Original article

Regulation of von Willebrand factor by ADAMTS13 ameliorates lipopolysaccharide-induced lung injury in mice

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Abstract

The relationship between von Willebrand factor (VWF) and inflammation has attracted considerable attention in recent years. VWF is stored in the Weibel–Palade bodies (WPBs) of endothelial cells (ECs), is released from WPBs in response to inflammatory stimuli, and is thought to contribute to inflammation by promoting leukocyte extravasation. In this study, mice were intratracheally injected with lipopolysaccharides to establish a lung injury model. The severity of lung inflammation was evaluated in mice with different genotypes (wild-type, *Vwf* $^{-/-}$, *Adamts13* $^{-/-}$) or mice treated with drugs that inhibit VWF function. Lung inflammation was significantly ameliorated in *Vwf* $^{-/-}$ mice as compared to wild-type mice. Furthermore, inflammation was significantly suppressed in wild-type mice treated with anti-VWF A1 antibody or recombinant human ADAMTS13 as compared with the untreated control group. The underlying mechanism appears to be an increased VWF/ADAMTS13 ratio at the site of inflammation and the interaction between blood cell components, such as leukocytes and platelets, and the VWF A1 domain, which promotes leukocyte infiltration into the lung. This study suggested that VWF-targeting agents, such as ADAMTS13 protein, may be a novel therapeutic option for treatment of pulmonary inflammatory diseases. (198 words)

INTRODUCTION

Von Willebrand Factor (VWF) is a multimeric glycoprotein synthesized in endothelial cells (ECs) and bone marrow megakaryocytes. It plays an important role in hemostasis. In ECs, the VWF protein is stored in a folded form in cytoplasmic granules called Weibel–Palade bodies (WPBs). Multimerized VWF proteins with high molecular weights are highly active. Various stimuli, such as inflammation and mechanical stress, induce the release of VWF to the lumen surface of endothelium. Exposed VWF multimers, which are extended by the shear stress of blood flow to capture platelets, are rapidly cleaved by ADAMTS13 (a disintegrin-like metalloproteinase with thrombospondin type 1 motifs 13) [1–4]. The cleavage of VWF is required to maintain appropriate hemostatic activity.

In recent years, much attention has been paid to the crosstalk between blood coagulation, inflammation, and immunity [5,6]. Coagulation factors act as inflammatory mediators or immune modifiers, and stimuli from inflammatory or immune responses activate coagulation. A specific locus in the A1 domain of VWF can bind to P-selectin glycoprotein ligand-1 (PSGL1) on leukocytes, facilitating leukocyte rolling on ECs [7]. Leukocyte recruitment is reduced in $Vwf^{-/-}$ mice as compared to $Vwf^{+/+}$ mice in a meningitis model [8], whereas inflammation is also suppressed in VWF-deficient status in a skin inflammation model [9].

Most coagulation-related factors are produced in the liver, particularly in hepatocytes, whereas VWF is synthesized in the ECs of systemic vascular networks. In this study, we focused on the role of VWF in the lungs because these are among the most vascularized organs. The lung is an organ that is exposed to physical mechanical stresses associated with respiratory movements and is a major route of bacterial and viral invasion. However, the role of VWF in local hemostasis and pulmonary inflammation has not yet been fully elucidated.

Therefore, in this study, we investigated the role of VWF in pulmonary hemostasis and

inflammation using a bacterial lipopolysaccharide (LPS)-induced acute bacterial mouse model.

MATERIALS AND METHODS

Animals

Vwf $^{-/-}$ mice, *Adamts13* $^{-/-}$ mice, and their genetically-matched control wild-type mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and National Institutes of Biomedical Innovation, Health and Nutrition (Osaka, Japan), respectively [10,11]. Generation of low tissue factor mice *(mF3* $^{-/-}$ */ hF3* $^{+/+}$) was described in previous studies [12,13]. All mice used in this study were of C57BL/6 background and were used for the experiments at 8–12 weeks of age. Mice were placed in cages in a temperature-controlled room with a 12-h light/dark cycle (8:00 a.m. lights on, 8:00 p.m. lights off). Food and water were provided *ad libitum*. Experimental protocols were developed in accordance with the guidelines outlined by the Institutional Animal Committee of Nara Medical University (No.12697 and 13452).

