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Critical role of von Willebrand factor and platelet interaction in venous thromboembolism

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Summary. It has been generally considered that platelets are less important in venous thrombus formation. However, clinical studies have shown an association between venous thromboembolism (VTE) and von Willebrand factor (VWF). We therefore investigated the contribution of VWF and platelet interaction to the onset of VTE using tissues from autopsies and from an animal model. An immunohistochemical study revealed that glycoprotein (GP) IIb/IIIa, fibrin, glycophorin A (erythrocyte-specific protein) and VWF were consistently localized in ilio-femoral venous thrombi and in pulmonary thromboemboli from 8 autopsied cases who died of VTE, and VWF was closely associated with GPIIb/IIIa and fibrin. Venous thrombi and pulmonary emboli contained significant amounts of GPIIb/IIIa and VWF, in addition to glycophorin A and fibrin, and the factors did not significantly differ between them. A rabbit model of VTE was developed by inserting a polyethylene tube into the iliac vein. The constituents of the induced thrombi were quite similar to those of human VTE. An antibody against VWF (AJW200), which inhibits interactions between the VWF A1 domain and platelet GPIb, significantly reduced venous thrombus formation and pulmonary thromboembolism in the model. These results suggest that VWF A1-platelet GPIb interaction plays a significant role in venous thrombus formation.

Key Words: Venous thromboembolism, Platelet, Fibrin, von Willebrand factor, Glycoprotein Ib

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Introduction

Venous thromboembolism (VTE), which clinically manifests as deep vein thrombosis (DVT) or pulmonary embolism (PE), is a major health problem, with an estimated incidence of 1 per thousand in the general population (Silverstein et al., 1998). Although the rate of fatal PE has declined from 6% to 2% over the last quarter of a century, about 30% of patients still die within 30 days of onset (Heit, 2002). Pulmonary emboli originate from deep leg vein thrombi at a rate of over 95%. Hypercoagulability, venous stasis and endothelial injury are historically important factors that are implicated in the genesis of venous thrombosis. Since the former two factors facilitate activation of the coagulation pathway, it is generally understood that venous thrombi are mainly composed of erythrocytes, with a large amount of fibrin and relatively few platelets, known as red thrombi (Stein and Evans, 1967; Beckering and Titus, 1969; Hirsh et al., 2001). Additionally, acquired and inherited risk factors activating the coagulation pathway have been identified, such as malignant disorders, long-term immobilization, deficiencies of protein C, protein S, and antithrombin, and a poor anticoagulant response to activated protein C (activated protein C resistance) (Hirsh et al., 2001; Heit, 2002). On the other hand, several clinical studies have revealed that elevated plasma levels of von Willebrand factor (VWF) and factor VIII (FVIII) are important risk factors for the development of venous thrombosis (Koster et al., 1995; Kraaijenhagen et al., 2000; Kyrle et al., 2000; Bombeli et al., 2002).

VWF is a large multimetric glycoprotein synthesized by endothelial cells and megakaryocytes. It mediates platelet adhesion and aggregation, and also acts as a

carrier for FVIII, protecting this coagulation factor from proteolysis by the activated protein C system (Weiss et al., 1977; Franchini and Lippi, 2006). The binding of VWF to glycoprotein (GP) Iba and GP IIb/IIIa plays a vital role in the initial stage of platelet adhesion and recruitment under rapid flow conditions, as well as thrombus growth on atherosclerotic lesions (Ruggeri, 1997; Yamashita et al., 2003; Goto, 2004; Mendolicchio and Ruggeri, 2005). Furthermore, a recent study revealed the role of VWF in platelet adhesion and subsequent thrombus growth in mouse mesenteric venules (Chauhan et al., 2007). However, the role of VWF in large vein thrombosis and in the development of VTE remains unknown.

The present study investigates the thrombus content of VTE from autopsies and assesses the role of VWF in venous thrombus formation and pulmonary embolism in a rabbit model of VTE.

