aggrecanase activity, suggesting that these ADAMTSs function through multiple exosites (35–39), as observed in the ADAMTS13-VWF system.

More than 80 causative mutations for congenital TTP have been identified in the ADAMTS13 gene (11, 40, 41), including 16 missense mutations within the DTCS region. These mutations are not restricted to a specific region but are located throughout the molecule, suggesting that most of the mutations cause some structural defect that affects proper folding and secretion (Table S2). The R349C and P353L mutants, however, are likely to affect enzymatic activity: Arg-349 is in exosite-1 and Pro-353 forms part of the potential substrate-binding S3′ pocket (Fig. 3*B*). Five polymorphisms have been identified within the DTCS region (Table S2). Approximately 10% of the Japanese population are heterozygous for P475S substitution, located in the V-loop (C_A), which reduces VWF-cleaving activity (40, 42). The P618A substitution reduces secretion efficiency in cultured cells (43). Both proline residues adopt the *cis* conformation and, therefore, substitution by nonproline residues would cause structural distortions.

Shear stress in the blood circulation controls the exposure of the cryptic scissile bond and exosite-binding regions in VWF to ADAMTS13. The M domain of ADAMTS13 is catalytically

active, whereas the noncatalytic domains display surface features that are optimized for recognizing an unfolded VWF A2 domain. Therefore, cleavage by ADAMTS13 is primarily dependent on shear-force-induced unfolding of the VWF molecule. The force-induced proteolysis observed for ADAMTS13-VWF represents a model for probing the molecular mechanisms underlying the translation of a mechanical stimulus into a chemical response in a biological system.

Materials and Methods

Preparation, Crystallization and Structural Analysis of ADAMTS13-DTCS. Production and crystallization of ADAMTS-DTCS has been described previously (44). Briefly, ADAMTS13-DTCS (residues 287–685), with a C-terminal tobacco etch virus proteinase cleavage site followed by tandem His-tag sequences, was expressed in CHO Lec 3.2.8.1 cells. After purification on a Ni-NTA column, ADAMTS13-DTCS was subjected to proteolysis with the tobacco etch virus proteinase and was further purified using HiTrap SP (GE Healthcare). ADAMTS13-DTCS crystals were obtained by the sitting drop vapor diffusion method, with drops containing 0.5 μL protein solution and 0.5 μL reservoir solution (26% (wt/vol) PEG1500, 100 mM Mes, pH 6.0) supplemented with 0.2 μL of 40% (wt/wt) pentaerythritol ethoxylate (3/4 EO/OH) (Hampton Research) equilibrated for several days at 293 K. Os-derivative crystals were obtained by soaking native crystals in reservoir solution supplemented with 1 mM $OsCl_3$ and 20% glycerol for several hours. Crystals were cryoprotected in reservoir solution supplemented with 20% glycerol and flash cooled under a stream of nitrogen gas at 100 K. All diffraction data were collected at the SPring-8 beamline BL41XU (Table S1). Details of structural analysis are described in *SI Text*.

Functional Analysis. Recombinant wild-type and 25 mutants of ADAMTS13-MDTCS (residues 75–685) with a C-terminal His-tag were prepared by transient expression using a cytomegalovirus promoter-driven expression vector and HeLa cells. The culture medium and cell lysates were collected 72 h posttransfection, and the expression levels were quantified by Western blotting using anti-His-tag (Fig. S6). For enzyme assays, culture medium (5 μL) containing equivalent amounts of ADAMTS13-MDTCSs was mixed with reaction mixture (95 μL) containing 2 μM fluorogenic substrate (FRETS-VWF73) (25), 10 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM $CaCl_2$, and 0.005% Tween-20. Initial velocities of the increase in fluorescence were determined for the enzymatic activity, and the relative activities of the mutants were calculated from a calibration curve for serially diluted wild-type ADAMTS13-MDTCS. The activity for each mutant was determined in duplicate or triplicate experiments.

ACKNOWLEDGMENTS. We thank M. Tomisako for her help in the crystallization, Y. Ben Ammar and the SPring-8 beamline staff for assistance with data acquisition, and D. Ginsburg (University of Michigan) for helpful comments on the manuscript. This work was supported, in part, by grants-in-aid from the Ministry of Health, Labor and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan, and the Takeda Science Foundation.

1. Porter S, Clark IM, Kevorkian L, Edwards DR (2005) The ADAMTS metalloproteinases. *Biochem J* 386:15–27.
2. Edwards DR, Handsley MM, Pennington CJ (2009) The ADAM metalloproteinases. *Mol Aspects Med* 29:258–289.
3. Sadler JE (1998) Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem* 67:395–424.
4. Ruggeri ZM (2003) Von Willebrand factor, platelets and endothelial cell interactions. *J Thromb Haemost* 1:1335–1342.
5. Sadler JE, Moake JL, Miyata T, George JN (2004) Recent advances in thrombotic thrombocytopenic purpura. *Hematology Am Soc Hematol Educ Program*, 407–423.
6. Dent JA, Berkowitz SD, Ware J, Kasper CK, Ruggeri ZM (1990) Identification of a cleavage site directing the immunochemical detection of molecular abnormalities in type IIA von Willebrand factor. *Proc Natl Acad Sci USA* 87:6306–6310.
7. Tsai HM, Sussman II, Nagel RL (1994) Shear stress enhances the proteolysis of von Willebrand factor in normal plasma. *Blood* 83:2171–2179.
8. Moake JL, et al. (1982) Unusually large plasma factor VIII:von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *N Engl J Med* 307:1432–1435.
9. Furlan M, et al. (1998) von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *N Engl J Med* 339:1578–1584.
10. Tsai HM, Lian EC (1998) Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. *N Engl J Med* 339:1585–1594.
11. Levy GG, et al. (2001) Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature* 413:488–494.
12. Soejima K, et al. (2001) A novel human metalloprotease synthesized in the liver and secreted into the blood: Possibly, the von Willebrand factor-cleaving protease? *J Biochem* 130:475–480.
13. Zheng X, et al. (2001) Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. *J Biol Chem* 276:41059–41063.
14. Soejima K, et al. (2003) ADAMTS-13 cysteine-rich/spacer domains are functionally essential for von Willebrand factor cleavage. *Blood* 102:3232–3237.
15. Zheng X, Nishio K, Majerus EM, Sadler JE (2003) Cleavage of von Willebrand factor requires the spacer domain of the metalloprotease ADAMTS13. *J Biol Chem* 278:30136–30141.
16. Ai J, Smith P, Wang S, Zhang P, Zheng XL (2005) The proximal carboxyl-terminal domains of ADAMTS13 determine substrate specificity and are all required for cleavage of von Willebrand factor. *J Biol Chem* 280:29428–29434.
17. Gao W, Anderson PJ, Sadler JE (2008) Extensive contacts between ADAMTS13 exosites and von Willebrand factor domain A2 contribute to substrate specificity. *Blood* 112:1713–1719.
18. Klaus C, et al. (2004) Epitope mapping of ADAMTS13 autoantibodies in acquired thrombotic thrombocytopenic purpura. *Blood* 103:4514–4519.
19. Luken BM, et al. (2005) The spacer domain of ADAMTS13 contains a major binding site for antibodies in patients with thrombotic thrombocytopenic purpura. *Thromb Haemost* 93:267–274.
20. Gerhardt S, et al. (2007) Crystal structures of human ADAMTS-1 reveal a conserved catalytic domain and a disintegrin-like domain with a fold homologous to cysteine-rich domains. *J Mol Biol* 373:891–902.
21. Mosyak L, et al. (2008) Crystal structures of the two major aggrecan degrading enzymes, ADAMTS4 and ADAMTS5. *Protein Sci* 17:16–21.
22. Takeda S (2009) Three-dimensional domain architecture of the ADAM family proteinases. *Semin Cell* Dev Biol 20:146–152.
23. Tan K, et al. (2002) Crystal structure of the TSP-1 type 1 repeats: A novel layered fold and its biological implication. *J Cell Biol* 159:373–382.
24. Takeda S, Igarashi T, Mori H, Araki S (2006) Crystal structures of VAP1 reveal ADAMs' MDC domain architecture and its unique C-shaped scaffold. *EMBO J* 25:2388–2396.
25. Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T (2005) FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. *Br J Haematol* 129:93–100.
26. Zanardelli S, et al. (2006) ADAMTS13 substrate recognition of von Willebrand factor A2 domain. *J Biol Chem* 281:1555–1563.
27. de Groot R, Bardhan A, Ramroop N, Lane DA, Crawley JT (2009) Essential role of the disintegrin-like domain in ADAMTS13 function. *Blood* 113:5609–5616.
28. Zhang Q, et al. (2009) Structural specializations of A2, a force-sensing domain in the ultralarge vascular protein von Willebrand factor. *Proc Natl Acad Sci USA* 106:9226–9231.
29. Furlan M, Robles R, Lämmle B (1996) Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. *Blood* 87:4223–4234.
30. Tsai HM (1996) Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. *Blood* 87:4235–4244.
31. Kokame K, Matsumoto M, Fujimura Y, Miyata T (2004) VWF73, a region from D1596 to R1668 of von Willebrand factor, provides a minimal substrate for ADAMTS-13. *Blood* 103:607–612.
32. Luken BM, et al. (2006) Amino acid regions 572–579 and 657–666 of the spacer domain of ADAMTS13 provide a common antigenic core required for binding of antibodies in patients with acquired TTP. *Thromb Haemost* 96:295–301.
33. Gao W, Anderson PJ, Majerus EM, Tuley EA, Sadler JE (2006) Exosite interactions contribute to tension-induced cleavage of von Willebrand factor by the antithrombotic ADAMTS13 metalloprotease. *Proc Natl Acad Sci USA* 103:19099–19104.
34. Wu JJ, Fujikawa K, McMullen BA, Chung DW (2006) Characterization of a core binding site for ADAMTS-13 in the A2 domain of von Willebrand factor. *Proc Natl Acad Sci USA* 103:18470–18474.
35. Tortorella M, et al. (2000) The thrombospondin motif of aggrecanase-1 (ADAMTS-4) is critical for aggrecan substrate recognition and cleavage. *J Biol Chem* 275:25791–25797.
36. Kashiwagi M, et al. (2004) Altered proteolytic activities of ADAMTS-4 expressed by C-terminal processing. *J Biol Chem* 279:10109–10119.
37. Gendron C, et al. (2007) Proteolytic activities of human ADAMTS-5: Comparative studies with ADAMTS-4. *J Biol Chem* 282:18294–18306.
38. Flannery CR, et al. (2002) Autocatalytic cleavage of ADAMTS-4 (Aggrecanase-1) reveals multiple glycosaminoglycan-binding sites. *J Biol Chem* 277:42775–42780.
39. Fushimi K, Troeberg L, Nakamura H, Lim NH, Nagase H (2008) Functional differences of the catalytic and non-catalytic domains in human ADAMTS-4 and ADAMTS-5 in aggrecanolytic activity. *J Biol Chem* 283:6706–6716.
40. Kokame K, et al. (2002) Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. *Proc Natl Acad Sci USA* 99:11902–11907.
41. Banno F, Miyata T (2008) in *Recent Advances in Thrombosis and Hemostasis 2008*, eds Tanaka K, Davie EW (Springer, Tokyo), pp 162–176.
42. Akiyama M, Kokame K, Miyata T (2008) ADAMTS13 P475S polymorphism causes a lowered enzymatic activity and urea lability in vitro. *J Thromb Haemost* 6:1830–1832.
43. Plaimauer B, et al. (2006) Modulation of ADAMTS13 secretion and specific activity by a combination of common amino acid polymorphisms and a missense mutation. *Blood* 107:118–125.
44. Akiyama M, Takeda S, Kokame K, Takagi J, Miyata T (2009) Production, crystallization and preliminary crystallographic analysis of exosite-containing fragment of human von Willebrand factor-cleaving proteinase, ADAMTS13. *Acta Crystallograph Sect F Struct Biol Cryst Commun F* 65:739–742.

