were negative, too. The platelet-associated IgG (PA-IgG) was high (3307 ng/10^7 cells, NR: 9–25 ng/10^7 cells). Evans syndrome was initially considered because of the weakly positive direct Coombs test and high titer of PA-IgG. Prednisolone was administered (1 mg/kg for 7 day), but in vain, and fluctuating consciousness disturbance appeared 2 weeks later. Red cell fragmentation and proteinuria became evident, and the direct Coombs test became negative. Now, the characteristic Moschowitz's pentad [4] was evident, and the patient was diagnosed as having TTP.

Assay of the plasma ADAMTS13 activity was then performed according to the method of Furlan et al. [1] with slight modification, as described in a recent publication [3]. Briefly, the pooled normal human plasma was serially diluted with 0.05 M Tris–HCL (pH: 7.4), and 10 μl of each dilution were mixed with 1 μl of 100 mM PMSF. Then 90 μl of the purified VWF (20 μg/ml) dissolved in a urea buffer (1.5 M urea, 0.05% NaN_3, 10 mM $BaCL_2$ and 5 mM Tris–HCL) were added to each mixture and incubated at 37 °C for 24 h. Subsequently, 10 μl of 100 mM EDTA were added to quench the enzyme activity. A portion of each reaction mixture was separated by SDS-1.2-% agarose gel electrophoresis, and then Western blotting and luminography visualized the VWF multimers. The inhibitory activity against ADAMTS13 activity was measured based on the methods described by Furlan et al. [1] and Tsai and Lian [2]. Briefly, a test sample was first heat-inactivated at 56 °C for 30 min. The supernatant or Tris-buffered saline (pH: 7.4) (control) was mixed with an equal volume of the pooled normal plasma, and then further incubated at 37 °C for 2 h. Next, the residual ADAMTS13 activity was measured as mentioned above. The inhibitory titer was also assayed using IgG purified from the patient's plasma by a protein A sepharose CL-6B column. One unit of the inhibitor was defined as the amount that reduces the ADAMTS13 activity to 50% of the control. The results demonstrated that the plasma ADAMTS13 activity was extremely low (<3% of the normal control) (Fig. 1), and the inhibitor against ADAMTS13 was positive in both the patient's heated plasma (2.0 Bethesda units/ml) and purified IgG (0.4 Bethesda units/mg IgG), thus confirming the diagnosis of TTP.

The patient responded well to plasma exchange (PE). In cases of acquired idiopathic TTP with a severe deficiency of ADAMTS13 due to the presence of its inhibitor, they are high responders to PE [3]. Soon after three times of PE, his consciousness level became clear, and his platelet count increased to more than 50×10^3/mm^3 (Fig. 1). The serum indirect bilirubin decreased to 2.2 mg/dl, and LDH returned to the normal range after five times of PE. TTP did not relapse until the patient died of liver failure on the 296th hospitalization day. In our patient, it was difficult to make an early diagnosis because of the weakly positive direct Coombs test in the first examination, but in the 2nd analysis, it was negative. We should notice that the diagnosis of TTP might be hampered by the clinical features accompanying hepatic failure similar to the pentad of the typical TTP. Consequently, determination of the ADAMTS13 activity and its inhibitor in the plasma is unambiguously important for confirming the diagnosis of TTP. Our experience, although it is still based on one case, may provide a strong impact on the diagnosis and treatment of TTP, as well as its pathogenesis in the patients with advanced liver cirrhosis.

Masato Yagita[1], Masahito Uemura[3],
Takefumi Nakamura[2], Akane Kunitomi[1],
Masanori Matsumoto[4], Yoshihiro Fujimura[4]
[1]*Department of Clinical Immunology and Hematology, Tazuke-Kofukai Medical Research Institute, Kitano Hospital, 2-4-20 Ohgi-machi, Kita-ku, Osaka 530-8480, Japan*
[2]*Department of Gastroenterology and Hepatology, Tazuke-Kofukai Medical Research Institute, Kitano Hospital, Osaka, Japan*
[3]*Third Department of Internal Medicine, Nara Medical University, Kashihara, Nara, Japan*
[4]*Department of Blood Transfusion Medicine, Nara Medical University, Kashihara, Nara, Japan*
E-mail address: mayagita@kitano-hp.or.jp

References

[1] Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle PA, Brenner B, et al. von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. N Engl J Med 1998;339:1578–1584.
[2] Tsai HM, Lian EC. Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. N Engl J Med 1998;339:1585–1594.
[3] Matsumoto M, Yagi H, Ishizashi H, Wada H, Fujimura Y. The Japanese experience with thrombotic thrombocytopenic purpura/hemolytic-uremic syndrome. Semin Hematol 2004;41:68–74.
[4] Moschowitz E. Hyaline thrombosis of the terminal arterioles and capillaries; a hitherto undescribed disease. Proc NY Pathol Soc 1924; 24:21–24.

doi:10.1016/j.jhep.2004.08.030

International Journal of
HEMATOLOGY

Case Report

Rituximab Provided Long-term Remission in a Patient with Refractory Relapsing Thrombotic Thrombocytopenic Purpura

Satoru Kosugi,[a] Masanori Matsumoto,[b] Yasushi Ohtani,[a] Hironori Take,[a]
Hiromichi Ishizashi,[b] Yoshihiro Fujimura,[b] Jun Kuyama[a]

[a]*Department of Internal Medicine, Toyonaka Municipal Hospital, Osaka;*
[b]*Department of Blood Transfusion Medicine, Nara Medical University, Nara, Japan*

Received November 29, 2004; received in revised form March 7, 2005; accepted March 8, 2005

Abstract

We describe a 69-year-old man with refractory relapsing thrombotic thrombocytopenic purpura (TTP) successfully treated with rituximab. The patient had once been successfully treated with plasmapheresis and vincristine, but he had relapsed after a short period. Although plasmapheresis, vincristine, and splenectomy could not achieve a consistent elevation of the platelet count, rituximab administration provided sustained remission for more than 7 months. Rituximab should be considered as a therapeutic alternative for refractory TTP.
Int J Hematol. 2005;81:433-436. doi: 10.1532/IJH97.04187

Key words: Thrombotic thrombocytopenic purpura (TTP); Rituximab; von Willebrand factor–cleaving protease (VWF-CP); Anti-ADAMTS13 autoantibody

1. Introduction

Thrombotic thrombocytopenic purpura (TTP) is an uncommon disorder characterized by the development of von Willebrand factor (VWF)-platelet–rich hyaline thrombi in the arterioles and capillaries [1]. TTP affects various organs (brain, heart, kidney, and others) and can have neurologic or cardiac complications with a fatal outcome.

Recent studies have shown that very large circulating multimers of VWF are responsible for TTP in adults [2] and that it is associated with severe deficiency of VWF-cleaving protease (or ADAMTS13, a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs) [2,3]. In most of the cases, the deficiency is due to an immunoglobulin G (IgG) autoantibody against ADAMTS13 that inhibits the activity of the protease [2,4]. The use of therapeutic plasma exchange (PE) has dramatically decreased the mortality rate of TTP [5]; however, relapse of the disease occurs in more than one third of the cases that achieve remission [6,7]. In the patients who develop multiple relapses or persistent disease, corticosteroids, vincristine (VCR), cyclophosphamide, azathioprine, high-dose intravenous IgG, cyclosporin A, and/or splenectomy have been used in combination with PE [8-11]. These treatment strategies have provided some improvement in the outcome. However, treating patients unresponsive to these therapies is still difficult.

Rituximab, a chimeric monoclonal antibody against CD20 that depletes B-cells in the circulation and in lymphoid tissues, has shown efficacy in the treatment of CD20-positive lymphoproliferative disorders [12]. Rituximab has also been shown to provide successful results in some patients with immune thrombocytopenic purpura or other antibody-mediated autoimmune diseases [13-15]. Because adult TTP is mostly derived from an autoimmune etiology, the use of rituximab would be promising. Recently, longstanding remission after the use of rituximab has been described in a few preliminary reports [16-21].

We describe a male patient who once had successfully been treated with VCR but who had relapsed after becoming refractory to the agent after a short period. Although he did not respond to splenectomy, rituximab administration provided a disease-free course for more than 7 months. We also detected an increase in ADAMTS13 activity and the disappearance of anti-ADAMTS13 autoantibodies after the use of rituximab. These findings suggest that rituximab could be a potent therapeutic option for refractory relapsing TTP.

Correspondence and reprint requests: Satoru Kosugi, MD, Department of Internal Medicine, Toyonaka Municipal Hospital, 1-14-1, Shibahara-cho, Toyonaka, Osaka, 560-8565, Japan; 81-6-6843-0101; fax: 81-6-6843-3739 (e-mail: gustav-k@syd.odn.ne.jp).

2. Case Report and Clinical Course

On August 15, 2003, a 69-year-old man was admitted to our hospital because of easy bruising and consciousness disturbance. He presented an elevated body temperature (37.2 C), confusion, and remarkable petechias or hematomas (on the forehead and extremities). The presence of hemolytic anemia was revealed with the following laboratory test results: hemoglobin level, 8.1 g/dL; total bilirubin level, 3.0 mg/dL, with an indirect bilirubin level of 2.7 mg/dL; and an elevated lactate dehydrogenase (LDH) level, 577 IU/L. Severe thrombocytopenia (5 10^9/L) and an elevated serum creatinine level (1.1 mg/dL) were also observed. Red blood cell fragmentation was not evident in the patient's blood smears. He had no history of diseases or medications that could cause secondary TTP, and other laboratory tests and radiographic examinations showed no sign of malignancies or collagen disorders. Therefore, the patient's disease was diagnosed as idiopathic TTP. PE in combination with plasma infusion (PI) and the administration of prednisolone (1 mg/kg) was started. Treatment with antiplatelet agents (100 mg aspirin and 300 mg dipyridamole) was then added the next day. After these therapies, the patient's consciousness improved remarkably, the LDH and bilirubin levels decreased to normal, and the platelet levels were elevated. However, a decrease in the frequency of the PE resulted in a relapse of the thrombocytopenia (Figure 1). We attempted steroid pulse therapy (methylprednisolone, 500 mg 3 days), but it failed. Because the patient had become dependent on PE, weekly VCR administration (1 mg/week) was started on September 22. After the third administration of the agent, the patient's platelet counts reached 180 10^9/L, and PE and PI treatments ceased (Figure 1).

The patient's remission continued for 4 months, and oral prednisolone therapy was tapered. However, the thrombocytopenia suddenly relapsed on March 2, 2004. Because PI with weekly VCR administration could not maintain the platelet levels, PE was readministered on March 17. After the fourth PE, a laparoscopic splenectomy was performed on March 25. However, the elevation of the platelet count was sustained for only a week, and then PE and PI had to be continued. This remarkable refractoriness prompted us to consider rituximab administration. After the patient gave signed informed consent, he received 4 weekly courses of 375 mg/m^2 rituximab from April 6. No complication was experienced during the treatment. After the first administration of the agent, additional PE was performed only once, PI was given only 4 times, and the patient's platelet counts never fell below 200 10^9/L. He has been disease free with

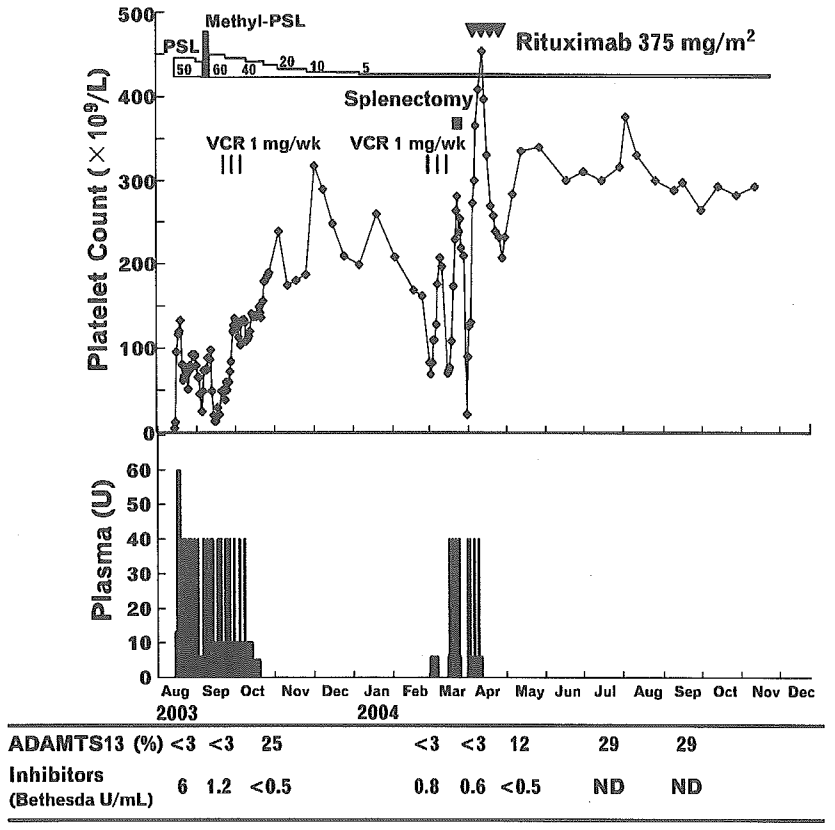

Figure 1. Clinical course of the patient. "Plasma" means the amount of fresh frozen plasma used for infusion or exchange. Therapeutic plasma exchange was performed when the amount of plasma was 40 units or greater. ADAMTS13 activity is presented as a percentage of that of normal human plasma. One Bethesda unit of inhibitor reduces the ADAMTS13 activity of an equal volume of normal plasma by 50%. PSL indicates prednisolone; Methyl-PSL, methylprednisolone pulse therapy (500 mg 3); VCR, vincristine; ND, not determined.

consistent platelet counts for more than 7 months (Figure 1). There has been no sign suggesting hemolysis after rituximab administration.

ADAMTS13 activities and its inhibitor levels were determined as previously described [22,23] on 11 occasions, and the data for 8 occasions are shown in Figure 1. At the onset, this patient had an extremely high titer of ADAMTS13 inhibitors, and we could not detect ADAMTS13 activities. PE, PI, and steroid administration achieved no increase in ADAMTS13 activity. During these therapies, ADAMTS13 inhibitor levels decreased gradually but remained detectable. The first course of VCR administration achieved an increase in ADAMTS13 activity with the disappearance of the inhibitors. However, after the relapse occurred, the second course of VCR with PI and splenectomy in combination with PE provided no beneficial change in either activity or inhibitor level. Reelevation of ADAMTS13 activity and the disappearance of ADAMTS13 inhibitors were observed only after rituximab administration. As of November 2004, a decrease in ADAMTS13 activity has not recurred.

3. Discussion

In this report we have described a patient with relapsing TTP that was refractory to PE, VCR therapy, and splenectomy but that was successfully treated with rituximab. Rituximab has provided the longest disease-free course in the case thus far.