Materials and methods

VTE in autopsies

We examined the ilio-femoral and pulmonary veins of 8 individuals who died of PE within 30 days of onset. Autopsies were performed after informed consent at Miyazaki University Hospital, Miyazaki Social Insurance Hospital, and Koga General Hospital. The institutional ethics committees approved the study protocol. The underlying diseases or states were malignancy (2), idiopathic (2), orthopedic surgery (1), immobility due to neurodegenerative disease (1), central vein catheterization (1), and nephrotic syndrome (1). Five of 8 patients had received anti-thrombotic drugs, including heparin, warfarin, tissue plasminogen activator and aspirin. Postmortem examinations identified both pulmonary thromboemboli and residual mural thrombi in the ilio-femoral veins of all 8 patients. The vessels with thrombi were fixed with 10% buffered formalin for 72 hours at room temperature, and then cut transversely and embedded in paraffin.

Light microscopy and immunohistochemistry of VTE

Serial, paraffin-embedded sections (3 μm thick) were morphologically evaluated by staining with hematoxylin and eosin/Victoria blue dye. The sections were also immunohistochemically stained using the following primary antibodies to platelet GPIIb/IIIa (Affinity Biologicals Inc., Hamilton, CA), fibrin (CHEMICON International, Temecula, CA), glycophorin A (a protein specific to erythrocyte, DAKO Japan, Kyoto, Japan) and VWF (The Binding Site, Birmingham, UK). The sections were stained with Envision (DAKO Japan) or with biotin-labeled secondary antibody (Jackson ImmunoResearch, Baltimore, MA). The activity of horseradish peroxidase was visualized using 3,3'-diaminobenzidine tetrahydrochloride, and the sections were faintly counterstained with Meyer's hematoxylin. Immunostaining controls included non-immune mouse IgG_1 or sheep serum instead of the primary antibodies. Immunopositive areas for each antibody were quantified using a color imaging morphometry system (Win Roof, Mitani, Fukui, Japan) (Yamashita et al., 2006).

Animal model of VTE

The Animal Care Committee of Miyazaki University approved the animal research protocols (1998-025-11). This investigation also conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Twenty-eight male Japanese white rabbits weighing 2.5 to 3.0 kg were fed with a conventional diet. All surgical manipulations proceeded under aseptic conditions and general anesthesia was accomplished by administering an intravenous injection of pentobarbital (25 mg/kg). A polyethylene tube (diameter, 76 μm Becton Dickinson, Franklin Lakes, NJ) was inserted via the femoral vein into the iliac vein under fluoroscopic guidance. The role of VWF in venous thrombus formation and pulmonary thromboembolism was evaluated by injecting an intravenous bolus of human anti-VWF monoclonal antibody (AJW200, at doses of 1.0 mg/kg) or saline (control) via the ear vein before inserting the polyethylene tube. This antibody reacts with the A1 domain of VWF in some species, including humans and rabbits (Kageyama et al., 2002). The antibody dosage used herein significantly inhibited platelet aggregation in the rabbits induced by botrocetin over the next 24 hours, but did not affect that induced by collagen (Yamashita et al., 2003). The rabbits were injected with heparin (500 U/kg, i.v.) after 4 or 24 hours, killed with an overdose of pentobarbital (60 mg/kg, i.v.) 5 minutes later and then perfused with 50 ml of 0.01 mol/L phosphate buffered saline.

Immunohistochemistry of rabbit venous thrombus and pulmonary embolus

Thrombi in the iliac veins were placed on dry paper and immediately weighed. The thrombi and lungs were fixed in 4% paraformaldehyde for 24 hours at 4°C. The lungs were cut into 3 parts, embedded in paraffin, sectioned (3 μm thick) and stained with hematoxylin and eosin/Victoria blue dye, as well as with antibodies against platelet GPIIb/IIIa (Affinity Biologicals Inc.), rabbit fibrin (a gift from Takeda Chemical Industries, Ltd. Osaka, Japan) (Kurokawa et al., 1991) and VWF (The Binding Site) as in the human samples. Pulmonary thromboemboli were counted in each section under light microscopy (4x magnification). Two investigators (S. G-M. and K.M.) who were blinded to the treatment assignments weighed the venous thrombi and counted the pulmonary emboli. Immunopositive areas for each