Venous Thromboembolism

— Deep Vein Thrombosis With Pulmonary Embolism, Deep Vein Thrombosis Alone, and Pulmonary Embolism Alone —

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Background There are few data on the differences between deep vein thrombosis (DVT) with pulmonary embolism (PE) (Group A) and without PE (Group B), and no recent data on the incidence of PE and DVT in Japan.
Methods and Results The symptoms and findings of the lower extremities and risks for venous thromboembolism were compared between Groups A and B, and the numbers of new patients with PE and those with DVT in 2006 were calculated. DVT was found equally in left and right legs in Group A, but more frequently in left legs than in right legs in Group B. Proximal thrombus was more frequent in Group A than in Group B, and the number of cases of symptoms resulting from DVT was less in Group A than in Group B. Proximal DVT, DVT in the right leg, no symptoms, and younger age were related to the presence of PE. The calculated number of new patients with PE per year was 7,864 (3,492 cases in 1996), and that with DVT per year was 14,674.
Conclusion DVT in patients with PE and those without PE differed in the site and symptoms. The calculated number of new patients with PE per year doubled in 1 decade in Japan. (*Circ J* 2009; **73:** 305–309)

Key Words: Deep vein thrombosis; Incidence; Pulmonary embolism; Symptoms; Venous thromboembolism

Pulmonary embolism (PE) and deep vein thrombosis (DVT) are thought to be the same disease with different presentation, and both have been handled as venous thromboembolism (VTE). Most cases of PE originate from DVT, so VTE is an important concept. However, there are no data on whether DVT with PE and DVT without PE have the same characteristics.

We reported the incidence of PE in 1996, 2000, and 2004[1–3] In 2004, 2 guidelines for VTE were published in Japan[4,5] generating increased interest in VTE.

The main purpose of this study was to clarify the different characteristics of DVT in cases with and without PE. The second purpose was to assess the recent incidence of PE and DVT in Japan.

Methods

The present study was approved by the Ethics Committee of Mie University. In July 2006, we sent questionnaires to the clinical departments (all departments of internal medicine, all departments of surgery, pediatrics, obstetrics and gynecology, orthopedics, otorhinolaryngology, ophthalmology, dermatology, and urology) of university schools of medicine or medical colleges and to hospitals with more than 100 beds in Japan. Based on the responses to the questionnaires, we assessed prospectively the number of new patients with PE from August 1, 2006 to September 30, 2006. The number of patients with PE (or DVT) per year was calculated as: the number of patients with PE (or DVT) per year = the number of patients with PE (or DVT) per 2 months × 6/the response rate[1–3]

PE was definitely diagnosed by (1) enhanced computed tomography, (2) pulmonary angiography, (3) pulmonary perfusion scintigraphy and/or pulmonary ventilation scintigraphy, (4) magnetic resonance imaging, or (5) autopsy. DVT was definitely diagnosed by (1) enhanced computed tomography, (2) venous ultrasonography, (3) contrast venography, (4) magnetic resonance venography, or (5) radioisotope venography. Major surgery was defined as abdominal surgery and/or surgery of more than 45 min duration within the previous 3 months[6–8] Immobilization was defined as strict bed rest for more than 3 continuous days within the previous 3 months[8]

We divided cases of VTE into 3 groups: DVT with PE, DVT alone, and PE alone.

Statistical Analysis

Analyses were performed using SPSS 15.0 (SPSS Inc, Chicago, IL, USA). All continuous variables were analyzed by Mann-Whitney test, and expressed as mean ± standard deviation. Non-ordinal categorical data were analyzed using the chi-square test. Multiple comparisons were performed using Bonferroni's modification. Potential risk factors for VTE were assessed using multiple logistic regression and the results were presented as estimated odds ratio (OR) with the corresponding 95% confidence intervals (CI). All significant tests were 2-tailed.

(Received April 16, 2008; revised manuscript received August 21, 2008; accepted September 11, 2008; released online December 18, 2008)
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Table 1 Patients' Backgrounds

	DVT with PE	*DVT alone*	*PE alone*
With patient profile (n)	*210*	*420*	*140*
Gender (M/F)	*87/123*	*140/280*	*44/96*
Age (years)	*63.9±15.5*	*66.3±15.9*	*67.6±15.0*
BMI (kg/m²)	*23.7±3.8[a]*	*23.3±4.2[b]*	*23.2±3.8[c]*

[a]n=199, [b]n=392, [c]n=129.
DVT, deep vein thrombosis; PE, pulmonary embolism; BMI, body mass index.

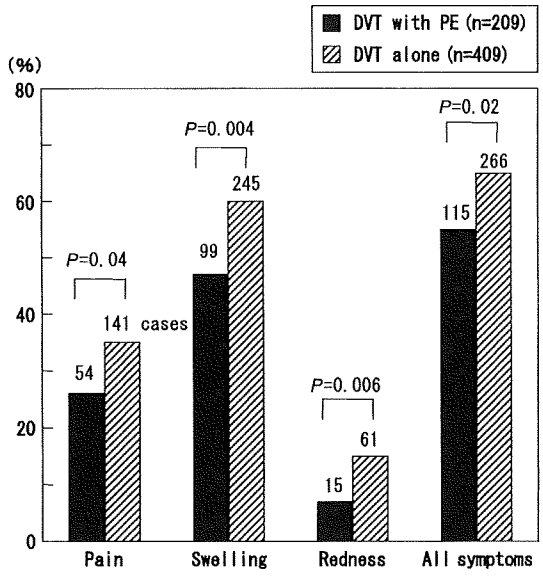

Fig 1. Symptoms of deep vein thrombosis (DVT). The number of cases is shown on each bar. PE, pulmonary embolism.

Results

Incidence of VTE

A total of 6,122 questionnaires were sent; 17 institutes were excluded from our analysis because they had closed or merged. We received 1,635 valid replies, giving a response rate of 26.8% (1,635/6,105). The number of patients newly diagnosed with PE was 351 during the 2 months of the present period, and that with DVT was 655. The estimated number of new patients with PE per year was 7,864 (95% CI: 6,572–9,155) and the incidence of PE was 61.9 (95% CI: 51.7–72.1) patients per 1,000,000 people per year in Japan. The estimated number of new patients with DVT per year was 14,674 (95% CI: 12,466–16,883) and the incidence of DVT was 115.5 (95% CI: 98.2–132.9) patients per 1,000,000 people per year in Japan.

Characteristics of DVT in Patients With and Without PE

Available cases with a detailed profile were 210 with both DVT and PE, 420 with DVT alone, and 140 with PE alone (**Table 1**). Symptoms resulting from DVT were more frequent in patients without PE, compared with those with PE (**Fig 1**). DVT was equally found in the left and right legs

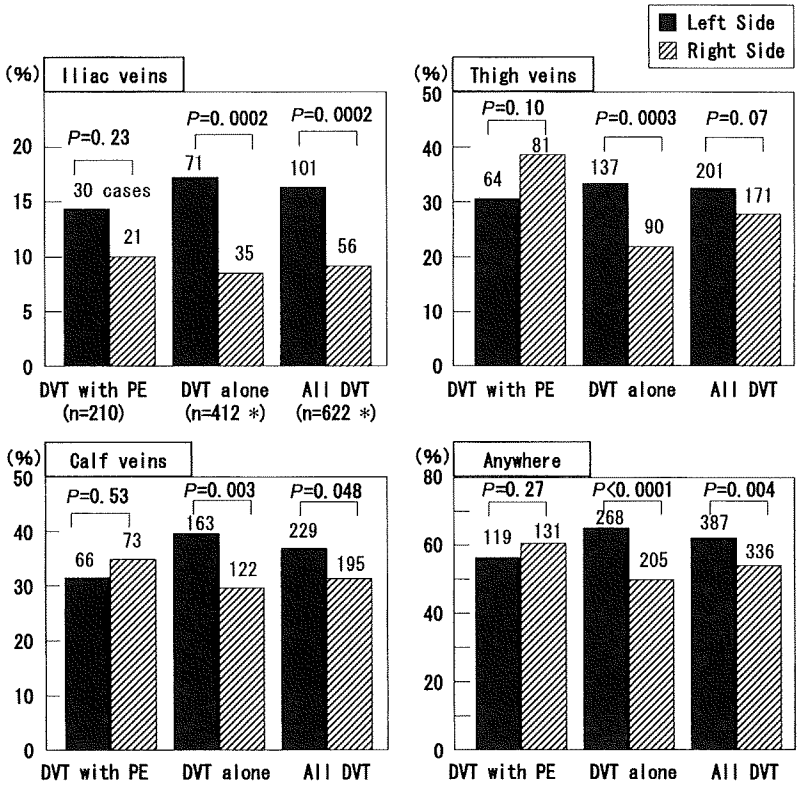

Fig 2. Location of deep vein thrombosis (DVT). The number of cases is shown on each bar. *Seven cases with DVT only in the upper extremities and one without data on DVT site were excluded. PE, pulmonary embolism.

Table 2 Diagnostic Techniques for DVT

	DVT with PE (n=210)	DVT alone (n=413*)	P value
Venous ultrasonography	132 (63%)	303 (73%)	0.008
CT	143 (68%)	180 (44%)	<0.0001
Contrast venography	18 (9%)	50 (12%)	0.22
MR venography	7 (3%)	22 (5%)	0.32
RI venography	3 (1%)	5 (1%)	1.00

CT, computed tomography; MR, magnetic resonance; RI, radioisotope. Other abbreviations see in Table 1.
**Seven cases with DVT only in the upper extremities were excluded.*

Table 3 Risk Factors for Venous Thromboembolism

	DVT with PE (n=210)	DVT alone (n=420)	PE alone (n=140)	P value
Prolonged immobilization	57 (27%)	101 (24%)	30 (21%)	0.46
Recent major surgery	54 (26%)	121 (29%)	40 (29%)	0.70
Cancer	48 (23%)	81 (19%)	23 (16%)	0.32
Recent major trauma and/or fracture	22 (11%)	47 (11%)	15 (11%)	0.96
Central venous catheter	7 (3%)	34 (8%)	9 (6%)	0.06
Pregnancy or postpartum	4 (2%)	14 (3%)	2 (1%)	0.34
Heart failure*	5 (2%)	22 (5%)	14 (10%)	0.009
Respiratory failure	5 (2%)	14 (3%)	8 (6%)	0.37
Cerebrovascular disease	10 (5%)	28 (7%)	8 (6%)	0.62
Connective tissue disease and/or steroid use	5 (2%)	11 (3%)	5 (4%)	0.79
Benign, large abdominal tumor	2 (1%)	9 (2%)	1 (1%)	0.33
No potential risk factors	42 (20%)	66 (16%)	28 (20%)	0.30

**P=0.10 between DVT with PE and DVT alone, P=0.003 between DVT with PE and PE alone. P=0.07 between DVT alone and PE alone. Abbreviations see in Table 1.*

Table 4 Multivariate Logistic Analysis of Relation to Presence of PE in Patients with DVT

	OR (95% CI)	P value
Age (10-year increments)	0.87 (0.77–0.99)	0.03
Male	1.12 (0.76–1.66)	0.57
No symptoms of DVT	2.05 (1.39–3.02)	0.0003
Right DVT	1.98 (1.22–3.19)	0.005
Left DVT	0.99 (0.61–1.60)	0.97
Proximal DVT[a]	1.79 (1.18–2.71)	0.006
BMI	1.03 (0.99–1.08)	0.16
Prolonged immobilization	1.2 (0.78–1.86)	0.41
Recent major surgery	0.83 (0.54–1.28)	0.40
Cancer	1.08 (0.68–1.70)	0.75
Recent major trauma and/or fracture	0.85 (0.46–1.54)	0.58
Central venous catheter	0.44 (0.19–1.00)	0.05
Pregnancy or postpartum	0.37 (0.10–1.32)	0.12
Heart failure	0.59 (0.20–1.68)	0.32
Respiratory failure	0.58 (0.18–1.92)	0.37
Cerebrovascular disease	0.66 (0.28–1.55)	0.34
Connective tissue disease and/or steroid use	1.48 (0.54–4.02)	0.45
Benign, large abdominal tumor	–	1.00

[a]Including IVC, iliac vein, and thigh veins.
OR, odds ratio; CI, confidence interval; IVC, inferior vena cava. Other abbreviations see in Table 1.

of patients with PE, but more frequently in the left than in the right leg of patients without PE (**Fig 2**). Proximal thrombus from the inferior vena cava to the popliteal vein was more frequent in patients with PE than in patients without PE (68% [142/210] vs 58% [240/412]; P=0.02).

