



Rapid Restoration of Thrombus Formation and High-Molecular-Weight von Willebrand Factor Multimers in Patients with Severe Aortic Stenosis After Valve Replacement

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Aim: Patients with severe aortic stenosis (AS) may have bleeding episodes due to the loss of high-molecular-weight (HMW) von Willebrand factor multimers (VWFMs). The absence of HMW-VWFMs and bleeding tendency are usually corrected after aortic valve replacement (AVR). To investigate the process of VWF recovery and symptoms in patients with severe AS, we analyzed changes in VWF antigen (VWF:Ag), ADAMTS13 activity (ADAMTS13:AC), and platelet thrombus formation under high shear stress conditions.

Methods: Nine patients with severe AS undergoing AVR were analyzed.

Results: Evident deficiency of HMW-VWFMs was observed in six patients before surgery, which was rapidly restored within 8 days after AVR. Median levels of VWF:Ag before surgery, on postoperative days (PODs) 1, 8, 15, and 22, and one year after AVR were 78.1%, 130%, 224%, 155%, 134%, and 142%, respectively. In contrast, ADAMTS13:AC was 50.5%, 35.5%, 25.5%, 25.1%, 30.3%, and 84.6%, respectively. Preoperative thrombus formation but not surface coverage was significantly lower than that on POD 22, which was considered as normal level in each patient. Compared with preoperative levels, thrombus volume was significantly lower on POD 1, but rapidly increased by POD 8.

Conclusion: Bleeding tendency and loss of HMW-VWFMs observed in patients with severe AS before surgery was rapidly corrected after AVR. Instead, patients were in a VWF-predominant state between POD 8 and 22.

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Key words: Aortic valve stenosis, Acquired von Willebrand syndrome, Thrombus formation, ADAMTS13

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Introduction

In 1958, Heyde described patients with calcific aortic valve stenosis (AS) who had massive gastrointestinal bleeding with no identifiable cause¹. Twenty-eight years later, gastrointestinal bleeding observed in patients with AS was attributed to submucosal angio-

dysplasia². Subsequently, King *et al.* reported an association between unexplained gastrointestinal bleeding and AS that resolved after aortic valve replacement (AVR)³. This unique bleeding syndrome, called Heyde's syndrome, is identified as acquired type 2A von Willebrand syndrome (AVWS). It is characterized by the loss of high-molecular-weight (HMW) von Willebrand factor multimers (VWFMs)⁴⁻⁶. This type of AVWS was also found in patients treated with ventricular assist devices⁷ and extracorporeal membrane oxygenation therapy⁸.

VWF is a multimeric glycoprotein that binds platelets at the site of vascular injury and promotes

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platelet–platelet interactions⁹). HMW-VWFMs are particularly important for hemostasis under high shear stress conditions. In addition, high shear stress induces structural changes in VWF and exposes the peptide bond between 1605 and 1606 of the A2 domain in VWF, the cleavage site of ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13)¹⁰. Consequently, plasma VWF contributes to two types of emergencies, bleeding and thrombosis¹¹. A recent study showed that lower plasma levels of ADAMTS13 are associated with arterial thrombosis¹². Therefore, the ratio of VWF to ADAMTS13 is sometimes used to evaluate the risk of thrombosis¹³.

In patients with AS, a significant negative correlation was observed *in vivo* between higher shear stress and loss of HMW-VWFMs⁵. Further, the absence of HMW-VWFMs and bleeding tendency are usually corrected by valve replacement⁵. These results suggest that high shear stress arising from passage through a narrowed aortic valve enhances proteolysis of VWF by ADAMTS13 and induces the loss of HMW-VWFMs. However, the process by which platelet thrombus formation is restored and HMW-VWFM recovery occurs after AVR in patients with severe AS has not been fully elucidated.

We investigated plasma levels of VWF antigen (VWF:Ag) with VWFM analysis, plasma levels of ADAMTS13 activity (ADAMTS13:AC), and platelet thrombus formation using a flow chamber system in patients with severe AS before and after valve replacement.

Materials and Methods

Patients

Nine patients who underwent AVR for isolated severe AS between January 2012 and December 2013 at Nara Medical University Hospital were included in this study. Exclusion criteria included acute infection, autoimmune disorder, malignancy, hemodialysis, and more than mild aortic valve regurgitation. Patients undergoing procedures such as coronary artery bypass grafting, mitral or tricuspid valve surgery, or aortic surgery in combination with AVR were also excluded.