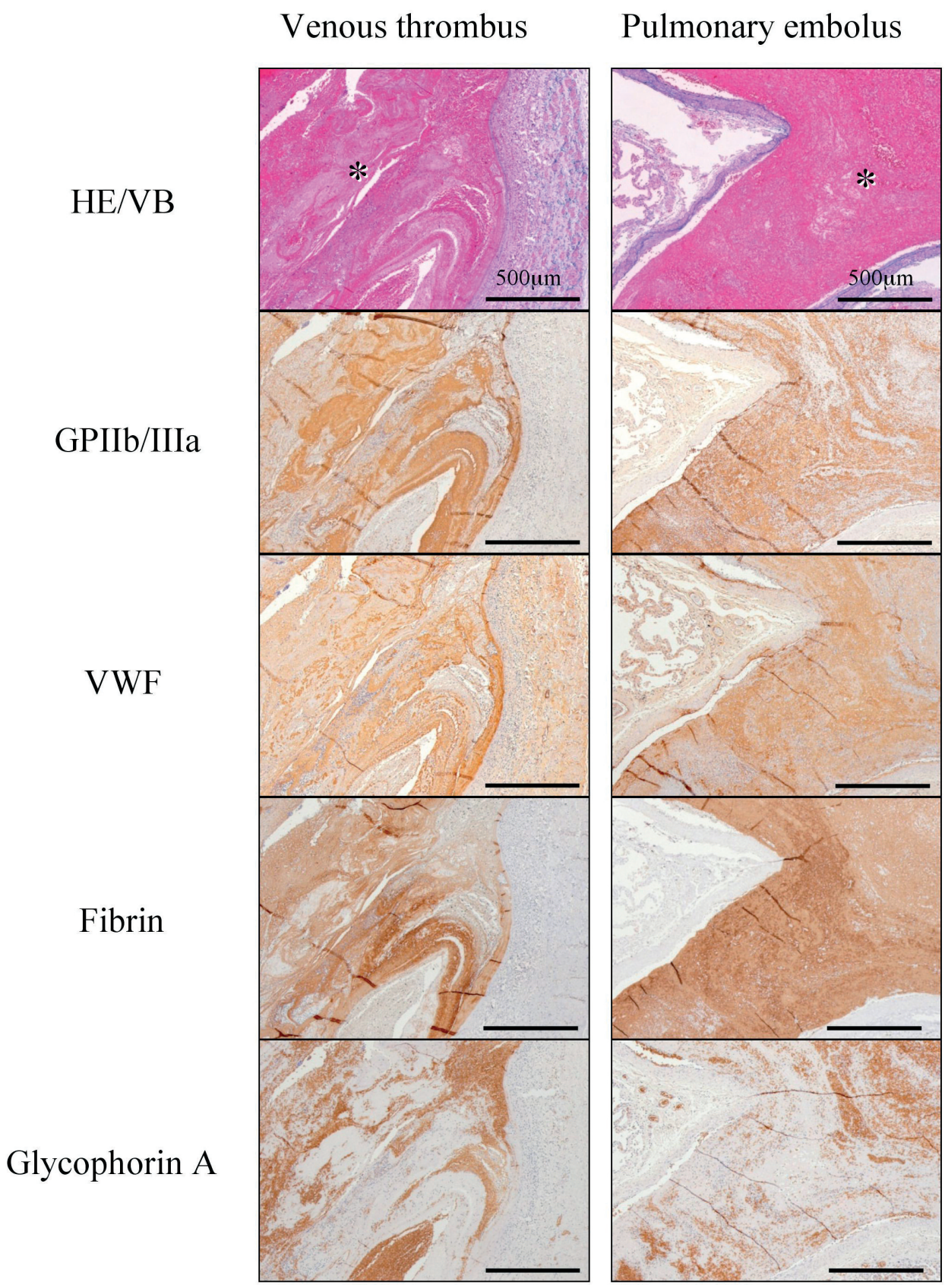

Fig. 1. Representative light and immunohistochemical microphotographs from a patient who died of venous thromboembolism. Thrombi (*) are immunopositive for GPIIb/IIIa, VWF, fibrin and glycophorin A. Immunohistochemical findings of all thrombi in other autopsies were similar.

antibody were quantified using a color imaging morphometry system (Win Roof, Mitani, Fukui, Japan) (Yamashita et al., 2006).

