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Variant Type 2B von Willebrand Disease; No Responsible Mutations at the von Willebrand Factor A1 Domain and the Surrounding Region

Ichiro FUSE[1], Wataru HIGUCHI[2], Yumiko UESUGI[3], and Yoshifusa AIZAWA[3]

[1]Division of Blood Transfusion, Niigata University Medical Hospital, [2]Division of Bone Marrow Transplantation, Niigata University Medical Hospital, [3]First Department of Internal Medicine, Niigata University School of Medicine, Niigata, Japan

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Summary. **We encountered a patient with variant of type 2B von Willebrand disease (vWD) who showed increased ristocetin-induced platelet aggregation, persistent thrombocytopenia, spontaneous platelet aggregation and normal vWF multimeric pattern in his plasma. However, we could not find any responsible mutations in or around the 505-695 disulfide loop (A1 loop) of the von Willebrand factor (vWF), at which all type 2B vWD candidate mutations have been reported to be clustered.**

This suggests that an unidentified vWF functional domain facilitating binding to platelet GPIb may exist at a site other than the A1 loop and the surrounding region.

Key words—type 2B von Willebrand disease, von Willebrand factor, A1 domain, gene analysis.

INTRODUCTION

Von Willebrand disease (vWD) is a hereditary hemorrhagic disorder due to either quantitative (type 1 and 3) or qualitative (type 2) abnormalities of the von Willebrand factor (vWF)[1]. Type 2B vWD is a subtype of vWD characterized by autosomal dominant inheritance and selective loss of the high molecular weight (HMW) vWF multimers from the plasma. In addition, type 2B vWF exhibits a markedly increased affinity for platelet glycoprotein Ib, resulting in increased ristocetin-induced platelet aggregation (RIPA) at low concentrations of ristocetin[2].

The vWF gene mutations responsible for type 2B vWD are nearly all clustered within the short peptide segment between Met 540 and Arg 578 located on the loop defined by the intracellular disulfide bond between Cys 509 and Cys 695 of the mature subunit of vWF (A1 loop)[3]. However, several mutations located outside the A1 loop (Pro 503 to Leu, His 505 to Asp, Leu 697 to Val, Ala 698 to Val at the surrounding region of the A1 loop) have also been identified as causing type 2B vWD[4-7].

In the present study, we report a variant of type 2B vWD characterized by persistent thrombocytopenia, enhanced RIPA, normal vWF multimeric distribution in plasma, and no responsible mutations in or around the A1 loop.

MATERIALS AND METHODS

Patient

The patient, an 80-year-old male, had a life-long history of a bleeding tendency. Low platelet counts were first detected when he was in his thirties. His father had also a life-long bleeding tendency and died of a cerebral hemorrhage at the age of 91 years. Of his two sons and one daughter, the second son had a bleeding tendency with low platelet counts. In December 1952, the patient was involved in a traffic accident and was admitted to a local hospital. He showed a marked bleeding tendency and required a blood transfusion. At that time, thrombocytopenia and a prolonged bleeding time were again detected, and he was diagnosed with chronic idiopathic thrombocytopenic purpura. In 1999, he suffered from periodontitis and

Correspondence: Ichiro Fuse M.D., Division of Blood Transfusion, Niigata University Medical Hospital, 1-754 Asahimachi, Niigata 951-8520, Japan.

required a tooth extraction. In consideration of his bleeding tendency, he consulted our department.

His platelet counts were always low ($4.5-7.0\times10^4/\mu l$) and bleeding time was longer than 15 min as measured by a modified Ivy technique. There were no morphologically abnormal platelets in his peripheral blood smear samples. The level of platelet associated IgG was normal (15.7 ng/10^7 platelets). The findings from repeated blood coagulation and fibrinolysis tests were as follows: prothrombin time, 11.5-15.5 sec (76.9-109.8%); activated partial thromboplastin time (APTT), 34.4-45.5 sec (normal control 31.2-34.6 sec); fibrinogen, 143-230 mg/dl; fibrinogen degradation products, 1.80-2.68 μg/ml. These findings indicate that the APTT was slightly prolonged. Dysfunction of the liver or enlargement of the spleen was not evident.