AS severity was assessed according to the European Association of Echocardiography/American Society of Echocardiography guidelines¹⁴. Briefly, severe AS was defined as an aortic valve area (AVA) < 1.0/cm² or a mean pressure gradient greater than 40 mmHg by Doppler echocardiography. None of the patients had a history of bleeding or received valve replacements with mechanical or bioprosthetic valves followed by administration of warfarin, anti-platelet agents, or both for prevention of thrombosis. Blood samples were col-

lected from these patients before AVR and on postoperative days (PODs) 1, 8, 15, and 22 and one year after valve replacement. Plasma levels of VWF:Ag and ADAMTS13:AC, VWFMs, and mural thrombus formation were measured using a flow chamber system. The ethics committee of Nara Medical University approved the study and written informed consent was obtained from all patients.

Echocardiographic Evaluation

Using an iE33 xMATRIX Echocardiography System (Philips Healthcare, Andover, MA, USA) or a Vivid E9 Ultrasound System (GE Healthcare, PA, USA), investigators assessed the preoperative hemodynamic performance of the aortic valve with transthoracic echocardiography. The mean and peak transvalvular pressure gradients were calculated with the modified Bernoulli equation, and the effective orifice area (EOA) was calculated using the continuity equation. Postoperative echocardiography was performed one month and one year after AVR. A size mismatch between the patient and the prosthesis was defined as an indexed EOA of < 0.85 cm² per square meter of body surface area (BSA).

VWF and ADAMTS13

Plasma levels of VWF:Ag were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using rabbit anti-human VWF polyclonal antiserum (DAKO, Glostrup, Denmark)¹⁵. The value obtained from normal individuals ($n=20$) using this assay was $102 \pm 33\%$. Plasma ADAMTS13:AC was determined using a commercially available chromogenic act-ELISA kit (Kainos Laboratories, Tokyo, Japan)¹⁶. The value obtained for normal individuals ($n=55$) using act-ELISA was $99 \pm 22\%$. The value of 100% was defined as the level of VWF:Ag and ADAMTS13:AC in pooled normal human plasma (NP). VWFM analysis was performed according to the method by Ruggeri and Zimmerman¹⁷ with modifications described by Warren *et al.*¹⁸. The experimental conditions, including western blotting with luminographic detection, were as previously described by Budde *et al.*¹⁹. Multimers were classified as HMW-VWFMs if there were >10 bands in the VWFM analysis²⁰. HMW bands that were not detected in NP were defined as unusually large (UL) VWFMs.

Platelet Thrombus Formation

Platelet thrombus formation was evaluated by thrombus generation under high shear stress conditions in a parallel plate flow chamber system^{21, 22}. Briefly, whole blood anticoagulated with argatroban was incubated with the fluorescent dye DiOC6 (1 μ M), and samples containing DiOC6-labeled platelets were per-

Table 1. Patient characteristics ($n=9$)

	median (range)
Age (years)	74 (69-78)
Sex (female/male)	5/4
BSA (m ²)	1.6 (1.3-1.8)
Underlying diseases	patient number
Hypertension	8
Diabetes mellitus	3
Dyslipidemia	6
Medication	
ACE-I/ARB	6
CCB	4
β -blocker	3
Statin	6

BSA: body surface area

ACE-I: angiotensin-converting enzyme inhibitor

ARB: angiotensin receptor blocker

CCB: calcium channel blocker

fused over a type I collagen-coated glass surface under a high shear rate (1500 s^{-1}) for 7 min. DiOC6 fluorescence corresponding to the platelets was examined at an excitation wavelength of 488 nm with a barrier filter at 500 nm. The area covered by adherent platelets (surface coverage) and the volume of each thrombus at 7 min after perfusion were evaluated using confocal laser scanning microscopy (CSLM; FV300; Olympus, Tokyo, Japan). Each flow chamber experiment was performed three times at each time point, and five areas ($211 \times 317 \text{ mm}$ each) randomly selected from each perfusion trial performed with whole blood from a patient with AS were evaluated.

Statistical Analysis

All continuous variables were expressed as medians (ranges). The Mann–Whitney U test was used to determine significant differences ($P < 0.05$) between groups. The Wilcoxon signed-rank test was used to compare values from different time points in each group. Discrete variables were compared using Fisher's exact test. Correlations between variables were assessed using Spearman's rank correlation test. Statistical analysis was performed using EZR on the R commander²³.

