Revised version (#TH-17-11-0778.R1)

Original Articles: Cellular Haemostasis and Platelets

von Willebrand factor aggravates hepatic ischemia-reperfusion injury by promoting neutrophil recruitment in mice

Yasuyuki Urisono[1], Asuka Sakata[2], Hideto Matsui[3], Shogo Kasuda[4], Shiro Ono[3], Kiyomi Yoshimoto[3], Kenji Nishio[3], Masayuki Sho[5], Masashi Akiyama[6], Toshiyuki Miyata[7], Kazuo Okuchi[1], Satoshi Nishimura[2] and Mitsuhiko Sugimoto[3]

Departments of Emergency and Critical Care Medicine[1], General Medicine[3] , Legal Medicine[4] and Surgery[5], Nara Medical University, Kashihara, Japan, Research Division of Cell and Molecular Medicine[2], Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan, and Departments of Molecular Pathogenesis[6], and Cerebrovascular Medicine[7], National Cerebral and Cardiovascular Center, Suita, Japan,

Running title: VWF aggravates mouse hepatic I/R injury

Word count (Summary: 204, text: 2669)

***footnote:** Supported in part by grants from the SENSHIN Medical Research Foundation (to M. Sugimoto), the Takeda Science Foundation (to M. Sugimoto), and by the MEXT KAKENHI (#15K10990 to K.N.).*

Correspondence to Mitsuhiko Sugimoto, MD, Department of General Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan; e-mail: sugi-ped@naramed-u.ac.jp; or Satoshi Nishimura, MD, PhD, Research Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan; e-mail: snishi-tky@umin.ac.jp.

Summary

Hepatic ischemia-reperfusion (I/R) injury is a serious liver damage that critically influences the clinical outcome of liver surgery or transplantation. Since recent studies indicated the critical involvement of VWF in reperfusion injuries of brain and myocardium, we hypothesised that VWF-dependent thrombotic or inflammatory responses also play a role in hepatic I/R injury. Using a mouse model of hepatic I/R injury, we explored the functional relevance of the von Willebrand factor (VWF)-ADAMTS13 axis in this pathologic condition. Time-course studies during hepatic I/R revealed significantly lower alanine aminotransferase (ALT) values, as well as greater hepatic blood flow, in VWF gene-deleted (KO) mice in comparison with wild-type (WT) mice. Histological analysis revealed a significantly lesser extent of neutrophil infiltration and hepatocellular necrosis in liver tissues of VWF-KO mice. Human recombinant ADAMTS13 significantly improved the impairment in ALT values and hepatic blood flow and decreased neutrophil infiltration within the liver tissue of WT mice. Real-time intravital imaging successfully visualised significantly reduced leukocyte–vessel wall interactions in I/R liver of VWF-KO mice. Taken together, our results indicate that VWF promotes neutrophil recruitment in ischemic mouse liver, critically aggravating reperfusion injury, and suggest that functional regulation of VWF by ADAMTS 13 represents a promising therapeutic option for hepatic I/R injury.

Key words: von Willebrand factor, ADAMTS13, hepatic ischemia-reperfusion injury, neutrophil recruitment

Introduction

Ischemia-reperfusion (I/R) injury causes serious liver damage during liver surgery and is a major cause of graft dysfunction after liver transplantation (1, 2). Although the mechanisms of hepatic I/R injury are complex, an excessive inflammatory response is assumed to play a role in pathogenesis (1-3). In this regard, accumulation and activation of neutrophils recruited into the liver vasculature have been implicated in hepatic microvascular dysfunction associated with reperfusion injury (3, 4).

von Willebrand factor (VWF) mediates platelet adhesion and aggregation, particularly in microcirculation systems under high shear stress conditions, and the VWF-cleaving protease ADAMTS13 downregulates in vivo VWF function to prevent thrombotic occlusion of the microvasculature due to excessive VWF functions (5-7). In addition to such thrombotic properties, recent mouse model studies of brain stroke and myocardial infarction revealed the involvement of VWF-dependent inflammatory responses in reperfusion injuries of brain and myocardium, respectively (8, 9).

Accordingly, we hypothesised that VWF-dependent thrombotic or inflammatory responses play a role in hepatic I/R injury. Using a mouse model of hepatic I/R injury, we explored the functional relevance of the VWF-ADAMTS13 axis in this pathologic condition.

