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.

Circ J 2009; **73:** 305–309

Venous Thromboembolism

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

Masahito Sakuma, MD; Mashio Nakamura, MD*; Norikazu Yamada, MD*; Satoshi Ota, MD*; Kunio Shirato, MD**; Takeshi Nakano, MD*; Masaaki Ito, MD**; Takao Kobayashi, MD†

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)
*Division of Internal Medicine, Onagawa Municipal Hospital, Onagawa, *Department of Cardiology, Mie University Graduate School of Medicine, Tsu, **Division of Internal Medicine, Saito Hospital, Ishinomaki and †Hamamatsu Medical Center, Hamamatsu, Japan*
Mailing address: Masahito Sakuma, MD, Division of Internal Medicine, Onagawa Municipal Hospital, 51-6 Horikiriyama, Washinokamihama, Onagawa 986-2243, Japan

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.

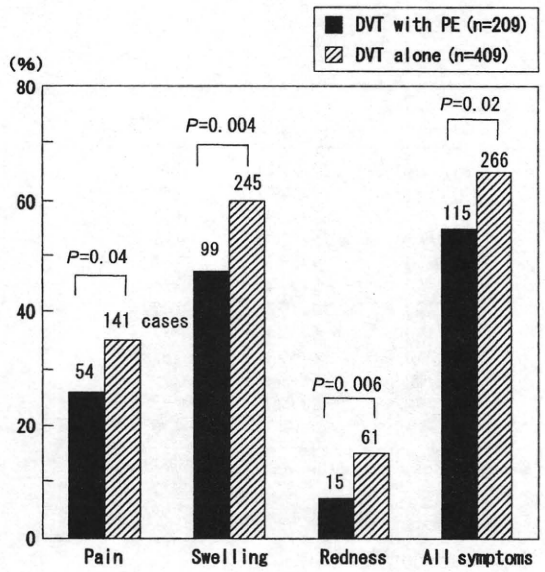

Fig 1. Symptoms of deep vein thrombosis (DVT). The number of cases is shown on each bar. PE, pulmonary embolism.

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

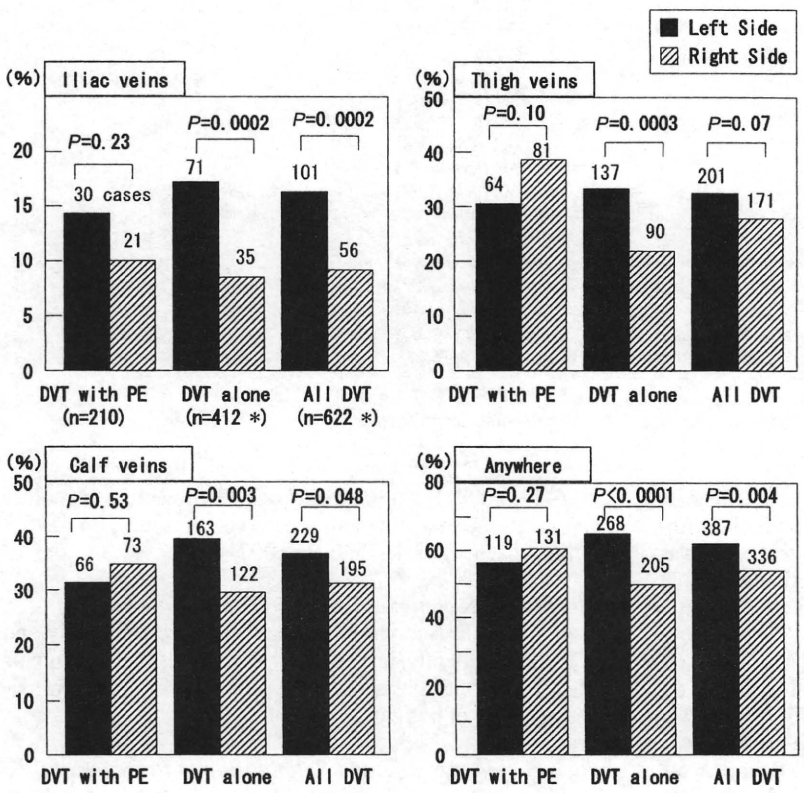

Fig 2. Location of deep vein thrombosis (DVT). The number of cases is shown on each bar. *Seven cases with DVT only in the upper extremities and one without data on DVT site were excluded. PE, pulmonary embolism.

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.

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

Tomio Kawasaki, MD, Ph.D

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.

Division of Cardiovascular Surgery, Department of Surgery, Osaka University Graduate School of Medicine, Osaka, Japan

Received: August 15, 2008 Accepted: January 9, 2009
Address for reprint requests to Tomio Kawasaki, MD, Ph.D: Division of Cardiovascular Surgery, Department of Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
Tel: +81-6-6879-3154, Fax: +81-6-6879-3163
E-mail: kawasaki@ck9.so-net.ne.jp

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).

A potential role for α-actinin in inside-out αIIbβ3 signaling

Seiji Tadokoro,[1,2] Tsuyoshi Nakazawa,[1] Tsuyoshi Kamae,[1] Kazunobu Kiyomizu,[1] Hirokazu Kashiwagi,[1] Shigenori Honda,[3] Yuzuru Kanakura,[1] and Yoshiaki Tomiyama[1,2]

[1]Department of Hematology and Oncology, Osaka University Graduate School of Medicine C9, Osaka, Japan; [2]Department of Blood Transfusion, Osaka University Hospital, Osaka, Japan; and [3]National Cardiovascular Center Research Institute, Osaka, Japan

Many different biochemical signaling pathways regulate integrin activation through the integrin cytoplasmic tail. Here, we describe a new role for α-actinin in inside-out integrin activation. In resting human platelets, α-actinin was associated with αIIbβ3, whereas inside-out signaling (αIIbβ3 activation signals) from protease-activated receptors (PARs) dephosphorylated and dissociated α-actinin from αIIbβ3. We evaluated the time-dependent changes of the αIIbβ3 activation state by measuring PAC-1 binding velocity. The initial velocity analysis clearly showed that PAR1-activating peptide stimulation induced only transient αIIbβ3 activation, whereas PAR4-activating peptide induced long-lasting αIIbβ3 activation. When αIIbβ3 activation signaling dwindled, α-actinin became rephosphorylated and reassociated with αIIbβ3. Compared with control platelets, the dissociation of α-actinin from αIIbβ3 was only transient in PAR4-stimulated $P2Y_{12}$-deficient platelets in which the sustained αIIbβ3 activation was markedly impaired. Overexpression of wild-type α-actinin, but not the mutant Y12F α-actinin, increased its binding to αIIbβ3 and inhibited PAR1-induced initial αIIbβ3 activation in the human megakaryoblastic cell line, CMK. In contrast, knockdown of α-actinin augmented PAR-induced αIIbβ3 activation in CMK. These observations suggest that α-actinin might play a potential role in setting integrins to a default low-affinity ligand-binding state in resting platelets and regulating αIIbβ3 activation by inside-out signaling. (*Blood.* 2011;117(1):250-258)

Introduction

Integrins and their ligands play key roles in development, immune responses, leukocyte traffic, hemostasis, and cancer and are at the core of numerous human diseases.[1] Many integrins are expressed with their extracellular domains in a default low-affinity ligand-binding state. The main platelet integrin, αIIbβ3, also known as GPIIb/IIIa, is present at a high density on circulating platelets. It is inactive on circulating platelets; if it were not, platelets would bind their main ligand, fibrinogen, from the plasma and aggregate, leading to thrombosis. This inactivation is important for the biologic function of integrins, as is most evident from assessments of their status on circulating blood cells. However, the molecular mechanisms of their being set to an inactive, low-affinity state remain unknown.

High-affinity ligand binding requires activation of integrins through conformational changes regulated by inside-out signaling.[2] Integrin cytoplasmic domains play a pivotal role in integrin signaling because the cytoplasmic tails of the integrin α and β subunits are directly accessible to the intracellular signaling apparatus, namely the integrin activation complex (IAC).[3] Moreover, ligand binding to the integrin induces outside-in signaling that leads to integrin clustering and subsequent recruitment of actin filaments to the integrin cytoplasmic domain. From the perspective that this recruitment occurs by a complex of interacting cytoskeletal proteins, many studies have focused on the components of the IAC, including talin, kindlin, filamin, and α-actinin. The binding of talin to the integrin β subunit cytoplasmic tail is a common final step in the activation process.[4-8] Kindlins bind to the more C-terminal of the NPxY motifs in β-integrin tails and modulate integrin activation.[9-11] Filamin binding to β integrin cytoplasmic tails is competitive with that of talin.[12] Although these studies suggest a model in which multiple proteins jockey for position on the β integrin tail, how cells orchestrate the process remains less well understood.