Results

Patient Characteristics

Characteristics of the nine patients are shown in **Table 1**. Patient age was relatively high (median, 74 years). As shown in **Table 2**, routine laboratory values were almost normal. Note that severe anemia with

Table 2. Laboratory data before aortic valve replacement ($n=9$)

	Median (range)
Hematological examination	
Red blood cell count ($10^{10}/\text{L}$)	397 (325-428)
Hematocrit (%)	38 (31-41)
Hemoglobin (g/dL)	12.7 (10.4-14)
Platelet counts ($10^9/\text{L}$)	168 (101-206)
PT-INR	1.0 (1.0-1.1)
APTT (second)	28 (26-35)
Fibrinogen (mg/dL)	290 (192-525)
Bleeding Time (minute)	2 (2-3.5)
Blood chemistry test	
Total bilirubin (mg/dL)	0.8 (0.5-1.6)
Lactate dehydrogenase (LDH, U/L)	216 (150-364)
Blood urea nitrogen (BUN, mg/dL)	20 (15-32)
Creatinine (CRE, mg/dL)	0.9 (0.5-1.2)
Albumin (Alb, g/dL)	4.3 (3.6-4.8)
Aspartate transaminase (AST, U/L)	21 (15-71)
Alanine transaminase (ALT, U/L)	23 (7-97)
Echocardiographic data	
Aortic valve area (cm ²)	0.6 (0.3-0.8)

PT-INR: prothrombin time- international normalized ratio

APTT: activated partial thromboplastin time

hemoglobin $< 10 \text{ g/dL}$ was not observed in these patients. As there was no apparent gastrointestinal bleeding, Heyde's syndrome could not be diagnosed. However, the aortic valve area was $< 1.0/\text{cm}^2$ in all patients, consistent with the criteria for severe AS.

Surgical Treatment

One patient received a mechanical valve (On-X Prosthetic Aortic Heart Valve, On-X Life Technologies, Austin, TX, USA), and the other eight patients received bioprosthetic valves (Perimount, Carpentier-Edwards, Irvine, CA, USA). The median blood loss during surgery and the first 24 postoperative hours was 998 mL (range, 170–2430 mL) and 744 mL (range, 380–1605 mL), respectively. No patient underwent mediastinal re-exploration for bleeding after surgery. All patients had an uneventful postoperative course. As shown in **Table 3**, the median peak aortic transvalvular pressure gradient was 98 mmHg before surgery, which improved dramatically except in Patient 9, who had a patient–prosthesis mismatch. In six patients, the mean preoperative transvalvular pressure gradient was over 40 mmHg, which is one of the diagnostic criteria for severe AS. The remaining three patients (Patients 5, 8, and 9) had a mean preoperative transvalvular gradient of $\leq 40 \text{ mmHg}$. These values quickly decreased to $< 20 \text{ mmHg}$, except in Patient 9, who had a patient–prosthesis mismatch. During the first year of follow-

Table 3. Changes of transthoracic echocardiographic parameters

Patient No	ABO blood type	Peak transvalvular gradient (mmHg)			Mean transvalvular gradient (mmHg)		
		Pre-operation	One month after operation	One year after operation	Pre-operation	One month after operation	One year after operation
1	A	98	11	12	54	5	6
2	O	100	15	26	54	7	11
3	AB	119	35	26	69	19	13
4	A	132	25	24	72	12	11
5	A	78	18	32	40	8	18
6	AB	117	19	19	64	9	9
7	A	79	21	20	46	10	9
8	A	54	22	25	32	12	12
9	B	62	51	52	40	25	26
Median		98	21	25	54	10	11
Maximum		132	51	52	72	25	26
Minimum		54	11	12	32	5	6

up after surgery, all patients, including the one with the mechanical prosthesis requiring anticoagulation, were asymptomatic. The indexed EOA for Patient 9 at one year was 0.5 cm² per square meter of BSA.