Materials and Methods

Mice

Wild-type (WT) and CAG-eGFP (GFP) mice were purchased from Japan SLC (Shizuoka, Japan), and VWF gene-deleted (KO) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). VWF-KO mice expressing GFP (GFP/VWF-KO mice) were generated by mating GFP and VWF-KO mice, and deletion of VWF was confirmed by genotyping. All mice used were of the C57BL/6 background.

Recombinant human ADAMTS13

Recombinant human ADAMTS13 (rADAMTS13) used in this study was described previously (10, 11). In brief, rADAMTS13, previously designated as MDTCS, spans from the metalloproteinase (M) domain to the spacer (S) domain (amino acid residues 75-685), and possesses VWF-cleaving activity equivalent to that of the full-length ADAMTS13 molecule, as determined by the FRETS-VWF73 assay (11, 12). In some experiments indicated, rADAMTS13 (3 μg/mouse equivalent to 2800 U/kg) was injected intravenously in WT mice 15 min before the I/R operation.

Mouse model of hepatic I/R injury

All animal experiments were approved by the Institutional Animal Care and Use Committee of Nara Medical University. Mouse experiments involving hepatic I/R injury were performed according to an established protocol (3, 13). Briefly, male mice (8-12 weeks of age) were anesthetized with sodium pentobarbital (60 mg/kg: subcutaneously injected), and a midline laparotomy was performed on a heating pad. Blood supply for the left lateral and median lobes of liver (approximately 70% of the liver mass) was interrupted

by cross-clamping the hepatic artery and portal vein with a microvascular atraumatic clip for 90 min. To prevent intestinal venous congestion, the caudal and right lateral lobes retained intact portal and arterial inflow and venous outflow. During the ischemic phase, hepatic blood flow (HBF), which was monitored on the surface of left lateral lobe by Laser Doppler flowmetry (ALF21, Advance Co, Tokyo, Japan), was maintained around 30% of pre-ischemic value. The clip was then removed to induce hepatic blood flow reperfusion. Peripheral blood was collected from the submandibular veins of mice 3h and 6h after reperfusion. After 24-h reperfusion, mice were sacrificed for blood collection and histological analysis of liver tissue. Excess blood loss was not observed in any mice throughout the surgical process.

Intravital microscopy

To visualise the ischemia-induced blood cell-vessel wall interactions in the liver microcirculation, we used an *in vivo* imaging system, according to an established experimental protocol described previously in detail (14-16), with minor modifications. Briefly, mice were anesthetized by the urethane injection (1.5 g/kg) after 2-h reperfusion following hepatic ischemia, and an incision was made so that the liver could be observed without being exteriorized. Mice were then subjected to real-time observation of liver vasculature by intravital microscopy (A1R MP, Nikon). RhodamineB-dextran (70 kDa, Sigma-Aldrich) showing blood flow, and Hoechst33342 staining nuclear DNA were injected via the tail vein, and the mice were secured to a heated stage (Thermoplate, Tokai Hit) as described (14-16). As a sham-operation control, the same intravital microscopy procedure was also performed without hepatic I/R operation in GFP or GFP/VWF-KO mice. In analyses of leukocyte-vessel wall interaction, 'rolling WBCs' were defined as those that moved at a velocity less than 80 μm/sec on the vessel wall, and 'adhering WBCs'

as those that remained stationary on vessel walls for more than 5 sec during a 5-min observation. 'Plugging WBCs' that shut down the blood flow of liver sinusoids were also calculated during 5-min observation periods. In analyses of platelet-vessel wall interaction, 'adhering platelets' were defined as those that remained stationary on vessel walls for more than 2 sec during 5-min observation. In addition, 'platelets adhering to WBCs' were defined as those moved along with WBCs in a flowing speed identical to that of WBCs, retaining the positional relationship between the platelet and WBC unchanged during 0.5-sec successive image frames. Thus, 'platelet-attached WBCs' were calculated during 5-min observation periods.

Statistics

All data are expressed as means ± standard deviation (SD). Differences between two groups of data were evaluated by Student's t-test. P values < 0.05 were considered statistically significant.