Values for factor VIII procoagulant activity (one-stage APTT assay using factor VIII-deficient substrate plasma, Behring Diagnostics GmbH, Germany), vWF antigen (vWF: Ag, Asserachrom vWF, Boehringer-Mannheim, USA), and ristocetin cofactor activity (von Willebrand Reagent, Dade Behring Marburg GmbH, Germany) were 56.0-73.3%, 73-90% and 76-96%, respectively, indicating the values to be subnormal or slightly lower than those within the normal ranges.

VWF multimer analysis

Multimeric composition of vWF was analyzed by electrophoresis in 0.1% SDS-2.5% agarose gel[8]. This was followed by Western blot detection with transfer of the multimers to a polyvinylidene difluoride membrane (Immobilon™, Millipore, Bedford, USA), reaction with a primary polyclonal rabbit antihuman antibody to vWF (Behring, Westwood, USA) and detection with a secondary goat antirabbit IgG alkaline phosphatase-conjugated antibody (Fisher Scientific, Pittsburgh, USA). The vWF multimeric pattern was compared with a normal human control sample.

Ristocetin-induced platelet aggregation study

Venous blood was collected into polyethylene tubes containing 1/10 vol of 3.8% sodium citrate from the patient and a normal volunteer after at least 12 h of fasting. Platelet-rich plasma (PRP) was obtained by centrifugation at 150 g for 10 min at room temperature. Platelet-poor plasma (PPP) was obtained by further centrifugation at 1,500 g for 10 min. In the normal control, platelet counts in the PRP were adjusted using PPP as a diluent, because the patient's platelet counts were low. The aggregation induced by ristocetin (Lundbeck, Copenhagen, Denmark) was measured in 200 μl of PRP by the turbidometric method described by Born[9] using a NKK-Hema-Tracer 1 aggregometer (NIKO Bioscience, Tokyo, Japan).

To investigate the effect of the patient's plasma on the low concentration of ristocetin (0.5 or 0.75 mg/ml)-induced aggregation in normal PRP, 100 μl of the patient's plasma was added to 100 μl of the normal PRP.

Mixing studies of ristocetin-induced platelet aggregation

Washed platelets were obtained as previously described[10]. Briefly, prostaglandin E_1 (PGE_1) (Sigma Chemical Co., St. Louis, USA) was added to PRP from a normal volunteer at a concentration of 0.5 μg/ml to prevent platelet aggregation during the subsequent manipulation. Platelets were then pelleted from PRP by centrifugation at 850 g for 15 min. The platelet pellet was washed with 10 mM Tris/1 mM EDTA/saline buffer at pH 7.5 twice and was finally suspended in patient PPP or normal PPP, and the induced platelet aggregation was assessed following the addition of ristocetin at 0.5 mg/ml.

Inhibition studies of ristocetin-induced platelet aggregation

Inhibition of ristocetin-induced platelet aggregation by anti-GPIb antibodies was tested by adding mouse monoclonal antibodies SZ2 (Immunotec, Marseilles, France)[11] at 20 μg/ml to $10\times10^4/\mu l$ healthy control platelets suspended in patient PPP. After the reaction mixture was allowed to stand for 3 min at 37℃, platelet aggregation was measured following the addition of ristocetin at 0.5 mg/ml.

Binding of patient's vWF: Ag to normal washed platelets

Two volumes of test plasma from the patient or normal subjects and one volume of ristocetin solution were mixed thoroughly. One volume of a suspension of normal washed platelets ($40\times10^4/\mu l$) in Tris-saline buffer containing 5 mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, USA) was then added and shaken gently. The mixture was incubated at 37℃ for 15 min without further shaking and then centrifuged at 2,500 g for 15 min. The residual concentration of vWF: Ag in the supernatant was measured and expressed as a percentage of the control value, in which the buffer was added in place of ristocetin.

Binding affinity of patient's washed platelets for normal vWF: Ag

Two volumes of pooled normal plasma and one volume of ristocetin solution were mixed thoroughly. One volume of platelet suspensions ($40\times10^4/\mu l$) prepared from normal subjects or the patient was added to this, shaken gently, and then rested at 37℃ for 15 min. After centrifugation at 2,500 g for 10 min, the supernatant was assayed for vWF: Ag.