α-Actinin plays multiple important roles in the cell.[13,14] It links the cytoskeleton to different transmembrane proteins in a variety of junctions, regulates activity of several receptors, and serves as a scaffold connecting the cytoskeleton to diverse signaling pathways. α-Actinin binds to the β integrin cytoplasmic tail,[15,16] and recent studies have shown that the interaction between α-actinin and the integrin β2 tail modulates integrin affinity.[17,18] For regulating α-actinin function, 4 main mechanisms have been identified to date: processing by proteases, binding to phosphatidylinositol intermediaries, phosphorylation by tyrosine kinases, and binding to calcium. For tyrosine phosphorylation regulation, a second wave of protein tyrosine phosphorylation that is strictly dependent on both ligand binding to αIIbβ3 and cytoskeleton organization was observed in platelets stimulated by thrombin, phorbol myristate acetate, or immobilized fibrinogen.[19] Platelet adhesion and spreading on fibrinogen, mediated by the integrin αIIbβ3, trigger a robust and sustained phosphorylation of focal adhesion kinase and α-actinin by outside-in signaling.[20,21] Focal adhesion kinase phosphorylates α-actinin, which lowers its affinity for actin.[22] Dephosphorylation of α-actinin is regulated by the protein tyrosine phosphatase (PTP) SHP-1 (also named PTP1C, SHPTP-1, SHP,

Submitted October 6, 2009; accepted October 1, 2010. Prepublished online as *Blood* First Edition paper, October 12, 2010; DOI 10.1182/blood-2009-10-246751.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

HCP, and PTPN6),[23] a main PTP expressed in platelets. Although the regulation of α-actinin by outside-in signaling has been well characterized, its role in inside-out signaling remains to be determined.

Here, we show that α-actinin is associated with resting αIIbβ3 in platelets. Inside-out signaling from thrombin receptors, protease-activated receptor 1 (PAR1) and PAR4, dephosphorylated and dissociated α-actinin from αIIbβ3. Protease-activated receptor 1–activating peptide (PAR1-AP) and PAR4-AP induce transient and sustained αIIbβ3 activation, respectively. When the αIIbβ3 activation signaling dwindled, α-actinin reassociated with αIIbβ3 on rephosphorylation. Our observations suggest an emergent picture of α-actinin as having a role in keeping integrins in a default low-affinity ligand-binding state and regulating integrin activation.

Methods

Preparation of human platelets

Platelets were taken from healthy donors as approved by the institutional review board of Osaka University and were prepared as described previously[24] with some modifications. In brief, venous blood was obtained from volunteers with acid citrate dextrose solution (National Institute of Health formula A) as an anticoagulant, used at a 1:6 vol/vol ratio. Platelet-rich plasma was obtained by centrifugation at 250*g* for 10 minutes. After incubation with 0.5μM prostaglandin E_1 for 15 minutes, platelets were isolated by centrifugation of the platelet-rich plasma at 750*g* for 10 minutes. The pellet was washed twice with PIPES (Piperazine-1,4-bis(2-ethanesulfonic acid)) saline buffer (0.15M NaCl, 20mM PIPES, pH 6.5). The washed platelets were resuspended in Walsh buffer (137mM NaCl, 2.7mM KCl, 1mM $MgCl_2$, 3.3mM NaH_2PO4, 3.8mM HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid], 0.1% glucose, 0.1% bovine serum albumin [BSA], pH 7.4) to a density of 4×10^8 platelets/mL and allowed to sit for 30 minutes before use.

Antibodies

The monoclonal anti–α-actinin antibody (BM-75.2) and anti–talin antibody (8d4) were purchased from Sigma-Aldrich. The monoclonal anti–α-actinin antibody (H-2) and horseradish peroxidase (HRP)–conjugated anti–mouse immunoglobulin M (IgM) were purchased from Santa Cruz Biotechnology. Anti–vasodilator-stimulated phosphoprotein (VASP) monoclonal antibody (IE273) was purchased from ImmunoGlobe. The antiphosphotyrosine (4G10) antibody and anti–phospho-VASP-Ser239 (16C2) were purchased from Upstate Cell Signaling Solutions. PAC-1, the monoclonal, ligand-mimetic, αIIbβ3-specific antibody that binds specifically to activated αIIbβ3, the allophycocyanin-conjugated monoclonal anti-CD25 antibody, allophycocyanin-conjugated anti-CD42b antibody, and phycoerythrin-conjugated anti-CD42b antibody were purchased from BD Biosciences. peridinin chlorophyll protein complex–cyanine 5.5 (PerCP-Cy5.5)–conjugated anti-CD25 antibody was purchased from eBioscience. The polyclonal anti-αIIbβ3 antibody was a gift from Dr Thomas J. Kunicki (The Scripps Research Institute), and the monoclonal anti-αIIbβ3 antibody that activates αIIbβ3, PT25-2,[25] was a gift from Drs. Makoto Handa and Yasuo Ikeda (Keio University). HRP-conjugated secondary antibodies, anti–mouse IgG (H+L), and anti–rabbit IgG (H+L) were purchased from Cell Signaling Technology. Fluorescein isothiocyanate (FITC)– or phycoerythrin-conjugated anti–mouse IgM (μ) was purchased from Caltag Laboratories.

Chemicals

PAR1-AP (SFLLRN), thrombin, and prostaglandin E_1 were purchased from Sigma-Aldrich. PAR4-AP (AYPGKF) was purchased from GenixTalk. AR-C69931MX, a $P2Y_{12}$-specific antagonist, was a gift from Astra-Zeneca. FK633, an αIIbβ3-specific antagonist,[26] was a gift from Astellas Pharma Inc. Protein phosphatase inhibitor-1 (PTPI-1) was purchased from Calbiochem.

Immunoprecipitation

Aliquots of washed platelets (4×10^8/mL) were incubated with PAR1-AP (25μM), PAR4-AP (150μM), or thrombin (0.2 U/mL) at room temperature. Reactions were stopped by lysis of platelets with an equal volume of 2 × neutral detergent lysis buffer (15mM HEPES, 150mM NaCl, 2% [vol/vol] Triton X-100, 10mM EGTA [ethylene glycol tetraacetic acid], and 1mM Na_3VO_4, pH 7.4, plus complete protease inhibitors purchased from Roche Applied Science). Insoluble debris was cleared from the lysate by centrifugation at 13 000*g* for 4 minutes at 4°C. Supernatants were precleared with protein G-sepharose (GE Healthcare) for 1 hour. Precleared lysates were added to the newly prepared protein G with 1 μg of antibody and incubated at 4°C with constant rotation. Immunoprecipitates were washed 3 times, and proteins were eluted from the beads by incubation of the immunoprecipitates with 20 μL of 3 × sodium dodecylsulfate (SDS) sample buffer (62.5mM Tris [tris(hydroxymethyl)aminomethane], pH 6.8, 25% [vol/vol] glycerol, 2% [vol/vol] SDS, 5mM 2-mercaptoethanol, and 0.01% bromophenol blue) at 96°C for 5 minutes.

Electrophoresis of proteins and immunoblotting

Proteins were separated by continuous SDS–polyacrylamide gel electrophoresis on 4%-20% gels and electrophoretically transferred to Immobilon-P phenylmethlsulfonyl fluoride membranes (Millipore). Membranes were blocked by incubation with 2% (wt/vol) BSA in TBST (150mM NaCl, 50mM Tris, and 0.1% [vol/vol] Tween 20, pH 7.4). Primary antibodies were diluted in 2% (wt/vol) BSA in TBST. After incubation with primary antibodies, membranes were washed with TBST and incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature. After further washing of the membrane, signals were detected by enhanced chemiluminescence. When necessary, membranes were immersed in Restore Western blot stripping buffer (Pierce Chemical) and incubated at room temperature for 30 minutes before extensive washing and reprobing with the appropriate antibody.

Cell culture, plasmids, and transfections

Mammalian expression plasmids, including pcDNA/α-actinin, were a gift from Dr Beatrice Haimovich (University of Medicine and Dentistry of New Jersey). The mutant α-actinin carrying a phenylalanine at position 12 (Y12F) was generated as described.[22] CMK cells were maintained in culture as described previously.[27] Ribavirin was not used in this study. The plasmid encoding the extracellular and transmembrane domains of the Tac subunit of the human interleukin-2 receptor was generated as described.[28] Nucleofection was performed with Nucleofector II (Amaxa Biosystems) according to the manufacturer's instructions. CMK cells were nucleofected with 10 μg/cuvette Tac subunit of the human interleukin-2 receptor–encoding plasmid and 20 μg/cuvette α-actinin–encoding plasmid. Cells were analyzed 20 hours after nucleofection. The short hairpin RNAs (shRNAs) lentiviral particules were generated as described.[4] 5′-GGAAGCCAGGCATGTGGTTCTGATCATTGG AAGCTTGCGATGATTAGGACTACATGCCTGTCTTCCTTTTTT-3′ and 5′-GGCCAGCTTCTCGTAGTCTTCCATAAGCTGAAGCTT GAGCTTATGGAGGATTATGAGAAGCTGGCTTTTTTT-3′ oligonucleotide sequences were used to construct control and α-actinin shRNA, respectively. The α-actinin shRNA sequence chosen is specific for human α-actinin-1 and is 82% conserved in the human α-actinin-4 nucleotide sequence. shRNA viral vectors were produced by cloning the siRNA cassette into the FG12 lentiviral transgene vector in which DsRed2 was substituted for enhanced green fluorescent protein. In plasmids encoding wild-type or mutant α-actinin, 3 silence mutations were generated to prevent annealing with the α-actinin shRNA.