Results

Effects of gene deletion of VWF or rADAMTS13 on hepatic blood flow (HBF) or serum alanine aminotransferase (ALT) during the reperfusion phase in mouse hepatic I/R injury

Experimental hepatic I/R was induced in 16 WT mice and 12 VWF- KO mice. In another group of WT mice (n=12), rADAMTS13 was administrated before I/R operation. During the ischemic phase, the HBF was maintained around 30% of pre-ischemic value for 90 min (Figure 1A). At 1h after reperfusion, HBF of WT (mean ± SD; 58 ± 5%) was apparently lower than that of KO mice (87 ± 11%). The HBF value of WT mice in which rADAMTS13 was injected (88 ± 21%) was comparable to that of KO mice. These values stayed nearly unchanged even after 24h of reperfusion (Figure 1A).

Peripheral blood was collected from the submandibular veins of mice 3h and 6h after reperfusion. The mice were sacrificed for blood collection and histological analysis of liver tissue 24h after reperfusion. The ALT values of KO mice were significantly ($*P < 0.05$) lower than those of WT mice at all time points examined (Figure 1B; WT: 6898 ± 3270, 8572±3453, and 1313 ± 621 IU/L vs. KO: 3043 ± 1320, 4276 ±3187, and 478 ± 330 IU/L at 3 h, 6h, and 24 h after reperfusion, respectively). In addition, the ALT values in WT mice were significantly ($*P < 0.05$) decreased by the rADAMTS13 infusion (Figure 1B; ALT values of WT + rADAMTS13; 4358 ±2788, 5552±4556, and 698 ± 664 IU/L at 3 h, 6h, and 24 h after reperfusion, respectively). In WT or VWF-KO mice, no significant increase or decrease were observed in both platelet and WBC counts at any time points examined during the hepatic I/R approach (results not shown).

These time-course analysis of HBF (Figure 1A) and serum ALT values (Figure 1B) demonstrated that liver damage during reperfusion was less extensive in VWF-KO mice than in WT controls, and the administration of rADAMTS13 improved significantly liver

damages in WT mice.

Histological analysis of liver tissues in WT mice with or without rADAMTS13 infusion or VWF-KO mice during hepatic I/R

Sinusoidal congestion, massive hepatocellular necrosis and vacuolization were remarkable in the liver sections of WT mice, albeit without typical thrombotic lesions of micro-vessels (Figure 2A). Liver damage was less severe in KO mice, in which the lobular architecture of hepatocytes was modestly preserved and cellular infiltration was reduced. Consistent with the ALT data (see Figure 1), rADAMTS13 infusion ameliorated liver damage in WT mice (Figure 2A). Statistical analysis of liver tissue (Figure2B) revealed also the significant reduction in neutrophil infiltration in VWF-KO mouse liver. In addition, we confirmed that administration of rADAMTS13 reduced significantly neutrophil infiltration in WT mice (Figure2B).

Intravital imaging for leukocyte-platelet-vessel wall interaction in mouse hepatic I/R liver

To elucidate cellular dynamics in the liver vasculature at the reperfusion phase following ischemia, we modified an intravital imaging system which was originally developed for *in vivo* observation of laser-induced thrombus formation (14-16).

Our novel system to visualise the ischemia-induced blood cell-vessel wall interactions revealed that leukocyte rolling and adhesion at the liver microcirculation were significantly reduced in VWF-KO mice (Figure 3A and Supplemental Video 1-2). Indeed, statistical analyses (Figure 3B-C) confirmed that numbers of both rolling and adhering leukocytes were significantly ($*P < 0.05$) less abundant in VWF-KO mice during 5-min observation periods. In addition, plugging leukocytes that shut down the bloodstream at the

smaller branched vessels were significantly (*$P < 0.05$) less abundant in VWF-KO mice (Figure 3D-E and Supplemental Video 1-2). In terms of platelet interaction with vessel walls, individual platelet adhesion was significantly (*$P < 0.05$) reduced in VWF-KO mice in comparison with GFP-control mice in the present I/R paradigm, while appreciable platelet aggregate formation on vessel walls were not detectable in both mice (Figure 4A-B). Further, platelet-leukocyte interaction which was grasped by the count of circulating platelet-attached WBC was also significantly (*$P < 0.05$) reduced in VWF-KO mice in the liver vasculatures (Figure 4A, 4C).