Coagulation parameters

Blood was collected from the central ear arteries of the rabbits into 3.8% sodium citrate (9:1 v/v). Plasma was prepared by centrifugation of citrated blood at 1,450 g for 10 minutes. The prothrombin time (PT) and activated partial thromboplastin time (aPTT) of plasma were measured in duplicate within 1 hour of blood collection using a coagulation timer (Behring Fibrintimer, Behring Diagnostics, Ahrensburg, Germany).

Statistical analyses

All data are presented as medians and interquartile ranges, individual dots, or means ± SD. The statistical significance of differences between measured parameters was evaluated using the Mann-Whitney U-test or the Kruskal-Wallis test with Dunn's multiple comparison test (GraphPad Prizm 4.03, GraphPad Software Inc., San Diego, CA). A P-value of <0.05 was considered significant.

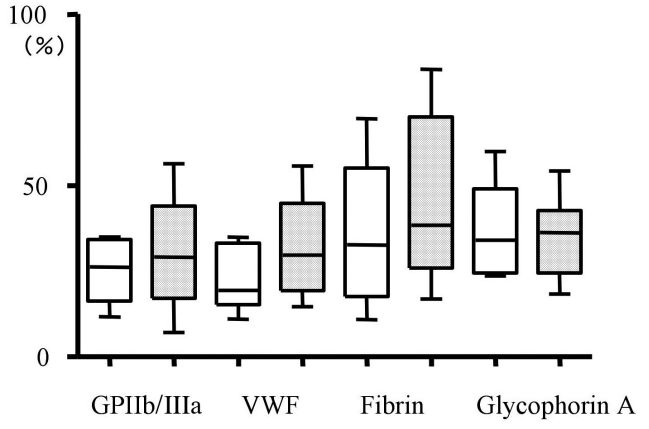

Fig. 2. Immunopositive areas (%) of human venous thrombus and pulmonary thromboembolus. Open box, venous thrombus; shaded box, pulmonary thromboembolus (n=8 each).

Results

Composition of thrombi in DVT and PE at autopsy

Large thrombotic emboli obstructed the main pulmonary artery or pulmonary artery bifurcation, and mural thrombi were found in the ilio-femoral veins of all autopsies. Immunohistochemical staining revealed the constitutive presence of GPIIb/IIIa, VWF, fibrin and glycophorin A in all of the venous mural thrombi and pulmonary emboli, and in closely associated areas that were immunopositive for GPIIb/IIIa, VWF and fibrin (Fig. 1). No immunoreaction was detectable when the primary antibodies were replaced with non-immune mouse IgG_1 or with non-immune sheep serum (data not shown). Both DVT and PE contained similar amounts of GPIIb/IIIa and VWF, in addition to glycophorin A and fibrin, with no significant differences among any of the factors (Fig. 2). The GPIIb/IIIa-, VWF-, fibrin- or glycophorin A-positive areas did not significantly differ between DVT and PE.

Iliac vein thrombus and pulmonary thromboembolus in rabbits

Iliac vein thrombi and pulmonary thromboemboli were generated by positioning a polyethylene tube in the iliac veins of rabbits for 4 or 24 hours. Immunohistochemical staining demonstrated the constitutive presence and close association of GPIIb/IIIa, VWF and fibrin in all thrombi (Fig. 3). The GPIIb/IIIa-, VWF-, or fibrin-positive areas did not significantly differ between rabbit venous thrombi and PE (Fig. 4). These findings were similar to those of human DVT and PE (Fig. 1). The thrombus weighted significantly more at 24 than at 4 hours (Fig. 5A).

To investigate the role of VWF in venous thrombus formation, anti-VWF antibody (AJW200; 1.0 mg/kg) was intravenously injected 1 hour before tube insertion. The antibody significantly reduced the weight of iliac vein thrombi at 4 and 24 hours and the numbers of pulmonary thromboemboli at 4 hours after insertion (Fig. 5). The antibody also tended to reduce the numbers of pulmonary thromboemboli at 24 hours, but the difference did not reach significance (p=0.097). Areas of VWF immunopositivity in venous thrombi did not significantly differ with or without AJW200 (Fig. 5C).

Table 1. Coagulation parameters in the rabbit model before, and 4 and 24 hours after polyethylene tube insertion.