Recent studies have revealed that most cases of adult TTP can be considered an autoimmune disorder caused by IgG autoantibodies against ADAMTS13 [2,4]. These autoantibodies work as an inhibitor that induces a deficiency of the protease. This autoimmune etiology of adult TTP suggests that TTP patients may respond to immunosuppressive therapies. In fact, immunosuppressive agents have been administered to PE-refractory or relapsing patients [8,9]. However, no standard guideline for the use of such agents has been established because of the rarity and heterogeneity of the condition. When patients become resistant to these agents, splenectomy has been considered the last option [10,11]. We unfortunately lack an effective strategy for the patients unresponsive even to splenectomy.

Because of its effectiveness against other autoimmune disorders [12-15], rituximab may be beneficial for treating TTP. Six reports have described the use of rituximab in refractory cases [16-21]. Table 1 summarizes these cases, including ours. Ten patients have been treated, and 9 complete responses have been achieved. Both a severe deficiency of ADAMTS13 activity and the presence of its inhibitors have been determined in 7 cases, all of which showed a decrease in inhibitor levels after rituximab administration. Six of these 7 patients achieved a complete response with increased activity of ADAMTS13. Only 1 patient did not achieve an increase in ADAMTS13 activity, and he needed a less intensive PE [16]. From these results, we hypothesized that rituximab eliminates B-cells responsible for TTP pathogenesis. In previous cases of other antibody-related disorders successfully treated with rituximab, rapid improvement with no great changes in immunoglobulin concentrations was observed. An increase in platelet counts in idiopathic thrombocytopenic purpura was achieved in a few weeks, a decrease in the proteinuria in idiopathic membranous nephropathy or a strength recovery with a decline of anti-GM1 ganglioside antibodies was achieved in a few months, and serum immunoglobulin levels in these cases did not fall below normal levels [13-15]. These results also suggest selective inhibition of the autoreactive clones that produce the pathogenic immunoglobulins. However, one may argue that the changes induced by rituximab were too rapid to be attributed only to B-cell depletion. Some other immune mechanisms coupled with rituximab administration may be responsible for the rapid and selective clinical effect.

It is tempting to conclude that the use of rituximab may facilitate remission, reduce the need for PE, and improve the outcomes of TTP cases. However, the number of reported cases is small, and the observation period has been relatively short. Whether rituximab is effective in all adult cases of TTP and when it should be used remain to be determined. In addition, the optimal dose of rituximab remains uncertain. Eight doses were needed in the first case of Gutterman et al

Table 1.
Summary of Previous Thrombotic Thrombocytopenic Purpura Cases Treated with Rituximab*

Report	Case No.	Rituximab Doses, n†	Concurrent Therapies	Levels after Rituximab Treatment		
				Response Duration, mo	ADAMTS13, %	Inhibitors, Bethesda U/mL
Gutterman et al [16]	1	8	None	CR (36+)	ND	ND
	2	8	None	CR (17), relapse	Increased (<10 22)	Decreased (1.3 0.25)
	3	4	None	PR	Unchanged (<10 <10)	Decreased (0.5 0.2)
Chemnitz et al [17]	1	4	VCR, PSL	CR (2+)	Increased (<1 100)	Decreased (0.8 <0.2)
	2	2	VCR, PSL	CR (12+)	ND	ND
Zheng et al [18]	1	6	Cy	CR (10+)	Increased (<10 17)	Decreased (6 <0.2)
Tsai and Shulman [19]	1	8	None	CR (10+)	Increased (<10 79)	Decreased (1.5 <0.2)
Yomtovian et al [20]	1	8	None	CR (15+)	Increased (<10 22)	Decreased (0.56 0.29)
Stein et al [21]	1	4	Cy	CR (6+)	ND	ND
This report	1	4	None	CR (7+)	Increased (<3 25)	Decreased (0.6 <0.5)

*ADAMTS13 indicates a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs; CR, complete response defined as a normal platelet count, stable hemoglobin level, and no symptoms or signs caused by thrombotic thrombocytopenic purpura; ND, not determined; PR, partial response defined as an increased platelet count with less intensive plasma exchange; VCR, vincristine; PSL, prednisolone; Cy, cyclophosphamide.
†Each rituximab dose was 375 mg/m^2.

and in the case of Yomtovian et al, and these patients needed PE even after the fourth rituximab administration [16,20]. On the other hand, the patient in the second case of Chemnitz et al showed complete remission after 2 doses without additional rituximab administration [17]. Because remission was achieved after 4 doses in the case of Tsai and Shulman [19], after 2 doses in the case of Stein et al [21], and after the first dose in our case, whether each patient needed the rituximab doses administered thereafter is unknown. For optimal treatment, rituximab doses might be adjusted according to the inhibitor levels in each case. A large clinical study is needed to answer these questions.

In summary, this is an interesting TTP case in which rituximab was the most effective agent during the treatment. Although only a small number of cases have suggested the efficacy of rituximab therapy, its use should be considered as an alternative for highly refractory cases. Establishment of strategies for the management of refractory TTP is urgent.

References

1. Bukowski RM. Thrombotic thrombocytopenic purpura: a review. *Prog Hemost Thromb.* 1982;6:287-337.
2. Furlan M, Robles R, Galbusera M, et al. von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *N Engl J Med.* 1998;339:1578-1584.
3. Furlan M, Robles R, Solenthaler M, Wassmer M, Sandoz P, Lammle B. Deficient activity of von Willebrand factor-cleaving protease in chronic relapsing thrombotic thrombocytopenic purpura. *Blood.* 1997;89:3097-3103.
4. Tsai HM, Lian EC. Antibodies to von Willebrand factor–cleaving protease in acute thrombotic thrombocytopenic purpura. *N Engl J Med.* 1998;339:1585-1594.
5. Rock GA, Shumak KH, Buskard NA, et al. Comparison of plasma exchange with plasma infusion in the treatment of thrombotic thrombocytopenic purpura: Canadian Apheresis Study Group. *N Engl J Med.* 1991;325:393-397.
6. Shumak KH, Rock GA, Nair RC. Late relapse in patients successfully treated for thrombotic thrombocytopenic purpura: Canadian Apheresis Group. *Ann Intern Med.* 1995;44:2836-2840.
7. Bell WR. Thrombotic thrombocytopenic purpura/hemolytic uremic syndrome relapse: frequency, pathogenesis, and meaning. *Semin Hematol.* 1997;34:134-139.
8. Kuwaan HC, Soff GA. Management of thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. *Semin Hematol.* 1997;34:159-166.
9. Allford SL, Hunt BJ, Rose P, Machin SJ. Guidelines on the diagnosis and management of the thrombotic microangiopathic haemolytic anaemias. *Br J Haematol.* 2003;120:556-573.
10. Aqui NA, Stein SH, Konkle BA, Abrams CS, Strobl FJ. Role of splenectomy in patients with refractory or relapsed thrombotic thrombocytopenic purpura. *J Clin Apheresis.* 2003;18:51-54.
11. Hovinga JAK, Studt JD, Biasiutti FD, et al. Splenectomy in relapsing and plasma-refractory acquired thrombotic thrombocytopenic purpura. *Br J Haematol.* 2004;89:320-324.
12. Boye J, Elter T, Engert A. An overview of the current clinical use of the anti-CD20 monoclonal antibody rituximab. *Ann Oncol.* 2003;14:520-535.
13. Levine TD, Pestronk A. IgM antibody-related polyneuropathies: B-cell depletion chemotherapy using rituximab. *Neurology.* 1999; 52:1701-1704.
14. Stasi R, Pagano A, Stipa E, Amadori S. Rituximab chimeric anti-CD20 monoclonal antibody treatment for adults with chronic idiopathic thrombocytopenic purpura. *Blood.* 2001;98:952-957.
15. Remmuzzi G, Chiuruchiu C, Abbate M, Brusegan V, Bontempelli M, Ruggenenti P. Rituximab for idiopathic membranous nephropathy. *Lancet.* 2002;360:923-924.
16. Gutterman LA, Kloster B, Tsai HM. Rituximab therapy for refractory thrombotic thrombocytopenic purpura. *Blood Cells Mol Dis.* 2002;28:385-391.
17. Chemnitz J, Draube A, Scheid C, et al. Successful treatment of severe thrombotic thrombocytopenic purpura with the monoclonal antibody rituximab. *Am J Hematol.* 2002;71:105-108.
18. Zheng X, Pallera AM, Goodnough LT, Sadler JE, Blinder MA. Remission of chronic thrombotic thrombocytopenic purpura after treatment with cyclophosphamide and rituximab. *Ann Intern Med.* 2003;138:105-108.
19. Tsai HM, Shulman K. Rituximab induces remission of cerebral ischemia caused by thrombotic thrombocytopenic purpura. *Eur J Haematol.* 2003;70:183-185.
20. Yomtovian R, Niklinski W, Silver B, Sarode R, Tsai HM. Rituximab for chronic recurring thrombotic thrombocytopenic purpura: a case report and review of the literature. *Br J Haematol.* 2004;124: 787-795.
21. Stein YG, Aeidman A, Fradin Z, Varon M, Cohen A, Mittelman M. Treatment of resistant thrombotic thrombocytopenic purpura with rituximab and cyclophosphamide. *Int J Hematol.* 2004;80:94-96.
22. Sugio Y, Okamura T, Shimoda K, et al. Ticlopidine-associated thrombotic thrombocytopenic purpura with an IgG-type inhibitor to von Willebrand factor-cleaving protease activity. *Int J Hematol.* 2001;74:347-351.
23. Kawahara M, Kanno M, Matsumoto M, Nakamura S, Fujimura Y, Ueno S. Diffuse neurodeficits in intravascular lymphomatosis with ADAMTS13 inhibitor. *Neurology.* 2004;63:1731-1733.

Original Paper

Pathophysiology of Haemostasis and Thrombosis

Pathophysiol Haemost Thromb 2005;34:35–40
DOI: 10.1159/000088546

Received: January 6, 2005
Accepted after revision: March 31, 2005

Platelets Treated with Ticlopidine Are Less Reactive to Unusually Large von Willebrand Factor Multimers than Are Those Treated with Aspirin under High Shear Stress

M. Matsumoto[a] S. Kawaguchi[b] H. Ishizashi[c] H. Yagi[a] J. Iida[b] T. Sakaki[b] Y. Fujimura[a]

Departments of [a]Blood Transfusion Medicine, [b]Neurosurgery and [c]Health Science, Nara Medical University, Kashihara, Japan

Key Words
Ticlopidine Aspirin von Willebrand factor
Cerebral ischemia

Abstract
Much attention has recently been focused on the interaction between unusually large von Willebrand factor multimers (UL-VWFM) and platelets under high shear stress in pathological thrombus formation. The antiplatelet drugs acetylsalicylic acid (aspirin) and a thienopyridine derivative (ticlopidine) are commonly used to treat cerebral ischemia but exert different effects on high-shear-stress-induced platelet aggregation (H-SIPA) in the plasma. To examine the effects of these drugs in the absence of plasma factors, we studied H-SIPA using washed platelets (WPs) and purified UL-VWFM. WPs were prepared from the blood of 9 aspirin-treated and 11 ticlopidine-treated patients with cerebral ischemia, and H-SIPA in the presence of UL-VWFM was measured using a cone plate aggregometer. Plasma levels of VWF antigen with its multimer analysis, ristocetin cofactor and VWF-cleaving protease (ADAMTS13) activity were also measured. Forty-six healthy volunteers from 2 age groups, 20–40 years (n = 20) and 41–60 years old (n = 26), were also tested as controls. H-SIPA was significantly inhibited for ticlopidine-treated platelets, but it was observed to a lesser extent for aspirin-treated platelets. For both groups, no difference in the plasma levels of VWF antigen, ristocetin cofactor and ADAMTS13 activity was noted. All patients possessed UL-VWFM, and it was detected in healthy volunteers with increasing frequency with increasing age. Under plasma-free conditions, platelets from aspirin-treated patients exhibit marginal but significant inhibition of H-SIPA. Furthermore, the presence of UL-VWFM in the plasma of patients and normal volunteers is directly related to their age rather than being a consequence of underlying disease.

Introduction

Antiplatelet drugs such as aspirin and thienopyridine derivatives are commonly used to treat patients with cerebral ischemia to prevent the occlusion of brain arteries. Aspirin inhibits thromboxane A_2 synthesis [1], and the thyenopyridine derivatives ticlopidine and clopidogrel block the ADP receptor $P2Y_{12}$ [2–4].

In 1994, Uchiyama et al. [5] reported that high-shear-stress-induced platelet aggregation (H-SIPA) using platelet-rich plasma (PRP) is enhanced in patients with cere-

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1424–8832/05/0341–0035$22.00/0

Accessible online at:
www.karger.com/pht

Yoshihiro Fujimura, MD
Department of Blood Transfusion Medicine, Shijyo-Cho 840
Kashihara City, Nara 634-8522 (Japan)
Tel. +81 744 22 3051, ext. 3289, Fax +81 744 29 0771
E-Mail yfujimur@naramed-u.ac.jp

Table 1. Patient characteristics

	Age years	Sex	Underlying disease	Complication
Ticlopidine				
1	67	M	TIA	DM
2	64	M	TIA	HT
3	66	F	TIA	–
4	53	M	TIA	DM, HT
5	54	M	RIND	–
6	65	M	RIND	DM, HL
7	60	M	TIA	–
8	31	F	TIA	–
9	56	M	TIA	HT
10	68	M	TIA	DM
11	77	M	TIA	HT
Aspirin				
1	65	F	TIA	HT
2	67	M	TIA	HT
3	65	M	TIA	–
4	52	M	TIA	–
5	63	M	TIA	DM, HL
6	41	M	TIA	–
7	52	F	TIA	HT
8	36	M	TIA	AP
9	67	M	TIA	DM

TIA = Transient ischemic attack; RIND = reversible ischemic neurological deficit; DM = diabetes mellitus; HT = hypertension; HL = hyperlipidemia; AP = angina pectoris.

bral ischemia due to the increase in large von Willebrand factor (VWF) multimers and that the enhancement of H-SIPA can be corrected by taking ticlopidine but not low-dose aspirin. H-SIPA is mediated by the interaction of the platelet receptors glycoprotein (GP) Ib and GP IIb/IIIa with VWF, which is a plasma GP exclusively synthesized in vascular endothelial cells and secreted into the circulation as unusually large VWF multimer (UL-VWFM). UL-VWFM most actively interacts with platelets and induces the formation of platelet thrombi under high shear stress conditions [6]. In the normal circulation, however, UL-VWFM is rapidly cleaved and degraded into smaller VWFM by the plasma protease VWF-cleaving protease (ADAMTS13), which attacks the Tyr842-Met843 bond [7].

In this study, we analyzed the effect of two antiplatelet drugs on H-SIPA using a washed platelet (WP) system and examined the UL-VWFM of patients with cerebral ischemia using SDS-0.9% agarose gel electrophoresis.