Discussion

Our results altogether clearly demonstrate the lesser liver damages in VWF-KO mice, in comparison with WT mice, in hepatic I/R injury. Given that VWF contributes to neutrophil accumulation within infarct lesion, which may aggravate acute myocardial infarction (9), the reduced neutrophil infiltration in KO mice is likely to explain beneficial effects on hepatic I/R injury.

However, it is important to take into account the fact that VWF-KO mice, in addition to completely lacking VWF, also lack intact Weibel-Palade bodies (17), resulting in impairment of P-selectin functions that are also important for leukocyte-vessel wall interactions. In this regard, previous studies demonstrated that blockage of VWF by the function-blocking antibody significantly decreased neutrophil extravasation in WT mice (18), and inversely, that administration of VWF in VWF-KO mice significantly increased tissue neutrophil accumulation (19), suggesting a substantial contribution of VWF in neutrophil recruitment. Indeed, effects of rADAMTS13 in the present study further support the above idea, while P-selectin also plays an important role in this context.

Although we could not detect any appreciable platelet aggregate formation in microvasculature in the present I/R paradigm, our novel intravital imaging system successfully revealed significantly decreased leukocyte-vessel wall interactions in VWF-KO mice. It should be noted, however, that due to some technical difficulties in observation of liver arterial vessels, our results had to be obtained in typical venous systems. Because the VWF functions are shear rate-dependent (5-7, 20), it is reasonable to assume that the contribution of VWF to leukocyte recruitment could be larger in liver arterial systems such as small arterioles or arterial capillaries, where blood flow creates greater shear stress.

Precise mechanisms for VWF-dependent leukocyte-vessel wall interaction in liver

vasculature remain to be addressed so far. In this regard, previous studies suggested that leukocyte could directly interact with VWF bound to endothelial cells via P-selectin glycoprotein ligand 1 and β2-integrins on leukocyte surfaces (21, 22). Since these leukocyte membrane receptors are known to bind to platelets as well, leukocytes can interact indirectly with vessel walls through platelet aggregates formed by VWF on endothelial cells (22-24). Indeed, our intravital imaging showed that both platelet interaction with vessel walls and WBCs are significantly reduced in VWF-KO mice (Figure 4), suggesting a relevant role of platelets on this disease state in a VWF-mediated manner. The latter mechanism associated with platelet aggregates may be more relevant under high shear stress conditions such as arterial liver sinusoids. Considering the shear rate-dependent property of VWF, platelet aggregates on VWF bound to stimulated endothelial cells could support subsidiary firm leukocyte adhesion against heightened rheological forces under such high shear stress conditions. In this regard, effects of rADAMTS13 in our study may imply a crucial role of ultra-large VWF strings bound to stimulated endothelial cells, which were released upon ischemia stimuli (24, 25).

In conclusion, VWF can promote leukocyte rolling and adhesion on endothelium in liver vasculature, perhaps in a concerted manner with P-selectin, eventually resulting in leukocyte extravasation and infiltration that cause liver tissue damages (Figure 5). Leukocyte capillary plugging, in addition to platelet micro-aggregates, contribute to microcirculation insufficiency. Thus, the series of VWF-dependent leukocyte recruitment are thought to play a determining role in the pathogenesis of hepatic I/R injury (Figure 5), while VWF-dependent hepatocellular apoptosis may also play a role in this context as previously suggested in the study for myocardial infarction (9). Because intentional ischemia and reperfusion, such as Pringle's maneuver, is indispensable for decreasing intraoperative bleeding during major liver surgeries (1, 2), the functional regulation of

VWF by ADAMTS 13, perhaps with minimized bleeding risk as previously suggested (20, 26), may have powerful therapeutic potential for hepatic I/R injury.

Acknowledgements

We thank Ayuri Nakamura for technical assistance.

Conflicts of interest

None declared.

References

1. Bahde R, Spiegel HU. Hepatic ischaemia–reperfusion injury from bench to bedside. *Br J Surg*. 2010; 97:1461-1475.

2. Peralta C, Jimenez-Castro MB, Gracia-Sancho J. Hepatic ischemia and reperfusion injury: Effects on the liver sinusoidal milieu. *J Hepatol*.2013; 59: 1094-1105

3. Abe Y, Hines IN, Zibari G, et al. Mouse model of liver ischemia and reperfusion injury: Method to study reactive oxygen and nitrogen metabolites in vivo. *Free Radic Biol Med*. 2009; 46: 1–7.