Changes in Platelet Count and Plasma Levels of VWF:Ag and ADAMTS13:AC

As shown in **Fig. 1**, the median platelet count dramatically decreased from $168 \times 10^9/\mu\text{L}$ before surgery to $72 \times 10^9/\mu\text{L}$ on POD 1 and gradually improved after POD 8. The median plasma level of VWF:Ag before surgery was 78.1%, which was lower than that in normal individuals. Plasma levels of VWF:Ag on POD 1, 8, 15, and 22 and one year after AVR were 130%, 224%, 155%, 134%, and 142%, respectively, which were significantly higher than preoperative levels. In contrast, median plasma ADAMTS13:AC before surgery was 50.5%, which was also relatively low (**Fig. 1**). Plasma ADAMTS13:AC on POD 1, 8, 15, and 22 and one year after surgery was 35.5%, 25.5%, 25.1%, 30.3%, and 84.6%, respectively. Plasma levels of ADAMTS13:AC gradually decreased until POD 15, whereas they increased to within the normal range (median, 84.6%) one year after AVR, which was significantly higher than values on POD 1, 8, 15, and 22. The ratios of VWF:Ag to ADAMTS13:AC before surgery, on POD 1, 8, 15, and 22, and one year after surgery were 1.6, 4.5, 8.1, 6.1, 4.1, and 1.64, respectively. On POD 8 and 15, the ratio was significantly higher than that before surgery and one year after AVR. Therefore, patients were assumed to be in a VWF-predominant state between POD 8 and 22, instead of having bleeding tendency before AVR.

VWFM Analysis

As shown in **Fig. 2**, we analyzed VWFMs in plasma samples before surgery, on POD 1, 8, 15, and 22, and one year after surgery. Of nine patients with severe AS, six had an evident deficiency of HMW-VWFMs before surgery. The remaining three patients (Patients 5, 8, and 9) had slightly decreased levels of HMW-VWFMs before surgery. These three patients had relatively mild findings of echocardiographic parameters (**Table 3**). In all nine patients, levels of HMW-VWFMs increased after surgery, and UL-VWFMs were detectable by POD 8 or 15. These results indicate that patients with severe AS had bleeding tendency before surgery, but the risk of thrombosis increased dramatically after surgery on POD 8 or 15. We found an evident deficiency of HMW-VWFMs in the patient with prosthesis size mismatch one year after AVR (Patient 9). The remaining eight patients did not have HMW-VWFM deficiency one year after surgery.

Thrombosis Formation Under High Shear Stress Conditions

We analyzed thrombosis formation under a high shear rate (1500 s^{-1}) at five time points: before surgery and on POD 1, 8, 15, and 22 using a flow chamber system. Three-dimensional images of mural thrombus generation on a collagen surface in Patient 4 are shown in **Fig. 3** as a representative case. Thrombus formation dramatically decreased on POD 1, compared with that before surgery, due to a decrease in platelet count associated with the effects of surgery, such as the use of an extracorporeal circulation device during surgery or dilution of blood by large transfused volumes.