Materials and Methods

Subjects

Twenty patients with cerebral ischemia who were being treated with antiplatelet drugs were enrolled in this study as listed in table 1 after informed consent had been obtained. Diagnoses were made based on neurological examinations, routine laboratory data, brain computed tomography, brain magnetic resonance imaging and cerebral angiography. None of the patients had findings of major vessel occlusions and neurological symptoms at the time of registration. They were not given any antiplatelet drugs for 2 weeks and any anticoagulants or fibrinolytic drugs for 24 h prior to the study. Then the patients were randomly allocated to receive 200 mg ticlopidine (Panaldin®, Daiichi Parmaceutial Corp.) or 81 mg aspirin (Bufferin®, Lion Corp.) by choosing the envelope including a card with a drug name, ticlopidine or aspirin. There was no statistically significant difference between these two groups concerning the demographics such as age, gender and clinical symptoms.

For each patient, 18 ml of blood was withdrawn by venipuncture from an antecubical vein using a 21-gauge needle before and days 7 after oral administration of ticlopidine or aspirin; the patient was then anticoagulated with a 1/10th volume of 3.8% Na_3-citrate. We chose day 7 after oral administration as the 2nd examination point because ticlopidine inhibits platelet aggregation after 3–5 days of use [8]. Five milliliters of the citrated blood were subsequently centrifuged at 3,000 *g* for 15 min at 4°C, and the platelet-poor plasma was separated and stored in aliquots at –80°C until use. The remaining 15 ml was used to prepare WPs as described below.

For the control experiments, citrated platelet-poor plasma was also prepared from two groups of healthy volunteers composed of 13 males and 7 females aged 20–40 years and 25 male and 1 female volunteers aged 40–61 years.

Preparation of UL-VWFM

Purification of VWF from cryoprecipitate was performed as described elsewhere [9]. Briefly, cryoprecipitate was prepared from 3 liters of outdated fresh frozen plasma, provided from the Japan Red Cross Blood Center by freezing at –80°C and thawing overnight at 4°C. After centrifugation at 7,500 *g* for 30 min at 4°C, the cryoprecipitate was then collected and dissolved in 300 ml of 25 mmol/l Tris-HCl buffer (pH 7.3) containing 0.5 mmol/l EDTA 4Na, 150 mmol/l NaCl and 1 mmol/l phenylmethylsulfonyl fluoride. Next, it was centrifuged at 7,500 *g* for 15 min at room temperature, and the supernatant was applied to a gelatin-sepharose 4B (Amersham Bioscience) column (Vt = 200 ml) at room temperature to remove fibronectin, after which the fall-through fractions were pooled. Following precipitation with 40% saturated $(NH_4)_2SO_4$ and centrifugation, the precipitate was separated and dissolved in 50 ml of 20 mmol/l imidazole-HCl buffer (pH 6.5) containing 20 mmol/l -aminocapronic acid, 1 mol/l NaCl and 10 mmol/l sodium citrate. It was then centrifuged again at 7,500 *g* for 15 min, and the resulting supernatant was applied to a sepharose 4B gel filtration column (5 × 100 cm, Amersham Bioscience). The eluate was collected in 8-ml volumes in separate tubes and dialyzed against phosphate-buffered saline (pH 7.3) at 4°C overnight, and the fractions containing UL-VWFM (fig. 1 and Results) were pooled and kept frozen in aliquots at –80°C. This purified material was used subsequently throughout this study.

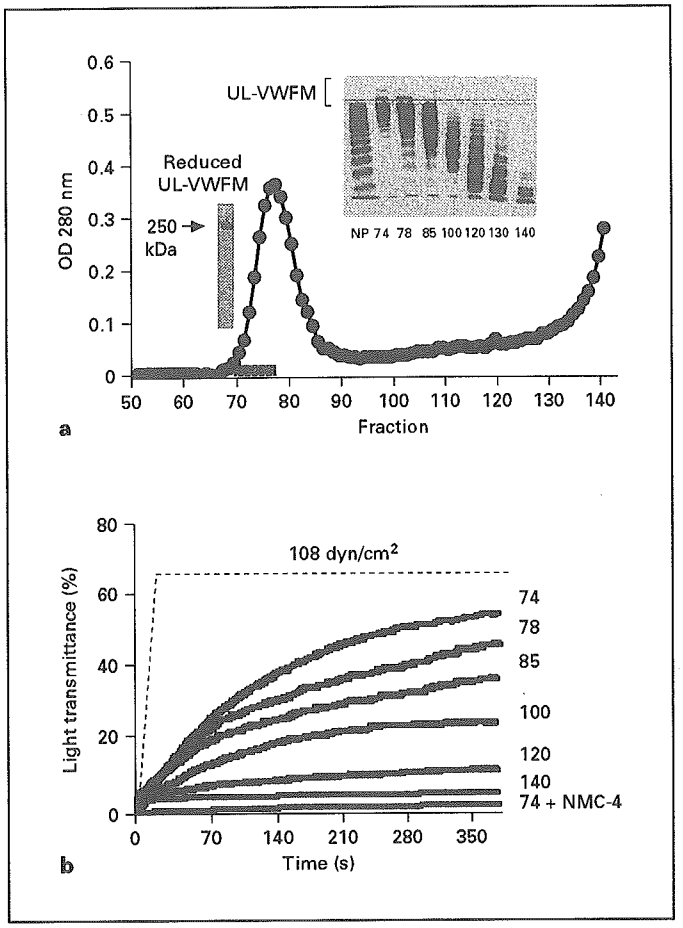

Fig. 1. Preparation of UL-VWFM. **a** Chromatographic separation of VWF on a Sepharose 4B column, where the black bar indicates the fractions which contain UL-VWFM. The right inset shows the results of the multimeric analysis performed by SDS-1.2% agarose gel electorophoresis. NP = Normal human plasma. To check the purity of VWF, the purified UL-VWF fraction was subjected to SDS-5% polyacrylamide gel electrophoresis under reducing conditions as shown in the left inset. **b** H-SIPA using normal WPs and the purified VWF fraction. H-SIPA using a mixture of normal WPs at a final concentration of 30 × 10^8/ml and each purified VWF fraction at a final concentration of 5 g/ml was dependent on its multimeric size.

Measurement of H-SIPA Using WPs

WPs were prepared under room temperature as follows [10]. First, PRP was produced by centrifugation at 200 *g* for 10 min using 15 ml citrated blood from patients as mentioned in the Subjects section and from a normal volunteer (blood type 0, Rho(D)+), and then acidified to pH 6.5 with acid citrate dextrose. The resulting platelets were separated from the PRP by centrifugation at 800 *g* for 10 min in the presence of 1 U/ml apyrase (Sigma-Aldrich) and 1 mmol/l prostaglandin E_1 (PGE_1; Sigma-Aldrich), and were then washed twice in platelet-washing buffer (113 mmol/l NaCl, 4.3 mmol/l K_2HPO_4, 4.2 mmol/l Na_2HPO_4, 24.4 mmol/l NaH_2PO_4 and 5.5 mmol/l glucose, pH 6.5) that contained 1 U/ml apyrase and 1 mmol/l PGE_1. The platelet pellets were next resuspended in HEPES-Tyrode's buffer (138 mmol/l NaCl, 2.8 mmol/l KCl, 2 mmol/l $CaCl_2$ and 10 mmol/l HEPES, pH 7.4) at a concentration of 3 × 10^8/ml and used within 2.5 h.

H-SIPA was measured using an argon-laser-assisted cone platelet aggregometer (Torey Medical Inc., Tokyo, Japan) [11] at room temperature. Before applying constant high shear stress at 108 dyn/cm^2, the purified UL-VWFM was added to the platelet suspensions at a final concentration of 5 g/ml in a total volume of 400 l. For some experiments, H-SIPA was measured in the presence of anti-VWF monoclonal antibody (NMC-4), for which the epitope resides on the VWF A1 domain and inhibits its binding to platelet GP Ib [9].

Additional Assays

Assays for VWF antigen [12] and ristocetin cofactor [13] were performed in addition to SDS-0.9% agarose gel electrophoresis followed by Western blotting with luminographic detection of VWFM [14, 15]. Plasma ADAMTS13 activity was assayed by the modified method of Furlan et al. [16] based on VWFM analysis [17]. The activity of pooled normal plasma was defined as 100%.

Statistical Analysis

Paired and unpaired comparisons between the two groups were performed using the Student's t test for which $p < 0.05$ was judged to indicate statistical significance. All experimental data are presented as means ± SD.

Results and Discussion

From SDS-1.2% agarose gel electrophoretic analysis, the initial half void volume fractions (F71–78) were found to possess UL-VWFM and also showed a single 250-kD band by SDS-5% polyacrylamide gel electrophoretic analysis under reducing conditions (fig. 1a). Furthermore, H-SIPA using a mixture of normal WPs and each purified VWF fraction at 5 g/ml final concentration was dependent on its multimeric size. Maximum aggregation was observed when using F74 VWF, and this was completely blocked by the anti-VWF monoclonal antibody NMC-4 at a final concentration of 10 g/ml (fig. 1) or with the anti-GP-Ib monoclonal antibody AP-1 at a final concentration of 10 g/ml (not shown). Thus, the pooled fractions (F71–78) were used as the source of UL-VWFM for subsequent studies of H-SIPA, and the percent light transmittance at 360 s after the generation of high shear stress at 108 dyn/cm^2 was recorded as the maximum aggregation value.

For the patients treated with ticlopidine, H-SIPA was dramatically but not totally inhibited, and the value was 43.8 ± 17.7% before and 21.2 ± 10.5% after treatment ($p < 0.001$) as shown in figure 2. Furthermore, for the patients treated with aspirin, H-SIPA was also inhibited to

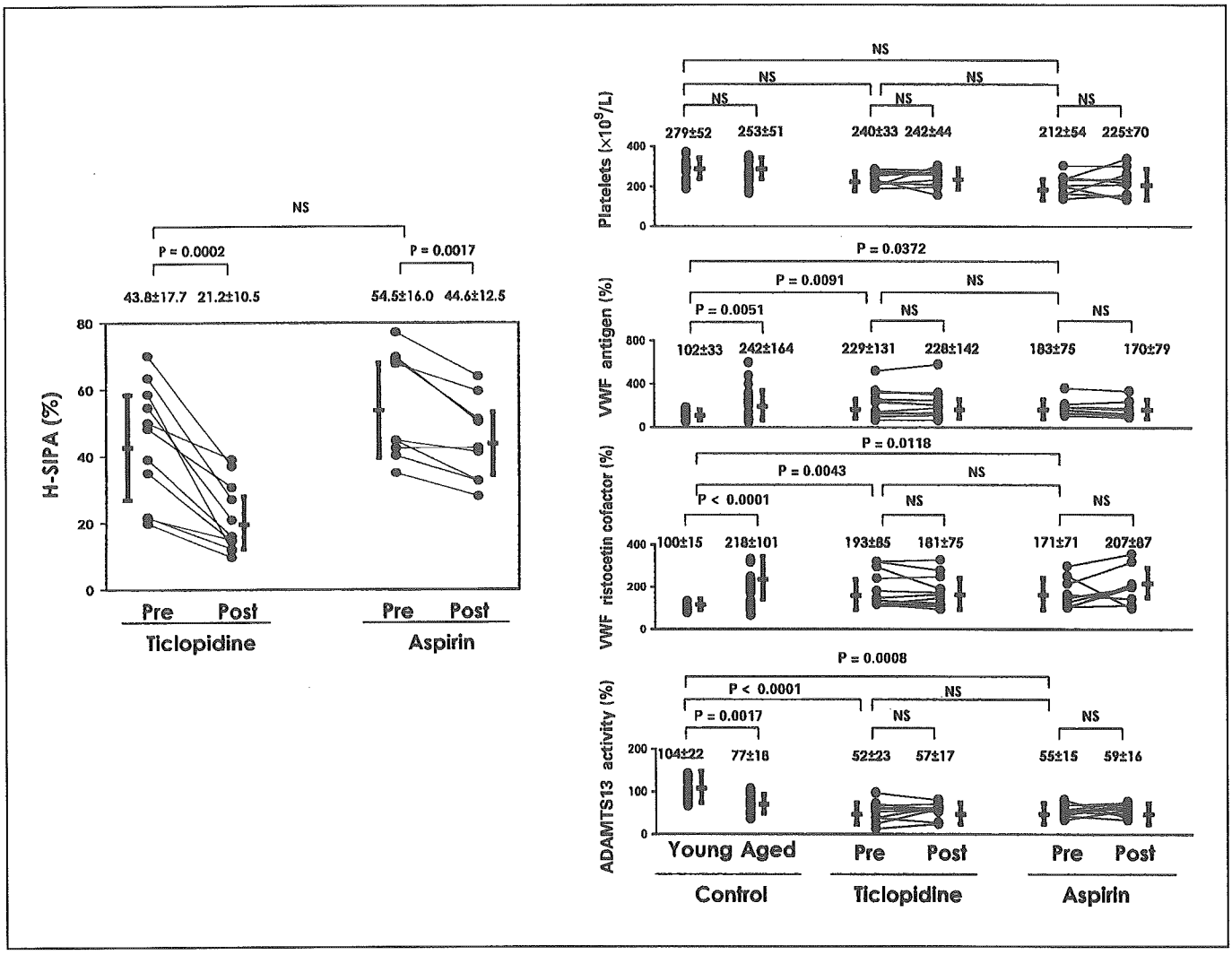

Fig. 2. Comparison of H-SIPA, platelets and VWF. For the patients treated with ticlopidine, H-SIPA was dramatically inhibited after treatment as was the case for patients treated with aspirin, although to a lesser extent. The plasma VWF antigen, ristocetin cofactor and ADAMTS13 activity in patients from both groups were significantly unchanged before and after treatment.

some extent but less than that after ticlopidine treatment, and the value was 54.5 ± 16.0% before and 44.6 ± 12.5% after treatment (p = 0.0017), which is in contrast to the results of Uchiyama et al. [3], who found a remarkable inhibition of H-SIPA in patients treated with ticlopidine but no significant inhibition in those treated with aspirin using a PRP system.