Relationship Between Symptoms of DVT and Age

Leg swelling (presence, 64.7±16.0 years; absence, 66.5±15.7; P=0.10) and redness (presence, 63.0±15.7 years; absence, 65.9±15.9; P=0.09) were found regardless of age in patients with DVT. Younger patients complained more about leg pain (complaint, 61.2±15.7 years; no complaint, 67.4±15.6; P<0.0001). All findings for DVT (objective or subjective) were greater in younger patients (presence, 64.4±15.8 years; absence, 67.4±15.9; P=0.007).

*Diagnostic Techniques for DVT (**Table 2**)*

Venous ultrasonography was used more frequently and CT less frequently in patients without PE than in patients with PE. Contrast venography was used in only approximately 10% of patients.

Risk Factors for VTE and Relationship to Presence of PE

There were no differences in the risk factors, except heart

Table 5 Management of Venous Thromboembolism

	[1]DVT with PE (n=210)	[2]DVT alone (n=420)	[3]PE alone (n=140)	P value*		
				[1] vs [2]	[1] vs [3]	[2] vs [3]
Heparin	*175 (83%)*	*243 (58%)*	*106 (76%)*	*<0.0001*	*0.30*	*0.0005*
Warfarin	*162 (77%)*	*282 (67%)*	*86 (61%)*	*0.03*	*0.006*	*0.66*
Anticoagulation						
Heparin→warfarin	*136 (65%)*	*173 (41%)*	*69 (49%)*	*<0.0001*	*0.02*	*0.30*
Heparin alone	*39 (19%)*	*70 (17%)*	*37 (26%)*	*1.00*	*0.26*	*0.04*
Warfarin alone	*26 (12%)*	*109 (26%)*	*17 (12%)*	*<0.0001*	*1.00*	*0.003*
Thrombolysis	*58 (28%)*	*55 (13%)*	*38 (27%)*	*<0.0001*	*1.00*	*0.006*
IVC filter	*110 (52%)*	*93 (22%)*	*22 (16%)*	*<0.0001*	*<0.0001*	*0.35*

**All P-values by chi-square analysis among 3 groups ([1], [2] and [3]) were less than 0.05. Multiple comparisons were performed using Bonferroni's modification.*
Abbreviations see in Tables 1, 4.

failure, among the 3 groups (patients with DVT and PE, those with DVT alone, and those with PE alone) (**Table 3**). Patients with DVT and PE were younger than those with DVT alone (63.9±15.5 years vs 66.3±15.9; P=0.04). PE was found in 30.5% of females with DVT and in 38.3% of males with DVT (P=0.053). Proximal DVT, DVT in the right leg, no symptoms, and younger age were independently related to the presence of PE in patients with DVT (**Table 4**).

*Management of VTE (**Table 5**)*

Heparin and thrombolysis were used less frequently in patients with DVT alone. Implantation of an inferior vena cava filter and chronic use of warfarin were more frequent in patients with DVT and PE. When limited to cases of DVT, inferior vena cava filters were used more often in cases of proximal DVT (OR, 3.51; 95% CI, 2.33–5.27; P<0.0001) and PE (OR, 3.71; 95% CI, 2.56–5.37; P<0.0001). Antiplatelet agents were administered in 8 patients (4%) with DVT and PE (aspirin in 8, ticlopidine in 2; 2 cases used both antiplatelet agents), 44 with DVT alone (aspirin in 36, ticlopidine in 6, cilostazol in 1, sarpogrelate in 1), and 9 (6%) with PE alone (aspirin in 8, ticlopidine in 1, beraprost in 2; 2 cases used 2 antiplatelet agents).

For DVT, catheter therapy was performed in 9 patients with DVT and PE, and in 8 patients with DVT alone. Surgery was performed in 3 patients with DVT and PE, and in 1 patient with DVT alone. On the other hand, for PE, catheter therapy was performed in 13 patients with DVT and PE, and in 7 patients with PE alone. Surgery was performed in 4 patients with DVT and PE, and in 4 patients with PE alone.

Discussion

Characteristics of DVT With and Without PE

DVT in patients with PE and those without PE differed in the site and symptoms. In particular, DVT was equally found in the left and right legs of patients with PE, but more frequently in the left than in the right leg in those without PE. Moreover, cases of symptoms resulting from DVT were less frequent in the presence of PE than in the absence of PE.

Ileofemoral DVT tends to occur in the left leg[9–12] whereas femoropopliteal DVT occurs equally in the right and left legs, and most are contiguous to calf thrombosis[9–12] Those previous reports and the present results suggest that DVT without PE is related to ileofemoral DVT, and that DVT with PE is related to femoropopliteal DVT.

DVT is more common on the left side[13] as observed in all of the present cases of DVT. In the present study, DVT with PE had no statistical difference in the rate of potential risk factors compared with DVT without PE.

Free-floating venous thrombi have a close relationship with PE compared with occlusive (no free-floating) thrombi[14] and the previous reports suggest that free-floating venous thrombi cause less symptoms from DVT than occlusive DVTs[14,15] On the other hand, most cases of symptomatic DVT have extensive occlusive proximal thrombi[9,16] The development of symptoms of DVT is thought to depend on the extent of thrombosis, the adequacy of collateral vessels, and the severity of associated vascular occlusion and inflammation[17] Leg edema is much more likely in contiguous thrombosis rather than with an isolated thrombus[18] DVT with PE has fewer symptoms, as shown in the present study, and resembles free-floating DVT.

Relationship to Presence of PE in Patients With DVT

Proximal DVT, DVT in the right leg and no symptoms of DVT were identified as independent of the presence of PE. Proximal DVT is often associated with acute PE[19–23] Embolic risk is low in calf-only DVT, but elevated in calf DVT with proximal (thigh) involvement[19] DVT in the right iliac vein is easily torn off and PE easily occurs because the right iliac vein is not compressed, unlike the left iliac vein. Most cases of DVT with no symptoms do not receive treatment and in such cases the DVT is found after PE occurs, which suggests that DVT showing few symptoms is a potential risk for PE. One of the candidate DVT is free-floating thrombi, but further study is needed to clarify this. Older patients with DVT have fewer symptoms and less incidence of PE; they may have fewer symptoms of PE and not be diagnosed as such, even if they have PE, but the real reason is unknown.

In the present study, the incidence of DVT was the same for the right and left legs in patients with PE, but multivariate logistic analysis revealed that DVT in the right leg was a risk for PE, because the left leg was prominent in all patients with DVT.

Diagnostic Techniques for DVT

Venous ultrasonography was used more frequently and CT less frequently in patients without PE than in patients with PE. Venous ultrasonography is noninvasive and convenient, and many diagnostic strategies for DVT use this method[5,24] CT has been used more recently for the diagnosis of PE in recent years[25] as its sensitivity for PE is not inferior to ventilation-perfusion lung scanning[26] CT has the merit that DVT is diagnosed at the same time, so many doctors in Japan may choose venous ultrasonography as the initial diagnostic method in patients suspected of having DVT, and CT in patients suspected of having PE.

Management of VTE

Heparin and thrombolysis were used less frequently in patients with DVT alone. Chronic use of warfarin was more frequent in patients with DVT and PE. Moreover, warfarin was used first more frequently without heparin in cases of DVT alone.

Implantation of an inferior vena cava filter was more frequently performed in patients with DVT and PE. When limited to cases of DVT, inferior vena cava filters were more frequently used in proximal DVT with PE. Recurrence of PE in a patient with PE would increase mortality, so inferior vena cava filters are used to prevent recurrent PE in patients with both DVT and PE.

Incidence of VTE

The calculated number of new patients with PE per year was 3,492 cases in 1996[1] and 7,864 in 2006 in the present study. The calculated number of new patients with PE per year increased 2.25-fold in 1 decade in Japan. These results are similar to the prevalence of PE estimated by the Ministry of Health, Labour and Welfare in Japan (3,000 patients in 1996 and 7,000 in 2005)?[27,28] The vital statistics were 1,410 deaths from PE in 1996, and 1,900 deaths in 2006?[29,30] Annual deaths from PE increased 1.35-fold in 1 decade, which was lower than the increment of diagnostic patients during the same period.

The calculated number of new patients with DVT per year was 14,674 in 2006, which is similar to the prevalence reported in 2005 (16,000 cases[28]).

Study Limitations

One limitation of the present study is the low response rate. Response rates for questionnaires regarding less common diseases are low in general. The response rate in studies on the incidence of PE performed by us was 40.7% in 1996, 30.6% in 2000, 29.8% in 2004, and 26.8% in the present study.

Our results may be affected by the timing of the diagnosis and examination of VTE. Moreover, symptoms of PE may mask symptoms of DVT, despite this being a prospective study. Therefore, additional examinations are necessary to confirm the present results.

Conclusion

DVT in patients with and without PE differs in its site and symptoms. The calculated number of new patients with PE per year doubled over 1 decade in Japan.

Acknowledgment

This study was partly supported by a grant from the Japanese Ministry of Health, Labor and Welfare for Blood Coagulation Abnormalities.