Polymerase chain reaction amplification and sequencing of vWF exon 28

Polymerase chain reaction (PCR) amplifications were performed on genomic DNA extracted from peripheral blood mononuclear cells. The 5' part of exon 28 of the vWF gene (residues 463-822) was amplified using the previously reported primers 226 and 227A[12]. These primers allow the specific amplification of the gene sequences without coamplification of the pseudogene sequences. Amplifications were performed using 2.5 U of Taq polymerase (Ampli Taq Gold, Perkin-Elmer Co. Ltd., NJ) dissolved in PCR buffer in a 100 μl reaction volume containing 0.2 mM of each dNTP and 0.5 μM of each primer. The PCR profile consisted of 10 min preincubation at 95℃ followed by 45 cycles of 1 min of denaturation at 94℃, 1 min of annealing at 60℃, 1.5 min of polymerization at 72℃, and finally 10 min of extension at 72℃. The PCR products were sequenced by the dideoxy chain termination method with a Model 310 automated DNA sequencer (Perkin-Elmer Japan Applied Biosystems Division, Tokyo, Japan).

RESULTS

VWF multimer analysis

The electrophoretic pattern obtained from the patient plasma showed a full range of vWF multimers, and did not give any evidence of a lack of high and intermediate molecular weight forms of vWF (Fig. 1). This indicates that the patient's vWF multimeric pattern was normal.

Ristocetin-induced platelet aggregation

The addition of ristocetin to the patient PRP resulted in platelet aggregation at concentrations (0.5 mg/ml) that never produced aggregation in normal subjects, and 1.0 mg/ml of ristocetin induced an almost full aggregation in the patient PRP (Fig. 2). This suggests that PRP from the patient was more sensitive to the induction of platelet aggregation by ristocetin. Furthermore, spontaneous platelet aggregation (SPA) was observed in the patient PRP (Fig. 3). These findings are typical of previous findings in type 2B and pseudo - or platelet-type vWD.

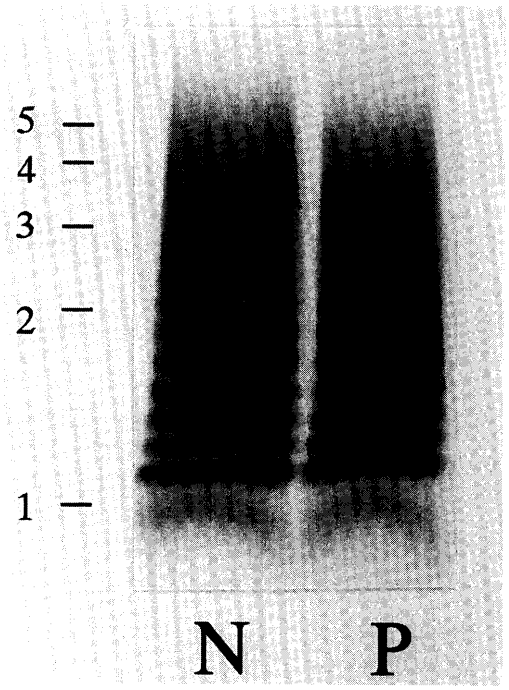

Fig. 1. 0.1% SDS-2.5% agarose gel electrophoresis of plasma vWF in the patient (P) and a normal control (N). The origin is at the top and the anode at the bottom. Samples from the patient and from a normal volunteer were probed with anti-vWF polyclonal antibodies. Numbers correspond to the various multimers of the vWF protomer numbered 1. Note that multimeric bands including the full range of vWF multimers are observed in the patient plasma as well as normal plasma.

Enhanced capacity of patient plasma to induce ristocetin aggregation

Addition of patient plasma (100 μl) to normal PRP (100 μl) did not cause SPA, but evoked platelet aggregation induced by low concentrations of ristocetin (0.5 and 0.75 mg/ml) (Fig. 4). In addition, ristocetin at 0.5 mg/ml induced platelet aggregation in the combination of normal washed platelets and patient PPP, and this aggregation was completely abolished by mouse monoclonal anti-GPIb antibodies SZ2 (Fig. 5). However, it was not inhibited by control mouse IgG1 (data not shown). These findings suggest that the patient plasma is responsible for the enhanced ristocetin-induced GPIb dependent aggregation.