Since H-SIPA in the PRP system is influenced by the nature of the platelets and multiple plasma factors, we analyzed the plasma VWF of both patient groups. As shown in figure 2, both groups exhibited a remarkably increased level of plasma VWF antigen and ristocetin cofactor compared with young healthy volunteers. The plasma VWF antigen and ristocetin cofactor of patients with cerebral ischemia were significantly unchanged before and after treatment, but a remarkable increase in both was observed in subjects of advanced age. Furthermore, a significant decrease in plasma ADAMTS13 activity compared with young healthy volunteers was noted for both patient groups, which was unchanged before and after treatment. A remarkable decrease was also observed for subjects of advanced age. The reason why the activity of plasma ADAMTS13 decreased in both patients with cerebral ischemia and advanced-age subjects is consid-

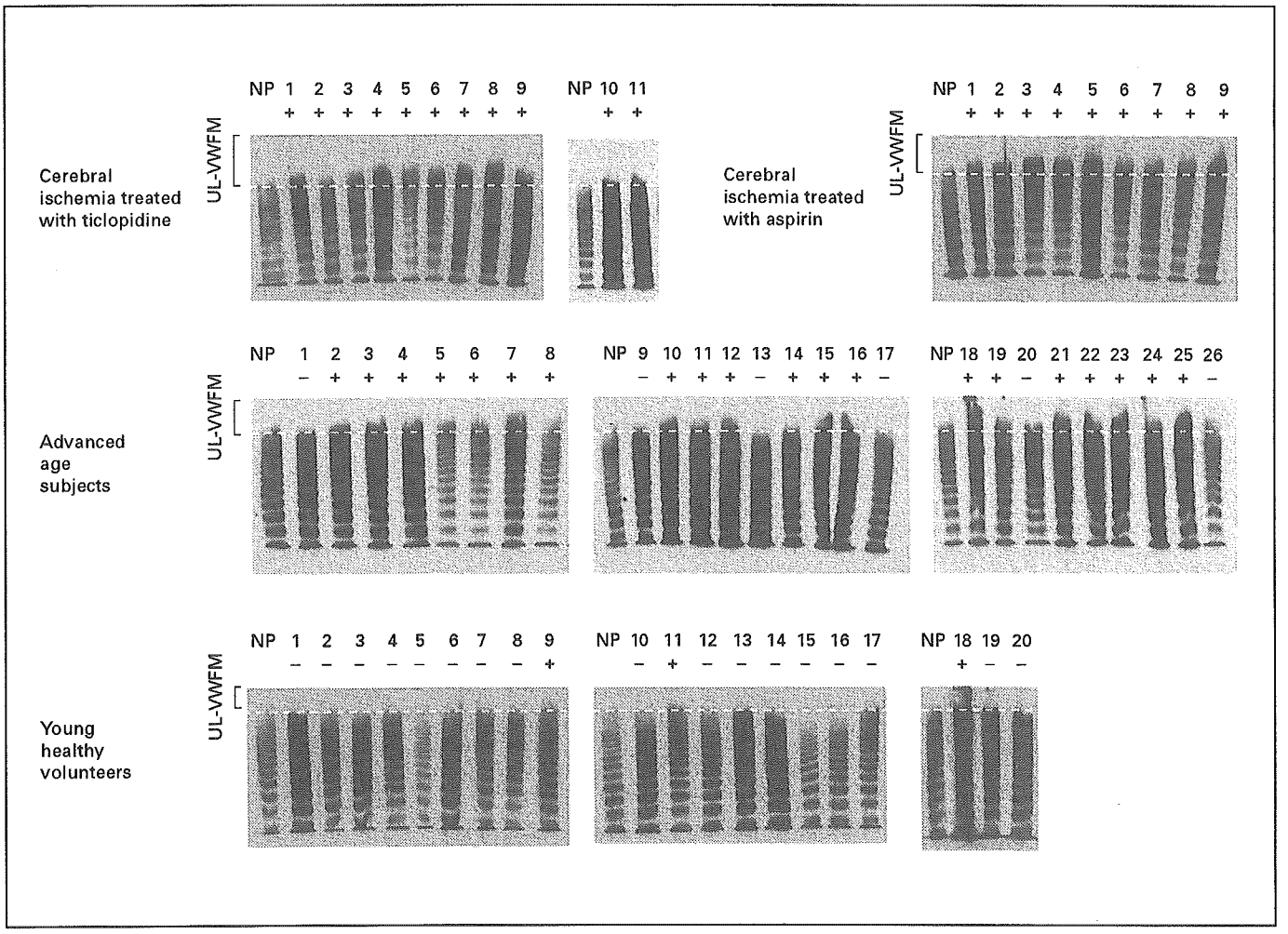

Fig. 3. The detection of UL-VWFM by SDS-0.9% agarose gel analysis. All patients with cerebral ischemia in both groups had UL-VWFM, and UL-VWFM was also detected in 20 of 26 of subjects of advanced age, whereas it was only seen in 3 out of 20 young healthy volunteers. NP = Normal human plasma.

ered to be decreased production of ADAMTS13 in the liver or consumption of ADAMTS13 to degrade very high amounts of VWF antigen. Mannucci et al. [18] speculated that the consumption of ADAMTS13 degraded a large amount of VWF antigen in order to explain the inverse correlation between ADAMTS13 activity and VWF antigen in healthy and various pathological conditions. Decreased activity of ADAMTS13 together with a large amount of VWF antigen may induce the appearance of UL-VWFM in plasma, which may result in a risk factor for cerebral ischemia to occur.

Our assay using the patient's WPs was successful at determining platelet function because it excluded the effect of plasma factors. Likewise, Sun et al. [19] have recently reported that something in the plasma was responsible for the phenomenon of shear aggregation 'aspirin resistance'.

Next, we examined the UL-VWFM of patients with cerebral ischemia by SDS-0.9% agarose gel electrophoresis as shown in figure 3. All patients with cerebral ischemia in both groups had UL-VWFM, and this confirmed the observed increase in plasma VWF antigen and ristocetin cofactor in patients with cerebral ischemia. UL-VWFM was also detected in 20 of 26 subjects of advanced age, whereas it was only seen in 3 of 20 young healthy volunteers.

Cerebral ischemia is caused by platelet thrombosis induced by high shear stress. Recent studies revealed that

VWF and its interaction with the platelet receptors GP Ib and GP IIb/IIIa play a role in platelet thrombosis induced by this type of stress [4]. In this study, we found that the enhancement of H-SIPA in patients with cerebral ischemia is corrected for by taking ticlopidine and aspirin, and that ticlopidine has a stronger effect than does aspirin. These drugs seem to solely affect platelet function. In addition, subjects of advanced age possessed UL-VWFM more frequently than did young subjects. These results indicate that people of advanced age may be more susceptible to developing cerebral ischemia.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology (to M.M., H.I. and Y.F.), and from the Ministry of Health and Welfare of Japan for Blood Coagulation Abnormalities H14-02 (to Y.F.).

References

1 Alberts MJ, Bergman DL, Molner E, Jovanovic BD, Ushiwata I, Teruya J: Antiplatelet effect of aspirin in patients with cerebrovascular disease. Stroke 2004;35:175–178.

2 Foster CJ, Prosser DM, Agans JM, Zhai Y, Smith MD, Lachowicz JE, Zhang FL, Gustafson E, Monsma FJ, Wiekowski MT, Abbondanzo SJ, Cook DN, Bayne ML, Lira SA, Chintala MS: Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. J Clin Invest 2001;107:1591–1598.

3 Turner NA, Moake JL, McIntire LV: Blockade of adenosine diphosphate receptors $P2Y_{12}$ and $P2Y_1$ is required to inhibit platelet aggregation in whole blood under flow. Blood 2001;98: 3340–3345.

4 Goto S, Noriko T, Eto K, Ikeda Y, Handa S: Functional significance of adenosine 5 -diphosphate receptor ($P2Y_{12}$) in the platelet activation initiated by binding of von Willebrand factor to platelet GP1b induced by conditions of high shear rate. Circulation 2002;105:2531–2536.

5 Uchiyama S, Yamazaki M, Maruyama S, Handa M, Ikeda Y, Fukuyama M, Itagaki I: Shear-induced platelet aggregation in cerebral ischemia. Stroke 1994;25:1547–1551.

6 Moake JL, Turner NA, Stathopoulos NA, Nolasco LH, Hellums JD: Involvement of large plasma von Willebrand factor (vWF) multimers and unusually large vWF forms derived from endothelial cells in shear stress-induced platelet aggregation. J Clin Invest 1986;78: 1456–1461.

7 Fujimura Y, Matsumoto M, Yagi H, Yoshioka A, Matsui T, Titani K: von Willebrand factor-cleaving protease and Upshaw-Schulman syndrome. Int J Hematol 2002;75:25–34.

8 Quinn MJ, Fitzgerald DJ: Ticlopidine and clopidogrel. Circulation 1999;100:1667–1672.

9 Fujimura Y, Usami Y, Titani K, Niinomi K, Nishino K, Takase T, Yoshioka A, Fukui H: Studies on anti-von Willebrand factor (vWF) monoclonal antibody NMC-4, which inhibits both ristocetin- and botrocetin-induced vWF binding to platelet glycoprotein Ib. Blood 1991;77:113–120.

10 Fujimura Y, Ikeda Y, Miura S, Yoshida E, Shima H, Nishida S, Suzuki M, Titani K, Taniuchi Y, Kawasaki T: Isolation and characterization of jararaca GPIb-BP, a snake venom antagonist specific to platelet glycoprotein Ib. Thromb Haemost 1995;74:743–750.

11 Ikeda Y, Handa M, Kawano K, Kamata T, Murata M, Araki Y, Anbo H, Kawai Y, Watanabe K, Itagaki I, Sakai K, Ruggeri ZM: The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. J Clin Invest 1991;87:1234–1240.

12 Bartlett A, Dormandy KM, Hawkey CM, Stableforth P, Voller A: Factor VIII-related antigen: Measurement by enzyme immunoassay. Br Med J 1976;24:994–996.

13 Macfarlane DE, Stibbe J, Kirby EP, Zucker MB, Grant RA, McPherson J: A method for assaying von Willebrand factor (ristocetin cofactor). Thromb Diath Haemorrh 1975;34: 306–308.

14 Ruggeri ZM, Zimmerman TS: Variant von Willebrand's disease: Characterization of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. J Clin Invest 1980;65: 1318–1325.

15 Budde U, Schneppenheim R, Plendl H, Dent J, Ruggeri ZM, Zimmerman TS: Luminographic detection of von Willebrand factor multimers in agarose gels and on nitrocellulose membranes. Thromb Haemost 1990;63:312–315.

16 Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle PA, Brenner B, Krause M, Scharrer I, Aumann V, Mittler V, Solenthaler M, Lammle B: Von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. N Engl J Med 1998;339:1578–1584.

17 Kinoshita S, Yoshioka A, Park Y-D, Ishizashi H, Konno M, Funado M, Matsui T, Titani K, Yagi H, Matsumoto M, Fujimura Y: Upshaw-Schulman syndrome revisited: A concept of congenital thrombotic thrombocytopenic purpura. Int J Hematol 2001;74:101–108.

18 Mannucci PM, Canciani MT, Forza I, Lussana F, Lattuada A, Rossi E: Changes in health and disease of the metalloprotease that cleaves von Willebrand factor. Blood 2001;98:2730–2735.

19 Sun L, Feng S, Resendiz JC, Lu X, Durante W, Kroll MH: Role of the Pyk2-MAP kinase-cPLA2 signaling pathway in shear-dependent platelet aggregation. Ann Biomed Eng 2004; 32:1193–1201.

Negative regulation of platelet function by a secreted cell repulsive protein, semaphorin 3A

Hirokazu Kashiwagi, Masamichi Shiraga, Hisashi Kato, Tsuyoshi Kamae, Naoko Yamamoto, Seiji Tadokoro, Yoshiyuki Kurata, Yoshiaki Tomiyama, and Yuzuru Kanakura

Semaphorin 3A (Sema3A) is a secreted disulfide-bound homodimeric molecule that induces growth cone collapse and repulsion of axon growth in the nervous system. Recently, it has been demonstrated that Sema3A is produced by endothelial cells and inhibits integrin function in an autocrine fashion. In this study, we investigated the effects of Sema3A on platelet function by using 2 distinct human Sema3A chimera proteins. We detected expression of functional Sema3A receptors in platelets and dose-dependent and saturable binding of Sema3A to platelets. Sema3A dose-dependently inhibited activation of integrin αIIbβ3 by all agonists examined including adenosine diphosphate (ADP), thrombin, convulxin, phorbol 12-myristate 13-acetate, and A23187. Sema3A inhibited not only platelet aggregation induced by thrombin or collagen but also platelet adhesion and spreading on immobilized fibrinogen. Moreover, Sema3A impaired αIIbβ3-independent spreading on glass coverslips and aggregation-independent granular secretion. Sema3A inhibited agonist-induced elevation of filamentous action (F-actin) contents, phosphorylation of cofilin, and Rac1 activation. In contrast, Sema3A did not affect the levels of cyclic nucleotides or agonist-induced increase of intracellular Ca^{2+} concentrations. Thus, the extensive inhibition of platelet function by Sema3A appears to be mediated, at least in part, through impairment of agonist-induced Rac1-dependent actin rearrangement. (Blood. 2005;106:913-921)

Introduction

Platelets play a crucial role not only in a hemostatic plug formation but also in a pathologic thrombus formation, particularly within atherosclerotic arteries subjected to high shear stress.[1,2] As an initial step in thrombogenesis, platelets adhere to altered vascular surfaces or exposed subendothelial extracellular matrices, then become activated and aggregate each other. These processes are primarily mediated by platelet surface glycoproteins such as GPIb-IX-V, integrin α2β1, GPVI, and integrin αIIbβ3.[3,4] Especially, integrin αIIbβ3 plays an essential part in aggregate formation and adhesive spreading of platelets during hemostasis.[5-7] Pathways that inhibit platelet function are as important as those that activate them. Endothelial cells produce 2 well-documented inhibitors of platelet activation and aggregation, prostaglandin I_2 (PGI_2) and nitric oxide (NO).[8] PGI_2 binds to a specific Gs-coupled receptor, thereby activating adenylate cyclase and cyclic adenosine monophosphate (cAMP)–dependent protein kinase or protein kinase A (PKA). NO activates soluble guanylate cyclase and cyclic guanosine monophosphate (cGMP)–dependent kinase or PKG. Ecto–adenosine diphosphatase (ADPase, CD39) located on the luminal surface of endothelial cells also inhibits platelet aggregation by decreasing the local concentration of ADP. Thus, endothelial dysfunction or damage promotes a prothrombotic state and may be involved in the pathogenesis of cardiovascular disorders, including atherosclerosis, diabetes mellitus, essential hypertension, hypercholesterolemia, and hyperhomocysteinemia.[8]

The semaphorin family comprises soluble and membrane-bound proteins that are defined by the presence of a conserved 500–amino acid semaphorin domain at their amino termini.[9] Class 3 semaphorins are secreted disulfide-bound homodimeric molecules, and Sema3A, a prototypic class 3 semaphorin, causes growth cone collapse and provides chemorepulsive guidance for migrating axons.[10-12] Cell surface receptor for Sema3A consists of a complex of 2 distinct transmembrane receptors, neuropilin-1 and plexin A (A1-A3).[10-13] Neuropilin-1 provides a binding site of Sema3A, while plexin A transduces the Sema3A signals into the cells through its cytoplasmic domain.[10-13] Although the intracellular signaling pathways evoked by Sema3A binding are not fully understood, plexins should interact with signaling molecules to regulate actin reorganization, since growth cone collapse is accompanied by rapid reorganization of the actin filaments normally present in lamellipodia or filopodia.[11,12] In this context, a Rho family small G-protein, Rac, has been identified as a potential regulator of semaphorin-dependent actin cytoskeletal dynamics.[11,12]

Although Sema3A function on neural development is studied intensively, its function in other organs is poorly understood. The fact that semaphorins are expressed in many different tissues suggests that they also play a role in systems other than

From the Department of Hematology and Oncology, Osaka University Graduate School of Medicine, Osaka, Japan; and the Department of Blood Transfusion, Osaka University Hospital, Osaka, Japan.