AJW200 (1.0 mg/kg i.v.; n=7)	Before	4h	24h
Prothrombin time (sec)	9.9±0.2	10.0±0.5	8.9±0.9
Activate partial thromboplastin time (sec)	18.4±0.3	16.8±0.8	16.3±1.5

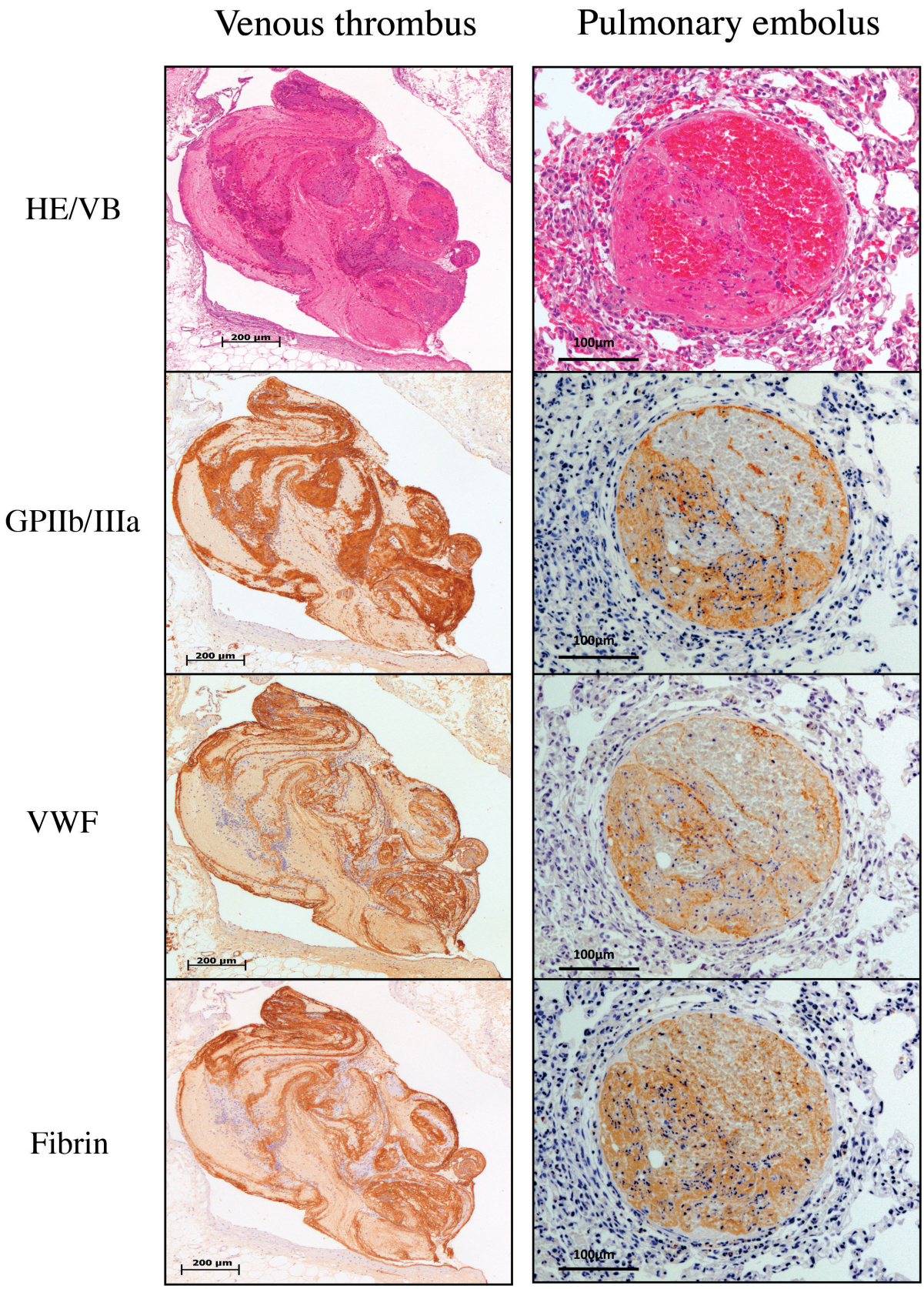

Fig. 3. Representative light and immunohistochemical microphotographs of thrombus and pulmonary thromboembolus 24 hours after polyethylene tube insertion into rabbit iliac veins. Thrombi are immunopositive for GPIIb/IIIa, VWF, and fibrin. Immunohistochemical findings were similar in all animals.