Flow cytometry and platelet aggregometry

Aliquots of washed platelets and FITC–PAC-1 were incubated with PAR1-AP (25μM), PAR4-AP (150μM), or thrombin (0.2 U/mL) at room temperature for various times. Binding of PAC-1 to platelets or CMK cells was assessed by flow cytometry with the use of FACSCalibur (Becton Dickinson).[29] The initial velocity of bound PAC-1 was analyzed as

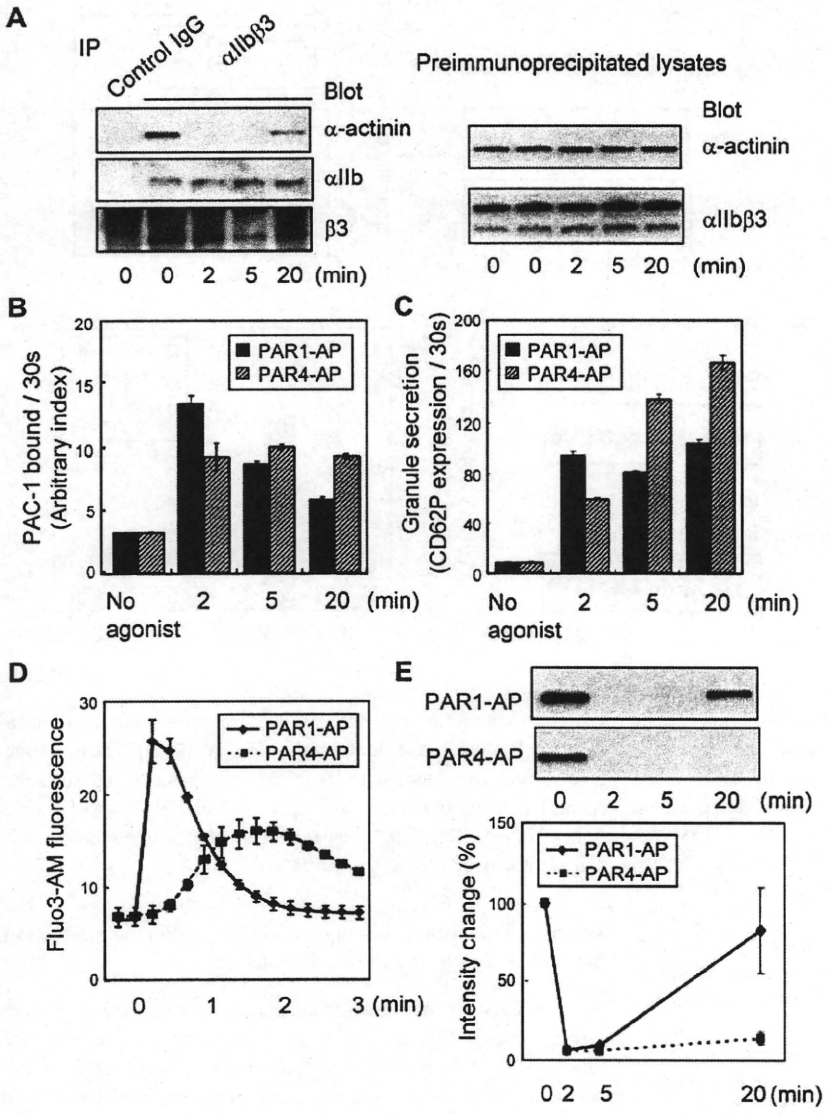

Figure 1. Dynamic changes in the interaction between αIIbβ3 and α-actinin in platelets. Washed human platelets were stimulated with PAR1-AP (25μM) or PAR4-AP (150μM) under nonstirring conditions for the time indicated. (A) αIIbβ3 was immunoprecipitated from lysates prepared from human platelets stimulated with PAR1-AP. Immunoprecipitates were then subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotted with anti–α-actinin antibody. Immunoblots were stripped and reprobed with anti-αIIbβ3 antibody. Preimmunoprecipitated lysates were also subjected to SDS-PAGE and immunoblotted with the same series of antibodies. (B) FITC–PAC-1 was added to the activated platelets after stimulation and incubated for 30 seconds to obtain the PAC-1 binding velocity at the time indicated. PAC-1 binding/30 seconds was normalized for integrin expression levels. (C) Phycoerythrin-conjugated anti-CD62P was added to the activated platelets and incubated for only 30 seconds to evaluate granule secretion. (D) Intracellular calcium mobilization was assessed by monitoring Fluo3-AM fluorescence by flow cytometry. (E) αIIbβ3 was immunoprecipitated then immunoblotted with anti–α-actinin antibody. Immunoblots shown are representative of 3 different experiments and analyzed by scanning densitometry and quantified with ImageJ (National Institutes of Health).

described.[30] In brief, washed platelets were mixed with PAR-AP at time "zero." At different time points from 2 minutes to 20 minutes, 20 μL of FITC–PAC-1 was added, and 30 seconds after the addition of PAC-1, 50 μL of platelet suspension was diluted into Walsh buffer, and bound PAC-1 was measured by flow cytometry. PAR1-AP–induced PAC-1 binding to CMK cells was analyzed on a gated subset of live (propidium iodide negative) differentiated (strongly $CD42b^+$) transfected (strongly $CD25^+$ or $DsRed2^+$) cells. Specific binding was assessed as total binding minus binding in the presence of 10μM FK633. Intracellular α-actinin expression was assessed by flow cytometry as previously described.[4] In brief, CMK cells were fixed with 0.5% paraformaldehyde, permeabilized with 0.05% saponin, and incubated for 30 minutes at room temperature with anti–α-actinin monoclonal antibody. After washing, the cells were incubated another 30 minutes with FITC-conjugated goat anti–mouse IgM. Cells were washed and resuspended in 500 μL of phosphate-buffered saline then analyzed by flow cytometry.

Presentation of data

Data are presented as mean ± SEM of ≥ 3 individual experiments from different blood donors. Analysis of statistical significance was performed with Student paired *t* tests, and differences were considered significant when $P < .05$. Immunoblots shown are representatives of 3 different experiments and were analyzed by scanning densitometry and quantified with ImageJ Version 1.40g (National Institutes of Health).

Results

Dynamic changes in the interaction between αIIbβ3 and α-actinin in platelets

Human washed platelets were stimulated with 25μM PAR1-AP under nonstirring conditions for ≤ 20 minutes to explore the role of α-actinin in inside-out signaling. Immunoprecipitation with polyclonal anti-αIIbβ3 followed by immunoblotting with anti–α-actinin showed that, in resting platelets, α-actinin was already associated with resting αIIbβ3 (Figure 1A). When platelets were stimulated with PAR1-AP, α-actinin was dissociated from αIIbβ3. Some actin-binding proteins moved to the Triton X-100–insoluble

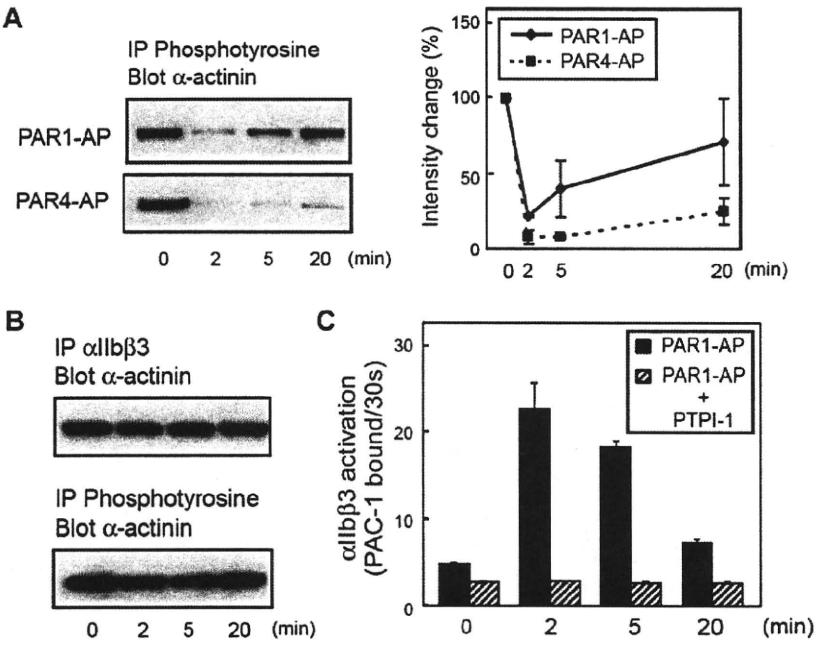

Figure 2. Kinetics of tyrosine phosphorylation of α-actinin during platelet activation and inhibition of SHP-1 by PTPI-1. Washed human platelets were stimulated with PAR1-AP (25μM) or PAR4-AP (150μM) for the time indicated. (A) Tyrosine-phosphorylated proteins were immunoprecipitated then immunoblotted with anti–α-actinin antibody. Immunoblots were analyzed by scanning densitometry and were quantified with ImageJ. (B) Washed human platelets were incubated at room temperature for 2 minutes in the presence of PTPI-1 (50μM). The platelets were then stimulated with PAR1-AP (25μM) for the time indicated. αIIbβ3 or tyrosine-phosphorylated proteins were immunoprecipitated then immunoblotted with anti–α-actinin antibody. (C) FITC–PAC-1 was added to the activated platelets after stimulation and incubated for 30 seconds to obtain the PAC-1 binding velocity at the time indicated. Error bars represent SEMs of 3 experiments.

fraction from the Triton X-100–soluble fraction in response to platelet activation; however, the amounts of α-actinin, talin, and αIIbβ3 in the Triton X-100–soluble fraction of PAR1-AP–activated platelets were similar to those of resting platelets under our experimental conditions. This finding suggests that the dissociation was not the result of the translocation of α-actinin into the Triton X-100–insoluble fraction. This dissociation remained unaffected even in the presence of 10μM of the αIIbβ3-specific peptidomimetic antagonist FK633, suggesting that the dissociation is independent of αIIbβ3-mediated outside-in signaling (data not shown). Interestingly, α-actinin rebound to αIIbβ3 at 20 minutes after PAR1-AP stimulation.