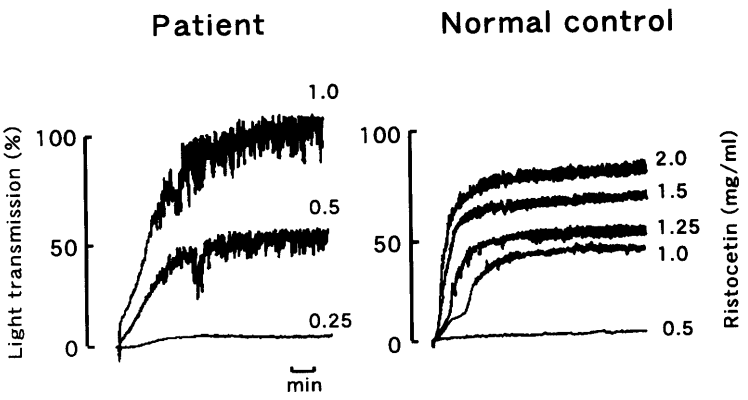

Fig. 2. Ristocetin-induced platelet aggregation in PRP from the patient and a normal control.

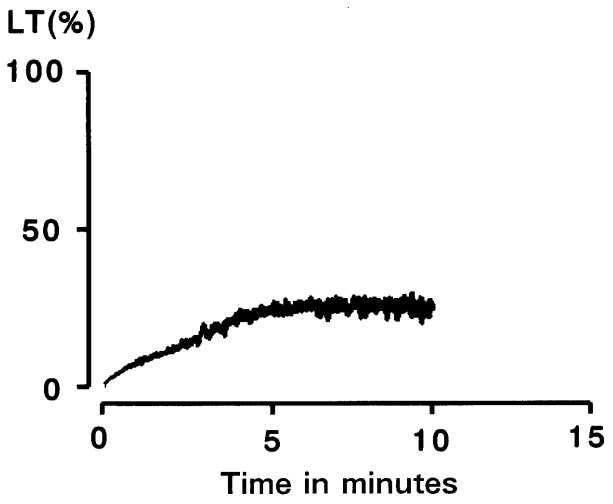

Fig. 3. Spontaneous platelet aggregation in the patient PRP.

Binding studies of vWF: Ag to platelets

Binding studies of vWF: Ag to platelets were performed in combinations of washed platelets and PPP from the patient and normal controls. Increased binding of patient vWF to normal washed platelets was observed at low concentrations of ristocetin (Fig. 6A). However, normal vWF did not show an increased binding to the patient platelets (Fig. 6B). These findings suggest that the patient vWF had a higher affinity for platelets.

Nucleic acid analyses

In this patient, we found two base substitutions located inside the A1 loop. One was a silent mutation (CCA→CCG, Pro574) and the other was a deduced amino acid substitution of Thr (ACC) for Ala618 (GCC). In addition, we found two base substitutions located outside the A1 loop, which resulted in amino acid substitutions of Asp709 to His and Val802 to Leu, respectively. However, these base substitutions were also identified in the genomic vWF genes isolated from twenty healthy controls. Therefore, all these amino acid substitutions can be considered polymorphisms and are not responsible for the novel disease phenotype observed in the present study[13].

DISCUSSION

The findings of the present study indicated that this patient exhibited features of type 2B vWD. This was demonstrated by the heightened response to ristocetin in PRP, the enhanced capacity of patient plasma to induce RIPA, and the increased binding of patient vWF to normal washed platelets in the presence of low concentrations of ristocetin. Pseudo-or platelet type vWD was excluded by demonstrating that washed patient platelets did not show an increased binding of normal vWF in the presence of ristocetin.