Coagulation parameters

Table 1 shows the coagulation parameters before and after AJW200 infusion. The antibody did not affect these parameters *ex vivo*.

Discussion

The present study demonstrated that the venous thrombi causing VTE are rich in not only erythrocytes and fibrin, but also in platelets and VWF, and that inhibition of the VWF A1 domain and platelet GPIb interaction reduces venous thrombus formation and pulmonary thromboembolism.

The traditional view is that deep venous thrombosis can occur in a relatively static environment and in various hypercoagulable states, and that such thrombi are thus composed of erythrocytes with a large amount of interspersed fibrin and relatively few platelets (Stein and Evans, 1967; Hirsh et al., 2001). However, our results showed that venous thrombi and pulmonary thromboemboli are composed of an admixture of erythrocytes, fibrin and platelets, and are richer in platelets and VWF than was previously thought. Staining methods could account for the different findings, since previous histopathological findings have been based on hematoxylin-eosin staining, whereas we used immunohistochemical detection with sensitive and specific antibodies. Although few histopathological studies have examined the composition of venous thrombi and pulmonary emboli, some have identified platelets in venous thrombi and in pulmonary thromboemboli. Sevitt (1974) showed that venous thrombi obtained from femoral valve pockets are usually dominated by red areas characterized by erythrocytes and fibrin, but many foci of platelets with fibrin borders were also present. Patterson (1969) reported that a platelet component is always present, particularly at the thrombus head and throughout the propagated tail, and concluded that arterial and venous thrombi have fundamentally similar morphologies. Although we did not obtain either VWF or FVIII levels from the autopsied patients, our results support this notion, as does recent clinical evidence that VWF is associated with VTE (Koster et al., 1995; Kraaijenhagen et al., 2000; Kyrle et al., 2000; Bombeli et al., 2002).

The venous thrombi and pulmonary thromboemboli in this study were rich in VWF, which is closely associated with platelets. These findings suggest that VWF plays a significant role in venous thrombus formation. The inhibition of VWF and platelet interaction with a monoclonal antibody against the VWF A1 domain (AJW200) obviously inhibited venous thrombus formation and pulmonary thromboembolism in our animal model. This finding indicated that VWF in venous thrombi is not merely incorporated but plays a functional role in this process. Accumulating evidence

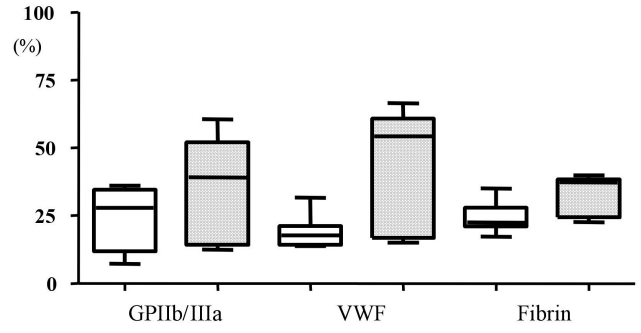

Fig. 4. Immunopositive areas (%) of rabbit venous thrombus and pulmonary thromboembolus. Open box, venous thrombus; shaded box, pulmonary thromboembolus (n=7 per group).