To clarify the physiologic relevance of the α-actinin dissociation to αIIbβ3 activation, we measured the amounts of PAC-1 binding after PAR1-AP or PAR4-AP stimulation. FITC–PAC-1 was incubated with activated platelets for 2 minutes, 5 minutes, and 20 minutes. Under PAR4-AP stimulation, the levels of PAC-1 binding increased as the incubation time extended. However, with PAR1-AP stimulation, the level of PAC-1 binding for a 20-minute incubation was similar to (or even lower than) that for a 5-minute incubation, suggesting that the number of activated αIIbβ3 molecules decreased at 20 minutes after PAR1-AP stimulation (supplemental Figure 1A, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

To evaluate more precisely the dynamic changes in the αIIbβ3 activation state, we performed initial velocity analysis for PAC-1 binding that has recently been developed.[30] In brief, FITC–PAC-1 was added to the activated platelets at the indicated time points after stimulation and incubated for only 30 seconds to obtain the PAC-1 binding velocity at the time points in question. The velocity of PAC-1 binding reflects the relative numbers of activated αIIbβ3 at those time points. PAC-1 binding was normalized for integrin expression levels. This initial velocity analysis clearly showed that PAR1 stimulation induced only transient αIIbβ3 activation, whereas PAR4 induced long-lasting αIIbβ3 activation (Figure 1B). Moreover, we assessed granule secretion and calcium mobilization under these conditions. These agonists induced different kinetics in CD62P expression and intracellular calcium mobilization, as detected by Fluo3-AM fluorescence (Figure 1C-D). These characteristics are consistent with the observed transient and sustained αIIbβ3 activation induced by PAR1-AP and PAR4-AP, respectively (Figure 1E). These data suggest that the dissociation of α-actinin may be related to αIIbβ3 activation.

Tyrosine phosphorylation of α-actinin regulates the interaction between α-actinin and αIIbβ3 in platelets

Because the α-actinin function appears to be regulated, in part, by tyrosine phosphorylation, we then examined the tyrosine phosphorylation state of α-actinin. The tyrosine-phosphorylated α-actinin was detectable as a faint band by immunoprecipitation with α-actinin antibody (BM-75.2 or H-2) followed by immunoblotting with 4G10 (supplemental Figure 1B). Accordingly, we modified our methods, and the platelet lysate was first subjected to immunoprecipitation with monoclonal antibody 4G10, followed by immunoblotting with anti–α-actinin antibody. In resting platelets, the anti–α-actinin antibody recognized the single 105-kDa protein (Figure 2A; supplemental Figure 1C). When stimulated with PAR1-AP, α-actinin was rapidly dephosphorylated. Although we have not excluded that this band was coprecipitated α-actinin with phosphoproteins, the phosphorylation profiles of immunoprecipitated α-actinin were almost the same as the faint band at 105 kDa in supplemental Figure 1B, suggesting that α-actinin itself was phosphorylated. α-Actinin was rephosphorylated at 20 minutes after PAR1-AP stimulation, whereas PAR4-AP induced sustained dephosphorylation of α-actinin. In addition to these agonists, we also examined adenosine diphosphate (ADP) and U46619-induced αIIbβ3 activation (supplemental Figure 2). ADP and U46619 induced transient and sustained αIIbβ3 activation, respectively. Again, α-actinin dissociation and de-phosphorylation induced by ADP and U46619 were transient and sustained, respectively. Thus, the kinetics of α-actinin phosphorylation seemed to synchronize with its interaction with αIIbβ3 (Figures 1E,2A).

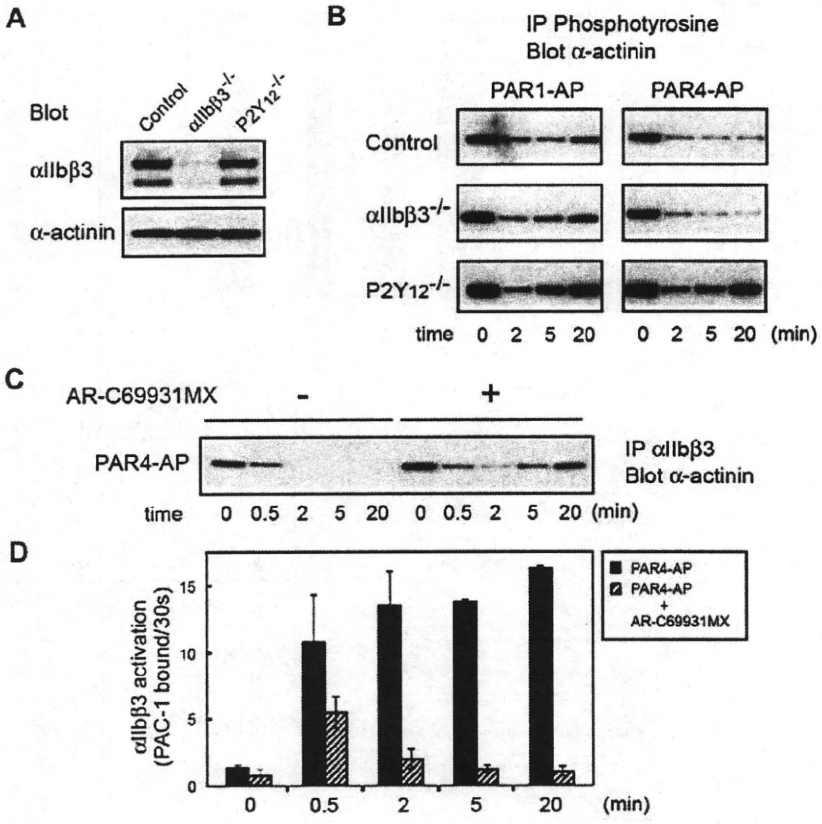

Figure 3. Changes in α-actinin phosphorylation and its interaction with αIIbβ3 in platelets from patients with Glanzmann thrombasthenia or $P2Y_{12}$ deficiency. (A) Platelet lysates from patients with Glanzmann thrombasthenia or patients with $P2Y_{12}$ deficiency were subjected to SDS–polyacrylamide gel electrophoresis and immunoblotted with anti-αIIbβ3 antibody or anti–α-actinin antibody. (B) Washed platelets were stimulated with PAR1-AP (25μM) or PAR4-AP (150μM) for the time indicated. Tyrosine-phosphorylated proteins were immunoprecipitated then immunoblotted with anti–α-actinin antibody. (C) Washed normal platelets were stimulated with PAR4-AP (150μM) for the time indicated after incubation with AR-C69931MX (1μM) for 2 minutes. αIIbβ3 was immunoprecipitated then immunoblotted with anti–α-actinin antibody. (D) Washed normal platelets were stimulated with PAR4-AP (150μM) for the time indicated after incubation with AR-C69931MX (1μM) for 2 minutes. FITC–PAC-1 was added to the activated platelets after stimulation and incubated for 30 seconds to obtain the PAC-1 binding velocity at the time indicated. Error bars represent SEMs of 3 experiments.

It has been shown that the PTP SHP-1 regulates the dephosphorylation of α-actinin.[23] To examine whether dephosphorylation of α-actinin regulates the dissociation of α-actinin from αIIbβ3 and integrin activation, we examined the effects of PTPI-1, a specific inhibitor of SHP-1[31] and PTP1B. Figure 2B shows that PTPI-1 inhibited the dephosphorylation of α-actinin. In addition, PTPI-1 markedly inhibited the activation of αIIbβ3 as well as the dissociation of α-actinin (Figure 2C). These results suggest that tyrosine phosphorylation of α-actinin regulates the interaction between α-actinin and αIIbβ3.