References

1. Kumasaka N, Sakuma M, Shirato K. Incidence of pulmonary thromboembolism in Japan. *Jpn Circ J* 1999; **63:** 439–441.
2. Kitamukai O, Sakuma M, Takahashi T, Kagaya Y, Watanabe J, Shirato K. Incidence and characteristics of pulmonary thromboembolism in Japan 2000. *Intern Med* 2003; **42:** 1090–1094.
3. Sugimura K, Sakuma M, Shirato K. Potential risk factors and incidence of pulmonary thromboembolism in Japan: Results from an overview of mailed questionnaires and matched case-control study. *Circ J* 2006; **70:** 542–547.
4. Editorial Committee on Japanese Guideline for Prevention of Venous Thromboembolism. Japanese Guideline for Prevention of Venous Thromboembolism. Medical Front International Ltd, Tokyo, 2004 (in Japanese).
5. The Japanese Circulation Society. Guidelines for the diagnosis, treatment and prevention of pulmonary thromboembolism and deep vein thrombosis (JCS 2004). *Circ J* 2004; **68**(Suppl): IV-1079–IV-1134 (in Japanese).
6. Nicolaides A, Breddin H, Fareed J, Goldhaber S, Haas S, Hull R, et al. Prevention of venous thromboembolism: International Consensus Statement: Guidelines compiled in accordance with the scientific evidence. *Int Angiol* 2001; **20:** 1–37.
7. Heit JA, Silverstein MD, Mohr DN, Petterson TM, O'Fallon WM, Melton LJ 3rd. Predictors of survival after deep vein thrombosis and pulmonary embolism. *Arch Intern Med* 1999; **159:** 445–453.
8. Quinn DA, Thompson BT, Terrin ML, Thrall JH, Athanasoulis CA, McKusick KA, et al. A prospective investigation of pulmonary embolism in women and men. *JAMA* 1992; **268:** 1689–1696.
9. Cogo A, Lensing AW, Prandoni P, Hirsh J. Distribution of thrombosis in patients with symptomatic deep vein thrombosis: Implications for simplifying the diagnostic process with compression ultrasound. *Arch Intern Med* 1993; **153:** 2777–2780.
10. Nylander G, Olivercrona H. The phlebographic pattern of acute leg thrombosis within a defined urban population. *Acta Chir Scand* 1976; **142:** 505–511.
11. Stamatakis JD, Kakkar VV, Lawrence D, Bentley PG. The origin of thrombi in the deep veins of the lower limb: A venographic study. *Br J Surg* 1987; **65:** 449–451.
12. Nicolaides AN, Kakkar VV, Field ES, Renney JT. The origin of deep vein thrombosis: A venographic study. *Br J Radiol* 1971; **44:** 653–663.
13. Ouriel K, Green RM, Greemberg RK, Clair DG. The anatomy of deep venous thrombosis of the lower extremity. *J Vasc Surg* 2000; **31:** 895–900.
14. Norris CS, Greenfield LJ, Herrmann JB. Free-floating ileofemoral thrombus: A risk of pulmonary embolism. *Arch Surg* 1985; **120:** 806–808.
15. Baldridge ED, Martin MA, Welling RE. Clinical significance of free-floating venous thrombi. *J Vasc Surg* 1990; **11:** 62–67.
16. Markel A, Manzo RA, Bergelin RO, Strandress ED Jr. Pattern and distribution of thrombi in acute venous thrombosis. *Arch Surg* 1992; **127:** 305–309.
17. Kearon C. Natural history of venous thromboembolism. *Circulation* 2003; **107**(Suppl): I-22–I-30.
18. Hill SL, Holtzman GI, Martin D, Evans P, Toler W, Goad K. The origin of lower extremity deep vein thrombi in acute venous thrombosis. *Am J Surg* 1997; **173:** 485–490.
19. Moser KM, LeMoine JR. Is embolic risk conditioned by location of deep venous thrombosis? *Ann Intern Med* 1981; **94:** 439–444.
20. Ferris EJ. Peripheral deep venous thrombosis and pulmonary thromboembolism: Correlative diagnostic evaluation. *Int Angiol* 1983; **2:** 85–98.
21. Dorfman GS, Cronan JJ, Tupper TB, Messersmith RN, Denny DF, Lee CH. Occult pulmonary embolism: A common occurrence in deep vein thrombosis. *Am J Roentgenol* 1987; **148:** 263–266.
22. Hiusman MV, Buller HR, ten Cate JW, van Roven EA, Vreeken J, Kersten MJ, et al. Unexpected high prevalence of silent pulmonary embolism in patients with deep venous thrombosis. *Chest* 1989; **95:** 498–502.
23. Monreal M, Ruiz J, Olazabal A, Arias A, Roca J. Deep venous thrombosis and the risk of pulmonary embolism: A systematic study. *Chest* 1992; **102:** 677–681.
24. Zieler BK. Ultrasonography and diagnosis of venous thromboembolism. *Circulation* 2004; **109**(Suppl): I-9–I-14.
25. Sakuma M, Okada O, Nakamura M, Nakanishi N, Miyahara Y, Yamada N, et al. Recent developments in diagnostic imaging techniques and management for acute pulmonary embolism: Multicenter registry by the Japanese Society of Pulmonary Embolism Research. *Intern Med* 2003; **42:** 470–476.
26. Anderson DR, Kahn SR, Rodger MA, Kovacs MJ, Morris T, Hirsch A, et al. Computed tomographic pulmonary angiography vs ventilation-perfusion lung scanning in patients with suspected pulmonary embolism. *JAMA* 2007; **298:** 2743–2753.
27. Statistics and Information Department, Minister's Secretariat, Ministry of Health, Labour and Welfare. Patient survey 1996. Tokyo, 1998.
28. Statistics and Information Department, Minister's Secretariat, Ministry of Health, Labour and Welfare. Patient survey 2005. Tokyo, 2007.
29. Statistics and Information Department, Minister's Secretariat, Ministry of Health, Labor and Welfare. Vital statistics of Japan 1996. Tokyo, 2008.
30. Statistics and Information Department, Minister's Secretariat, Ministry of Health, Labor and Welfare. Vital statistics of Japan 2006. Tokyo, 2008.

Biochemical and Biophysical Research Communications 378 (2009) 716–720

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Generation of megakaryocytes and platelets from human subcutaneous adipose tissues

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ARTICLE INFO

Article history:
Received 14 November 2008
Available online 6 December 2008

Keywords:
Adipocyte precursor cells
Megakaryocytes
Platelets

ABSTRACT

Recent advances in regenerative medicine have created a broad spectrum of stem cell research. Among them, tissue stem cell regulations are important issues to clarify the molecular mechanism of differentiation. Adipose tissues have been shown to contain abundant preadipocytes, which are multipotent to differentiate into cells including adipocytes, chondrocytes, and osteoblasts. In this study, we have first shown that megakaryocytes and platelets can be generated from adipocyte precursor cells. Human adipocyte precursor cells were cultured in conditioned media for 12 days to differentiate adipocytes, followed by 12 days of culture in media containing thrombopoietin. The ultrastructures of adipocyte precursor cell- and bone marrow CD34-positive cell-derived megakaryocytes and platelets were similar. In addition, adipocyte precursor cell-derived platelets exhibited surface expression of P-selectin and bound fibrinogen upon stimulation with platelet agonists, suggesting that these platelets were functional. This is the first demonstration that human subcutaneous adipocyte precursor cells can generate megakaryocyte and functional platelets in an *in vitro* culture system.

Blood platelets are small non-nucleated cellular fragments derived from megakaryocytes (MKs) that play an essential role in hemostatic plug formation as well as the pathogenesis of arterial thrombosis [1,2]. The molecular mechanisms of differentiation from hematopoietic stem cells into MKs and platelets have been extensively studied. However, many unresolved problems have been remained. Regarding the practical problem, platelet transfusion has been considered to be less than ideal [3,4]. Because platelet concentrates have short storage life, the supply of platelet concentrates has been limited. Furthermore, platelet transfusion is associated with the risks of viral or bacterial infection and serious immune reactions.

Development of *in vitro* culture systems to produce a large number of platelets from stem cells is a research of current interest, because its development is very important to study the molecular mechanism of platelet production or to prepare platelet concentrates. So far, it was reported that platelets were generated *in vitro* from hematopoietic stem cells and embryonic stem (ES) cells. The traditional method using hematopoietic stem cells is the most facile protocol to produce platelets *in vitro*. However, the difficulty in obtaining the sufficient number of hematopoietic stem cells from bone marrow or peripheral blood, particularly in humans, has been pointed out. The culture system to produce ES cell-derived platelets *in vitro* has been recently developed, and it has been shown that abundant platelets from ES cells could be obtained *in vitro*. However, sophisticated experimental technique is required to achieve it.

Adipose tissues are recently highlighted as a source of preadipocytes, some with very restricted potential and others with multipotency for tissue engineering and regenerative medicine [5,6]. Adipose-derived stem cells have several common stem cell surface markers, such as CD9, CD29, CD106, CD146, and Stro-1. To date, experimental studies have shown that subcutaneous adipose tissues have the ability to differentiate into the cell lineage for adipocyte, chondrocyte, myocyte, and osteoblast [5]. Although human adipose tissues include a CD34-positive cell population, i.e., cell population with surface marker for hematopoietic stem cells [7], this fact prompted us to investigate whether platelets could be produced through the adipose tissues. In addition, advantage of using subcutaneous adipose tissues for platelet production is obvious, since subcutaneous adipose tissues are easily obtained and available in large amounts. Here, we have first described an *in vitro*

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doi:10.1016/j.bbrc.2008.11.117

culture system to produce MKs and platelets from adipocyte precursor cells obtained from human subcutaneous adipose tissues.

Materials and methods

Cell culture. Primary human adipocyte precursors from subcutaneous adipose tissues (Cambrex Bio Science Inc., Walkersville, MD) were cultured using preadipocyte growth medium-bullet kit® (Cambrex Bio Science Inc.), for differentiation into mature adipocytes according to the manufacturer's protocol. The media to differentiate into "mature adipocyte" was designated as "MA media". The density of adipocyte precursor cells used in this study was 1.5×10^5 cells/well of a 6-well plate in 2 ml of MA media. By day 12, approximately 80% of the cells showed differentiation into mature adipocytes based on their morphology and positive Oil Red-O staining. Quantification of Oil Red-O stained cells was performed by the manual counting of positive stained cells under the microscope. Quantitative analysis of differentiated cell was also performed using AdipoRed test kit (Cambrex Bio Science Inc.), according to the manufacturer's protocol. Cells (1.5×10^5 cells/well of a 6-well plate) were then cultured in conditioned media, which contained thrombopoietin (TPO), prepared by modification of the method by Zauli et al. and Kerrigan et al., to further differentiate the cells into MK lineages for 12 days [8,9]. The media containing "TPO" to differentiate into MK lineages was designated as "TPO media". Briefly, the modified conditioned medium was Iscove's modified Dulbecco's medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin G sodium, 0.1 mg/ml streptomycin sulfate, 0.5% bovine serum albumin, 4 μg/ml LDL cholesterol, 200 μg/ml iron-saturated transferrin, 10 μg/ml insulin, 50 μM 2-β-mercaptoethanol, 20 μM each nucleotide, 20 μM dNTP, and 50 ng/ml TPO (a gift from KIRIN Brewery Ltd., Japan). MKs and platelets derived from normal human bone marrow CD34-positive cell (Cambrex Bio Science Inc.) were also obtained in the liquid culture system using the conditioned medium for MK lineages, and these cells were used as controls for ultrastructural analysis and flow cytometric analysis (as described below).

Ultrastructural analysis. The methods used for ultrastructural analyses of MKs and platelets derived from adipocyte precursor cells were essentially as described previously [10,11]. MKs and platelets generated from human bone marrow CD34-positive cells were also analyzed ultrastructurally as control. For immuno-electron microscopic analyses, we used anti-von Willebrand factor antibody (Dako Japan, Tokyo, Japan) as a primary antibody, and AuroProbe EM Goat anti-Rabbit IgG (GE Healthcare Life Sciences, Buckinghamshire, UK) was used as secondary antibody.