Mice experiments and tissue collection

Mice were anesthetized by isoflurane inhalation (Pfizer, New York, NY, USA) and intratracheally instilled with lipopolysaccharide from *Escherichia coli* O111:B4 (LPS; Sigma-Aldrich, St. Louis, MO, USA) via the nostrils at a dose of 2 mg/kg (approximately 50 μL). The same volume of phosphate-buffered saline (PBS, Fujifilm, Tokyo, Japan) was used as a control. Recombinant full-length human ADAMTS13 protein (RHA) [14] or the murine anti-human VWF A1 domain antibody NMC/VW4 [15–17] were intravenously injected into the retro-orbital plexus at doses of 80 μg/kg or 0.4 mg/kg body weight (approximately 200 or 100 μL), respectively, immediately before LPS instillation. The concentration of ADAMTS13 in the RHA solution was determined using ELISA as described below. The same volume of PBS

and/or normal mouse IgG (Fujifilm) were used as a control for RHA and NMC/VW4.

Three days after LPS or PBS instillation, the mice were euthanized by exsanguination and cervical dislocation under isoflurane anesthesia, after terminal blood was drawn from the inferior vena cava using 3.8% sodium citrate (Muto Pure Chemicals, Tokyo, Japan). Bronchoalveolar lavage fluid (BALF) and lung tissue samples were collected. BALF was collected from mice by postmortem lavage with 3 × 900 μL of ice-cold PBS. BALF samples were centrifuged (500 *g*, 20 min, 4°C), and the supernatant and the cell pellets were separated. The cell pellets were resuspended in 200 μL of PBS. The number of red blood cells (RBCs) and white blood cells (WBCs) in the resuspended cell pellets was determined using an automated blood cell counter (Thinka CB-1010, Arkray, Kyoto, Japan). After BALF collection, the lung tissues were extracted, minced, immediately frozen in liquid nitrogen, and stored at -80°C until used. The citrated whole blood samples were centrifuged (4500 *g*, 15 min, 22°C) after blood cell counting with an automated blood cell counter. Then, the plasma samples were stored at -80°C until used.

RNA isolation and real-time PCR

Total RNA was isolated from lung tissues by using the TRIzol™ method (Thermo Fisher Scientific, Waltham, MA, USA). One microgram of total RNA was reverse transcribed into cDNA using a high-capacity RNA-to-cDNA™ kit (Thermo Fisher Scientific). VWF, pro-inflammatory cytokines, and chemokine mRNA expression levels were measured by quantitative real-time PCR (qPCR) using StepOne Plus (Applied Biosystems, Waltham, MA, USA). The TaqMan probes and primers used for the analysis of these genes were chosen from the TaqMan Gene Expression Assay (Applied Biosystems), as listed in Supplementary Table 1.

Enzyme-linked immunosorbent assay

The protein levels of VWF in the plasma and BALF supernatants were assessed using a mouse Von Willebrand Factor A2 ELISA kit (ab208980; Abcam, Cambridge, UK). Protein levels of pro-inflammatory cytokines and chemokines in the BALF supernatants were assessed by DuoSet® ELISA kits (R&D Systems, Minneapolis, MN, USA) as listed in Supplementary Table 2. All BALF samples were evaluated without dilution and assays were performed according to the manufacturer's instructions. Plasma levels of ADAMTS13 were determined by chromogenic ADAMTS13-act–enzyme-linked immunosorbent assay (ELISA) (Kainos, Tokyo, Japan) [18].