Submitted October 26, 2004; accepted April 10, 2005. Prepublished online as *Blood* First Edition Paper, April 14, 2005; DOI 10.1182/blood-2004-10-4092.

Supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture in Japan; the Yamanouchi Foundation for Research and Metabolic Disorders, Tsukuba, Japan; and Mitsubishi Pharma Research Foundation, Osaka, Japan.

Reprints: Hirokazu Kashiwagi, Department of Hematology and Oncology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan; e-mail: kashi@hp-blood.med.osaka-u.ac.jp.

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 U.S.C. section 1734.

the nervous system.[12] Indeed, in addition to neural abnormalities, mice lacking a functional *Sema3A* gene have abnormalities in their heart and visceral tissues, suggesting that Sema3A signaling might be indispensable for normal development in several organs.[14,15] Very recently, Serini et al reported that semaphorins are also involved in angiogenesis.[16] They showed that endothelial cells generate chemorepulsive autocrine signals of class 3 semaphorins that localize at nascent adhesive sites in spreading endothelial cells.[16] Interestingly, Sema3A inhibits the integrin-mediated adhesion to extracellular matrix and impedes their directional motility, which could explain the aberrant vascularization that is observed in Sema3A-deficient mice.[16] Others also showed that plexin signaling negatively regulates integrin-based adhesive complexes, which leads to the inhibition of cell adhesion, lamellipodia formation, and cell migration.[17]

Since integrin αIIbβ3 is essential for platelet function and endothelial cells express Sema3A, we sought to investigate the effects of Sema3A on platelet function. In this study, we demonstrate that Sema3A binds to platelets and inhibits αIIbβ3 activation extensively. Sema3A also inhibits platelet aggregate formation and platelet adhesion and spreading on immobilized fibrinogen. Moreover, Sema3A inhibits αIIbβ3-independent spreading on glass coverslips and aggregation-independent granular secretion. Further investigation of signaling pathways demonstrates that Sema3A markedly impairs agonist-induced Rac1-dependent actin rearrangement.

Materials and methods

Reagents

Recombinant human Sema3A fused to human Fc fragment (Sema3A/Fc) was obtained from R&D Systems (Minneapolis, MN). A construct consisting of the human *Sema3A* cDNA fused to the catalytic domain of human placental alkaline phosphatase (*AP*) cDNA was prepared as previously described using the pAP-tag2 expression vector (GenHunter, Nashville, TN).[10] The plasmid was transfected to 293T cells by Lipofectamine2000 (Invitrogen, Carlsbad, CA), and recombinant Sema3A/AP was purified from cultured medium using anti–human AP monoclonal antibody–conjugated sepharose beads (clone 8B6) and dialyzed against phosphate-buffered saline (PBS). Human IgG (hIgG) and human placental AP were used for controls of Sema3A/Fc and Sema3A/AP, respectively. Purity of Sema3A/Fc and Sema3A/AP was confirmed by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining (SilverSNAP Stain Kit; Pierce, Rockford, IL). Convulxin was kindly provided by Dr M. Moroi (Department of Protein Biochemistry, Institute of Life Science, Kurume University, Fukuoka, Japan). Fibrinogen was purchased from Calbiochem (San Diego, CA) and was labeled with fluorescein isothiocyanate (FITC), as previously described.[18] Type I collagen was obtained from MC Medical (Tokyo, Japan). A hybridoma producing IV.3, a mouse monoclonal antibody specific for human Fcγ-RIIA (CD32), was obtained from American Type Culture Collection (Rockville, MD) and IV.3 Fab fragments were generated as described previously.[19] All other reagents were purchased from Sigma (St Louis, MO), unless otherwise indicated.

Platelet preparation

Washed platelets were prepared as described previously.[20] In brief, 6 vol freshly drawn venous blood from healthy volunteers was mixed with 1 vol acid-citrate-dextrose and centrifuged at 250*g* for 10 minutes to obtain platelet-rich plasma (PRP). After a 5-minute incubation with 1 μM prostaglandin E_1 (PGE_1) and 1 U/mL apyrase, the PRP was centrifuged at 750*g* for 10 minutes, washed once with citrate buffer containing 1 μM PGE_1 and 1 U/mL apyrase, and resuspended in an appropriate buffer. Washed platelets were rested for 30 minutes at 37°C before use in any experiments. In all experiments using Sema3A/Fc, platelet FcγRIIA receptor was blocked by preincubation with 20 μg/mL IV.3 Fab.

Platelets for RNA extraction were prepared as described previously.[21] In brief, to remove the contaminated leukocytes, PRP was passed through a leukocyte removal filter (Sepacell PL-5A; Asahi Medical, Tokyo, Japan), which can remove more than 99.9% of contaminated leukocytes.[21]

Detection of binding of Sema3A to platelets and Sema3A receptors in platelets

For detection of binding of Sema3A/Fc to platelets, 5×10^5 washed platelets in Walsh buffer (137 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 3.3 mM NaH_2PO_4, 3.8 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 0.1% glucose, 0.1% bovine serum albumin [BSA], pH 7.4) were incubated with various concentrations of Sema3A/Fc for 30 minutes at room temperature and washed once with citrate buffer. Then, platelets were resuspended in PBS with FITC-labeled anti–human Fc for 20 minutes, followed by flow cytometric analysis. For detection of the binding of Sema3A/AP, 5×10^6 platelets were incubated with various concentrations of Sema3A/AP for 30 minutes at room temperature. After washing with citrate buffer, AP activity was measured using disodium phenylphosphate as a substrate (Sanko Jun-yaku, Tokyo, Japan). The number of Sema3A binding sites was estimated by the maximum AP activity of Sema3A/AP obtained from standard AP activity. In some experiments, platelets were first incubated with 125 μg/mL Sema3A/Fc or hIgG for 10 minutes. After washing, platelets were incubated with 10 μg/mL Sema3A/AP for another 30 minutes, and AP activity was measured.

Western blotting and flow cytometry of neuropilin-1 were performed with mouse anti–neuropilin-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously.[22,23] Horseradish peroxidase (HRP)–conjugated anti–mouse IgG (New England Biolabs, Beverly, MA) and Alexa488-conjugated anti–mouse IgG (Molecular Probes, Eugene, OR) were used as secondary antibodies for Western blotting and flow cytometry, respectively. Reverse transcriptase–polymerase chain reaction (RT-PCR) for detection of plexin-A1, -A2, and -A3 was performed as described.[16] In brief, RNA was extracted by a Trizol reagent (Invitrogen), and cDNA was synthesized using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). RT products were amplified in a PCR reaction with a Taq polymerase (Takara ExTaq; Takara Bio, Shiga, Japan). Primer sequences and PCR conditions were described previously.[16]

Activation of αIIbβ3 by various agonists

Activation state of αIIbβ3 was monitored by binding of a ligand-mimetic antibody, PAC-1, or soluble fibrinogen under flow cytometric analysis as described previously.[21,22,24] In brief, 5×10^5 platelets in Walsh buffer were preincubated with Sema3A/Fc or Sema3A/AP for 10 minutes, followed by incubation with agonists and FITC-conjugated PAC-1 (BD Biosciences, Franklin Lakes, NJ) or FITC-fibrinogen for 20 minutes at room temperature. Then, platelets were diluted to 500 μL with Walsh buffer and analyzed immediately on flow cytometry (FACScan; BD Japan, Tokyo, Japan).

Platelet aggregation study

Platelet aggregation was monitored using a platelet aggregometer (model 313M; MC Medical) at 37°C with a stirring rate at 1000 rpm, as previously described.[20] In brief, Sema3A/AP- or AP-treated platelets were suspended in modified Tyrode buffer containing 1 mM $MgCl_2$ at the concentration of $2 \times 10^5/\mu L$. After addition of $CaCl_2$ at the final concentration of 1 mM and incubation for one minute at 37°C, aggregation was initiated by addition of agonists.

Platelet granular secretion

Granular secretion was monitored by FITC-CD62P (Immunotech, Marseille, France) and phycoerythrin-conjugated CD63 (Immunotech) binding to platelets under flow cytometry as described previously.[25]

Adhesion to immobilized fibrinogen or glass coverslips

Adhesion of platelets to immobilized fibrinogen was assessed as described previously.[26] In brief, a 96-well polystyrene plate (Greiner Japan, Tokyo, Japan) was coated with fibrinogen at the various concentrations in PBS for 16 hours at 4°C. Platelets (1.25×10^6) in Tyrode buffer (137 mM NaCl, 12 mM $NaHCO_3$, 2.6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 0.1% glucose, 0.1% BSA, pH 7.4) were incubated with 20 μg/mL Sema3A/Fc or hIgG for 10 minutes at room temperature, and then they were placed on each well followed by incubation for one hour at room temperature. After washing 3 times with PBS to remove nonadherent platelets, adhered platelets were quantified by measuring endogenous cellular acid phosphate activity.[27] Relative adhesion to the maximum binding was calculated by dividing the acid phosphatase activity of adherent platelets by that of nontreated platelets adhered on the 10 μg/mL fibrinogen.

Morphologic study of adhered platelets was performed as described previously.[28] In brief, glass coverslips were coated with 20 μg/mL fibrinogen for 16 hours at 4°C, and then washed with PBS. After incubation with Sema3A/Fc or hIgG, 2×10^6 platelets in Tyrode buffer were incubated on the fibrinogen-coated coverslips for 45 minutes at 37°C or on the nontreated coverslips for 10 minutes at room temperature. Nonadherent platelets were washed away and adherent cells were stained with tetramethylrhodamine B isothiocyanate–conjugated phalloidin. Platelet spreading was observed under a florescence microscope (PROVIS AX-80; Olympus, Tokyo, Japan).

Quantification of F-actin contents

Filamentous actin (F-actin) content was analyzed by flow cytometry with bodipy-phallacidin as described previously.[28] In brief, after incubation with 20 μg/mL Sema3A/Fc or hIgG, platelets in Walsh buffer were stimulated with a 30-second incubation with 30 μM protease-activated receptor 1 (PAR1)–thrombin receptor-activating peptide (TRAP) or 0.5 U/mL thrombin at 37°C. Then, platelets were fixed with 4 vol of 2.6% glutaraldehyde in 5.3 mM EDTA (ethylenediaminetetraacetic acid) for 2 hours at 37°C. After washing twice with PBS, the platelets were resuspended to half their initial volume and incubated at 37°C either with 3.3 μM bodipy-phallacidin (Molecular Probes) or bodipy-phallacidin in the presence of a 300-fold molar excess of unlabeled phallacidin. After 30 minutes, the platelets were washed twice with PBS and platelet fluorescence was analyzed in the fluorescence intensity 1 (FL1) channel of the flow cytometer. Specific phallacidin binding was obtained by subtraction of mean fluorescence intensity of FL1 with unlabeled phallacidin from that of FL1 without unlabeled phallacidin.

Detection of phosphorylation of cofilin and activated Rac1

After incubation with 20 μg/mL Sema3A/Fc or hIgG for 10 minutes at room temperature, 1×10^7 platelets in Walsh buffer were incubated with 0.5 U/mL thrombin for the indicated times at 37°C without stirring. Then, cells were lysed with SDS sample buffer with 5% β-mercaptoethanol (β-ME). Proteins were resolved on a 15% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA). Phosphorylated cofilin was detected by using anti–phosphocofilin antibody (Cell Signaling Technology, Beverly, MA). After stripping the membrane with a stripping buffer (Restore Western Blot Stripping Buffer; Pierce), the membrane was rehybridized with anticofilin antibody (BD Biosciences). Optical density of the bands was measured by National Institutes of Health (NIH) Image software (Bethesda, MD). After calibrating the density of phosphorylated cofilin with that of total cofilin, relative increase of phosphorylated cofilin against that of IgG-treated platelets without agonist stimulation was calculated.

Detection of activated Rac1 was performed using a kit of pull-down assay according to the manufacturer's directions (EZ-Detect Rac1 Activation Kit; Pierce). In brief, Sema3A/Fc- or hIgG-treated platelets in Walsh buffer were incubated with 30 μM PAR1-TRAP for the indicated times at 37°C without stirring. Then, cells were lysed with 0.5% Triton-X100 lysis buffer. Guanosine triphosphate (GTP)–form of Rac1 was pull-downed by incubation with glutathione-S–transferase (GST)–p21-activated kinase 1 (PAK1)–p21-binding domain (PBD) and glutathione beads for one hour at 4°C. After washing with lysis buffer, precipitates were eluted with SDS sample buffer with β-ME, followed by electrophoresis on a 12% SDS-PAGE gel. After transfer to a PVDF membrane, Rac1 was detected by a mouse anti-Rac1–specific antibody. Total Rac1 was detected by

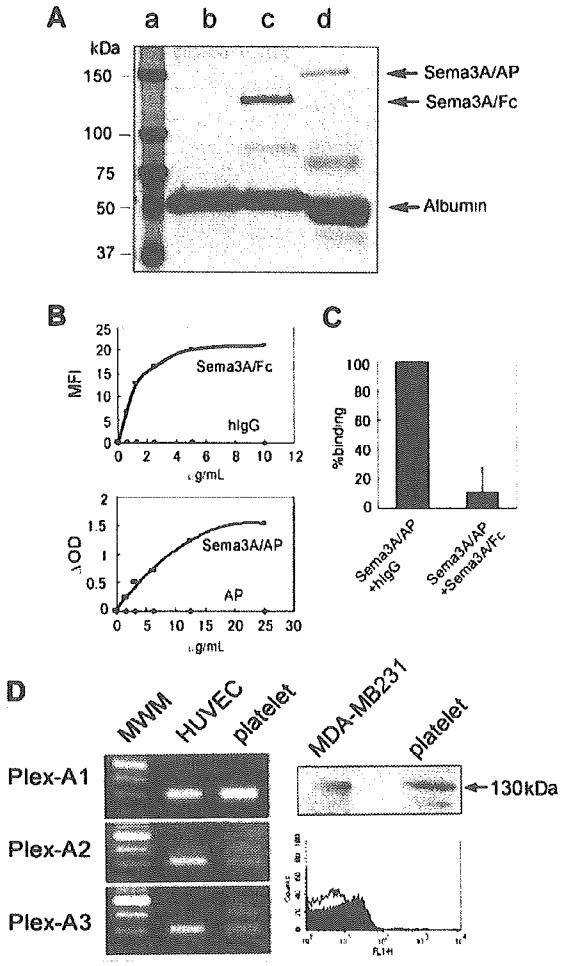

Figure 1. Detection of Sema3A binding to platelets and expression of Sema3A receptors in platelets. (A) Silver stain of purified Sema3A fusion proteins; 0.25 μg of Sema3A/Fc (~ 125 kDa, lane c) and Sema3A/AP (~ 150 kDa, lane d) were loaded on a 7.5% SDS-PAGE gel under reducing conditions and silver staining was performed. Sema3A/Fc and Sema3A/AP samples contain BSA as a carrier protein. In lane c, only BSA was loaded. Molecular weight marker was loaded in lane a. (B) Binding of Sema3A/Fc or Sema3A/AP to platelets. Washed platelets (5×10^5) were incubated with Sema3A/Fc or hIgG, followed by incubation with FITC anti–human Fc. Sema3A/Fc binding was detected by flow cytometry, and mean fluorescence intensity (MFI) was plotted in the top panel. Washed platelets (5×10^8) were incubated with Sema3A/AP or AP, and after washing, AP activity was measured using disodium phenylphosphate as a substrate. Change in optical density (ΔOD) was plotted on the bottom panel. Shown are representative results of 3 independent experiments. (C) Inhibition of Sema3A/AP binding by Sema3A/Fc. Washed platelets were first incubated with 125 μg/mL hIgG or Sema3A/Fc. Then, platelets were incubated with 10 μg/mL Sema3A/AP, and AP activity was measured. Shown is mean and SE of relative binding to hIgG-incubated sample of 3 independent experiments. (D) Expression of plexin-A1, -A2, or -A3 in platelets was detected by RT-PCR assay (left). Human umbilical vein endothelial cell (HUVEC) was used as a positive control. Expression of neuropilin-1 in platelets was detected by Western blotting and flow cytometric analysis (right). In Western blotting, neuropilin-1 expression was detected by anti–neuropilin-1 antibody, followed by incubation with HRP anti–mouse IgG. MDA-MB231 was used as a positive control. In flow cytometry, platelets were incubated with mouse monoclonal anti–neuropilin-1 antibody (filled curve) or control antibody (MOPC21; open curve), followed by incubation with Alexa488-conjugated anti–mouse IgG. MWM indicates molecular weight marker.

electrophoresis of total lysates on an SDS-PAGE gel followed by detection with the Rac1-specific antibody.