Flow cytometric analysis. Flow cytometric analysis was performed to characterize the adipocyte precursor cell-derived MKs and platelets using anti-CD41 (also known as glycoprotein IIb or integrin α_{IIb}) antibody (MBL international corporation, Boston, MA), anti-CD42a (also known as glycoprotein Ibβ) antibody (MBL international corporation), anti-CD42b (also known as glycoprotein Ibα) antibody (MBL international corporation), and propidium iodide (PI) (Sigma, St. Louis, MO). Analysis of the surface marker and DNA content by interaction with PI was performed essentially as described previously [10]. Briefly, the cells stained by anti-CD41 antibody were resuspended in PI (50 μg/ml in 0.1% sodium citrate) containing 20 μg/ml RNAase (Sigma), and the samples were incubated for 60 min at 37 °C. The MKs and platelets were counted by flowcytometry on day 12 after differentiation into MK lineages using the relative value of 10^7 adipose cells on day 0 versus megakaryocyte-sized CD41(+) cells and platelet-sized CD41(+) cells [10], respectively. In pilot study, we observed that the cell population of megakaryocyte-sized CD41(+) cells contained nuclei, as assessed by interaction with PI [10]. Therefore, megakaryocyte-sized CD41(+) cells were defined as adipose tissue-derived MKs in this study. For functional assay, the surface expression of P-selectin was examined in adipocyte precursor cell-derived platelets stimulated with thrombin (final concentration, 5 U/ml) for 5 min. Fluorescein isothiocyanate-conjugated anti-P-selectin antibody was purchased by BD Biosciences (Franklin Lakes, NJ). Adipocyte precursor cell-derived platelets were stimulated with thrombin (final concentration, 5 U/ml) in the presence of 100 μg/ml Alexa Fluor 488-labeled human fibrinogen (Molecular Probes, Eugene, OR) for 5 min, and the sample mixture diluted with Hepes buffer was analyzed by flow cytometry. Isotype controls were used in all flow cytometric analyses.

Results

Primary human adipocyte precursors from subcutaneous adipose tissues were cultured in MA media to differentiate into mature adipocytes. In quantitative analysis of differentiated cells, a cytoplasmic triglyceride accumulation was measured, and the relative fluorescence level to the value of undifferentiated cells was 0.007 ± 0.002 (mean ± SD), 2.620 ± 0.806, 4.012 ± 0.978, and 5.903 ± 1.606 for day 0, day 3, day 8, and day 12, respectively. By day 12, approximately 80% of the cells showed differentiation based on their morphology and positive Oil Red-O staining (Fig. 1). Cells were then cultured in TPO media for further differentiation of the cells into MK lineages. In this *in vitro* liquid culture system, human subcutaneous adipocyte precursor cells were differentiated into MK lineage cells, capable of producing platelets. Their morphological features were assessed by electron microscopy and immuno-electron microscopy. Human bone marrow CD34-positive cell-derived MKs and platelets were also examined as controls (electoronic supplementary material 1(a) and (b)). Adipocyte precursor cultured cell-derived MKs exhibited characteristic organelles, such as granules, demarcation membranes, and lobulated nuclei (Fig. 2(A)), and adipocyte precursor cell-derived platelets showed quite similar characteristics to those from CD34-positive cell-derived platelets in that contained granules, mitochondria, and open canalicular system (Fig. 2(B)). These findings of the adipocyte precursor cell-derived cells were consistent with those for the bone marrow CD34-positive cell-derived MKs and platelets. In addition, immuno-electron microscopic study with anti-von Willebrand factor antibody was conducted to further characterize the adipocyte precursor cell-derived MKs. It was clearly demonstrated that the adipocyte precursor cell-derived MKs contained von Willebrand factor in granule assessed by immunochemical analysis (Fig. 2(C)). This finding of the adipocyte

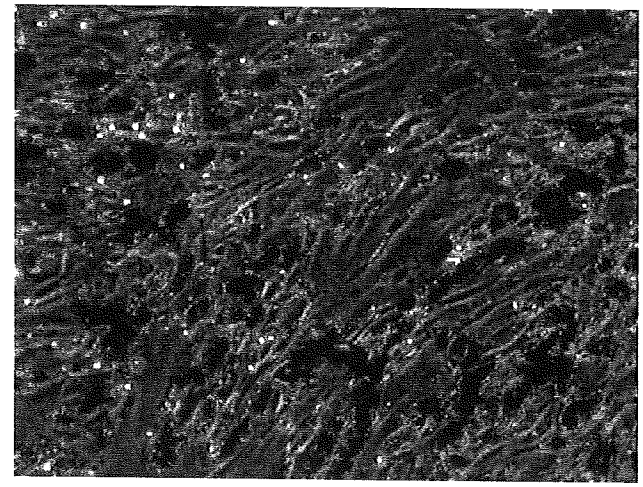

Fig. 1. Differentiated into adipocytes were stained with Oil Red-O.

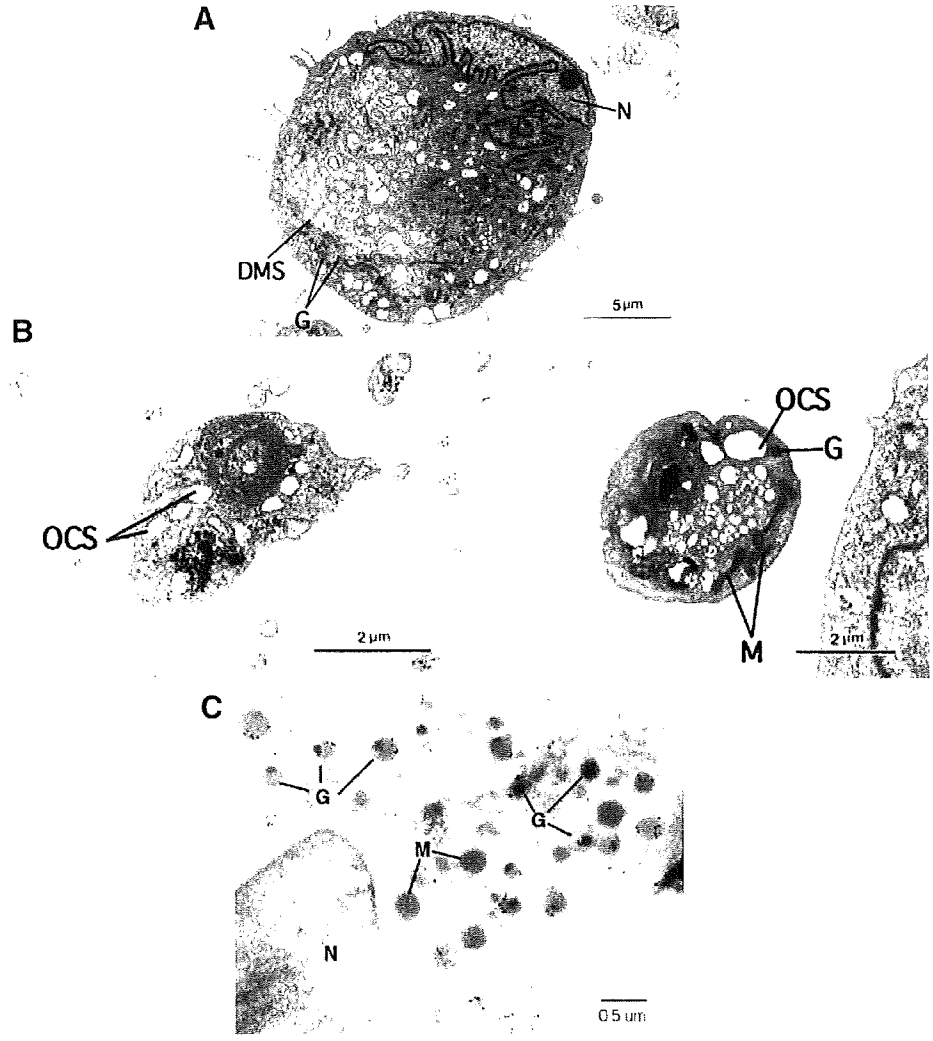

Fig. 2. (A–C) The morphology of (A) megakaryocytes and (B) platelets derived from adipocyte precursor cells was analyzed using transmission electron microscopy. Regarding megakaryocytes, typical organelles such as granules (G), demarcation membrane system (DMS), and nuclei (N) were evident in adipocyte precursor cell-derived megakaryocytes. Also, adipocyte precursor cell-derived platelets contained granules, mitochondria (M), and open canalicular system (OCS). (C) von Willebrand factor expression in adipocyte precursor cell-derived megakaryocytes was shown by immuno-electron microscopy with anti-von Willebrand factor antibody.

precursor cell-derived cells was consistent with that for the bone marrow CD34-positive cell-derived MKs (electoronic supplementary material 1(c)).

The MKs and platelets were counted by flow cytometry on day 12 after differentiation into MK lineages using the relative value of 10^7 adipose cells on day 0 versus megakaryocyte-sized CD41(+) cells and platelet-sized CD41(+) cells, respectively. The number of MK and platelet produced was approximately $2 \times 10^6 \pm 2500$ and $15 \times 10^4 \pm 270$, respectively. The DNA ploidy of the adipocyte precursor cell-derived MKs ranged from 2 N to 16 N (electoronic supplementary material 2(a)). In addition to the expression of CD41, expressions of other platelet specific receptors were clearly observed by flow cytometry using antibodies for CD42a and CD42b (electoronic supplementary material 2(b)–(e)). These findings of the adipocyte precursor cell-derived cells were consistent with those for the bone marrow CD34-positive cell-derived MKs (electoronic supplementary material 3 (a)–(e)). We also performed a functional assay by flow cytometry. Surface expression of P-selectin, a platelet activation marker, and fibrinogen binding to platelets were examined in adipocyte precursor cell-derived platelets stimulated with thrombin (final concentration, 5 U/ml). Binding of anti-P-selectin specific antibody and Alexa Fluor 488-labeled fibrinogen to platelet-sized CD41(+) cells were clearly demonstrated in response to thrombin. Approximately 30% of platelet-sized CD41(+) cells expressed on the cell surface in response to thrombin (electoronic supplementary material 4(a)). Fibrinogen binding upon thrombin stimulation was observed to be approximately 35% of platelet-sized CD41(+) cells (electoronic supplementary material 4(b)). Although the values of P-selectin expression and fibrinogen binding showed markedly different between the stimulated and unstimulated cells, the values did not have significantly differences ($p = 0.3029$ for P-selectin surface expression, $p = 0.0562$ for fibrinogen binding). These results suggest that the adipocyte precursor cell-derived platelets were functional.

Discussion

In the present study, we developed a novel system in which adipocyte precursor cells in human subcutaneous adipose tissue were successfully differentiated into MK lineages in an *in vitro* liquid culture system in the presence of TPO. Adipose tissues are recently considered to contain the abundant and accessible source of preadipocytes with the restricted potential and multipotent cells for tissue engineering and regenerative medicine. So far, experimental studies showed that subcutaneous adipose tissues had the ability to differentiate the cell lineage for

dipocyte, cardiomyocyte, chondrocyte, endothelial, myocyte, neuronal-like, and osteoblast [12]. This study is the first to demonstrate platelet production from human subcutaneous adipocyte precursor cells *in vitro*. Previous studies showed that platelets were generated *in vitro* from ES cells and hematopoietic stem cells from bone marrow, peripheral blood, and cord blood. Regarding the experiments using ES cells, the cells are unlimited sources, and the *in vitro* differentiation system for MK lineage from ES cells is a powerful tool to obtain abundant modified MKs and platelets, however, the experiments to generate platelets from ES cells need higher experimental techniques. On the other hand, generation of platelets from hematopoietic stem cells is technically easier, because hematopoietic stem cells have higher potential for differentiation into MK lineage. However, it is extremely difficult to obtain sufficient amounts of hematopoietic stem cells from typical sources, such as bone marrow cells. This study obtained MKs and functional platelets from human subcutaneous adipocyte precursor cells. The fact that expression of CD34, a cell surface marker for hematopoietic stem cell, in adipose tissues was demonstrated in several papers prompted us to investigate the capability of subcutaneous adipocyte precursor cells to produce MKs and platelets. We therefore tried to develop a novel system in which adipocyte precursor cells in human subcutaneous adipose tissue are differentiated into MK lineage *in vitro*. Because subcutaneous adipose tissues are easily obtained and available in large amounts, these tissues are abundant and easily accessible source materials for generation of platelet *in vitro*. Moreover, the present method does not need complicated techniques.