However, in contrast to typical type 2B vWD, the present case did not show a lack of HMW vWF multimers in plasma despite the presence of SPA and persistent thrombocytopenia. This suggests that the patient vWF had sufficient capacity to cause SPA *in vitro* and thrombocytopenia *in vivo*, but its large multimer could not easily be cleared from the plasma. The normal vWF multimeric pattern in type 2B vWD has been reported in several cases: type 2B New York[14], type 2B Malmö[15] and type 2B Hiroshima[16]. However, persistent thrombocytopenia was

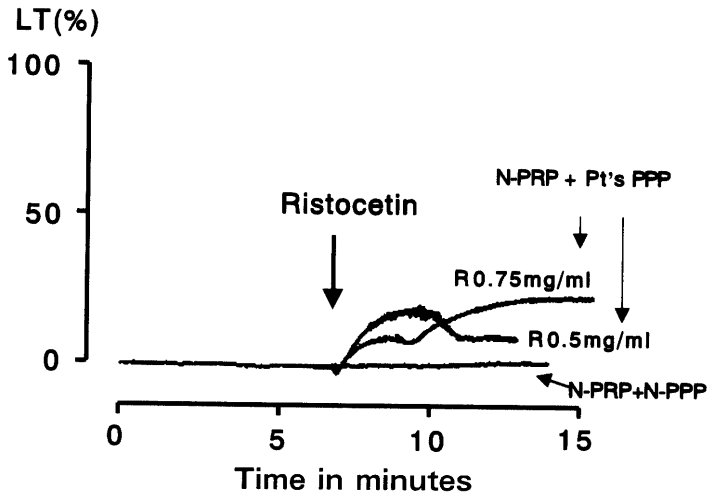

Fig. 4. Enhancement of ristocetin-induced platelet aggregation in PRP from a normal subject by patient plasma.
One hundred microliters of normal or patient's plasma was added to 100 μl of normal PRP, and spontaneous platelet aggregation (SPA) was observed in each mixture. After that, low concentrations of ristocetin (0.5 and 0.75 mg/ml) were added to the mixture. The patient's plasma did not cause SPA, but 0.5 or 0.75 mg/ml of ristocetin induced platelet aggregation in the mixture consisting of normal PRP and patient plasma, which was not observed in the mixture consisting of normal PRP and normal plasma.

not found in patients with type 2B New York or type 2B Malmö. Im type 2B Hiroshima, chronic thrombocytopenia was found, but not SPA. Taking these reports into consideration, the present case demonstrates features other than those in previously reported patients.

In type 2B vWD, most of the vWF mutations identified are located within the A1 loop. Four different mutations located outside the A1 loop have also been identified, as described in the Introduction. With respect to type 2B vWD showing a normal vWF multimeric pattern in plasma, Pro 503 to Leu located outside the A1 loop was reported to be responsible in type 2B New York and type 2B Malmö[4]. In type 2B Hiroshima, the deduced amino acid substitution of Thr for Ala618 in the A1 loop, which was also present in our case, was found. However, it was polymorphic, and no responsible mutations were found at the A1 loop or the surrounding region. In the present case, we could not find any responsible mutations at the A1 loop or the surrounding region as have been reported in type 2B vWD. In view of this, the present case closely resembles type 2B Hiroshima.

It is generally assumed that the binding of vWF to the subendothelium would be followed by a conformational change[17] resulting in the direct binding of the larger multimers to platelet GPIb. The GPIb-binding domain was identified on a 52/48-kD tryptic fragment beginning with amino acid residue 449[18]. Furthermore, residues 494-511 and 692-708 have been

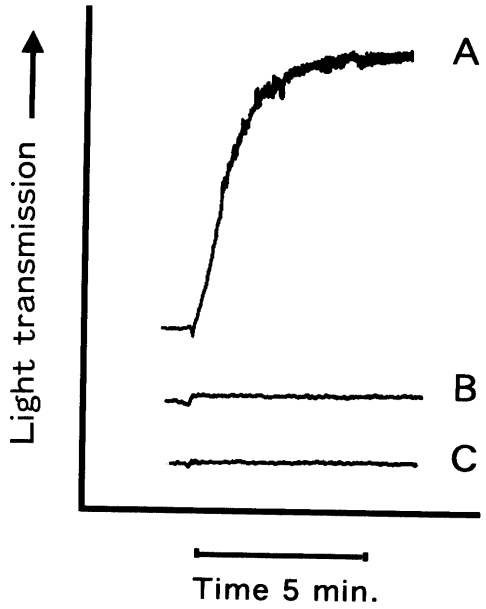

Fig. 5. Mixing studies of ristocetin (0.5 mg/ml)-induced platelet aggregation and the effect of monoclonal antibodies against GPIb on the aggregation. **A.** Normal platelets+patient PPP. **B.** Normal platelets+normal PPP. **C.** Normal platelets+patient PPP+anti-GPIb monoclonal antibodies (SZ2, 10 μg/ml).