Interaction between α-actinin and integrin in platelets from a patient with Glanzmann thrombasthenia or $P2Y_{12}$ deficiency

To examine further the role of α-actinin in inside-out signaling, we analyzed platelets from a patient with Glanzmann thrombasthenia or $P2Y_{12}$-ADP receptor deficiency.[24] Figure 3A shows the expression profiles of αIIbβ3 and α-actinin in both patients. In Glanzmann thrombasthenia platelets, the phosphotyrosine profile of α-actinin was almost the same as that of control platelets under both PAR1-AP and PAR4-AP stimulation, confirming that αIIbβ3 outside-in signaling does not mediate these changes. In sharp contrast, PAR4-AP stimulation failed to induce the sustained dephosphorylation of α-actinin in $P2Y_{12}$-deficient platelets. Similarly, compared with control platelets, dephosphorylation of α-actinin induced by PAR1-AP was also disrupted at earlier time points in $P2Y_{12}$-deficient platelets (Figure 3B). This early disruption of the dephosphorylation of α-actinin is consistent with our previous finding that $P2Y_{12}$-mediated signaling is essential for sustained αIIbβ3 activation.[29] Indeed, the blockade of $P2Y_{12}$ with AR-C69931MX impaired the PAR4-AP–induced sustained αIIbβ3 activation, leading to the reassociation of α-actinin with αIIbβ3 (Figure 3C-D).

We have previously reported that the sustained αIIbβ3 activation induced by thrombin could be disrupted by inhibiting $P2Y_{12}$-mediated signaling even after thrombin stimulation.[29] Washed platelets were stimulated with thrombin at 0.2 U/mL. Two minutes later, we added AR-C69931MX (1μM) to the thrombin-stimulated platelets. We confirmed that AR-C69931MX still disrupted the sustained αIIbβ3 activation even under these conditions (Figure 4A). Interestingly, the sustained dephosphorylation and the dissociation of α-actinin from αIIbβ3 were disrupted by adding AR-C69931MX (Figure 4B-C). The blockade of $P2Y_{12}$ was confirmed by the VASP phosphorylation state.[32] These results suggest that the interaction between α-actinin and αIIbβ3 is, at least in part, regulated by $P2Y_{12}$-mediated signaling but not by αIIbβ3 outside-in signaling.

α-Actinin modulates agonist-induced αIIbβ3 activation in CMK cells

Finally, we used direct genetic manipulation to examine the effect of α-actinin on αIIbβ3 activation in human megakaryoblastic cell line CMK cells. Because the tyrosine residue at position 12 was reported as the main site of tyrosine phosphorylation in α-actinin,[23] we overexpressed wild-type α-actinin and Y12F α-actinin in CMK cells. As shown in Figure 5A, the expression of α-actinin was increased to 130%-170% of endogenous levels without affecting αIIbβ3 expression. Next, we examined the interaction between α-actinin and αIIbβ3 in CMK cells. Like platelets and primary megakaryocytes,[4] CMK cells can activate αIIbβ3 by the stimulation of physiologic agonists such as PAR1-AP.[27,30] Similar to platelets, α-actinin was associated with αIIbβ3 in unstimulated CMK cells. When stimulated with PAR1-AP, α-actinin dissociated from αIIbβ3. Overexpression of wild-type α-actinin, but not the

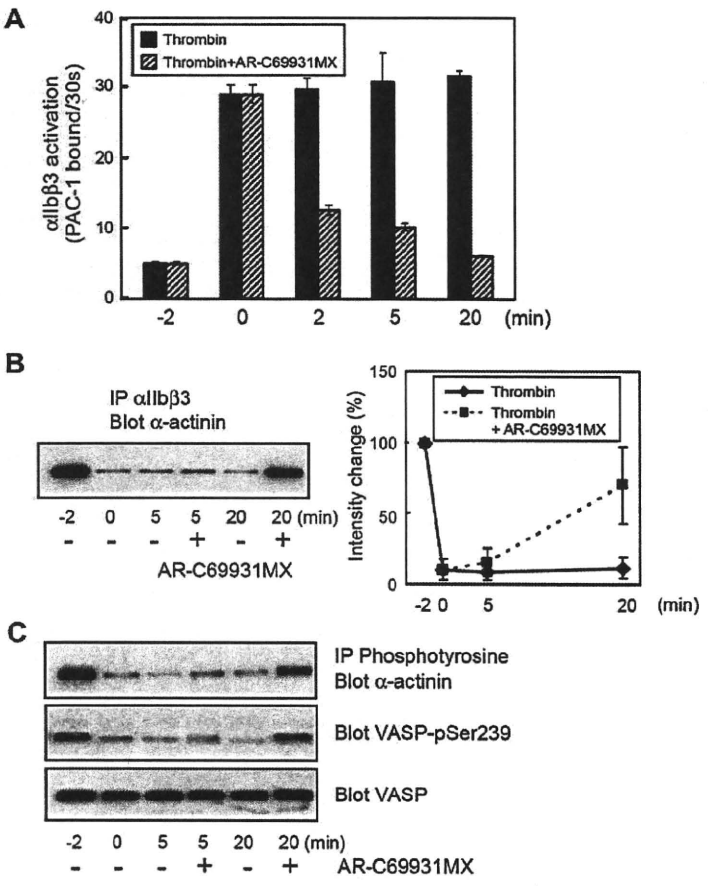

Figure 4. Effects of the blockade of $P2Y_{12}$ on α-actinin and αIIbβ3. Washed human platelets were incubated with thrombin (0.2 U/mL). The $P2Y_{12}$ antagonist, AR-C69931MX (1μM), was added after 2 minutes of thrombin stimulation. (A) FITC–PAC-1 was added to the activated platelets after stimulation and incubated for 30 seconds to obtain the PAC-1 binding velocity at the time indicated. (B) αIIbβ3 was immunoprecipitated then immunoblotted with anti–α-actinin antibody. (C) Tyrosine-phosphorylated proteins were immunoprecipitated then immunoblotted with anti–α-actinin antibody. Preimmunoprecipitated lysates were also subjected to SDS–polyacrylamide gel electrophoresis and immunoblotted with anti–phospho-VASP (Ser239) antibody or anti-VASP antibody. Error bars represent SEMs of 3 experiments.

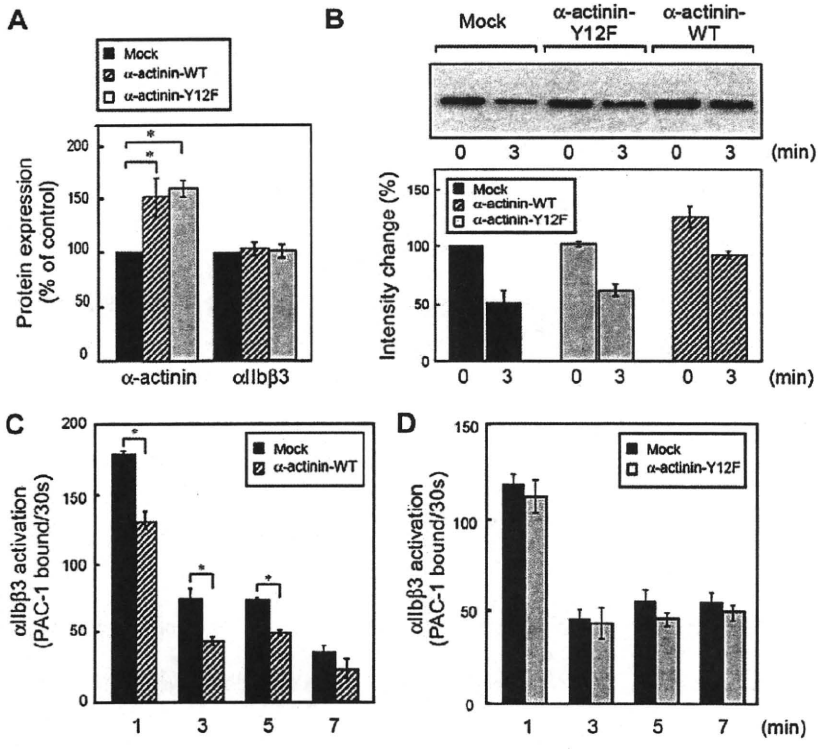

Figure 5. Effects of α-actinin on αIIbβ3 inside-out signaling in CMK cells. Human megakaryoblastic CMK cells were transiently transfected with plasmids encoding for α-actinin and Tac subunit of the human interleukin-2 receptor. Protein expression and integrin activation were assessed 20 hours after transfection. (A) Intracellular α-actinin and surface αIIbβ3 were determined by flow cytometry. Bar charts represent specific antibody binding to highly transfected cells (CD25-allophycocyanin fluorescence > 50) normalized to mock plasmid (pcDNA3.1)–transfected cells. Transfected CMK cells were incubated with PAR1-AP (50μM) for the time indicated. αIIbβ3 was immunoprecipitated then immunoblotted with anti–α-actinin antibody(B). Bar charts represent PAC-1 binding to wild-type α-actinin (C) or mutant α-actinin (D) transfected cells. Error bars represent SEMs of 3 experiments. *$P < .05$.