Intracellular Ca^{2+} mobilization

Intracellular Ca^{2+} concentrations in fluo-3–loaded platelets were assessed under flow cytometry as described previously.[29] In brief, platelets were labeled with 5 μM fluo-3-AM (Wako Pure Chemical, Osaka, Japan) at 37°C for 15 minutes. After incubation with 20 μg/mL Sema3A/Fc or hIgG, 5×10^5 platelets in 200 μL Walsh buffer were subjected to flow cytometry analysis. After the determination for about 10 seconds of baseline fluo-3 fluorescence from the platelet population, cell aspiration into the flow cytometry was briefly paused, and 1:10 volume of 5 U/mL thrombin was added. The acquisition was then resumed, and changes in log fluorescence versus time were recorded. For each plot, rectangular analysis regions were defined over the time axis, and mean florescence intensity was calculated with CellQuest software (BD Japan).

Quantification of platelet cyclic nucleotide levels

For cAMP quantification, 1.6×10^6 platelets in Walsh buffer were incubated with 20 μg/mL Sema3A/Fc or hIgG for 10 minutes at room temperature. Iloprost (20 μg/L; Cayman Chemical, Ann Arbor, MI) was used as an agonist for activation of adenylate cyclase. ADP (5 μM) was added to the platelet samples and incubated for 2 minutes at room temperature to study inhibition of adenylate cyclase. After lysing platelets, cAMP contents were measured by an enzyme immunoassay kit according to the manufacturer's directions (Biotrak cAMP EIA System; Amersham, Piscataway, NJ). For cGMP quantification, 3.6×10^6 platelets in Walsh buffer were incubated with 20 μg/mL Sema3A/Fc or hIgG for 10 minutes at room temperature, and cGMP contents were measured by an EIA kit (Biotrak cGMP EIA System; Amersham).

Statistical analysis

Experimental differences over the controls were analyzed by the Student *t* test. Probability values of *P* less than .05 were considered significant.

Results

Binding of Sema3A to platelets and expression of Sema3A receptors in platelets

We used 2 distinct Sema3A chimera proteins in this study: recombinant human Sema3A fused to human Fc fragment (Sema3A/Fc) or to the catalytic domain of human placental alkaline phosphatase (Sema3A/AP) (Figure 1A). We first investigated the binding of Sema3A to platelets. As shown in Figure 1B (upper), Sema3A/Fc bound to platelets in a dose-dependent and saturable manner. Sema3A/AP also bound to the platelets in basically the same manner as Sema3A/Fc, although it needed about 2-fold concentrations, compared with Sema3A/Fc, to saturate the binding to platelets (Figure 1B lower). About 90% of the Sema3A/AP binding was inhibited by preincubation with excess amounts of Sema3A/Fc, confirming the specificity of Sema3A binding to platelets (Figure 1C). We estimated the binding sites of Sema3A were approximately 8000 (7980 ± 500, n = 4) per platelet.

Next, we examined expression of Sema3A receptors in platelets. Western blotting and flow cytometric analysis revealed that neuropilin-1 was expressed in platelets (Figure 1C). Plexin expression was examined by RT-PCR assay, using platelet samples in which the contaminated leukocytes were removed by a leukocyte removal filter. As shown in Figure 1C, plexin-A1 and low levels of plexin-A2 and plexin-A3 were expressed in platelets. These results suggest that platelets express functional Sema3A receptors.

Effects of Sema3A on αIIbβ3 activation by various agonists and platelet aggregation

Since Sema3A inhibits integrin function in endothelial cells,[16] we examined the effects of Sema3A on integrin αIIbβ3 activation using a ligand-mimetic antibody, PAC-1. Sema3A/Fc dose-dependently inhibited PAC-1 binding induced by all agonists examined, including agonists that act via G-protein–coupled receptors (ie, ADP, thrombin, and U46619) and convulxin, which acts via G-protein–uncoupled receptor, GPVI (Figure 2A; Table 1). Sema3A/Fc inhibited A23187- and phorbol 12-myristate 13-acetate (PMA)–induced PAC-1 binding, suggesting that Sema3A inhibits αIIbβ3 activation mainly downstream of intracellular calcium mobilization and protein kinase C activation. Sema3A/AP also inhibited αIIbβ3 activation by thrombin and ADP (Figure 2B), indicating that the inhibitory effects were caused by the Sema3A domain, not by the fused Fc or AP domain. Sema3A/Fc inhibited a physiologic ligand, soluble fibrinogen binding to platelets after

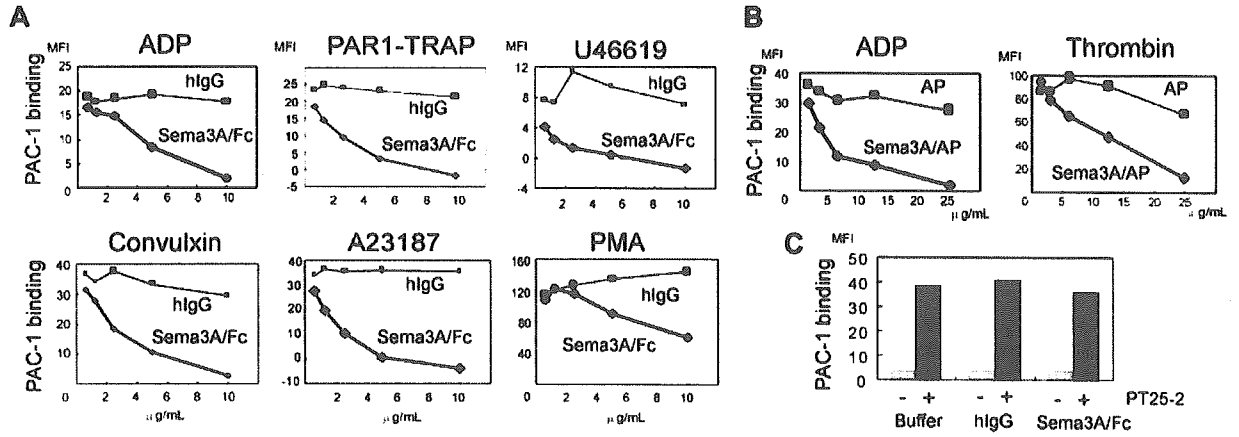

Figure 2. Inhibition of αIIbβ3 activation by Sema3A. (A) Washed platelets preincubated with the indicated concentrations of Sema3A/Fc (◆ and bold lines) or hIgG (■ and thin lines) were activated with ADP (5 μM), PAR1-TRAP (15 μM), U46619 (2 μM), convulxin (5 ng/mL), A23187 (2.5 μM), or PMA (200 nM). Activated αIIbβ3 was detected by binding of FITC–PAC-1. Shown are representative results of 3 to 5 independent experiments. (B) Washed platelets were preincubated with Sema3A/AP (◆ and bold lines) or AP (■ and thin lines) and activated by ADP (5 μM) or thrombin (0.5 U/mL), and then FITC–PAC-1 binding was detected. Shown are representative results of 3 independent experiments. (C) PBS-, hIgG-, or Sema3A/Fc-treated platelets were incubated with or without an αIIbβ3-activating antibody, PT25-2, and PAC1 binding was examined. Shown are representative results of 3 independent experiments.

Table 1. Inhibition of agonist-induced PAC1 binding by Sema3A

Agonist	Concentration (% inhibition)
ADP, μM	5 (90.4 ± 12.1)
PAR1-TRAP, μM	15 (115.2 ± 10.2)
Thrombin, U/mL	0.5 (97.4 ± 3.3)
U46619, μM	2 (112.5 ± 9.3)
Convulxin, ng/mL	5 (94.7 ± 6.0)
A23187, μM	2.5 (106 ± 5.5)
PMA, nM	200 (58.1 ± 14.3)

Platelets preincubated with 10 μg/mL Sema3A/Fc were activated with indicated agonists, and FITC-PAC1 binding was detected as demonstrated in Figure 2. Percent inhibition of mean fluorescent intensity against hIgG-treated platelets was calculated. Data represent mean ± SE of at least 3 independent experiments.

ADP and PAR1-TRAP stimulation, as well as PAC-1 binding (data not shown). PAC-1 binding with PT25-2, an anti-αIIbβ3 antibody that induces activated conformation of αIIbβ3 without intracellular signaling, was unaffected by preincubation with Sema3A (Figure 2C), indicating that Sema3A does not disturb PAC-1 binding competitively to its receptor. Since activation of αIIbβ3 leads to ligand binding and platelet aggregate formation, we studied the effects of Sema3A on platelet aggregation. Sema3A/AP impaired aggregate formation in low concentrations of collagen and thrombin (Figure 3), although it was hard to detect the inhibitory effects of Sema3A on platelet aggregation in high concentrations of the agonists.

Effects of Sema3A on granular secretion

We examined effects of Sema3A binding to platelets on granular secretion after ADP and thrombin stimulation. Surface expression of CD62P and CD63 was used for monitoring the secretion of alpha granule and dense or lysosome granule, respectively.[30] Sema3A/Fc dose-dependently inhibited surface expression of both CD62P and CD63 after ADP and thrombin stimulation without stirring, indicating that Sema3A inhibits aggregation-independent granule secretion induced by platelet agonists (Figure 4).

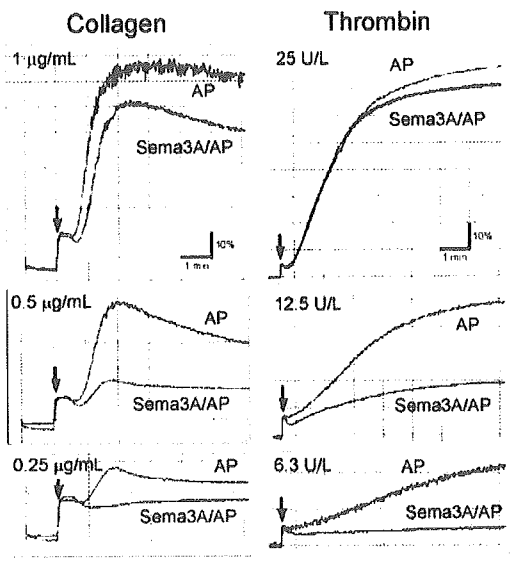

Figure 3. Inhibition of platelet aggregation by Sema3A. Washed platelets preincubated with 20 μg/mL Sema3A/AP or AP were activated with the indicated concentrations of collagen (left column) or thrombin (right column). Platelet aggregation was monitored using a platelet aggregometer. Arrow indicates the addition of agonists. Shown are representative results of 3 independent experiments.

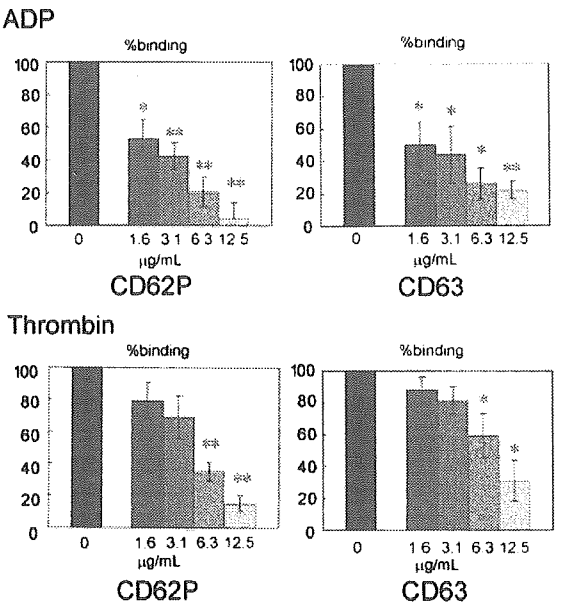

Figure 4. Inhibition of agonist-induced granular secretion by Sema3A. Washed platelets were preincubated with the indicated concentrations Sema3A/Fc, and then activated with ADP (5 μM) or thrombin (0.5 U/mL). Granular secretion was assessed by FITC-CD62P and PE-CD63 binding to platelets, and percent binding against hIgG-treated platelets was calculated. Shown are mean ± SE of percent binding of 3 independent experiments. $^{*}P < .05$; $^{**}P < .01$.

Effects of Sema3A on platelet adhesion and spreading

We next examined the effects of Sema3A on platelet adhesion to immobilized fibrinogen or nonspecific glass coverslips under static conditions. Quantification of adhered platelets by acid phosphatase method showed that preincubation with 20 μg/mL Sema3A/Fc led to about 20% reduction in platelet adhesion at every concentration of fibrinogen examined (Figure 5A). Microscopic examination demonstrated that after 45 minutes of incubation on 20 μg/mL fibrinogen, more than 80% of platelets showed full spreading (Figure 5Bi). In sharp contrast, spreading of Sema3A-treated platelets was markedly impaired (Figure 5Bii). The inhibition of platelet spreading by Sema3A was not αIIbβ3 specific, since Sema3A also inhibited platelet spreading on noncoated glass coverslips (Figure 5Biii-iv).