In this study, we obtained approximately 2×10^6 MKs and 15×10^4 platelets from 10^7 adipocyte precursor cells. On a similar culture scale, 10^5 human ES cells generated approximately 5–20×10^3 CD41a(+)/CD42b(+) cells [13] or >2–5×10^5 MKs [14], and 10^7 bone marrow mononuclear cells generated approximately 6×10^4 megakaryocyte-sized CD41(+) cells (unpublished observations), although it is difficult to compare precisely the efficiency of platelet production between various stem cell sources. Also, 10^4 murine ES cells were reported to generate approximately 10^8 platelets [10,15], and 10^5 human ES cells generated approximately 10^5 platelets [14].

The important question has remained, which cell population in human adipocyte precursor cells can be responsible for generation of MKs and platelets. The critical single cell experiment definitely is necessary to define the cells in human adipocyte precursor cells which may differentiate into MKs and platelets. The present experimental condition made it possible to produce a large amount of MKs and platelets: adipocyte precursor cells were initially cultured in MA media for 12 days to differentiate into mature adipocytes, followed by further 12 days of culture in TPO media to differentiate into MK lineages. In a pilot study using small amount of adipocyte precursor cells (approximately 10^5 cells), we first cultured in the MA media to differentiate into mature adipocytes. After designated days of culture, i.e., day 0, day 6, day 9, and day 12, subsequent culture in TPO media for 12 days was performed, and the number of MKs demonstrated as CD41-positive large-sized cells was counted. It was found that the number of MKs was unmeasurable, i.e., CD41 (+) cells < Isotype control (+) cells, 3500, 7900, and 9500, in day 0, 6, 9, and 12 samples, respectively. Also, we obtained MKs and platelets from a mouse preadipocyte cell line, 3T3-L1 cells, using this experimental system (data not shown). These might have a suggestion that the observed MKs and platelets were obtained from adipocyte precursor cells and not other cell types.

The present study did not show the reason why the culture condition that approximately 80% of the cells showed differentiation into mature adipocytes have effect on cell differentiation into MK lineage. Because TPO acts through c-mpl to stimulate the proliferation and maturation of MKs and their progenitors, the c-mpl expression was investigated in two cell populations, adipocyte differentiated and undifferentiated cells. It was clearly demonstrated that the cell population, which approximately 80% of the cells showed differentiation into mature adipocytes, showed small, but detectable amount of c-mpl as assessed by real-time quantitative PCR, whereas the c-mpl expression in undifferentiated cells showed undetectable level (data not shown). Thus, cell population, which approximately 80% of the cells showed differentiation into mature adipocytes, was considered to have the ability for responsiveness to thrombopoietin, although we did not show which cells have the c-mpl.

In the present culture system, subcutaneous adipocyte precursor cells were cultured in MA media for 12 days to differentiate into mature MKs. By day 12, approximately 80% of cells showed differentiation into mature adipocytes, and the remaining cells consisted of mostly preadipocytes. All of these cells were then cultured in TPO media to differentiate into MK lineages. Although the cell population that can be responsible for generation of MKs and platelets was not identified in these cells, the cells which approximately 80% of cells showed mature adipocytes were used as materials to further differentiate into MK lineages. Thus, we should describe the possibility that mature adipocytes have potencies to differentiate into other cell types. In mammals, although terminally differentiated cells have been considered to have no ability of dedifferentiation, there were several reports that terminally differentiated cells have the potential to differentiate into other cells under specific-experimental conditions. It was demonstrated that adipocyte-derived cells showed characteristics of adipogenic progenitors [16–18]. Yagi et al. reported that a preadipocyte cell line was established from mouse mature adipocytes [19]. Nobusue et al. showed to establish a preadipocyte cell line from mature adipocytes of green fluorescent protein transgenic mice [20]. Justesen et al. demonstrated that human adipocytes transdifferentiated to bone-forming cells [21]. Matsumoto et al. showed that human mature adipocyte-derived dedifferentiated fat cells had the potential to differentiate into adipocytes, chondrocytes, and osteoblastes [22]. Also, Kazama et al. reported that dedifferentiated fat cells derived from mature adipocytes had the ability to differentiate into skeletal myocytes [23]. Regarding other cases for dedifferentiation, McGann et al. reported that terminally differentiated mouse C2C12 myotubes showed to have the ability of dedifferentiation [24]. Chen et al. showed that the mouse C2C12 cells had dedifferentiated phenotypes [25]. Also, induced pluripotent stem (iPS) cells were generated from adult human dermal fibroblasts, fetal human fibroblasts, and human newborn foreskin fibroblasts, although the generation of iPS cells requires virus-mediated transfection of transcriptional factors [26,27],

Further study is necessary to identify which cell population can produce MKs and platelets from human subcutaneous adipocyte precursor cells, and to characterize human platelets generated from subcutaneous adipocyte precursor cells.

Disclosure of conflict of interests

All authors state that they have no conflict of interest.

Acknowledgments

The authors would like to thank Dr. Andrew D. Leavitt, University of California, San Francisco, CA, for providing the detailed protocol on *in vitro* differentiation into megakaryocyte lineages from bone marrow mononuclear cells.

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (#18209021).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.11.117.

References

[1] G. Davi, C. Patrono, Platelet activation and atherothrombosis, N. Engl. J. Med. 357 (2007) 2482–2494.
[2] D. Varga-Szabo, I. Pleines, B. Nieswandt, Cell adhesion mechanisms in platelets, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 403–412.
[3] K. Kaushansky, Historical review: megakaryopoiesis and thrombopoiesis, Blood 111 (2008) 981–986.
[4] D.F. Stroncek, P. Rebulla, Platelet transfusions, Lancet 370 (2007) 427–438.
[5] C.T. Gomillion, K.J. Burg, Stem cells and adipose tissue engineering, Biomaterials 27 (2006) 6052–6063.
[6] L. Casteilla, C. Dani, Adipose tissue-derived cells: from physiology to regenerative medicine, Diabetes Metab. 32 (2006) 393–401.
[7] C. Sengenès, K. Lolmède, A. Zakaroff-Girard, R. Busse, A. Bouloumié, Preadipocytes in the human subcutaneous adipose tissue display distinct features from the adult mesenchymal and hematopoietic stem cells, J. Cell. Physiol. 205 (2005) 114–122.
[8] G. Zauli, A. Bassini, M. Vitale, D. Gibellini, C. Celeghini, E. Caramelli, S. Pierpaoli, L. Guidotti, S. Capitani, Thrombopoietin enhances the alpha IIb beta 3-dependent adhesion of megakaryocytic cells to fibrinogen or fibronectin through PI 3 kinase, Blood 89 (1997) 883–895.
[9] S.W. Kerrigan, M. Gaur, R.P. Murphy, S.J. Shattil, A.D. Leavitt, Caspase-12: a developmental link between G-protein-coupled receptors and integrin alphaIIbbeta3 activation, Blood 104 (2004) 1327–1334.
[10] T.T. Fujimoto, S. Kohata, H. Suzuki, H. Miyazaki, K. Fujimura, Production of functional platelets by differentiated embryonic stem (ES) cells in vitro, Blood 102 (2003) 4044–4051.
[11] H. Suzuki, T. Kaneko, T. Sakamoto, M. Nakagawa, T. Miyamoto, M. Yamada, K. Tanoue, Redistribution of alpha-granule membrane glycoprotein IIb/IIIa (integrin alpha IIb beta 3) to the surface membrane of human platelets during the release reaction, J. Electron Microsc. (Tokyo) 43 (1994) 282–289.
[12] J.M. Gimble, A.J. Katz, B.A. Bunnell, Adipose-derived stem cells for regenerative medicine, Circ. Res. 100 (2007) 1249–1260.
[13] M. Gaur, T. Kamata, S. Wang, B. Moran, S.J. Shattil, A.D. Leavitt, Megakaryocytes derived from human embryonic stem cells: a genetically tractable system to study megakaryocytopoiesis and integrin function, J. Thromb. Haemost. 4 (2006) 436–442.
[14] N. Takayama, H. Nishikii, J. Usui, H. Tsukui, A. Sawaguchi, T. Hiroyama, K. Eto, H. Nakauchi, Generation of functional platelets from human embryonic stem cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors, Blood 111 (2008) 5298–5306.
[15] Y. Matsubara, M. Murata, H. Suzuki, T. Kamata, A. Shimizu, A.D. Leavitt, Y. Ikeda, Ultrastructural evidences of caspase-dependent platelet generation in ES cell-differentiation system, Blood 6 (Suppl.) (2005) 1009a.
[16] H. Sugihara, N. Yonemistu, S. Miyabara, K. Yun, Primary cultures of unilocular fat cells: characteristics of growth in vitro and changes in differentiation properties, Differentiation 31 (1986) 42–49.
[17] H. Sugihara, N. Yonemistu, S. Miyabara, S. Toda, Proliferation of unilocular fat cells in the primary culture, J. Lipid Res. 28 (1987) 1038–1045.
[18] M. Shigematsu, H. Watanabe, H. Suguhara, Proliferation and differentiation of unilocular fat cells in the bone marrow, Cell. Struct. Funct. 24 (1999) 89–100.
[19] K. Yagi, D. Kondo, Y. Okazaki, K. Kano, A novel preadipocyte cell line established from mouse adult mature adipocytes, Biochem. Biophys. Res. Commun. 321 (2004) 967–974.
[20] H. Nobusue, T. Endo, K. Kano, Establishment of a preadipocyte cell line derived from mature adipocytes of GFP transgenic mice and formation of adipose tissue, Cell Tissue Res. 332 (2008) 435–446.
[21] J. Justesen, S.B. Pederson, K. Stenderup, M. Kassem, Subcutaneous adipocytes can differentiate into bone-forming cells in vitro and in vivo, Tissue Eng. 10 (2004) 381–391.
[22] T. Matsumoto, K. Kano, D. Kondo, N. Fukuda, Y. Iribe, N. Tanaka, Y. Matsubara, T. Sakuma, A. Satomi, M. Otaki, J. Ryu, H. Mugishima, Mature adipocyte-derived differentiated fat cells exhibit multilineage potential, J. Cell. Physiol. 215 (2008) 210–222.
[23] T. Kazama, M. Fujie, T. Endo, K. Kano, Mature adipocytes-derived dedifferentiated fat cells can transdifferentiate into skeletal myocytes in vitro, Biochem. Biophys. Res. Commun. 317 (2008) 769–774.
[24] C.J. McGann, S.J. Odelberg, M.T. Keating, Mammalian myotube dedifferentiation induced by newt regeneration extract, Proc. Natl. Acad. Sci. USA 98 (2001) 13699–13704.
[25] S. Chen, Q. Zhang, X. Wu, P.G. Schultz, S. Ding, Dedifferentiation of lineage-committed cells by a small molecule, J. Am. Chem. Soc. 126 (2004) 410–411.
[26] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, Induction of pluripotent stem cells from adult human fibroblasts by defined factors, Cell 131 (2007) 861–872.
[27] J. Yu, M.A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J.L. Frane, S. Tian, J. Nie, G.A. Jonsdottir, V. Ruotti, R. Stewart, I.I. Slukvin, J.A. Thomson, Induced pluripotent stem cell lines derived from human somatic cells, Science 318 (2007) 1917–1920.