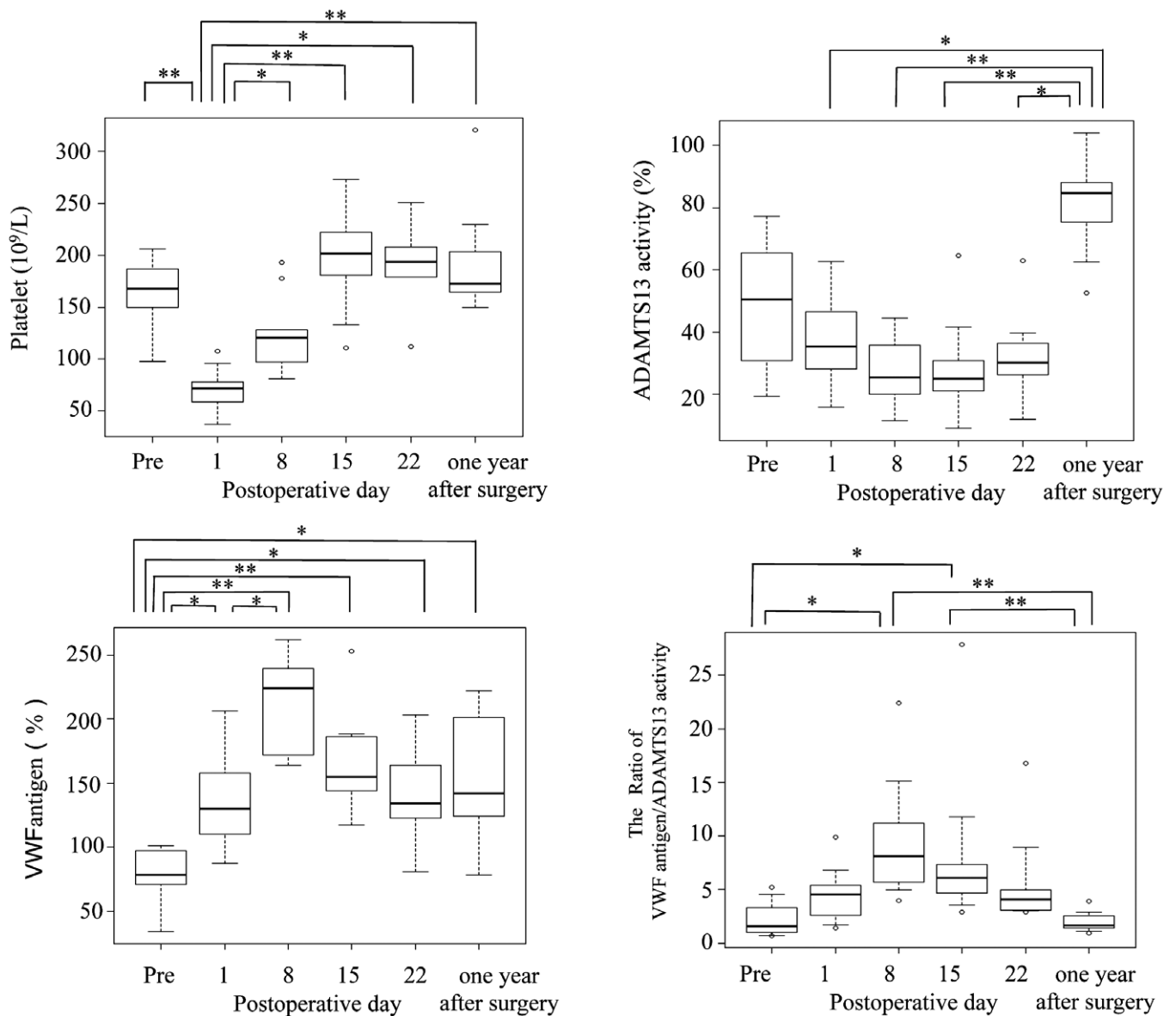


Fig. 1. Sequential changes in platelet count, plasma levels of von Willebrand factor (VWF) antigen, and ADAMTS13:AC before and after valve replacement

Median platelet count dramatically decreased from before surgery to postoperative day (POD) 1, and gradually increased after POD 8. Median plasma levels of VWF:Ag before surgery were relatively low compared with those in normal individuals. Levels on POD 1, 8, 15, and 22 and one year after surgery were significantly higher than preoperative levels ($P < 0.05$). Plasma levels of ADAMTS13:AC before surgery were relatively low, and levels on POD 1, 8, 15, and 22 were clearly lower than those one year after aortic valve replacement. The ratios of VWF:Ag to ADAMTS13:AC dramatically increased between POD 8 and 22. * $P < 0.05$, ** $P < 0.01$

However, thrombus formation recovered by POD 8.

A quantitative analysis of surface coverage and thrombus volume at 7 min after perfusion is shown in **Fig. 4**. Surface coverage on POD 1 was significantly lower compared with that before surgery ($P < 0.05$). Compared with POD 1, surface coverage was significantly higher ($P < 0.05$) on POD 8, 15, and 22. However, preoperative values were the same as those on

POD 22. With regard to platelet thrombus formation, thrombus volume was significantly lower on POD 1 and higher on POD 8, 15, and 22 compared with that before AVR ($P < 0.05$, **Fig. 4**). These results indicate that surface coverage before surgery was not significantly lower, but thrombus volume before surgery was significantly lower than that on POD 22, which is considered normal in each patient.