Effects of Sema3A on agonist-induced cytoskeleton rearrangement of platelets

The remarkable inhibition of platelet spreading by Sema3A suggests that Sema3A affects cytoskeletal rearrangement of platelets. To address the hypothesis, we quantified F-actin contents in platelets using bodipy-phallacidin and flow cytometry. Thrombin and PAR1-TRAP induced elevation of F-actin as demonstrated,[28] and Sema3A significantly impaired the elevation of agonist-induced F-actin elevation (Figure 6A). Cofilin is a protein that promotes severing and depolymerization of F-actin,[31,32] and involvement of cofilin in Sema3A signaling has been demonstrated.[31] Therefore, we next examined phosphorylation of cofilin after PAR1-TRAP stimulation. Sema3A decreased the level of phosphorylated cofilin in both resting and PAR1-TRAP–stimulated platelets, suggesting that Sema3A may keep cofilin in the dephosphorylated, activated state and increase severing of F-actin (Figure 6B). Since phosphorylation of cofilin is regulated by LIM kinase,[31,32] an effecter of Rac-PAK signaling pathway,[33] and the involvement of

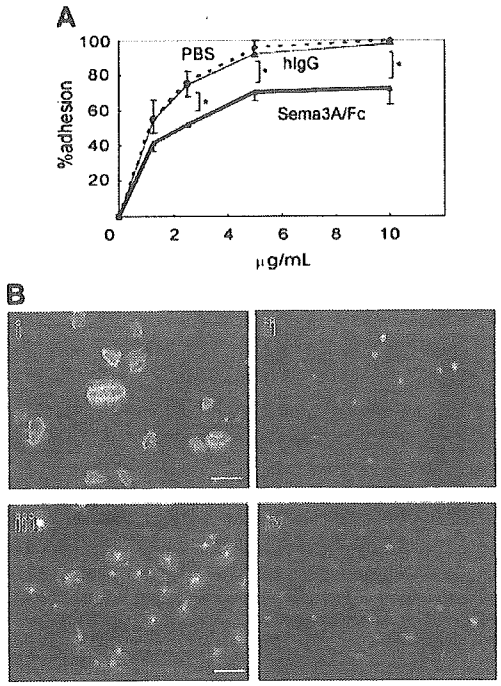

Figure 5. Effects of Sema3A on platelet adhesion and spreading. (A) Washed platelets were incubated with 20 μg/mL Sema3A/Fc (bold line) or hIgG (thin solid line), or PBS (dashed line), and then placed on the various concentrations of immobilized fibrinogen for one hour. After washing with PBS to remove nonadherent platelets, adhered platelets were quantified by acid phosphatase method. Mean and SE of percent adhesion of 3 independent experiments was plotted. $^*P < .05$. (B) Sema3A-treated platelets (ii,iv) or hIgG-treated platelets (i,iii) were placed on fibrinogen-coated (i-ii) or nontreated (iii-iv) glass coverslips. Adhered platelets were stained with TRICT (tetramethylrhodamine-5(and 6)-isothiocyanate)–phalloidin. Images were captured with a CCD camera (DP-70; Olympus) mounted on an Olympus AX-80 fluorescence microscope equipped with a 100 ×/1.30 oil immersion objective lens. Images were acquired with Olympus DP Controller software and processed with Adobe Photoshop Elements 2.0 (Adobe Systems, San Jose, CA). Original magnification × 1000, and bar in panel Bi represents 10 μm.

Rac in semaphorin signaling is well demonstrated,[11,12] we examined the effects of Sema3A on Rac1 activation by PAR1-TRAP. Consistent with previous reports,[34,35] PAR1-TRAP induced rapid activation of Rac1 in platelets at the maximum in 30 seconds, and Sema3A almost completely inhibited the Rac1 activation induced by PAR1-TRAP (Figure 6C). These results suggest that Sema3A inhibits agonist-induced actin rearrangement via Rac1-dependent pathway including phosphorylation of cofilin.

Some reports demonstrated that Sema3A affects another cytoskeletal component, microtubule rearrangement.[36,37] However, we did not observe any apparent effects of Sema3A on tubulin staining in platelets (data not shown).

Effects of Sema3A on Ca^{2+} and cyclic nucleotide signaling in platelets

To examine whether Sema3A may affect Ca^{2+} signaling, fluo-3–loaded platelets were stimulated with thrombin and intracellular Ca^{2+} concentrations were monitored under flow cytometry. Thrombin induced rapid increase in intracellular Ca^{2+} concentrations in control platelets as described,[20] and Sema3A/Fc did not affect the thrombin-induced increase in intracellular Ca^{2+} concentrations (Figure 7).

Since the best characterized platelet inhibitory signaling pathways are cyclic nucleotide pathways,[38] we finally examined the effects of Sema3A on cyclic nucleotides in platelets. Sema3A did not increase the basal cAMP level in nonstimulated platelets per se (Table 2). Stable prostacyclin, iloprost, elevates intracellular cAMP, and addition of ADP impairs the iloprost-induced cAMP elevation by inhibiting adenylate cyclase.[39] Again, Sema3A treatment did not change cAMP contents in iloprost- and ADP-treated platelets (Table 2). Sema3A also had no effects on basal cGMP contents, whereas sodium nitroprusside, a stimulator of NO/protein kinase G pathway, induced elevation of cGMP contents (Table 3). Moreover, a nitric oxide synthase (NOS) inhibitor, L-nitroarginine methyl ester, or a NO-donor, L-arginine, had no effects on the inhibition of αIIbβ3 activation by Sema3A (data not shown).[40] These results suggest that neither cAMP nor cGMP is involved in inhibition of platelet function by Sema3A.

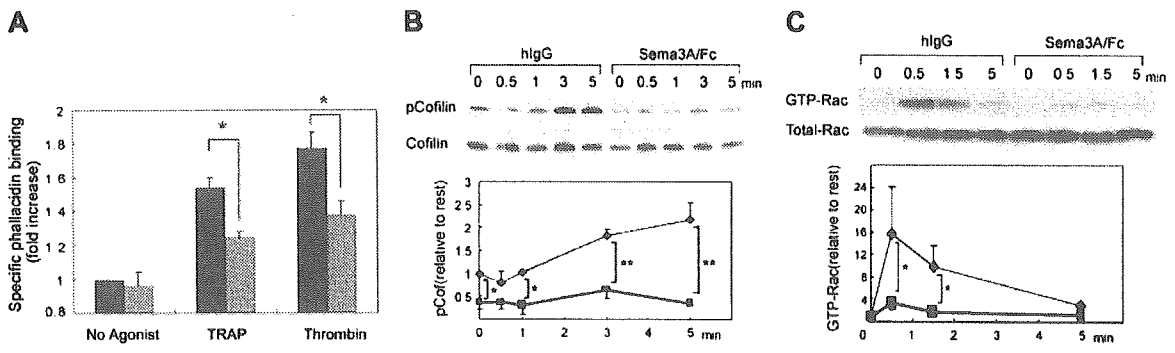

Figure 6. Effects of Sema3A on F-actin contents, cofilin phosphorylation, and Rac1 activation. (A) Sema3A/Fc- (gray bars) or hIgG-treated (black bars) platelets were activated by 30 μM PAR1-TRAP or 0.5 U/mL thrombin at 37°C for 30 seconds without stirring. After fixation, F-actin was stained with bodipy-phallacidin. Specific phallacidin binding was obtained by subtraction of FL1 fluorescence with a 300-fold more excess of unlabeled phallacidin from FL1 fluorescence without unlabeled phallacidin, and fold increase against fluorescence of no agonist sample was calculated. Data represent mean and SE of 3 independent experiments. $^*P < .05$. (B) Sema3A/Fc- or hIgG-treated platelets were activated with 30 μM PAR1-TRAP for the indicated time at 37°C without stirring. Then, cells were lysed and SDS-PAGE was performed. Phospho-cofilin was detected by anti–phospho-cofilin–specific antibody. After stripping, total cofilin was detected by anticofilin antibody. Optical density of the bands was measured by NIH Image software, and relative increase against phospho-cofilin in IgG-treated platelets without thrombin was calculated. Mean and SE of 3 independent experiments was plotted in bottom panel. $^*P < .05$; $^{**}P < .01$. (C) Sema3A/Fc- or hIgG-treated platelets were activated with 30 μM PAR1-TRAP for the indicated time at 37°C without stirring. GTP-form of Rac1 was precipitated by incubation with GST-PAK1-PBD and glutathione beads. After SDS-PAGE electrophoresis, Rac1 was detected by a Rac1-specific antibody. Total Rac was detected by electrophoresis of total lysates on an SDS-PAGE gel followed by detection with the same antibody. Optical density of the bands was measured by NIH Image software, and relative increase against GTP-Rac in IgG-treated platelets without thrombin was calculated. Sema3A/Fc is indicated by ■ and bold lines; hIgG, by ◆ and thin lines. Mean and SE of 3 independent experiments was plotted in lower panel. $^*P < .05$.

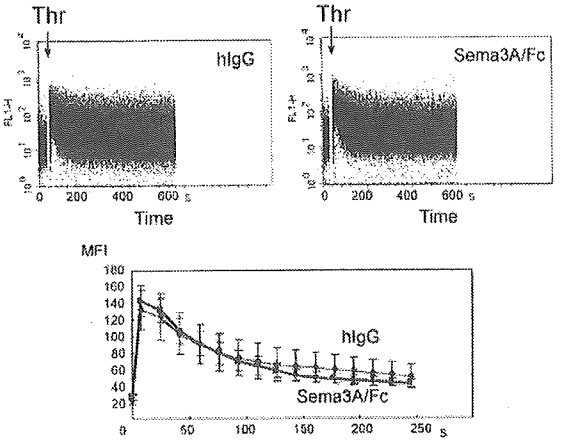

Figure 7. Effects of Sema3A on thrombin-induced increase of intracellular Ca^{2+} concentrations. Fluo-3–labeled platelets were incubated with 20 μg/mL Sema3A/Fc or hIgG. After the determination for about 10 seconds of baseline fluo-3 fluorescence from the platelet population, cell aspiration into the flow cytometry was briefly paused, and 1:10 volume of 5 U/mL thrombin (Thr) was added. The acquisition was then resumed, and changes in log fluorescence versus time were recorded (top panels). For each plot, rectangular analysis regions were defined over the time axis, and mean fluorescence intensity was calculated. Mean ± SE of 3 independent experiments was plotted in bottom panel. Bold and thin lines represent Sema3A/Fc and hIgG, respectively.

Discussion

In this report, we demonstrated for the first time the binding of Sema3A on platelets and extensive inhibitory effects of Sema3A on platelet function. As reported in endothelial cells,[16] Sema3A inhibited integrin-mediated function in platelets (ie, inhibition of αIIbβ3 activation and platelet aggregate formation, and adhesion and spreading on immobilized fibrinogen). However, Sema3A also inhibited αIIbβ3-independent adhesion and spreading on non-treated glass coverslips and aggregation-independent granular secretion. Although the most potent platelet inhibitory pathways are cyclic nucleotide pathways,[38] we did not detect any effect on cAMP and cGMP contents by Sema3A treatment. Thrombin-induced Ca^{2+} signaling was also unaffected by Sema3A treatment.

Sema3A markedly impaired αIIbβ3-independent as well as αIIbβ3-dependent platelet spreading. We demonstrated that Sema3A inhibited the increase of F-actin contents after thrombin or PAR1-TRAP stimulation. Thus, Sema3A inhibited adhesion-induced and agonist-induced actin rearrangement. Furthermore, Sema3A inhibited phosphorylation of cofilin and Rac1 activation after PAR1-TRAP stimulation. Several reports revealed that Rac1 activation is necessary for platelet actin assembly and lamellipodia formation after agonist stimulation.[34,35,41] Therefore, marked impairment of Rac1 activation is very likely to account for the Sema3A-induced impairment of actin rearrangement and spreading in platelets. There were 2 major downstream effectors of Rac1 identified, PAK and WAVEs ([Wiskott-Aldrich syndrome protein] WASP family Verprolin-homologous proteins).[42] Several PAK substrates or binding partners have been implicated in the effects of PAK, including filamin, LIM kinase, myosin, and paxillin.[43] Among them, LIM kinase phosphorylates and inactivates cofilin, a protein that promotes severing and depolymerization of F-actin.[31,32] Consistent with the inhibition of Rac1 activation, Sema3A inhibited phosphorylation of cofilin in both resting and activated platelets, suggesting that Sema3A increases severing and depolymerization of F-actin by keeping cofilin in the activated state. Rac1 inhibition by Sema3A might affect the activation of another major downstream effector of Rac1, WAVEs. WAVEs, also known as Scar, belong to the WASP family and activate actin-related protein 2 and 3 (Arp2/3) complex, resulting in nucleating actin polymerization.[43] Others and we have demonstrated that platelets contain WAVE isoforms and may regulate lamellipodia formation.[44,45] Therefore, it is also likely that Sema3A may inhibit actin rearrangement via inhibition of WAVE-dependent initiation of actin polymerization.

Table 2. Effects of Sema3A on cAMP

	cAMP, pmol/10^8 platelets
hIgG, 20 μg/mL	1.06 ± 0.19*
Sema3A/Fc, 20 μg/mL	1.00 ± 0.68*
Iloprost, 20 μg/L	45.94 ± 5.31
Iloprost, 20 μg/L + ADP, 5 μM + hIgG, 20 μg/mL	7.34 ± 0.47†
Iloprost, 20 μg/L + ADP, 5 μM + Sema3A/Fc, 20 μg/mL	5.66 ± 0.90†

Data represent the mean ± SE of 3 independent experiments.
*P = .94.
†P = .24.

Table 3. Effects of Sema3A on cGMP

	cGMP, pmol/10^8 platelets
hIgG, 20 μg/mL	0.83 ± 0.08*
Sema3A/Fc, 20 μg/mL	0.86 ± 0.04*
Sodium nitroprusside, 1 mM	5.56 ± 0.83

Data represent the mean ± SE of 3 independent experiments.
*P = .70.

In contrast to our results, it has been demonstrated that Rac1 activation is essential for Sema3A-induced growth cone collapse in neural cells,[46,47] and Sema3A-induced phosphorylation of cofilin is necessary for the process.[48] However, in these reports, the authors analyzed direct signaling induced by the binding of Sema3A. In this study, we analyzed the effects of Sema3A binding on agonist-induced signaling in platelets. Interestingly, Aizawa et al also found that phosphorylated cofilin was subsequently dephosphorylated within 5 minutes at the neural growth cone and the phosphorylated level of cofilin decreased to 0.16-fold of that of untreated growth cone,[48] which is consistent with our observation that cofilin is dephosphorylated in Sema3A-treated platelets. Signaling pathways from semaphorin receptors to Rac have not been fully understood even in neural cells.[11,12] Human plexin-B1, a receptor for Sema4D, and fly plexin B interact with activated Rac directly, and it has been suggested that these plexins sequester activated Rac and antagonize its signaling pathway.[49-51] Very recently Turner et al reported the association of activated Rac and the cytoplasmic tail of plexin-A1,[52] although others failed to detect the interaction.[50,53] Further studies are necessary to reveal the mechanism of regulation of Rac by Sema3A in platelets.