Histological analyses

LPS- or PBS-treated mice were euthanized in the same manner 3 days after instillation, as mentioned above. The lungs were inflated by intratracheal injection of 500 μL of 10% formalin neutral buffer solution (Fujifilm). The lungs were excised and fixed in 10% formalin. After paraffin embedding, lung sections were subjected to hematoxylin and eosin (HE) staining and VWF immunostaining using an anti-human VWF antibody (A0082, Dako, Santa Clara, CA, USA). Images were captured and analyzed using a light microscope (BZ-X710; Keyence, Osaka, Japan). Quantitative analyses of the obtained images were performed using Image J software and the Hybrid Cell Count module built into the Keyence microscope system.

Vascular permeability

Pulmonary vascular permeability after LPS instillation was analyzed by two different methods, measuring the lung wet/dry ratio and FITC-dextran [19]. To measure the lung wet/dry ratio, LPS- or PBS-treated mice were anesthetized and the lungs were perfused with 10 mL of PBS through the right ventricle on day 3. After euthanasia, the whole lung was collected and

weighed to determine the “wet weight.” After drying at 55°C for 48 h, the dried lung was weighed as “dry weight”, and then the wet/dry ratio was calculated. In the FITC-dextran method, LPS- or PBS-treated mice were retro-orbitally injected with FITC-dextran (MW:70000, Sigma–Aldrich) at a dose of 20 mg/kg body weight (approximately 200 μL) at day 3. One hour later, the mice were euthanized and BALF and whole blood were collected. The concentration of FITC-dextran in BALF supernatants and plasma was calculated from their luminescence (λ em. 522 nm/ λ ex. 498 nm). The FITC-dextran concentration in the BALF supernatants was normalized to the concentration in plasma.

Statistical analyses

All statistical analyses were performed using GRAPHPAD PRISM (version 5.01, GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard deviation. Two-group comparisons of continuous data were performed using two-tailed Student’s *t*-tests. For multiple-group comparisons, data were analyzed using one-way or two-way analyses of variance (ANOVAs) and Bonferroni correction for repeated measures over time. A *P*-value of < 0.05 was considered statistically significant.

Results

Vwf gene expression in different organs

First, we investigated *Vwf* mRNA levels in different organs, including the brain, lung, heart, liver, kidney, intestine, and spleen of wild-type C57BL/6 mice (Figure 1A). The highest level of *Vwf* mRNA expression was observed in the lungs, likely due to the high levels of vasculature in the lungs.

Time course of LPS-induced lung injury model

Next, LPS (2 mg/kg) was intratracheally instilled in wild-type C57/BL6 mice and their body weight and WBC count in the BALF were assessed before and 1, 2, 3, and 4 days after instillation. The peak of both the WBC count increase and of body weight decrease were observed on day 3, suggesting that the inflammation induced by LPS instillation was most prominent around day 3. Therefore, we used day 3 in subsequent experiments (Figure 1D). Both the WBC count and body weight returned to baseline by day 7, and no mice died during the observation period.

Expression of VWF in the murine LPS-induced lung injury model

Changes in VWF expression in the murine LPS-induced lung injury model were evaluated using wild-type C57/BL6 mice. *Vwf* mRNA and VWF protein levels in the lungs and VWF protein levels in the BALF supernatant and plasma were quantified using qPCR and ELISA, respectively. Interestingly, *the Vwf* mRNA levels in the lungs of LPS-treated mice were significantly lower than those in the PBS-treated control group (Figure 2A). In contrast, the amount of VWF protein in the BALF supernatant was significantly higher in the LPS-treated groups than in the PBS-treated group (Figure 2B). There were no significant differences in the plasma VWF protein levels between the PBS- and LPS-treated groups (Figure 2C). To evaluate the localization of the VWF protein in the lungs, VWF was stained with an anti-VWF antibody. In both groups, VWF was strongly detected in vascular endothelial cells and weakly stained in alveolar epithelial cells (Figure 2D). In the LPS group, infiltration of the VWF protein into the lung parenchyma was slightly enhanced. The thickness of VWF-stained area of randomly selected large blood vessels in the lungs of LPS-treated groups was significantly reduced compared with those of PBS-treated group (2.26 ± 0.65 μm and 1.31 ± 0.49 μm for PBS- and LPS-treated group, respectively) (Figure 2E). Taken together, these results suggested that intratracheally instilled LPS stimulated lung endothelial cells to release intracellularly

stored VWF into the parenchyma and airspace.