4. Yadav S, Howell DN, Steeber DA, et al. P-selectin mediates reperfusion injury through neutrophil sequestration in the warm ischemic mouse liver. *Hepatology*. 1999; 29:1494-1502.

5. Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell interactions. *J Thromb Haemost*. 2003;1:1335-1342.

6. Sadler JE. Von Willebrand factor, ADAMTS13, and thrombotic thrombocytopenic purpura. *Blood*. 2008;112:11-18.

7. Tsai HM. Shear stress and von Willebrand factor in health and disease. *Semin Thromb Hemost*. 2003; 29:479-488.

8. Fujioka M, Hayakawa K, Mishima K, et al. ADAMTS13 gene deletion aggravates ischemic brain damage: a possible neuroprotective role of ADAMTS13 by ameliorating postischemic hypoperfusion. *Blood*. 2010; 115:1650-1653.

9. De Meyer SF, Savchenco AS, Haas MS, et al: Protective anti-inflammatory effect of ADAMTS13 on myocardial ischemia/reperfusion injury in mice. *Blood.* 2012; 120: 5217-5223.

10. Akiyama M, Nakayama D, Takeda S, et al. Crystal structure and enzymatic activity of an ADAMTS-13 mutant with the East Asian-specific P475S polymorphism. *J Thromb Haemost.* 2013; 11: 1399-1406.

11. Doi M, Matsui H, Takeda H, et al: ADAMTS13 safeguards the myocardium in a mouse model of myocardial infarction. *Thromb Haemost.* 2012; 108:1236-1238.

12. Kokame K, Nobe Y, Kokubo Y, et al. FRETS-VWF73. A first fluorogenic substrate for ADAMTS13 assay. *Br J Haematol.* 2005; 129: 93-100.

13. Akahori T, Sho M, Hamada K, et al. Importance of proliferator-activated receptor-γ in hepatic ischemia/reperfusion injury in mice. *J Hepatol.* 2007; 47: 784-792

14. Takizawa H, Nishimura S, Takayama N, et al. Lnk regulates integrin alphaIIbbeta3 outside-in signaling in mouse platelets, leading to stabilization of thrombus development in vivo. *J Clin Invest.* 2010; 120:179–190.

15. Nishimura S, Manabe I, Nagasaki M, et al. In vivo imaging visualizes discoid platelet aggregations without endothelium disruption and implicates contribution of inflammatory cytokine and integrin signaling. *Blood.* 2012;119: e45-e56.

16. Nishimura S, Nagasaki M, Kunishima S, et al. IL-1α induces thrombopoiesis through megakaryocyte rupture in response to acute platelet needs. *J Cell Biol.* 2015; 209: 453-466.

17. Denis CV, André P, Saffaripour S, et al. Defect in regulated secretion of P-selectin affects leukocyte recruitment in von Willebrand factor-deficient mice. *Proc Natl Acad Sci USA.* 2001; 98: 4072-4077.

18. Petri B, Broermann A, Li H, et al. von Willebrand factor promotes leukocytes extravasation. *Blood.* 2010; 116: 4712-4719.

19. Kasuda S, Matsui H, Ono S, et al. Relevant role of von Willebrand factor in neutrophil recruitment in a mouse sepsis model involving cecal ligation and puncture. *Haematologica.* 2016; 101: e52-e54.

20. Shida Y, Nishio K, Sugimoto M, et al. Functional imaging of shear-dependent activity of ADAMTS13 in regulating mural thrombus growth under whole blood flow conditions. *Blood.* 2008; 111:1295-1298.

21. Pendu R, Terraube V, Christophe OD, et al. P-selectin glycoprotein ligand 1 and beta2-integrins cooperate in the adhesion of leukocytes to von Willebrand factor. *Blood.* 2006;108:3746-3752.

22. Swystun LL, Liaw PC. The role of leukocytes in thrombosis. *Blood.* 2016; 128: 753-762.

23. Drakeford C, O'Donnell JS. Targeting von Willebrand factor-mediated inflammation. *Arterioscler Thromb Vasc Biol.* 2017;37:1590-1591.