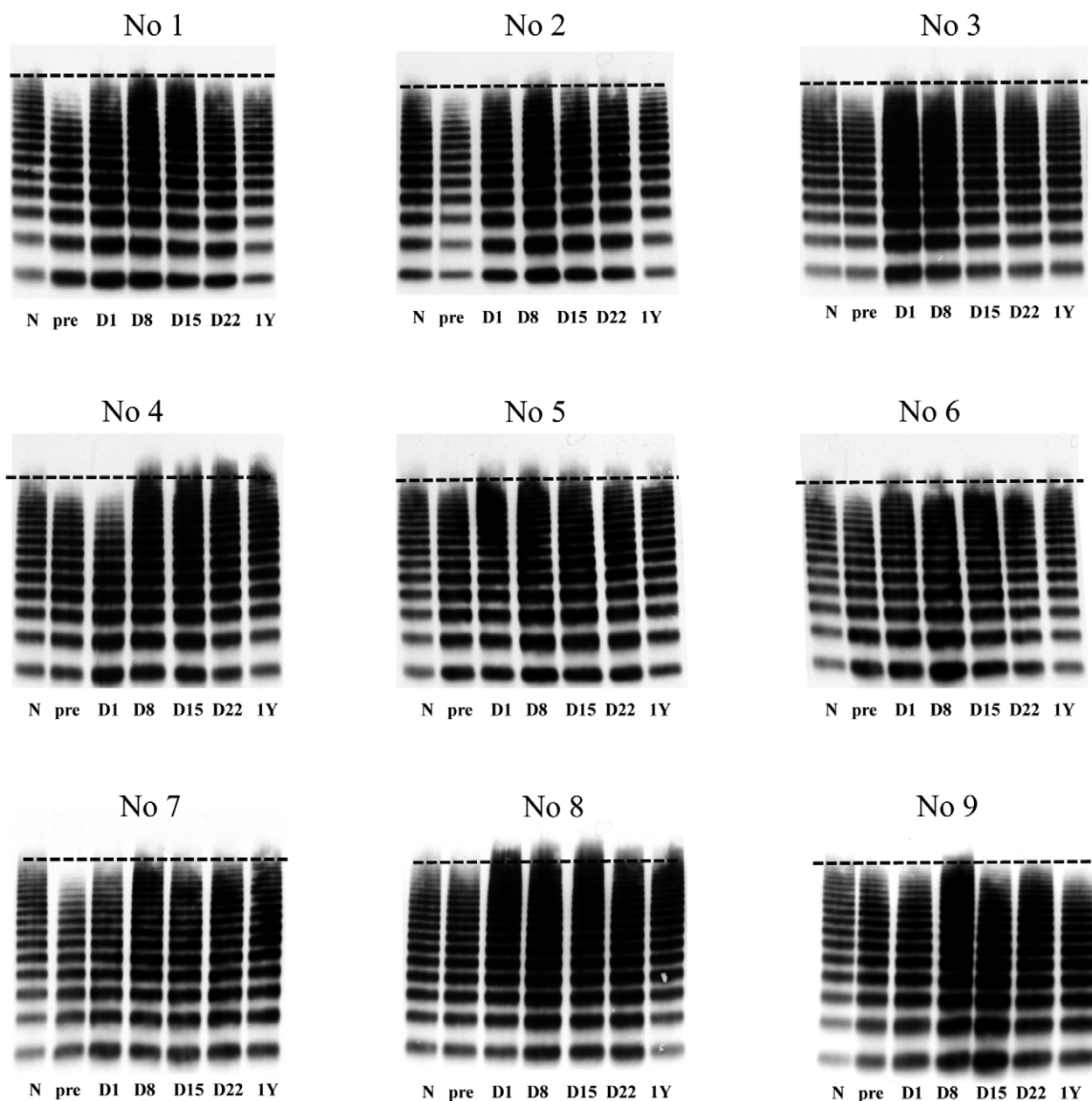


Fig. 2. von Willebrand factor multimer (VWFM) analysis

We performed VWFM analysis using plasma collected before surgery (pre), on postoperative days (D) 1, 8, 15, and 22, and one year (1Y) after aortic valve replacement. N indicates normal plasma. Of nine patients with severe aortic stenosis, six had evident deficiency of HMW-VWFMs before surgery. The remaining three (Patients 5, 8, and 9) had slightly decreased levels of HMW-VWFMs before surgery. In all patients, UL-VWFMs were detectable on D 8 or 15.

Discussion

Recent studies have reported that patients with severe AS have impaired platelet function under high shear stress conditions^{24, 25}. These hemostatic abnormalities are completely corrected by AVR^{24, 25}. However, the correction process has not been fully elucidated. Therefore, we performed serial assessments of mural

thrombus formation along with an analysis of VWFMs and plasma levels of VWF:Ag and ADAMTS13:AC before and after AVR in patients with isolated severe AS.