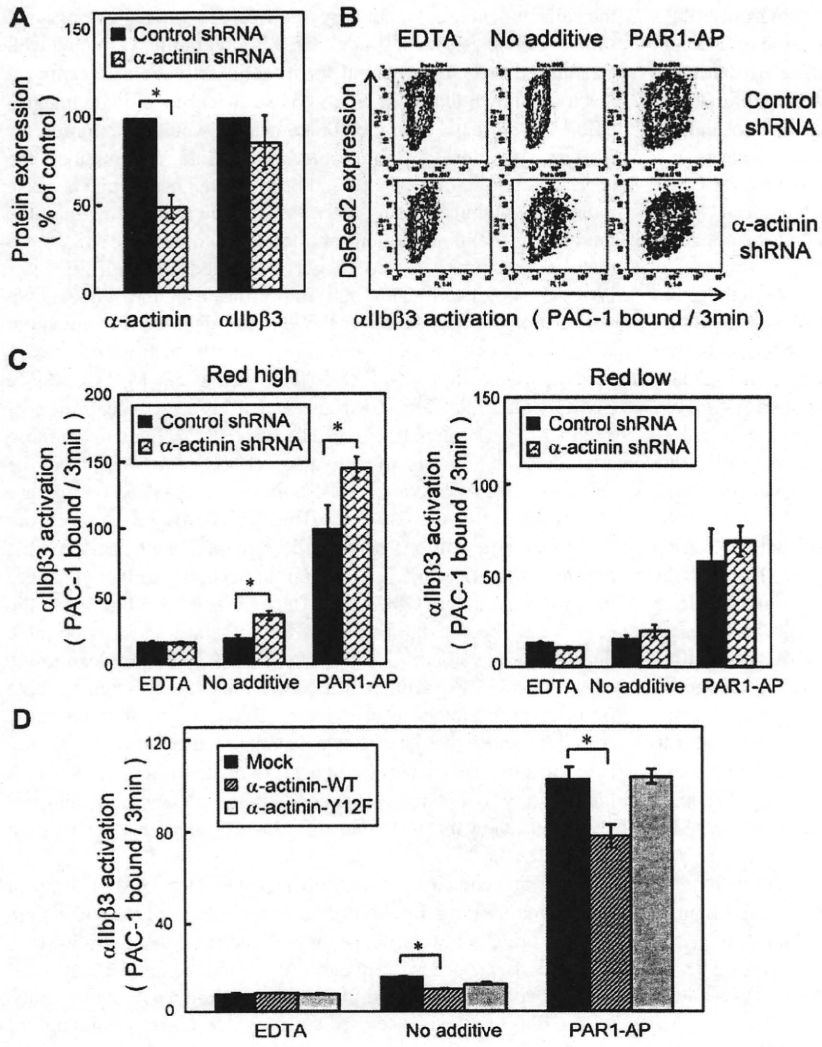

Figure 6. Knockdown of α-actinin augmented PAR1-AP–induced PAC-1 binding in CMK. Lentiviral particles encoding a shRNA for α-actinin or a control shRNA were transduced to CMK cells. (A) Intracellular α-actinin and surface αIIbβ3 were determined by flow cytometry. (B) CMK cells were stimulated with PAR1-AP (50μM) under nonstirring conditions with FITC–PAC-1 for 3 minutes. In contour plots, PAC-1 binding is shown on the x-axis, and transduction of the lentiviral particles is estimated by DsRed2 expression on the y-axis. (C) Bar charts represent PAC-1 binding to highly transduced cells (DsRed2 fluorescence > 250) and to less transduced cells (DsRed2 fluorescence < 50). (D) α-Actinin shRNA–transduced CMK cells were transiently transfected with plasmid encoding for wild-type or mutant α-actinin. Cells were stimulated with PAR1-AP (50μM) under nonstirring conditions with FITC–PAC-1 for 3 minutes. Error bars represent SEMs of 3 experiments in PAR1-AP and EDTA and of 8 experiments in no additive condition. $*P < .05$.

mutant Y12F α-actinin, increased the basal association between α-actinin and αIIbβ3 (Figure 5B).

We then performed PAC-1 binding velocity analysis on the gated subset of live, transfected, and CD42b$^+$ CMK cells stimulated with PAR1-AP, as described in the experimental procedures. CMK cells bound little PAC-1 in the absence of agonists. When stimulated with PAR1-AP, most mock-transfected cells bound PAC-1 (supplemental Figure 3A). Overexpression of wild-type α-actinin decreased PAC-1 binding velocity (Figure 5C) as well as the amounts of PAC-1 binding in the conventional PAC-1 binding assay (supplemental Figure 3C). The α-actinin expression has a concentration-dependent effect. High expression of α-actinin suppressed PAC-1 binding induced by PAR1-AP, but low expression of α-actinin did not (supplemental Figure 3B). In contrast, overexpression of the mutant Y12F α-actinin did not decrease PAC-1 binding velocity (Figure 5D). To examine further the effects of α-actinin, α-actinin expression was knocked down by shRNA in CMK cells. Knockdown was maximal at 10 days after infection, and shRNA induced 40%-60% reduction in the α-actinin expression (Figure 6A). Although adequate α-actinin reduction may not be obtained in CMK cells, decreased level of α-actinin augmented αIIbβ3 activation induced by PAR1-AP. Again, transduction levels (DsRed2 expression) have a concentration-dependent effect. High expression of DsRed2 augmented PAC-1 binding induced by PAR1-AP, but low expression of DsRed2 did not (Figure 6B-C). In contrast to PAR1-AP, PAR4-AP induced little PAC-1 binding velocity in CMK cells when stimulated with such high concentration as 1mM (supplemental Figure 4A). We assessed whether knockdown of α-actinin affects this PAR4-AP condition in CMK. PAC-1 binding induced by PAR4-AP was significantly augmented in the knockdown cells as well as PAR1-AP (supplemental Figure 4B). Finally wild-type α-actinin and Y12F α-actinin were overexpressed in α-actinin shRNA-transduced CMK cells. Wild-type α-actinin, but not the mutant Y12F α-actinin, normalized the augmented αIIbβ3 activation induced by PAR1-AP in CMK cells (Figure 6D). These results suggest that the binding of α-actinin might be involved in αIIbβ3 activation.

Discussion

Here, we focused on α-actinin as a member of the IAC and assessed its potential role in integrin-reversible activation. Resting αIIbβ3 was already associated with α-actinin, and inside-out

signaling by PARs induced a dissociation of α-actinin from αIIbβ3. This dissociation was regulated by dephosphorylation of α-actinin and associated with reversible αIIbβ3 activation, as evidenced by the stimulation of PAR1-AP and PAR4-AP. Overexpression of wild-type α-actinin, but not a mutant with a tyrosine-phosphorylation defect (Y12F), inhibited αIIbβ3 activation in a megakaryocytic cell line, CMK, in which αIIbβ3 is activated by PAR1-AP. Knockdown of α-actinin augmented PAR-AP–induced PAC-1 binding in CMK. Thus, our observations suggest that α-actinin may play a role in keeping αIIbβ3 in a low-affinity state.

Thrombin activates human platelets through proteolytic activation of 2 protease-activated receptors, PAR1 and PAR4.[33] PAR1 is a high-affinity receptor for platelet activation at low concentrations of thrombin, whereas PAR4 is a low-affinity receptor that mediates thrombin signaling at high concentrations. Consistent with previous reports,[34,35] we showed distinct kinetics of signaling from these PARs by evaluation of intracellular calcium mobilization and P-selectin translocation. PAR1 triggered a rapid and transient increase in intracellular calcium, whereas PAR4 triggered a slower but more prolonged response. Accordingly, we used PAR1-AP and PAR4-AP to examine the role of α-actinin in integrin activation. To assess the activation state of αIIbβ3 more precisely, we performed initial velocity analysis at a specific time point. The kinetic approach with the use of flow cytometry has been proposed for assessing the dynamics of αIIbβ3 activation,[36] and we have modified it as the initial velocity analysis for assessing reversible αIIbβ3 activation. On-rate of PAC-1 binding reflects the number of activated receptors, and the initial velocity analysis showed that PAR1 stimulation induced only transient activation. In addition, only 25% of αIIbβ3 was kept activated at 20 minutes after stimulation compared with the amount of activated αIIbβ3 at 2 minutes after stimulation.

In contrast, PAR4 induced long-lasting αIIbβ3 activation, and the amount of activated αIIbβ3 was almost the same at 2 minutes and 20 minutes after stimulation. The association/dissociation behavior of α-actinin with αIIbβ3 is apparently related to the distinct αIIbβ3 activation kinetics induced by PAR1-AP, PAR4-AP, ADP, and U46619.

We have shown that continuous interaction between released endogenous ADP and $P2Y_{12}$ is critical for sustained αIIbβ3 activation induced by thrombin.[29] In this context, the sustained αIIbβ3 activation induced by PAR4-AP was markedly impaired in $P2Y_{12}$-deficient platelets. In addition, the dissociation and dephosphorylation of α-actinin induced by PAR4-AP as well as by PAR1-AP was markedly impaired. Moreover, after αIIbβ3 activation was completed by thrombin stimulation, the addition of AR-C69931MX disrupted the dissociation and dephosphorylation of α-actinin as well as the sustained αIIbβ3 activation. PTPI-1, an inhibitor of SHP-1, inhibited the dissociation as well as the dephosphorylation of α-actinin induced by PAR1-AP. These data suggest a close association between the dissociation and dephosphorylation of α-actinin and regulation of these changes, at least in part, by $P2Y_{12}$-mediated signaling.