Ann Vasc Dis Vol.2, No.1; 2009; pp17–20
Online April 15, 2009
doi:10.3400/avd.AVDsa08019

Special Article

Medical Standards Seen from the Perspective of Changes in Academic Society Themes: Investigation of a Lawsuit Concerning the Prevention of Venous Thromboembolism

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Objective: **To determine whether a violation of the standard of care for prevention of pulmonary embolism by preventing deep vein thrombosis occurred in 1999.**
Materials and methods: **Themes from past general meetings of the three societies that comprise the Japanese Board of Cardiovascular Surgery that pertained to venous thromboembolism from 1999 to 2006 were examined and analyzed for an appeal hearing to determine whether a violation had occurred.**
Results: **The first pertinent session on a method for the prevention of pulmonary embolism was presented in 2006 by the Japanese Society for Vascular Surgery. Thus, the medical treatment performed in this case did not violate the standard of care in 1999.**
Conclusion: **The "standard of medical treatment at the time", can be discerned by tracing consensus agreement at session meetings. If the consensus from each session is recorded, a more detailed analysis can be made of the agreement reached by board members.**

Key words: accident, cognitive impairment, guideline, session-theme, message

Introduction

The judicial system must make many decisions on whether or not a medical procedure performed at a defending institution strayed from the expected "standard of medical care of the time". Since legal judges are not medical experts, serious consideration is given to the written opinions of the plaintiff, the defendant's doctors, or the official judgment of an impartial third party. These written opinions and judgments must give a true and unbiased evaluation based on the medical standards of the time. However, medicine continues to develop daily; it is a process of reflection and renewal, and thus treatments themselves are fluctuating entities. For this reason, each medical judgment is different. As medical standards shift, an ambiguity in judgment arises, and this leads to a distrust of medical care.

The establishment of medical standards should be based on the consensus of the medical community. Themes (session theme) that are recurrently adopted at medical conventions provide suitable material for the consideration of medical standards. Repetition of a theme is a good indicator of the value of a theme's investigation. Consensus opinions reached on debated themes reflect the standard of medical care of the time (of course, dissenting opinions often exist).

In order to analyze the standard of medical care, it is imperative to understand this progression of themes. How they originate, how they are selected, how problems are presented, and how themes are accepted by the societies' members.

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Materials and Methods

Selection of Session Theme

Sessions pertaining to the direct connection between deep vein thrombosis and pulmonary embolism were investigated. Further, sessions reviewed were those in which a medical society consensus could be formulated. These were: symposiums, workshops, consensus meetings, plenary sessions, president demanded sessions, debate sessions and panel discussions, all given at general assemblies. All other sessions were omitted.

The Societies Focused Upon

The majority of Japanese venous disease patients are cared for by vascular surgeons, with most practitioners belonging to the Japanese Society for Vascular Surgery. Licensing in this specialty is handled solely by the Japanese Board of Cardiovascular Surgery. The three bodies that compose this board–namely, the Japanese Society for Vascular Surgery, the Japanese Society for Cardiovascular Surgery and the Japanese Association for Thoracic Surgery–were the objects of this study. The time span of this investigation was from 1999 to 2006.

Outline of the Case Judged and Judgment Method

The central point of the case being appealed was whether or not the measures employed for prevention of pulmonary embolism at the time were appropriate. In 1999, the patient was admitted to a local general hospital for surgery. The patient's obesity and a long surgical procedure posed a risk for deep vein thrombosis, however, no swelling of the lower extremities existed and the patient displayed no deep vein thrombosis. Post-surgery, the patient suddenly developed a pulmonary embolism. The expert opinion written for the plaintiff stated that this was caused by the hospital's negligence in not following preventative measures as per the guidelines of 1999. The courts then compared the open prevention measures policy of the defendant hospital and the actual level of practice provided, with those of other local hospitals of comparable status.

There was little objective data reflecting the "standard of medical care of the time" for the defendant hospital and those of comparable scale. Therefore, "pulmonary embolism prevention concepts" that would create the "standard of medical care" of the time were examined. The preventative strategies for pulmonary embolism included the "early diagnosis and treatment of deep vein thrombosis" and "the prevention of deep vein thrombosis itself". The former had been hastily established by Japanese pulmonary specialists of the time. The latter was a method practiced in the western world, however the designated appropriate dosage amounts for heparin differed for Japan and the west. Analysis of the results of session themes as objective data was used to determine which method was the "standard of medical care" in 1999. This result was given to the court.

Results

Session Themes

Themes from the General Assembly's collections with the phrases "deep vein thrombosis" or "pulmonary embolism" in the title were examined. The Japanese Association for Thoracic Surgery had no relevant topics, and the Japanese Society for Cardiovascular Surgery had one session from 2005 entitled "Guidelines for Treatment of Venous Thromboembolism". The Japanese Society for Vascular Surgery had five sessions in which the prevention, diagnosis and treatment of pulmonary embolisms were dealt with (**Table 1**).

Also, of these three societies, the first and only to have a session theme on "deep vein thrombosis prevention" as a means of pulmonary embolism prevention was the Japanese Society for Vascular Surgery ("Inspection and Problems of the Guidelines for Venous Thromboembolism"). It became clear that prior to this, the Japanese Society for Vascular Surgery was vigorously centered on the established method of "early diagnosis and treatment of deep vein thrombosis" and that this was the standard method for prevention of pulmonary embolism (**Table 1**).

Conclusion of Suit

It was clear that the 1999 "standard of medical care" for prevention of pulmonary embolism was, the "early diagnosis and treatment of deep vein thrombosis". The investigation into "prevention of pulmonary embolism through the prevention of deep vein thrombosis" was not undertaken by the medical community until 2006. Further, the first guideline relating to the prevention of venous thrombosis, created by the Japanese Circulation Society in 2004, did not exceed a translation of western guidelines and was not born of the structure of Japanese illness.[1] This did not gain consensus from the medical community, and could only be regarded as falling within the confines of an informational reference. It follows that prior to 2004, even though the writing referred to as a guideline in 1999 touched on the "prevention of pulmo-

Table 1 Session themes of society meetings related to venous thromboembolism

Japanese Society for Vascular Surgery								
	1999	2000	2001	2002	2003	2004	2005	2006
Symposium	–	–	Etiology, treatment, and long-term result of deep vein thrombosis	–	–	–	–	–
Pannel Discussion	–	–	–	–	–	Deep vein thrombosis: Thorough discussion of diagnosis and treatment	–	–
President Demand	–	–	–	–	Treatment of pulmonary thromboembolism 1	–	–	Inspection and problems of the guidelines for venous thromboembolism
President Demand	–	–	–	–	Treatment of pulmonary thromboembolism 2	–	–	–

The first and only session theme on "deep vein thrombosis prevention" as a means of pulmonary embolism was the Japanese Society for Vascular Surgery with "Inspection and Problems of the Guidelines for Venous Thromboembolism" in 2006. The Japanese Association for Thoracic Surgery had no relevant topics and the Japanese Society for Cardiovascular Surgery had one session on 2005 entitled "Guidelines for Treatment of Venous Thromboembolism".

nary embolism through the prevention of deep vein thrombosis", this did not reflect the "standard of medical care" of the country at the time.

Summarily, preventative measures for pulmonary embolism in 1999 were the "early diagnosis and treatment of deep vein thrombosis", and not the "prevention of pulmonary embolism through the prevention of deep vein thrombosis". The suit concluded that it could not be said that the "defendant hospital was liable for the development of the pulmonary embolism" through not taking measures to prevent deep vein thrombosis at the time.

Discussion

Features of a Society's Specialty

Of the three associations that focus on the specialty of venous diseases and compose the Japanese Board for Cardiovascular Surgery, much activity is centered on the Japanese Society for Vascular Surgery. However, there are many specialists who, due to many restrictions, are unable to obtain a position on the Japanese Board for Cardiovascular Surgery. This is a reflection of the thought that society expects someone other than cardiovascular surgeons to be specialists in vascular disease.

Standard of Medical Care

From a medical viewpoint, the "Standard of medical care", is not the average level of care, nor is it a minimal level that hospitals must maintain. Rather, it is a provisional goal used by the judicial system designed to raise the level of care provision of all members of the medical society to the highest level".

Incidentally, the judicial system defines "standard of medical care" as "the standard of medical treatment claimed by the medical institution (at the time) based on contractual medical care (at the time)". Medicine must continually improve, and "the expectation held by patients that the medical institution through its nature seeks to improve binds (patients and hospitals to) a medical contract". Through this premise, the "standard of medical care" is fixed until such time as there is a medical association presentation or addendum report. The famous Supreme Court decision of Heisei 7 (1995) (a case concerning the photocoagulation procedures on infants with retinopathy) used this type of basic understanding in its ruling.[1] In that instance, the conscientious "cooperative aim" towards the improvement of medicine legally became a "cooperative duty". However, through legal findings, the judicial system continues to demand a clarifica-

tion of the "standard of medical care" from the medical world and the judicial system further demands "the conscious use of legal findings in medical reports".[2] Furthermore, the only reason that messages from the medical profession do not reach judicial ears is that there are doctors that continue to deliver erroneous messages.[3, 4] Therefore medical experts (not board certified medical experts), must return to the judicial system the most common medical understanding of the field based on current objective data. Session theme analysis is useful for this process.

The Significance of Session Themes

Session theme analysis holds more utility than just the clarification of guideline problems. From the results of session theme analysis it could be seen that neither the Japanese Association for Thoracic Surgery nor the Japanese Society for Cardiovascular Surgery held themes on venous disease, and that such knowledge was not jointly accumulated by the members of these associations. Conversely, with respect to the Japanese Circulation Society's 2004 "Guideline Pertaining to Pulmonary Embolism and Deep Vein Thrombosis Diagnosis, Treatment and Prevention", the Japanese Association for Thoracic Surgery and the Japanese Society for Cardiovascular Surgery are both listed as joint research associations. The Japanese Society for Vascular Surgery, however, is not. Associations operate by obtaining member consensus. The public must see that an association is part of a joint research effort, and that an association bears responsibility to the guidelines decided, and that the members of the association abide by these same guidelines. The only guideline definition supported by the Japanese Society for Vascular Surgery clarified here, received consensus from members of the society.

Conclusion

From investigation of the progression of session themes, the society's members' understanding of the standard of medical care of the time and the society's transformation over time became clear. It is believed that the recording of each future session's progressive conditions, problem points, resources, and reasons for discontinuation, would assist better understanding of societies' opinions based on their member consensus.

Acknowledgements

The author would like to thank Doctor Toshiteru Ohnisi, Chairperson of the board of Directors, and Kousei Igakukai, for their warmhearted advice and encouragement. This research received a scientific research grant from the Ministry of Health, Labor and Welfare. Thank you also to Mr. C.S. Magor for his advice on the English translation.