We found that thrombus volume, one indicator of platelet aggregation *ex vivo*, was reduced in patients with severe AS before AVR. In contrast, the platelet adhesion area was not significantly lower before surgery. Mural thrombus formation at the site of vascular

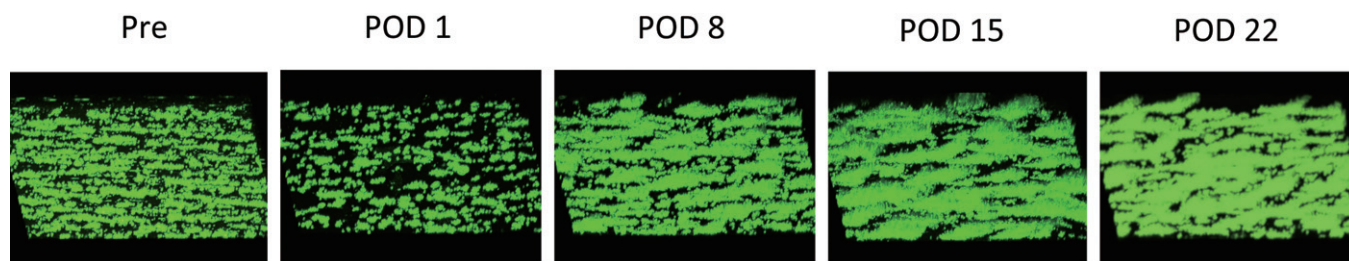


Fig. 3. Three-dimensional images of mural thrombus generation on a collagen surface

Representative images from Patient 4 are shown. Less thrombus formation occurred on postoperative day (POD) 1 compared with that prior to surgery. However, the ability to form thrombi was quickly recovered by POD 8 and increased up to POD 22 after aortic valve replacement.

injury contributes to the arrest of bleeding. During the initial stages of thrombus formation, platelet adhesion to denuded vessel walls is initiated by VWF binding to platelet glycoprotein (GP) Ib/IX/V complexes under high shear stress conditions²⁶. This is followed by platelet activation and aggregation, which require an association between GPIIb/IIIa and VWF^{27, 28}. In patients with AS, gastrointestinal bleeding events have been rarely reported, despite a high frequency of HMW-VWFM loss⁷. After AVR, we found that both surface coverage and thrombus volume were remarkably reduced on POD 1 as a result of the artificial effects of AVR. Low platelet count, a consequence of the use of an extracorporeal circulation device during surgery, and dilution of blood by large transfused volumes during surgery likely contributed to reductions in platelet count. After POD 8, the surface coverage area was almost the same as that before surgery. However, thrombus volume before surgery was significantly lower than baseline values after POD 22. These results suggest that it is platelet aggregation that is mostly impaired in patients with severe AS. Panzer *et al.*²⁴ also reported that levels of HMW-VWFMs were diminished, and that the loss of HMW-VWFMs affected platelet aggregation more than platelet adhesion in patients with AS, using the cone and plate analyzer Impact-R. They speculated that low-to-intermediate molecular weight VWFMs might be sufficient for platelet adhesion, but HMW-VWFMs are required for platelet aggregation.

Deficiencies in HMW-VWFMs were observed in six of nine patients with AS before surgery. The remaining three patients did not have any evident lack of HMW-VWFMs. Both peak and mean transvalvular gradients of these three patients were the lowest despite an AVA of $<1.0/\text{cm}^2$, which meets the criteria for severe AS. These findings are consistent with the results reported by Vincentelli *et al.*⁵ The percentage of HMW-VWF is inversely correlated with mean transvalvular gradient in patients with severe AS. In

six patients, HMW-VWFM deficiency was completely resolved after POD 8. Moreover, all nine patients had UL-VWFMs on POD 8 or 15. Some patients continued to have UL-VWFMs from POD 15 to one year after AVR. One patient (Patient 9) did not have HMW-VWFMs one year after surgery due to a patient–prosthesis mismatch.

Plasma levels of VWF:Ag gradually increased until POD 8 and then gradually decreased. VWF:Ag levels were within the normal range one year after AVR. In contrast, plasma levels of ADAMTS13:AC were relatively low before surgery and gradually decreased until POD 15. At one year after AVR, plasma levels of ADAMTS13:AC increased to the normal range. These results indicate that ADAMTS13:AC was low due to the consumption in cleaving VWF during the preoperative and perioperative periods. To evaluate levels of both VWF and ADAMTS13 together, the ratio of VWF to ADAMTS13 has been used in systemic inflammation²⁹ and liver disease¹³. We previously reported that a ratio of VWF:Ag to ADAMTS13:AC above 5 might be a risk factor for thromboembolic events in patients after hematopoietic stem cell transplantation³⁰. In this study, the ratio of VWF:Ag to ADAMTS13:AC before surgery was 1.56. It dramatically increased to 9.5 on POD 8 and decreased to 5.4 on POD 22. Finally, it decreased to 1.64 at one year after AVR. Patients were in a VWF-predominant state between POD 8 and 22, and we may pay attention to the thrombosis rather than bleeding even in the early stage after surgery.