24. Bernardo A, Ball C, Nolasco L, Choi H, Moake JL, Dong JF. Platelets adhered to endothelial cell bound ultra-large von Willebrand factor strings support leukocyte tethering and rolling under high shear stress. *J Thromb Haemost.* 2005;3:562-570.

25. Dhanesha N, Prakash P, Doddapattar P, et al. Endothelial Cell-Derived von Willebrand factor is the major determinant that mediates von Willebrand factor-dependent acute ischemic stroke by promoting postischemic thrombo-inflammation. *Arterioscler Thromb Vasc Biol*. 2016;36:1829-1837.

26. Zhao B-Q, Chauhan AK, Canault M, et al. von Willebrand factor-cleaving protease ADAMTS13 reduces ischemic brain injury in experimental stroke. *Blood.* 2009;114:3329-3334.

Figure Legends

Figure 1. Effects of gene deletion of VWF or rADAMTS13 on hepatic blood flow (HBF) and serum alanine aminotransferase (ALT) in mouse hepatic I/R injury. Hepatic ischemia and reperfusion (I/R) was induced in WT mice and VWF- KO mice (see the upper horizontal bar representing the I/R protocol). In another group of WT mice, rADAMTS13 was injected intravenously 15 min before the I/R operation. (A) Effects of gene deletion of VWF or rADAMTS13 on HBF during the reperfusion phase. HBF at 1h, 3h, and 24h after reperfusion were expressed as the percentage of pre-ischemic value (mean ± SD). During the ischemic phase, HBF was maintained around 30% of pre-ischemic value for 90 min. At 1h after reperfusion, HBF of WT (♦) was apparently lower than that of KO mice (Δ). The HBF value of WT mice in which rADAMTS13 was injected (○) was comparable to that of KO mice. These values stayed nearly unchanged even after 24h of reperfusion. (B) Effects of gene deletion of VWF or rADAMTS13 on serum ALT during the reperfusion phase. Bars indicate the mean ± SD of ALT values at the indicated time points. Note that the ALT values of KO mice were significantly lower than those of WT mice at all time points examined. Note also that the ALT values in WT mice were significantly decreased by the rADAMTS13 infusion.

Figure 2. Histological analysis of liver tissues in WT mice with or without rADAMTS13 infusion or VWF-KO mice during hepatic I/R. Mice were sacrificed for histological analysis of liver tissue 24h after reperfusion. (A) Images of samples stained with hematoxylin-eosin (original magnification, 100× and 200×) are each representative of five independent mouse samples. Sinusoidal congestion, massive hepatocellular necrosis and vacuolization were remarkable in the liver sections of WT mice (left panels). Liver

damage was less severe in KO mice (middle panels). rADAMTS13 infusion ameliorated liver damage in WT mice (right panels). (B) Statistical analyses of infiltration of neutrophils in liver tissues of WT mice with or without rADAMTS13 infusion, or VWF-KO mice during hepatic I/R. The number of multinucleated cells (corresponding to neutrophils) was measured in defined areas within the above images; each bar represents the mean (±SD) number of 15 areas (1 mm^2 each; three areas were randomly selected from five individual mouse samples). These analyses indicate that neutrophil numbers in liver tissues were significantly ($*P < 0.05$) reduced in VWF-KO mice or after administration of rADAMTS13 to WT mice ($*P < 0.05$).

Figure 3. Intravital imaging of liver vasculature in mouse hepatic I/R: Effects of gene deletion of VWF on leukocyte-vessel wall interaction. Experimental conditions for hepatic ischemia and reperfusion (I/R) are essentially the same as those described in the Figure 1 legend. After 2-h reperfusion following hepatic ischemia, GFP-control (n=5) or GFP/VWF-KO (n=4) mice were subjected to real-time observation of liver vasculature by intravital microscopy. As a sham-operation control, the same intravital microscopy procedure was also performed without hepatic I/R operation in GFP (n=3) or GFP/VWF-KO (n=3) mice. (A) Intravital images of hepatic vein branches. Cells are visualized in green. Injected dextran (red) shows blood flow, and Hoechst (blue) indicates nuclear DNA (see also Supplemental Video1-2). White arrows indicate rolling or adhering leukocytes (WBC) on vessel walls. (B, C) Statistical analyses of rolling or adhering WBCs during hepatic I/R. Rolling or adhering WBCs were calculated during 5-min observation of hepatic vessels in the intravital images shown in the left panels (six and 14 vessels out of five GFP-control and four GFP/VWF-KO mice that underwent the hepatic I/R operation were examined, respectively; and four and eight vessels out of three GFP-control and three GFP/