Effects of VWF deficiency on LPS-induced lung injury

To determine if VWF contributes to pulmonary hemostasis in the LPS-induced lung injury model, RBC counts in the BALF of *Vwf*$^{+/+}$ and *Vwf*$^{-/-}$ mice were determined at 3 days after LPS instillation (Figure 3A and 3B). As a positive control for this experiment, low tissue factor mice (*mF3* $^{-/-}$ / *hF3* $^{+/+}$), which possess < 1% of normal tissue factor expression levels, were similarly evaluated. As shown in Figure 3B, low tissue factor mice exhibited significant pulmonary hemorrhage, as evidenced by an increase in the number of RBCs in the BALF and its red color, as compared to control mice (*mF3* $^{+/+}$ / *hF3* $^{+/+}$), as previously reported [20]. On the other hand, *Vwf*$^{-/-}$ mice showed no increase in pulmonary hemorrhage, as compared to control *Vwf*$^{+/+}$ mice (Figure 3A).

Next, we evaluated the gene expression of the pro-inflammatory cytokines IL6 (*Il6*), TNFα (*Tnf*), and IL1β (*Il1b*), and chemokines MCP1 (*Ccl2*), KC (*Cxcl1*), and MIP2α (*Cxcl2*) in the lung tissue to investigate the effect of VWF-deficiency on LPS-induced pulmonary inflammation (Figure 3C). mRNA expression of all these pro-inflammatory cytokines and chemokines assessed were elevated by LPS instillation in both the *Vwf*$^{+/+}$ and *Vwf*$^{-/-}$ mice groups. However, *Vwf*$^{-/-}$ mice exhibited lower levels of the mRNAs than did the *Vwf*$^{+/+}$ mice.

Protein levels of IL6, TNFα, IL1β, MCP1, and KC in the BALF supernatant were quantified by ELISA (Figure 3D). No significant differences in the protein levels of these inflammatory mediators were found between *Vwf*$^{+/+}$ and *Vwf*$^{-/-}$ mice after administration of LPS, but all the values tended to be lower in *Vwf*$^{-/-}$ mice than in *Vwf*$^{+/+}$ mice.

Next, we evaluated the lung vascular permeability of LPS- or PBS-treated mice using two different methods: measurement of the lung wet/dry ratio and FITC-dextran. Vascular permeability is generally increased by inflammatory stimuli, such as LPS. Organs with enhanced vascular permeability become edematous and their wet weight increases. We compared the lung wet/dry ratios between *Vwf*$^{+/+}$ and *Vwf*$^{-/-}$ mice (Figure 3E). In the second method, FITC-dextran was intravenously injected into LPS- or PBS-treated mice and the amount of dextran extravasated into the BALF was quantified and compared between *Vwf*$^{+/+}$ and *Vwf*$^{-/-}$ mice (Figure 3F). In both methods, the levels of vascular permeability were enhanced by LPS instillation, but the degree of increase in *Vwf*$^{-/-}$ mice was significantly lower than those in *Vwf*$^{+/+}$ mice.

To analyze the extravasation of leukocytes, hematoxylin and eosin-stained lung sections were observed microscopically. Figure 3G shows representative tissue sections of LPS- and PBS-treated, *Vwf*$^{+/+}$ and *Vwf*$^{-/-}$ mice. The number of mononuclear cells extravasated into lung parenchyma was higher in LPS-treated *Vwf*$^{+/+}$ mice than in LPS-treated *Vwf*$^{-/-}$ mice (Figure 3H).

Taken together, our data showed that VWF-deficiency is associated with reduced LPS-induced lung vascular permeability, leukocyte extravasation, and pulmonary inflammation.

LPS-induced lung injury in *Vwf*$^{+/-}$ mice.