Cellular control of integrin activation requires transmission of a signal from the cytoplasmic tails to the extracellular domains. One may argue that the activation state of αIIbβ3 is an intrinsic state of the integrin itself and not a property of platelets per se.[37] However, this concept is based on the property of exogenous αIIbβ3 expressed on Chinese hamster ovary cells which is not activated by several platelet agonists. In this study, we have demonstrated that endogenous αIIbβ3 expressed on CMK cells can be activated by PAR-APs. Recent studies have identified some intracellular adapters, enzymes, and substrates necessary for αIIbβ3 activation and the tails function as regulatory scaffolds.[38] Among cytoplasmic proteins associated with β3 tail, talin[4,8] and kindlins[9] are now well established as being essential for integrin activation. Recently, it has been shown that talin binding is sufficient to activate integrin αIIbβ3[39] and that a talin membrane contact would be required for integrin activation.[39,40] In this context, α-actinin may modulate the accessibility of talin to the β3 tail or to plasma membrane because putative α-actinin binding sites have been reported within the membrane proximal region of the β3 cytoplasmic tail.[15,41] In platelets α-actinin was no longer associated with αIIbβ3 after PAR-AP stimulation. This may imply that α-actinin knockdown would not further enhance the extent of integrin activation. However, α-actinin knockdown significantly augmented PAC-1 binding induced by 1mM PAR4-AP as well as 50μM PAR1-AP in CMK. Unlike platelets, CMK needed a higher concentration of agonists to get enough levels of activated αIIbβ3. Even under those conditions, the levels of activated αIIbβ3 were less, and the sustained time of activation was shorter than platelets. From these observations, we assume that αIIbβ3 in CMK cells under our experimental conditions may not be fully activated. It is possible that this is the reason why α-actinin knockdown further enhances integrin activation in CMK cells. Thus, the α-actinin binding to the β3 tail may keep αIIbβ3 in a low-affinity state, and it is possible that the dissociation of α-actinin from the β3 tail may lead to the easy accessibility of talin to the β3 tail by inside-out signaling. Like β3 integrin, β2 integrins can change affinity on a subsecond time scale.[1] Recent studies have shown that intermediate-affinity lymphocyte function-associated antigen-1 bound to α-actinin, whereas high-affinity lymphocyte function antigen-1 bound to talin.[17,18] These data show that α-actinin and talin regulate integrin activation differently.

α-Actinin colocalizes with actin and stabilizes the actin filament web in nonmuscle cells.[13] It has been suggested that most α-actinin forms a bridge between the actin filaments and that there is some α-actinin associated with integrin at the end of actin filaments.[14] Indeed, we have confirmed that most α-actinin was not associated with αIIbβ3, as evidenced by the presence of large amounts of α-actinin in the supernatant even in immunoprecipitates of αIIbβ3 from resting platelets (data not shown). In the fibroblast, the phosphorylation of α-actinin reduces its affinity for actin and prevents its localization to focal adhesion plaques.[42] Another report showed that the phosphorylation of α-actinin might serve to modulate the coupling/uncoupling of integrins to the cytoskeleton in platelets.[23] Outside-in signaling involving interaction of αIIbβ3 with its immobilized ligand fibrinogen triggers tyrosine phosphorylation and cytoskeletal reorganization in platelets.[20] Pathologic shear forces, such as those encountered in stenotic midsized coronary and cerebral arteries, directly affect ligand-dependent αIIbβ3 outside-in signaling, and a recent study showed that pathologic shear stress induced dissociation of α-actinin from the β3 tail, which is αIIbβ3 dependent.[43] Our observations described here are clearly different phenomena from those induced by outside-in signaling; an αIIbβ3-specific antagonist, FK633, did not inhibit the dissociation, and dephosphorylation was induced even in thrombasthenic platelets. Thus, both inside-out signaling and outside-in signaling regulate the dissociation of α-actinin from αIIbβ3.

Integrin function is a complex process regulated by the balance of positive and negative regulatory proteins. Several factors have been identified as positive and negative regulators of αIIbβ3.[44,45] Platelet agonists, such as thrombin and ADP, induce αIIbβ3 activation, whereas various vasodilators released from the endothelial cells keep αIIbβ3 inactive in circulating blood. In addition to platelet agonists and vasodilators, the balance of positive and negative

regulatory proteins in IAC may regulate αIIbβ3 activation. Our observations described here suggest that α-actinin may act as a negative regulator in resting platelets. Although further work is needed to determine each specific role of α-actinin in platelets, the α-actinin–integrin interaction might be added to the discussion of new potential targets for atherothrombotic disease therapies.

Acknowledgments

This work was supported by Grant-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology in Japan, from the Ministry of Health, Labor and Welfare in Japan; and "Academic Frontier" Project in Japan.

Authorship

Contribution: S.T. designed and performed experiments, analyzed data, and wrote the paper; T.N., T.K., and K.K. helped perform parts of the experiments; H.K. and S.H. provided feedback and experimental advice; and Y.K. and Y.T. supervised the project and reviewed the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Seiji Tadokoro, Department of Hematology and Oncology, Osaka University Graduate School of Medicine C9, 2-2 Yamada-oka, Suita Osaka 565-0871, Japan; e-mail: tadokoro@hp-blood.med.osaka-u.ac.jp.