VWF-KO mice that underwent the sham operation, respectively). Bars indicate the mean (±SD) number of rolling or adhering WBCs. Note that both rolling and adhering WBCs were significantly (*$P < 0.05$) less abundant in VWF-KO mice. (D) Plugging WBCs at hepatic vein branches in intravital images. Images were taken from similar locations as in the videos described in Figure 3A (see Supplemental Video 1-2). Large vessels located in the center of images are hepatic venules, and the smaller branched vessels are liver sinusoids. White arrows indicate plugging WBCs that shut down the blood flow of liver sinusoids during 5-min observation periods. (E) Statistical analyses of plugging WBCs during hepatic I/R. Plugging WBCs were calculated during 5-min observation of hepatic vessels in the intravital images shown in the left panels. Bars indicate the mean (±SD) number of plugging WBCs. Note that plugging WBCs were significantly (*$P < 0.05$) less abundant in VWF-KO mice.

Figure 4. Intravital imaging of liver vasculature in mouse hepatic I/R: Effects of gene deletion of VWF on the platelet interaction with vessel walls or leukocytes. The origin of Images shown in this figure were the same videos described in the Figure 3 (see Supplemental Video 1-2). (A) Intravital images of hepatic vein branches in GFP-control mice that underwent the hepatic I/R operation. The right image shows the same location of left image taken after 0.5 second. Lower insets represent each corresponding high-power field of indicated portion of upper images, in which green arrows indicate platelets adhering to vessel walls and orange arrows indicate platelets which attached to WBCs. Thus, blue arrows indicate platelet-attached WBCs. (B) Statistical analyses of adhering platelets or platelet-attached WBCs during hepatic I/R. Platelet count adhering to vessel walls (left panel) were calculated during 5-min observation period of hepatic vessels in the intravital microscopy (see Supplemental Video 1-2). Platelet-attached WBCs (right panel) are

expressed as the percentage of the total flowing WBCs recognized during 5-min observation period. Bars indicate the mean ±SD, respectively. Note that both the platelet interaction with vessel walls and WBCs are significantly (*$P < 0.05$) reduced in VWF-KO mice in the hepatic I/R approach.

Figure 5. Schematic summary of VWF-dependent mechanisms for hepatic ischemia-reperfusion injury. Ischemic stimuli activate sinusoidal endothelial cells (EC) in liver vasculature, resulting in the release of larger VWF multimer from EC and plasma VWF deposition on activated EC. Then, platelet adhesion and aggregation proceeds on VWF bound to EC. At the reperfusion phase, neutrophil rolling and adhesion are promoted on VWF bound to EC in the presence or absence of platelet aggregates, resulting in neutrophil extravasation and infiltration that cause liver tissue damages. Leukocyte capillary plugging, in addition to platelet micro-aggregates, contribute to microcirculation insufficiency. ADAMTS13 downregulates the VWF functions by cleaving larger VWF multimer, reducing neutrophil recruitment and liver I/R injury.

Extra table

1. **'What is known on this topic'**

   · Hepatic ischemia-reperfusion (I/R) injury is serious liver damage that critically influences the clinical outcome of liver surgery or transplantation.

   · Although the mechanisms of hepatic I/R injury are complex, an excessive inflammatory response is assumed to play a role in pathogenesis

   · Recent studies indicated the critical involvement of VWF in reperfusion injuries of brain and myocardium

2. **'What this paper adds'**

   · Using a mouse experimental model of hepatic ischemia-reperfusion (I/R) injury, we demonstrated the relevant role of von Willebrand factor (VWF) that aggravates I/R injury in mouse liver.

   · Our novel intravital imaging system successfully enabled real-time visualisation for ischemia-induced leukocyte-vessel wall interaction in liver micro-vasculature, revealing a crucial involvement of VWF-dependent leukocyte recruitment in hepatic I/R injury.

   · Our results suggest that the functional regulation of VWF by ADAMTS 13 may have powerful therapeutic potential for hepatic I/R injury.