To investigate whether the suppression of inflammation by VWF-deficiency (Figure 3) was dependent on the level of VWF expression, we generated *Vwf*$^{+/-}$ mice by crossbreeding

Vwf$^{+/+}$ and *Vwf*$^{-/-}$ mice. The mice were then subjected to LPS instillation. The lung mRNA levels and BALF protein levels of inflammatory cytokines and chemokines in *Vwf*$^{+/+}$, *Vwf*$^{+/-}$, and *Vwf*$^{-/-}$ mice were quantified by qPCR (Supplementary Figure 1A) and ELISA (Supplementary Figure 1B), respectively. In terms of both mRNA and protein expression, *Vwf*$^{+/-}$ mice demonstrated intermediate values between *Vwf*$^{+/+}$ and *Vwf*$^{-/-}$ mice (slightly closer to *Vwf*$^{-/-}$ mice), suggesting that LPS-induced lung inflammation is quantitatively dependent on the expression level of VWF.

Effects of genetic lack of ADAMTS13 and administration of recombinant human ADAMTS13 protein on LPS-induced lung injury

ADAMTS13 regulates the size of VWF multimers in the blood by cleaving highly active VWF multimers to an appropriate size. Its deficiency results in an excess of ultra-large VWF multimers (UL-VWFM), leading to a prothrombotic state. LPS was administered to *Adamts13*$^{-/-}$ and *Adamts13*$^{+/+}$ mice to determine if the induced pulmonary inflammation was exacerbated in the presence of excess amounts of UL-VWFM in the blood. We observed no difference in the expression of inflammatory mediators in *Adamts13*$^{-/-}$ mice as compared to *Adamts13*$^{+/+}$ mice (Figure 4A). Furthermore, the protein levels of pro-inflammatory cytokines and chemokines were similar in *Adamts13*$^{+/+}$ and *Adamts13*$^{-/-}$ mice (Figure 4B). Taken together, *Adamts13*$^{+/+}$ and *Adamts13*$^{-/-}$ mice showed no significant differences in terms of LPS-induced pulmonary inflammation.

We speculated that, under inflammatory conditions, ADAMTS13 is overwhelmed by the released VWF. Therefore, we determined whether the administration of exogenous ADAMTS13 could suppress LPS-induced inflammation by cleaving locally released UL-VWFM. Recombinant human ADAMTS13 (RHA, 80 μg/kg) was injected into wild-type C57/BL6 mice prior to LPS instillation. In a preliminary experiment in which *Adamts13*$^{-/-}$ mice were intravenously administered with RHA (80 μg/kg) and their plasma levels of ADAMTS13 were serially measured using specific ELISA, we observed approximately 5.5 μg/mL of plasma concentration of ADAMTS13 immediately after injection and the levels decreased to 0.26 μg/mL at 72 hours after administration. No ADAMTS13 levels were detected in the plasma obtained before RHA injection (Supplementary Figure 2). The mRNA expression levels of inflammatory cytokines and chemokines in the lungs of RHA-administered LPS-treated wild-type mice were lower than those in the lungs of saline-administered LPS-treated wild-type mice. In particular, *Il6* mRNA was significantly decreased following RHA treatment (Figure 4C). This trend was particularly pronounced for the protein levels of inflammatory cytokines and chemokines in BALF (Figure 4D). These results supported the hypothesis that local VWF/ADAMTS13 imbalance exacerbates the inflammatory response.

Suppression of LPS-induced lung injury by VWF antibody administration.