References

1. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell.* 2002;110(6):673-687.
2. Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood.* 2004;104(6):1606-1615.
3. O'Toole TE, Katagiri Y, Faull RJ, et al. Integrin cytoplasmic domains mediate inside-out signal transduction. *J Cell Biol.* 1994;124(6):1047-1059.
4. Tadokoro S, Shattil SJ, Eto K, et al. Talin binding to integrin beta tails: a final common step in integrin activation. *Science.* 2003;302(5642):103-106.
5. Moser M, Legate KR, Zent R, Fässler R. The tail of integrins, talin, and kindlins. *Science.* 2009; 324(5929):895-899.
6. Wegener KL, Partridge AW, Han J, et al. Structural basis of integrin activation by talin. *Cell.* 2007;128(1):171-182.
7. Petrich BG, Marchese P, Ruggeri ZM, et al. Talin is required for integrin-mediated platelet function in hemostasis and thrombosis. *J Exp Med.* 2007; 204(13):3103-3111.
8. Nieswandt B, Moser M, Pleines I, et al. Loss of talin1 in platelets abrogates integrin activation, platelet aggregation, and thrombus formation in vitro and in vivo. *J Exp Med.* 2007;204(13):3113-3118.
9. Moser M, Nieswandt B, Ussar S, Pozgajova M, Fässler R. Kindlin-3 is essential for integrin activation and platelet aggregation. *Nat Med.* 2008; 14(3):325-330.
10. Ma YQ, Qin J, Wu C, Plow EF. Kindlin-2 (Mig-2): a co-activator of beta3 integrins. *J Cell Biol.* 2008; 181(3):439-446.
11. Harburger DS, Bouaouina M, Calderwood DA. Kindlin-1 and -2 directly bind the C-terminal region of beta integrin cytoplasmic tails and exert integrin-specific activation effects. *J Biol Chem.* 2009;284(17):11485-11497.
12. Kiema T, Lad Y, Jiang P, et al. The molecular basis of filamin binding to integrins and competition with talin. *Mol Cell.* 2006;21(3):337-347.
13. Otey CA, Carpen O. alpha-Actinin revisited: a fresh look at an old player. *Cell Motil Cytoskeleton.* 2004;58(2):104-111.
14. Sjöblom B, Salmazo A, Djinović-Carugo K. alpha-Actinin structure and regulation. *Cell Mol Life Sci.* 2008;65(17):2688-2701.
15. Otey CA, Pavalko FM, Burridge K. An interaction between alpha-actinin and the beta1 integrin subunit in vitro. *J Cell Biol.* 1990;111(2):721-729.
16. Pavalko FM, LaRoche SM. Activation of human neutrophils induces an interaction between the integrin beta2-subunit (CD18) and the actin binding protein alpha-actinin. *J Immunol.* 1993;151(7):3795-3807.
17. Smith A, Carrasco YR, Stanley P, Kieffer N, Batista FD, Hogg N. A talin-dependent LFA-1 focal zone is formed by rapidly migrating T lymphocytes. *J Cell Biol.* 2005;170(1):141-151.
18. Stanley P, Smith A, McDowall A, Nicol A, Zicha D, Hogg N. Intermediate-affinity LFA-1 binds alpha-actinin-1 to control migration at the leading edge of the T cell. *EMBO J.* 2008;27(1):62-75.
19. Shattil SJ, Ginsberg MH, Brugge JS. Adhesive signaling in platelets. *Curr Opin Cell Biol.* 1994; 6(5):695-704.
20. Haimovich B, Lipfert L, Brugge JS, Shattil SJ. Tyrosine phosphorylation and cytoskeletal reorganization in platelets are triggered by interaction of integrin receptors with their immobilized ligands. *J Biol Chem.* 1993;268(21):15868-15877.
21. Izaguirre G, Aguirre L, Ji P, Aneskievich B, Haimovich B. Tyrosine phosphorylation of alpha-actinin in activated platelets. *J Biol Chem.* 1999;274(52):37012-37020.
22. Izaguirre G, Aguirre L, Hu YP, et al. The cytoskeletal/non-muscle isoform of alpha-actinin is phosphorylated on its actin-binding domain by the focal adhesion kinase. *J Biol Chem.* 2001;276(31):28676-28685.
23. Lin SY, Raval S, Zhang Z, et al. The protein-tyrosine phosphatase SHP-1 regulates the phosphorylation of alpha-actinin. *J Biol Chem.* 2004; 279(24):25755-25764.
24. Shiraga M, Miyata S, Kato H, et al. Impaired platelet function in a patient with P2Y12 deficiency caused by a mutation in the translation initiation codon. *J Thromb Haemost.* 2005;3(10):2315-2323.
25. Tokuhira M, Handa M, Kamata T, et al. A novel regulatory epitope defined by a murine monoclonal antibody to the platelet GPIIb-IIIa complex (alphaIIbbeta3 integrin). *Thromb Haemost.* 1996; 76(6):1038-1046.
26. Honda S, Tomiyama Y, Aoki T, et al. Association between ligand-induced conformational changes of integrin alphaIIbbeta3 and alphaIIbbeta3-mediated intracellular Ca^{2+} signaling. *Blood.* 1998;92(10): 3675-3683.
27. Kashiwagi H, Shiraga M, Honda S, et al. Activation of integrin alphaIIbbeta3 in the glycoprotein Ib-high population of a megakaryocytic cell line, CMK, by inside-out signaling. *J Thromb Haemost.* 2004;2(1):177-186.
28. LaFlamme SE, Akiyama SK, Yamada KM. Regulation of fibronectin receptor distribution. *J Cell Biol.* 1992;117(2):437-447.
29. Kamae T, Shiraga M, Kashiwagi H, et al. Critical role of ADP interaction with P2Y12 receptor in the maintenance of alphaIIbbeta3 activation: association with Rap1B activation. *J Thromb Haemost.* 2006;4(6):1379-1387.
30. Shiraga M, Kamae T, Akiyama M, et al. P2Y12-independent transient activation and P2Y12-dependent prolonged activation of platelet integrin alphaIIbbeta3 [abstract]. *Blood.* 2006;108(11):435a. Abstract 1509.
31. Arabaci G, Guo XC, Beebe KD, Coggeshall KM, Pei D. alpha-Haloacetophenone derivatives as photoreversible covalent inhibitors of protein tyrosine phosphatases. *J Am Chem Soc.* 1999; 121(21):5085-5086.
32. Aleil B, Ravanat C, Cazenave JP, Rochoux G, Heitz A, Gachet C. Flow cytometric analysis of intraplatelet VASP phosphorylation for the detection of clopidogrel resistance in patients with ischemic cardiovascular diseases. *J Thromb Haemost.* 2005;3(1):85-92.
33. Kahn ML, Zheng YW, Huang W, et al. A dual thrombin receptor system for platelet activation. *Nature.* 1998;394(6694):690-694.
34. Covic L, Gresser AL, Kuliopulos A. Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. *Biochemistry.* 2000;39(18):5458-5467.
35. Shapiro MJ, Weiss EJ, Faruqi TR, Coughlin SR. Protease activated receptor 1 and 4 are shut off with distinct kinetics after activation by thrombin. *J Biol Chem.* 2000;275(33):25216-25221.
36. Frojmovic M, Wong T, van de Ven T. Dynamic measurements of the platelet membrane glycoprotein IIb-IIIa receptor for fibrinogen by flow cytometry, I: methodology, theory and results for two distinct activators. *Biophys J.* 1991;59(4):815-827.
37. O'Toole TE, Loftus JC, Du XP, et al. Affinity modulation of the alphaIIbbeta3 integrin (platelet GPIIb-IIIa) is an intrinsic property of the receptor. *Cell Regul.* 1990;1(12):883-893.
38. Legate KR, Fässler R. Mechanisms that regulate adaptor binding to beta-integrin cytoplasmic tails. *J Cell Sci.* 2009;122(Pt 2):187-198.
39. Ye F, Hu G, Taylor D, et al. Recreation of the terminal events in physiological integrin activation. *J Cell Biol.* 2010;188(1):157-173.
40. Lau TL, Kim C, Ginsberg MH, Ulmer TS. The structure of the integrin alphaIIbbeta3 transmembrane complex explains integrin transmembrane signalling. *EMBO J.* 2009;28(9):1351-1361.
41. Lyman S, Gilmore A, Burridge K, Gidwitz S, White GCII. Integrin-mediated activation of focal adhesion kinase is independent of focal adhesion formation or integrin activation. Studies with activated and inhibitory beta3 cytoplasmic domain mutants. *J Biol Chem.* 1997;272(36):22538-22547.
42. von Wichert G, Haimovich B, Feng GS, Sheetz MP. Force-dependent integrin-cytoskeleton linkage formation requires downregulation of focal complex dynamics by Shp2. *EMBO J.* 2003;22(19):5023-5035.
43. Feng S, Lu X, Reséndiz JC, Kroll MH. Pathological shear stress directly regulates platelet alphaIIbbeta3 signaling. *Am J Physiol Cell Physiol.* 2006;291(6):C1346-C1354.
44. Tomiyama Y, Shiraga M, Kashiwagi H. Positive and negative regulation of integrin function. In: Tanaka K, Davie EW, eds. *Recent Advances in Thrombosis and Hemostasis 2008.* New York, NY: Springer;2008:243-252.
45. Brass LF, Zhu L, Stalker TJ. Minding the gaps to promote thrombus growth and stability. *J Clin Invest.* 2005;115(12):3385-3392.

Int J Hematol (2011) 93:106–111
DOI 10.1007/s12185-010-0731-5

CASE REPORT

Molecular analysis of a patient with type I Glanzmann thrombasthenia and clinical impact of the presence of anti-αIIbβ3 alloantibodies

Hirokazu Kashiwagi · Kazunobu Kiyomizu · Tsuyoshi Kamae · Tsuyoshi Nakazawa · Seiji Tadokoro · Shuji Takiguchi · Yuichiro Doki · Yuzuru Kanakura · Yoshiaki Tomiyama

Received: 28 October 2010 / Revised: 12 November 2010 / Accepted: 12 November 2010 / Published online: 8 December 2010

Abstract The occurrence of transfusion-related alloimmunization against αIIbβ3 is a major concern in patients with Glanzmann thrombasthenia (GT). However, few data are available about molecular defects of GT patients with anti-αIIbβ3 alloantibodies as well as clinical impact of these antibodies on platelet transfusion. Here, we report a case of type I GT with anti-HLA and anti-αIIbβ3 alloantibodies, who underwent laparoscopic total gastrectomy due to gastric cancer. We found a novel β3 nonsense mutation, 892C > T (Arg272X), and the patient was homozygous for the mutation. Laparoscopic gastrectomy was successfully performed with continuous infusion of HLA-matched platelet concentrates and bolus injection of recombinant factor VIIa at 2 h intervals. Total bleeding was 370 mL and no red-cell transfusion was necessary. Flow cytometric analysis employing anti-αIIbβ3 monoclonal antibody revealed that the transfused platelet count was maintained around 20–30 × 10^9/L during the operation and 10 × 10^9/L on the following day. Flow cytometric analysis also showed that transfused platelets retained normal reactivity to ADP stimulation. These results indicate that flow cytometry is useful to assess survival and function of transfused platelets in GT patients with anti-αIIbβ3 antibodies.

Keywords Glanzmann thrombasthenia · Anti-αIIbβ3 alloantibody · Mutation · Platelet transfusion · Recombinant factor VIIa

H. Kashiwagi · K. Kiyomizu · T. Kamae · T. Nakazawa · S. Tadokoro · Y. Kanakura · Y. Tomiyama
Department of Hematology and Oncology, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan

S. Takiguchi · Y. Doki
Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan

Y. Tomiyama (✉)
Department of Blood Transfusion, Osaka University Hospital, 2-15 Yamada-oka, Suita, Osaka 565-0871, Japan
e-mail: yoshi@hp-blood.med.osaka-u.ac.jp

1 Introduction

Glanzmann thrombasthenia (GT) is a rare hereditary bleeding disorder that is due to a quantitative and/or qualitative defect in integrin αIIbβ3 [glycoprotein (GP) IIb/IIIa, CD41/CD61]. GT is classified by the content of αIIbβ3: type I with <5% of normal controls, type II with 5–20%, and variant type with qualitative defect [1]. Clinical presentation includes mild to severe mucosal bleeds, traumatic or surgical hemorrhage, and occasional life-threatening bleeding episodes. Although treatment such as local measures and/or antifibrinolytics agents may be beneficial for mild bleeding, platelet transfusion is often necessary for severe bleeding, delivery and surgery. However, repeated transfusions may induce alloantibodies against HLA and/or αIIbβ3, leading to refractoriness to platelet transfusion. Moreover, it may be possible that anti-αIIbβ3 antibodies functionally block the binding of physiological ligands to αIIbβ3 on transfused platelets [2]. Treatment of GT patients with alloantibodies against αIIbβ3 are challenging. Removal of antibodies by plasma pheresis or immunoadsorption may be effective, although the procedure may present difficulties [3, 4]. In such cases, recombinant factor VIIa (rFVIIa) could represent an alternative. Tengborn and Petruson [5] reported the first successful case of rFVIIa treatment for epistaxes of a GT