Note From the Author

The preceding material first appeared in Japanese: Kawasaki, Tomio. 2008. Gakkai seshion no tehma hensen kara mita iryou suijyun–joumyaku kessensyou ni okeru iryousosyou no kentou [Standards of Medical Treatment Seen from the Standpoint of Changes in Academic Society Session Themes: Settlement of a Lawsuit Concerning Medical Treatment of Venous Thrombosis]. Jpn J Vasc Surg. 17, 7–12, 2008.

REFERENCES

1) Utsugi S, Machino S, Hirabayashi K, et al. Ijihou hanrei hyakusen [Top 100 Medical Precedents]. Jurist. 2006; **183**: 142–4 (in Japanese).
2) Fukuda T, Takase K. 2004. Iryousosyou to senmon jyouhou [Medical Suits and Expert Information]. Tokyo, Japan: Hanrei Taimuzusya, 2004 (in Japanese).
3) Kawasaki T. Minjisosyou ni okeru kouteki iryou kanntei ha nanino tameni okonawareru noka? [Why do Public Medical Judgments in Civil Suits Occur?] Jurist. 2007; **1327**: 2–6 (in Japanese).
4) Kawasaki T. Iryou funnsou ni mirareru "Ninshiki no soui" ha naze kaisyou sarenai noka? [Universal Differences in Perception and Legal System] Law and Technology. 2007; **37**: 29–37 (in Japanese).

Ⅷ．血　液

特発性血小板減少性紫斑病

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疑うポイント

◇血小板のみの著明な減少．赤血球系，白血球系の数や形態異常を認めない（ときに失血性または鉄欠乏性貧血を伴うことがある）
◇皮下出血や歯肉出血など皮膚粘膜出血が主症状．関節内出血は通常認めない
◇薬剤性，SLE，リンパ増殖性疾患，HIV 感染症，肝硬変などに起因する血小板減少症を除外すべきである
◇EDTA 依存性偽性血小板減少症を除外する
◇血栓性血小板減少性紫斑病やヘパリン起因性血小板減少症など，血小板輸血を避けるべき疾患を除外する
◇活動性の出血を認める場合のみならず，鼻出血や口腔内の出血や血腫を認める場合は，ただちに治療を開始する

Ⅰ．症例提示

症　例

症例は 35 歳，女性．生来健康で，とくに易出血性を自覚したことはなかった．1 週間前より発熱および感冒様症状があり，近医にて抗菌薬と消炎剤を投与されていた．2〜3 日前より四肢を中心に青あざを認めるようになったため，近医を再受診した．著明な血小板減少（0.5 万/μl）を指摘され，同日当院へ紹介となる．当院での身体所見にて，四肢および体幹部に点状出血や斑状出血を認め，口腔内に血腫を認めたため緊急入院となる．

入院時の身体所見および検査所見：意識清明．口腔内に血腫あり．四肢および体幹部に点状出血および斑状出血を認める．検尿，異常なし．赤血球 415 万/μl，Hb 12.9 g/dl，Ht 38%，白血球 6,800/μl，血小板 0.4 万/μl，白血球分画異常なし．

PT 71%，APTT 34 sec，FDP(D ダイマー)0.2 μg/ml．生化学検査(肝機能，腎機能検査)異常なし．

血小板輸血を行ったあと，骨髄穿刺を施行した．有核細胞数 16 万/μl，顆粒球系，赤血球系分画に異常なく，また形態異常を認めず．巨核球数は 200/μl と増加していた．

Ⅱ．診　　断

診断のポイント

特発性血小板減少性紫斑病(idiopathic thrombocytopenic purpura：ITP)は，他の基礎疾患や薬剤などの原因が明らかではないにもかかわらず，血小板の破壊が亢進し血小板減少をきたす後天性の疾患である．その原因が血小板に対する自己抗体であるため，欧米では特発性(idiopathic)というよりは，免疫性(immune)あるいは自己免疫性(autoimmune)という表現が用いられることが多い．

皮下出血，歯肉出血，鼻出血，性器出血など，皮膚粘膜出血が主症状である．ITP では血友病など，凝固因子異常症にみられる関節内出血や筋肉内出血は，きわめてまれである．また，凝固系の異常は必ず否定すべきである．

実地診療において ITP の診断はいまだ除外診断が主であるため，赤血球系(鉄欠乏性貧血は可)や白血球系に異常がないことを確認すべきである．薬剤性，SLE，リンパ増殖性疾患，HIV 感染症，肝硬変などに起因する血小板減少症も除外すべきである．上記に示した自験例の場合，抗菌薬も投与されていたため疑わしい薬剤はすべて中止した．また，骨髄穿刺は必須ではないが，高齢者で骨髄異形成症候群が否定できない場合は，積極的に骨髄穿刺を行うべきである．Table 1 に，厚生労働省の難治疾患克服研究事業「血液凝固異常症に関する調査研究」班の ITP に関しての新たな診断基準案を示している("専門家へのコメント"参照)．

血小板数が 3～5 万/μl 以下の症例で無症状の場合や，検査コメントに血小板凝

Table 1. 慢性 ITP の診断基準(案)

1) 血小板減少(10 万/μl 以下)
2) 末梢血塗抹標本は正常
3) 以下の検査の内 3 項目以上を満たす
   ① 貧血がない
   ② 白血球減少がない
   ③ 末梢血中の抗 GPⅡb-Ⅲa 抗体産生 B 細胞の増加
   ④ 血小板関連抗 GPⅡb-Ⅲa 抗体の増加
   ⑤ 網状血小板比率の増加
   ⑥ 血漿 TPO は軽度上昇にとどまる(<300 pg/ml)
4) 他の免疫性血小板減少性紫斑病(SLE，リンパ増殖性疾患，HIV 感染症，肝硬変，薬剤性など)を除外できる

集とある場合は，EDTA 依存性偽性血小板減少症を除外すべきである．末梢血用スピッツの抗凝固薬 EDTA-2K により，血小板が凝集し自動血球計算器において白血球と認識され，見かけ上血小板数低値となることがある．そのため，塗抹標本や抗凝固薬なしの採血直後に測定し，血小板数が正常であることを確認する．この場合，治療は不要である．

ITP はその発症様式とその経過により，急性型と慢性型に分類されるが，発症時に分類することはきわめて困難である．一般的に急性型の場合，出血症状が強い場合が多い．

専門家へのコメント

PAIgG(platelet-associated IgG，血小板関連 IgG)の測定は，2006 年に保険収載されている．ITP においてはその 90% 以上の症例において PAIgG が上昇しており，その疾患感受性は高いが，PAIgG は血小板に結合した(あるいは付着した)非特異的な IgG も測定するため，再生不良性貧血などの血小板減少時にも PAIgG が高値になることがあり，その特異性は低く 27% とも報告されている．そのため，PAIgG が高値であるだけで ITP とは診断できない．

ITP の病態では幼若血小板を反映する網状血小板が増加しているが，血小板産生を反映する血漿トロンボポエチン(TPO)濃度は正常あるいは軽度増加するに留まる．一方，再生不良性貧血などの血小板産生障害の場合，血漿 TPO 濃度は著明に増加する．また，血小板自己抗体として，血小板膜 GPⅡb-Ⅲa や GPⅠb-Ⅸに対する抗体が証明できれば ITP 診断の特異性は高い．しかしながら，抗体の検出感度が約 50% と低いのが弱点である．これらの ITP の補助診断検査はいまだ研究室レベルであり，実地診療には応用されていない．

知っておきたい語句

◇血栓性血小板減少性紫斑病(TTP)

本疾患は，von Willebrand 因子の切断酵素である ADAMTS13 に対するインヒビター(自己抗体)により生じる血栓性疾患であり，血小板減少に加え溶血性貧血や腎機能障害を呈する．本疾患では，血小板減少に対し血小板輸血は，病状を悪化させるため避けるべきである．血漿交換(＋ステロイド)が第一選択である(TTP の項を参照されたい)．

◇ヘパリン起因性血小板減少症

ヘパリン投与によりヘパリン-血小板第 4 因子複合体に対して抗体が産生され，その抗体が血小板を活性化させ血小板減少をきたす病態で，血栓性疾患である．2 万/μl 以下になることはまれである．血小板輸血は避けるべきである．

Ⅲ. 治　　療

治療のポイント

活動性出血を認める場合は当然であるが，血小板数が2万/μl以下で鼻出血や口腔内の出血や血腫を認めた場合，脳出血や消化管出血の危険性があるため，入院させ治療をすぐに開始すべきである．一方，血小板数が2～3万/μlで無症状の場合は，必ずしも入院の必要はなく外来で対応しうる(Table 2，Fig. 1)．

重篤な出血がある場合，まず血小板輸血を行う．しかしながら輸血された血小板はすぐに破壊されるため，同時にガンマグロブリン大量療法(IVIg)とステロイド治療を開始する．緊急の場合はステロイドパルス療法が主となる．ある程度時間に余裕があるなら，経口でprednisolone(体重あたり0.5～1 mg)を開始する．

緊急性がなく，時間的に余裕がある場合，「血液凝固異常症に関する調査研究」班からは*H. pylori*除菌療法を試みるとのガイドライン案が提唱されている．*H. pylori*感染ITPにおいて，除菌療法奏効例のうち約60～70%において血小板増加が認められる(保険適用外)．

Table 2. ITPの初回治療指針(米国血液学会ガイドライン)

血小板数	<2万/μl	2～3万/μl	3～5万/μl
出血傾向なし	ステロイド	無治療 or ステロイド*	無治療 or ステロイド**
紫斑のみ	ステロイド	ステロイド*	無治療 or ステロイド**
粘膜出血 (口腔，鼻腔，性器)	入院管理 ステロイド	ステロイド	ステロイド
重篤な出血 (頭蓋内，消化管)	入院管理 IVIg ステロイド	入院管理 IVIg ステロイド	入院管理 ステロイド IVIg

*60歳以上の高齢者あるいは高血圧，潰瘍性疾患，活動的な生活者など，出血のリスクファクターを有する場合は治療する．

**高血圧，潰瘍性疾患，活動的な生活者など，出血のリスクファクターを有する場合は治療する．

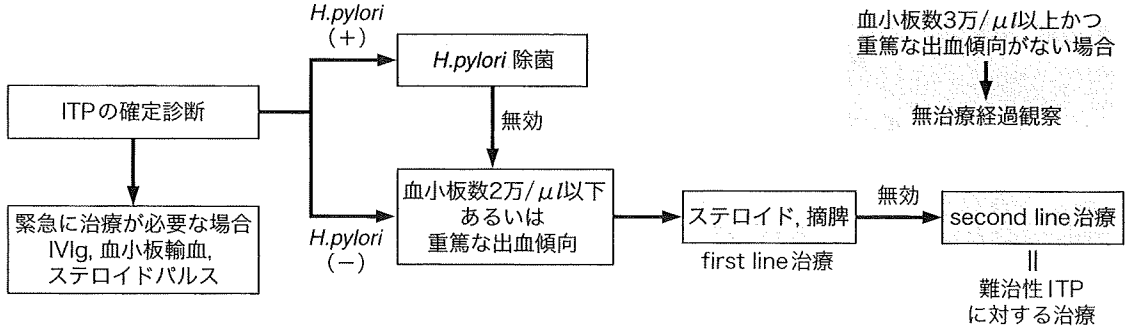

Fig. 1. ITP治療ガイドライン(案)