There are some limitations to this study. First, the number of patients analyzed in this study was relatively small in a single hospital. Therefore, potential bias might exist and the impact of this study could be limited. However, it is very difficult to perform the VWFM analyses and thrombosis formation in a larger scale of patients because VWFM analysis is a time-consuming examination and platelet thrombus formation should be performed within 3 h of collecting

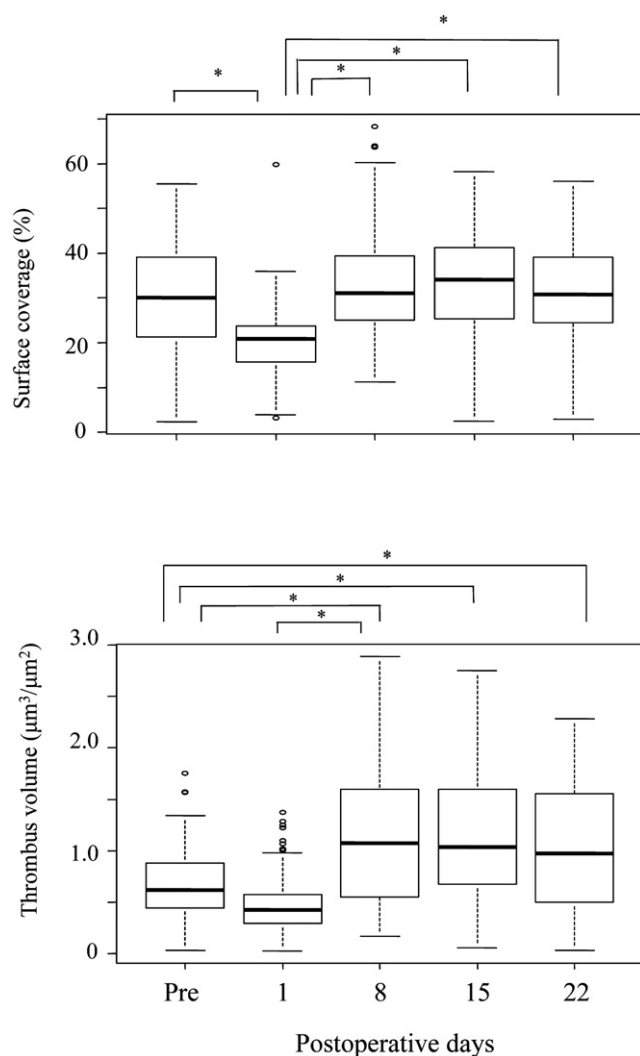


Fig. 4. Changes in percentage of surface coverage and thrombus volume before and after valve replacement

A quantitative analysis of surface coverage and volume of thrombus formation at 7 min after perfusion is shown. Surface coverage was significantly lower on postoperative day (POD) 1 compared with that prior to surgery, but increased significantly on POD 8, 15, and 22. However, preoperative values were similar to those on POD 22, which were considered the normal level for each patient. Compared with preoperative values, thrombus volume was significantly lower on POD 1 and higher on POD 8, 15, and 22. Preoperative thrombus volume but not surface coverage was significantly lower compared with POD 22 reference levels. * $P < 0.05$

blood. Second, blood samples from patients were collected every 7 days in the perioperative period. Since the factors analyzed in this study such as VWF and ADAMTS13 seemed to change over a short period of time, we might miss the correct sequence of change. Finally, VWF activity was not analyzed in this study. The adhesive activity of VWF depends on its molecular size. Therefore, we could speculate the VWF activ-

ity using the result of VWF analysis.

In conclusion, we found less platelet thrombus formation in patients with severe AS before surgery, but the ability to form platelet thrombi, along with levels of HMW-VWFMs, were rapidly restored by POD 8. We should pay attention to thrombotic events rather than bleeding between the second and third weeks after AVR in patients with severe AS.

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Conflicts of Interest

MM is on a clinical advisory board for Baxalta.

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