Is the impairment of actin rearrangement via inhibition of Rac1 responsible for Sema3A-induced extensive negative regulation of platelet function other than platelet spreading? To investigate the role of actin rearrangement on platelet function, effects of cytochalasins or latrunculin A, inhibitors of actin polymerization, have been studied.[54-58] There are some discrepancies in these reports, mainly because of the differences in experimental conditions; some reports demonstrated that high concentrations of cytochalasins inhibited agonist-induced αIIbβ3 activation and platelet aggregation, indicating that de novo actin polymerization affects activation of αIIbβ3,[54,55,58] whereas low concentrations of cytochalasin D and latrunculin A activated αIIbβ3.[57] Integrin activating inside-out

signaling consists of 2 aspects: conformational change that regulates integrin affinity and integrin clustering that regulates its avidity.[5,7] αIIbβ3 clustering may be promoted by actin cytoskeletal rearrangement, although conformational change seems to be the dominant way in αIIbβ3 activation.[59] Moreover, recent reports revealed that talin binding to integrin cytoplasmic tails is essential for integrin activation.[60,61] Since talin links integrin to actin filaments in clustering of integrins into adhesion complexes,[62,63] defects of actin polymerization may impair broad aspects of integrin signaling. However, impairment of actin rearrangement does not appear to be the sole mechanism of Sema3A inhibition of platelet function, since, in contrast to Sema3A, cytochalasins have no inhibitory effects on granular secretion.[55,58] Rac1 regulates many cellular activities besides cytoskeletal rearrangement, such as cell polarity and vesicle trafficking in other cells.[42] Moreover, Sema3A may act via Rac1-independent pathways (eg, the collapsin response mediator protein (CRMP)–mediated pathway).[12] These hypotheses remain to be determined.

It has been well documented that endothelial cells negatively regulate platelet function by secreted PGI_2, NO, and membrane-bound ecto-ADPase.[8] Since Sema3A is also produced in endothelial cells and inhibits platelet function extensively, Sema3A may contribute to maintain blood flow in normal, injured, or newly synthesized vessels by keeping platelets in the resting state. Moreover, since Sema3A appears to inhibit platelet function via unique Rac1-dependent pathway, modulation of Sema3A-inducing signaling pathway may be a new target of antiplatelet therapy.

In conclusion, we demonstrated that Sema3A binds to platelets and inhibits platelet function extensively. The inhibition of platelet function by Sema3A appeared to be mediated, at least in part, through impairment of agonist-induced Rac1-dependent actin rearrangement. We believe that these results reveal a new Sema3A function on thrombosis and hemostasis, and a unique inhibitory signaling pathway evoked by Sema3A binding to platelets.

Acknowledgment

We are grateful to Dr M. Moroi (Department of Protein Biochemistry, Institute of Life Science, Kurume University, Fukuoka, Japan) for providing us with convulxin.

References

1. Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes. N Engl J Med. 1992;326:242-250.
2. Antithrombotic Trialists' Collaboration. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ. 2002;324:71-86.
3. Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. Cell. 1998;94:657-666.
4. Tomiyama Y, Shiraga M. Shattil SJ. Platelet membrane proteins as adhesion receptors. In: Gresele P, Page C, Fuster V, Vermylem J, eds. Platelets in Thrombotic and Non-Thrombotic Disorders. Cambridge, United Kingdom: Cambridge University Press; 2002:80-92.
5. Shattil SJ, Kashiwagi H, Pampori N. Integrin signaling: the platelet paradigm. Blood. 1998;91: 2645-2657.
6. Tomiyama Y. Glanzmann thrombasthenia: integrin αIIbβ3 deficiency. Int J Hematol. 2000;72: 448-454.
7. Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. Blood. 2004;104:1606-1615.
8. Pearson JD. Endothelial cell function and thrombosis. Baillieres Best Pract Res Clin Haematol. 1999;12:329-341.
9. Pasterkamp RJ, Kolodkin AL. Semaphorin junction: making tracks toward neural connectivity. Curr Opin Neurobiol. 2003;13:79-89.
10. Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD. Neuropilin is a semaphorin III receptor. Cell. 1997;90:753-762.
11. Tamagnone L, Comoglio PM. Signalling by semaphorin receptors: cell guidance and beyond. Trends Cell Biol. 2000;10:377-383.
12. Goshima Y, Ito T, Sasaki Y, Nakamura F. Semaphorins as signals for cell repulsion and invasion. J Clin Invest. 2002;109:993-998.
13. Antipenko A, Himanen JP, van Leyen K, et al. Structure of the semaphorin-3A receptor binding module. Neuron. 2003;39:589-598.
14. Behar O, Golden JA, Mashimo H, Schoen FJ, Fishman MC. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. Nature. 1996;383:525-528.
15. Taniguchi M, Yuasa S, Fujisawa H, et al. Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. Neuron. 1997;19:519-530.
16. Serini G, Valdembri D, Zanivan S, et al. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. Nature. 2003;424:391-397.
17. Barberis D, Artigiani S, Casazza A, et al. Plexin signaling hampers integrin-based adhesion, leading to Rho-kinase independent cell rounding, and inhibiting lamellipodia extension and cell motility. FASEB J. 2004;18:592-594.
18. Honda S, Tomiyama Y, Aoki T, et al. Association between ligand-induced conformational changes of integrin $\alpha_{IIb}\beta_3$ and $\alpha_{IIb}\beta_3$-mediated intracellular Ca^{2+} signaling. Blood. 1998;92:3675-3683.
19. Tomiyama Y, Tsubakio T, Piotrowicz RS, Kurata Y, Loftus JC, Kunicki TJ. The Arg-Gly-Asp (RGD) recognition site of platelet glycoprotein IIb-IIIa on nonactivated platelets is accessible to high-affinity macromolecules. Blood. 1992;79:2303-2312.
20. Shiraga M, Tomiyama Y, Honda S, et al. Involvement of Na^+/Ca^{2+} exchanger in inside-out signaling through the platelet integrin $\alpha_{IIb}\beta_3$. Blood. 1998;92:3710-3720.
21. Kashiwagi H, Honda S, Tomiyama Y, et al. A novel polymorphism in glycoprotein IV (replacement of proline-90 by serine) predominates in subjects with platelet GPIV deficiency. Thromb Haemost. 1993;69:481-484.
22. Kashiwagi H, Tomiyama Y, Tadokoro S, et al. A mutation in the extracellular cysteine-rich repeat region of the β_3 subunit activates integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. Blood. 1999;93:2559-2568.
23. Kashiwagi H, Shiraga M, Honda S, et al. Activation of integrin $\alpha_{IIb}\beta_3$ in the glycoprotein Ib-high population of a megakaryocytic cell line, CMK, by inside-out signaling. J Thromb Haemost. 2004;2: 177-186.
24. Kashiwagi H, Schwartz MA, Eigenthaler M, Davis KA, Ginsberg MH, Shattil SJ. Affinity modulation of platelet integrin $\alpha_{IIb}\beta_3$ by β_3-endonexin, a selective binding partner of the β_3 integrin cytoplasmic tail. J Cell Biol. 1997;137:1433-1443.
25. Hayashi S, Oshida M, Kiyokawa T, et al. Determination of activated platelets: evaluation of methodology and application for patients with idiopathic thrombocytopenic purpura. Rinsho Byori. 2001;49:1287-1292.
26. Kiyoi T, Tomiyama Y, Honda S, et al. A naturally occurring Tyr143Hisα_{IIb} mutation abolishes $\alpha_{IIb}\beta_3$ function for soluble ligands but retains its ability for mediating cell adhesion and clot retraction: comparison with other mutations causing ligand-binding defects. Blood. 2003;101:3485-3491.
27. Bellavite P, Andrioli G, Guzzo P, et al. A colorimetric method for the measurement of platelet adhesion in microtiter plates. Anal Biochem. 1994;216: 444-450.
28. Leng L, Kashiwagi H, Ren XD, Shattil SJ. RhoA and the function of platelet integrin $\alpha_{IIb}\beta_3$. Blood. 1998;91:4206-4215.
29. do Ceu Monteiro M, Sansonetty F, Goncalves MJ, O'Connor JE. Flow cytometric kinetic assay of calcium mobilization in whole blood platelets using Fluo-3 and CD41. Cytometry. 1999;35:302-310.
30. Rendu F, Brohard-Bohn B. Platelet organelles. In: Gresele P, Page C, Fuster V, Vermylem J, eds. Platelets in Thrombotic and Non-Thrombotic Disorders. Cambridge, United Kingdom: Cambridge University Press; 2002:104-112.
31. Arber S, Barbayannis FA, Hanser H, et al. Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature. 1998;393:805-809.
32. Yang N, Higuchi O, Ohashi K, et al. Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature. 1998;393: 809-812.
33. Edwards DC, Sanders LC, Bokoch GM, Gill GN. Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nat Cell Biol. 1999;1:253-259.
34. Azim AC, Barkalow K, Chou J, Hartwig JH. Activation of the small GTPases, rac and cdc42, after ligation of the platelet PAR-1 receptor. Blood. 2000;95:959-964.
35. Vidal C, Geny B, Melle J, Jandrot-Perrus M, Fontenay-Roupie M. Cdc42/Rac1-dependent activation of the p21-activated kinase (PAK) regulates human platelet lamellipodia spreading: implication of the cortical-actin binding protein cortactin. Blood. 2002;100:4462-4469.
36. Fritsche J, Reber BF, Schindelholz B, Bandtlow CE. Differential cytoskeletal changes during growth cone collapse in response to hSema III and thrombin. Mol Cell Neurosci. 1999;14:398-418.
37. Gu Y, Ihara Y. Evidence that collapsin response mediator protein-2 is involved in the dynamics of

microtubules. J Biol Chem. 2000;275:17917-17920.

38. Schwarz UR, Walter U, Eigenthaler M. Taming platelets with cyclic nucleotides. Biochem Pharmacol. 2001;62:1153-1161.
39. Gabbeta J, Yang X, Kowalska MA, Sun L, Dhanasekaran N, Rao AK. Platelet signal transduction defect with Gα subunit dysfunction and diminished $G\alpha_q$ in a patient with abnormal platelet responses. Proc Natl Acad Sci U S A. 1997;94:8750-8755.
40. Freedman JE, Loscalzo J, Barnard MR, Alpert C, Keaney JF, Michelson AD. Nitric oxide released from activated platelets inhibits platelet recruitment. J Clin Invest. 1997;100:350-356.
41. Hartwig JH, Bokoch GM, Carpenter CL, et al. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. Cell. 1995;82:643-653.
42. Burridge K, Wennerberg K. Rho and Rac take center stage. Cell. 2004;116:167-179.
43. Takenawa T, Miki H. WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. J Cell Sci. 2001;114:1801-1809.
44. Oda A, Miki H, Wada I, et al. WAVE/Scars in platelets. Blood. Prepublished on August 3, 2004, as DOI 10.1182/blood-2003-04-1319. (Now available as Blood. 2005;105:3141-3148.
45. Kashiwagi H, Shiraga M, Kato H, et al. Expression and subcellular localization of WAVE isoforms in the megakaryocyte/platelet lineage. J Thromb Haemost.2005;3:361-368.
46. Jin Z, Strittmatter SM. Rac1 mediates collapsin-1-induced growth cone collapse. J Neurosci. 1997;17:6256-6263.
47. Kuhn TB, Brown MD, Wilcox CL, Raper JA, Bamburg JR. Myelin and collapsin-1 induce motor neuron growth cone collapse through different pathways: inhibition of collapse by opposing mutants of rac1. J Neurosci. 1999;19:1965-1975.
48. Aizawa H, Wakatsuki S, Ishii A, et al. Phosphorylation of cofilin by LIM-kinase is necessary for semaphorin 3A-induced growth cone collapse. Nat Neurosci. 2001;4:367-373.
49. Hu H, Marton TF, Goodman CS. Plexin B mediates axon guidance in Drosophila by simultaneously inhibiting active Rac and enhancing RhoA signaling. Neuron. 2001;32:39-51.
50. Vikis HG, Li W, He Z, Guan KL. The semaphorin receptor plexin-B1 specifically interacts with active Rac in a ligand-dependent manner. Proc Natl Acad Sci U S A. 2000;97:12457-12462.
51. Driessens MH, Hu H, Nobes CD, et al. Plexin-B semaphorin receptors interact directly with active Rac and regulate the actin cytoskeleton by activating Rho. Curr Biol. 2001;11:339-344.
52. Turner LJ, Nicholls S, Hall A. The activity of the plexin-A1 receptor is regulated by Rac. J Biol Chem. 2004;279:33199-33205.
53. Zanata SM, Hovatta I, Rohm B, Puschel AW. Antagonistic effects of Rnd1 and RhoD GTPases regulate receptor activity in semaphorin 3A-induced cytoskeletal collapse. J Neurosci. 2002;22:471-477.
54. Peerschke EI. Observations on the effects of cytochalasin B and cytochalasin D on ADP- and chymotrypsin-treated platelets. Proc Soc Exp Biol Med. 1984;175:109-115.
55. Lefebvre P, White JG, Krumwiede MD, Cohen I. Role of actin in platelet function. Eur J Cell Biol. 1993;62:194-204.
56. Torti M, Festetics ET, Bertoni A, Sinigaglia F, Balduini C. Agonist-induced actin polymerization is required for the irreversibility of platelet aggregation. Thromb Haemost. 1996;76:444-449.
57. Bennett JS, Zigmond S, Vilaire G, Cunningham ME, Bednar B. The platelet cytoskeleton regulates the affinity of the integrin $\alpha_{IIb}\beta_3$ for fibrinogen. J Biol Chem. 1999;274:25301-25307.
58. Natarajan P, May JA, Sanderson HM, Zabe M, Spangenberg P, Heptinstall S. Effects of cytochalasin H, a potent inhibitor of cytoskeletal reorganisation, on platelet function. Platelets. 2000;11:467-476.
59. Hato T, Pampori N, Shattil SJ. Complementary roles for receptor clustering and conformational change in the adhesive and signaling functions of integrin $\alpha_{IIb}\beta_3$. J Cell Biol. 1998;141:1685-1695.
60. Tadokoro S, Shattil SJ, Eto K, et al. Talin binding to integrin β tails: a final common step in integrin activation. Science. 2003;302:103-106.
61. Calderwood DA. Integrin activation. J Cell Sci. 2004;117:657-666.
62. Brown NH, Gregory SL, Rickoll WL, et al. Talin is essential for integrin function in Drosophila. Dev Cell. 2002;3:569-579.
63. Jiang G, Giannone G, Critchley DR, Fukumoto E, Sheetz MP. Two-piconewton slip bond between fibronectin and the cytoskeleton depends on talin. Nature. 2003;424:334-337.