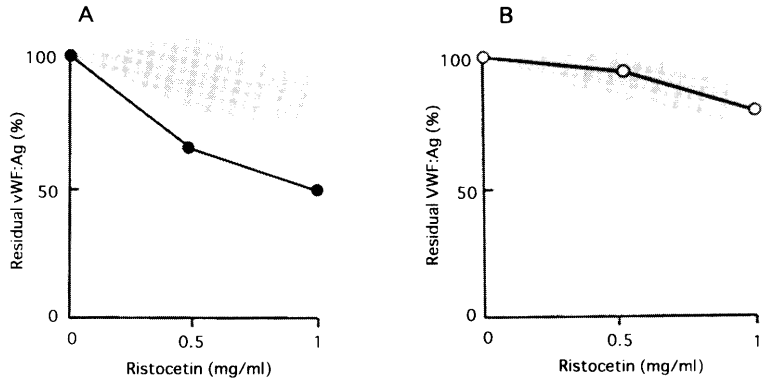

Fig. 6. The binding of patient vWF to normal washed platelets (**A**) and the binding of normal vWF to washed patient platelets (**B**) in the presence of ristocetin.
Washed platelets from normal subjects or the patient were incubated with normal or patient plasma for 15 min at 37℃. Platelets were separated and residual vWF: Ag in the supernatant was assayed and expressed as a percentage of the control value, in which ristocetin was replaced by a buffer. The normal ranges obtained from 10 normal subjects are shown by the dotted areas. (●—●), patient PPP+normal washed platelets (Mean of duplicate), (○—○), patient washed platelets+normal PPP (Mean of duplicate).

reported to play a role in the conformation of the A1 loop by limiting exposure of the GPIb-binding site[2,5-7,20,21]. However, no mutations were found at these sites in the present study.

To date, there have been many studies on the molecular bases needed for the function of vWF. However, almost all the information deals with the basal subunit[19] and we do not have detailed knowledge about the three-dimensional structure of important functional domains of vWF, including the A1 loop. Considering that the conformational change of vWF is important to bind to GPIb, it is likely that a new and unidentified functional vWF domain, which induces the conformational change of the A1 loop to result in facilitative binding to platelets, is responsible for this disease phenotype. Recently, Obert et al. [22] reported that the conformational changes in the A3 domain of vWF were important to modulate the interaction of the A1 loop with platelet GPIb. Further investigation will be needed to clarify the mechanism of enhanced vWF-GPIb binding in the present patient.

In conclusion, although no responsible mutations at the A1 loop or the surrounding region could be identified in the patient vWF, this study provides new insights into vWF-GPIb binding physiology.

REFERENCES

1) Sadler JE: A revised classification of von Willebrand disease. For the subcommittee on von Willebrand factor of the scientific and standardization committee of the international society on thrombosis and haemostasis. *Thromb Haemost* **71**: 520-525, 1994.
2) Ginsburg D, Sadler JE: Von Willebrand disease: A database of point mutations, insertions and deletions. *Thromb Haemost* **69**: 177-184, 1993.
3) Meyer D, Fressinaud E, Gaucher C, Lavergne JM, Hibert L, Ribba AS, Jorieux S, Mazurier C: INSERM network on molecular abnormalities in von Willebrand disease: Gene defects in 150 unrelated French cases with type 2 von Willebrand disease: from the patient to the gene. *Thromb Haemost* **78**: 451-456, 1997.
4) Holmberg L, Dent JA, Schneppenheim R, Budde U, Ware J, Ruggeri ZM: von Willebrand factor mutation enhancing interaction with platelets in patients with normal multimeric structure. *J Clin Invest* **91**: 2169-2177, 1993.
5) Rabinowitz I, Randi AM, Shindler KS, Tuley EA, Rustagi PK, Sadler JE: Type IIB mutation His-505→Asp implicates a new segment in the control of von Willebrand factor binding to platelet glycoprotein Ib. *J Biol Chem* **268**: 20497-20501, 1993.
6) Hilbert L, Gaucher C, de Romeuf C, Horellou MH, Vink T, Mazurier C: Leu 697→Val mutation in mature von Willebrand factor is responsible for type IIB von Willebrand disease. *Blood* **83**: 1542-1550, 1994.
7) Hilbert L, Gaucher C, Mazurier C: Effects of different amino acid substitution in the leucine 649-proline 708 segment of recombinant von Willebrand factor. *Br J Haematol* **91**: 983-990, 1995.
8) Ruggeri ZM, Zimmerman TS: Variant von Willebrand's disease. *J Clin Invest* **65**: 1318-1325, 1980.