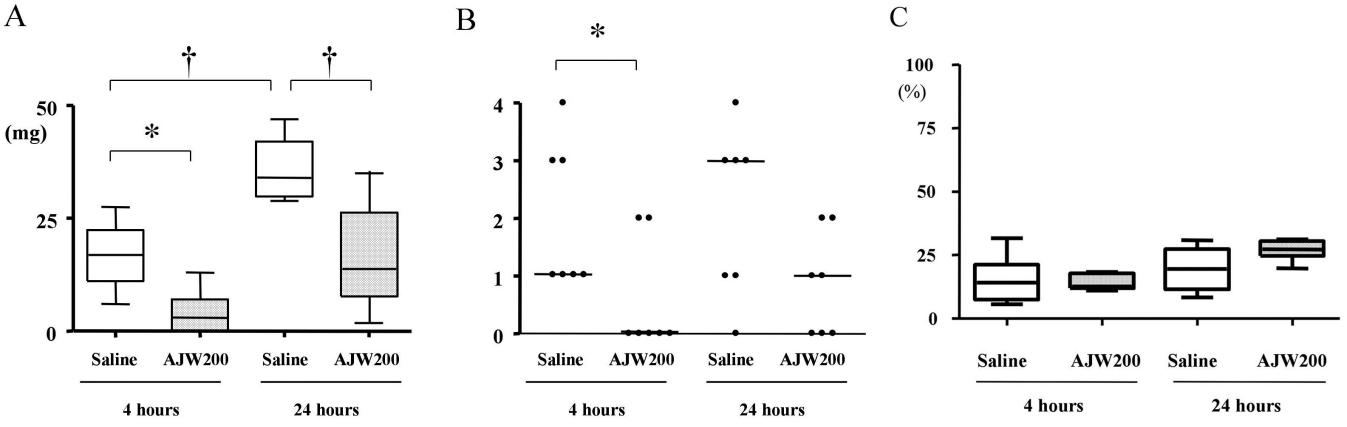

Fig. 5. Venous thrombus weight **(A)**, number of pulmonary thromboemboli **(B)**, and VWF-immunopositive areas (%) in rabbit venous thrombi **(C)** 4 or 24 hours after polyethylene tube insertion into rabbit iliac veins. Bar indicates median; *: $p<0.05$; †: $p<0.01$; n=7 per group.

has indicated that interaction between the VWF A1 domain and platelet GPIb, which is an initial and essential step in platelet adhesion, is particularly modulated under rapid flow conditions (Ruggeri, 1997; Goto, 2004; Mendolicchio and Ruggeri, 2005). However, some studies have shown that the interaction works even under static conditions in vitro (Savage et al., 1992) or venous condition (Andre et al., 2000). We previously reported that VWF contributes to fibrin-rich arterial thrombus propagation even when flow is reduced or disturbed (Yamashita et al., 2004). Moreover, Chauhan et al. recently demonstrated that VWF and FVIII are independently required to form stable occlusive thrombi in mouse mesenteric venules (Chauhan et al., 2007). These findings suggest that VWF within thrombus contributes to venous thrombus formation *in vivo*.

The inhibition of interaction between platelet GPIb and the VWF A1 domain reduced venous thrombus formation to some extent. Inhibition of the A1 or the A3 domain of VWF does not affect thrombus volume on the collagen surface at low shear rates (Ruggeri, 1997). On the other hand, inhibition of GPIb and a lack of VWF reduce thrombus formation on the fibrin surface at a low shear rate (Hantgan et al., 1990). Moreover, VWF is required for platelet tethering on platelet monolayers at a low shear rate (Kulkami et al., 2000). These and our results suggest that VWF contributes to platelet adhesion on thrombus, rather than on the vascular surface under low shear conditions.

The composition of thrombi in human DVT and PE did not significantly differ (Fig. 2). We considered that erythrocytes and fibrin might be more abundant in thrombi of PE than of DVT, because they originate from the tail portion of thrombi in DVT. Further examination is required to clarify this issue, because our autopsy study has obvious methodological limitations. The number of autopsies was small and we examined only residual mural thrombi (after PE onset) in the ilio-femoral veins in a limited number of histological specimens. Therefore, the present findings might not reflect the total areas of venous thrombi and pulmonary emboli. In addition, the patients had various underlying diseases and had received anti-thrombotic drugs that could affect the composition of thrombi. Therefore, we might have overestimated the role of VWF and underestimated the effects of underlying diseases and anti-thrombotic therapies. Despite these limitations, thrombi in DVT and PE always contained a considerable amount of platelets and VWF, suggesting that the size, rather than the content of venous thrombi, is crucial to the development of pulmonary embolism.

In summary, our autopsy and animal studies demonstrated that venous thrombi and pulmonary emboli are rich in VWF and platelets, as well as erythrocytes and fibrin, and that VWF might contribute to venous thrombus growth as well as the development of pulmonary emboli.

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