9) Born GVR: Aggregation of blood platelets by ADP and its reversal. *Nature* **194**: 927-929, 1962.
10) Fuse I, Hattori A, Mito M, Higuchi W, Yahata K, Shibata A, Aizawa Y: Pathogenetic analysis of five cases with a platelet disorder characterized by the absence of thromboxane A_2 (TXA_2)-induced platelet aggregation in spite of normal TXA_2 binding activity. *Thromb Haemost* **76**: 1080-1085, 1996.
11) Ruan C, Du X, Xi X, Castaldi PA, Berndt MC: A murine antiglycoprotein Ib complex monoclonal antibody, SZ2, inhibits platelet aggregation induced by both ristocetin and collagen. *Blood* **69**: 570-577, 1987.
12) Mancuso DJ, Tuley EA, Westfield LA, Lester-Mancuso TL, Le Beau MM, Sorace JM, Sadler JE: Human von Willebrand factor gene and pseudogene: structural analysis and differentiation by polymerase chain reaction. *Biochemistry* **30**: 253-269, 1991.
13) Sadler JE, Ginsburg D: A database of polymorphism in the von Willebrand factor gene and pseudogene. *Thromb Haemost* **69**: 185-191, 1993.
14) Weiss HJ, Sussman II: A new von Willebrand variant (Type I, New York): Increased ristocetin-induced platelet aggregation and plasma von Willebrand factor containing the full range of multimers. *Blood* **68**: 149-156, 1986.
15) Holmberg L, Berntorp E, Donner M, Nilsson IM: von Willebrand's disease characterized by increased ristocetin sensitivity and the presence of all von Willebrand factor multimers in plasma. *Blood* **68**: 668-672, 1986.
16) Takimoto Y, Imanaka F: Type 2B Hiroshima: a variant of von Willebrand disease characterized by chronic thrombocytopenia and the presence of all von Willebrand factor multimers in plasma. *Int J Hematol* **70**: 127-131, 1999.
17) Bolhuis PA, Sakariassen KS, Sander HJ, Bouma BN, Sixma JJ: Binding of factor VIII-von Willebrand factor to human arterial subendothelium precedes increased platelet adhesion and enhances platelet spreading. *J Lab Clin Med* **97**: 568-576, 1981.
18) Fujimura Y, Titani K, Holland LZ, Russell SR, Roberts JR, Elder JH, Ruggeri ZM, Zimmerman TS: von Willebrand factor. A reduced and alkylated 52/48-kDa fragment beginning at amino acid residue 449 contains the domain interacting with platelet glycoprotein Ib. *J Biol Chem* **261**: 381-385, 1986.
19) Girma JP, Meyer D, Verweij CL, Pannekoek H, Sixma JJ: Structure-function relationship of human von Willebrand factor. *Blood* **70**: 605-611, 1987.
20) Matsushita T, Sadler JE: Identification of amino acid residues essential for von Willebrand factor binding to platelet glycoprotein Ib: Charged-to-alanine scanning mutagenesis of the A1 domain of human von Willebrand factor. *J Biol Chem* **270**: 13406-13414, 1995.
21) Sugimoto M, Mohri H, Mcclintock RA, Ruggeri ZM: Identification of discontinuous von Willebrand factor sequences involved in complex formation with botrocetin. A model for the regulation of von Willebrand factor binding to platelet glycoprotein Ib. *J Biol Chem* **266**: 18172-18178, 1991.
22) Obert B, Houllier A, Meyer D, Girma JP: Conformational changes in the A3 domain of von Willebrand factor modulate the interaction of the A1 domain with platelet glycoprotein Ib. *Blood* **93**: 1959-1968, 1999.