To investigate the VWF-mediated inflammatory mechanism further, we intravenously administered anti-human VWF monoclonal antibody (NMC/VW4) to wild-type C57/BL6 mice prior to LPS administration. NMC/VW4 recognizes the A1 domain of VWF and strongly inhibits the VWF-platelet glycoprotein (GP) Ib interaction. This antibody also reacts with murine VWF. Interestingly, the mRNA expression of inflammatory cytokines in the lungs of NMC/VW4-LPS-treated mice was significantly lower than that in PBS-LPS-treated mice (Figure 5A). A similar trend was observed for chemokine mRNAs levels (Figure 5A). Although

no significant differences were found in BALF protein levels, all the values tended to be lower in NMC/VW4-LPS-treated mice than in PBS-LPS-treated mice. (Figure 5B). Of note, no significant enhancement of pulmonary hemorrhage was observed even in the NMC/VW4-LPS-treated mice (data not shown). The results shown in Figures 5A and 5B were very similar to those obtained in the experiment using *Vwf* $^{-/-}$ mice (Figure 3C and 3D). These anti-inflammatory effects by NMC/VW4 were not observed when normal mouse IgG was used instead of NMC/VW4 (Supplementary figure 3), indicating the observed anti-inflammatory effects were exerted by VWF-inhibiting activity of NMC/VW4, not by non-specific IgG reaction.

Overall, these results suggested that the VWF-mediated enhancement of inflammation can be partly attributed to the interaction between the VWF A1 domain and platelet GPIb.

DISCUSSION

In the present study, we demonstrated that VWF promoted inflammatory reactions using the LPS-induced lung injury model in mice. This is consistent with previous reports that VWF acts as a pro-inflammatory promoter [7–9,21]. In addition, we clarified that intravenous administration of either recombinant human ADAMTS13 protein or the A1 domain-selective anti-VWF antibody suppressed lung inflammation in wild-type mice, suggesting that VWF-mediated progression of inflammation was due to a bias in the functional VWF/ADAMTS13 abundance ratio in the blood in the lungs, followed by an increased interaction between the VWF A1 domain and WBCs.

In the pulmonary inflammation model used in the present study, VWF was not required for hemostasis. In contrast, TF-deficiency was associated with increased pulmonary hemorrhage in this model. Increased hemorrhage increases the number of leukocytes in the

alveolar space as well as inflammation. *Vwf*$^{-/-}$ mice do not exhibit increased hemorrhage in the LPS-induced injury model and therefore the increased inflammation observed in these mice cannot be attributed to a hemorrhage-dependent increase in leukocytes. VWF-deficiency reduced the LPS-induced increase in the mRNA expression of pro-inflammatory cytokines and chemokines in the lungs (Figure 3C). Notably, *Vwf* $^{+/-}$ mice exhibited an intermediate phenotype (Supplementary Figure 1A). These results supported the involvement of VWF in the progression of lung inflammation.

The protein levels of inflammatory cytokines and chemokines in BALF did not differ as much as did the mRNA levels between *Vwf*$^{+/+}$ and *Vwf*$^{-/-}$ mice, but the protein levels in *Vwf*$^{-/-}$ mice consistently tended to be lower than those in *Vwf*$^{+/+}$ mice. However, it is difficult to provide a reasonable explanation for this discrepancy. Because only proteins exudated into the bronchial lumen were included in the BALF, the ELISA values may not have reflected the condition of the whole lung. It is notable that *Vwf*$^{+/+}$ mice showed significantly increased vascular permeability as compared to *Vwf*$^{-/-}$ mice (Figure 3E, 3F).

ADAMTS13-deficiency is associated with high levels of highly active UL-VWFM, leading to a pathological prothrombotic status. Previous reports have demonstrated that ADAMTS13-deficient mice, due to their potential prothrombotic tendency, have higher morbidity and mortality rates than wild-type mice in various disease models, such as ischemia-reperfusion of the brain [22,23] or the heart [24–26]. Therefore, we expected that ADAMTS13-deficient mice would exhibit increased pulmonary inflammation in an LPS-induced lung inflammation model. Unexpectedly, however, no significant differences in inflammation were observed between *Adamts13*$^{+/+}$ mice and *Adamts13*$^{-/-}$ mice (Figure 4A, 4B). A possible

explanation for this is that local VWF release in the lungs of LPS-treated mice overwhelms the inhibitory capacity of endogenous ADAMTS13. To test this hypothesis, we examined whether intravenous administration of exogenous recombinant human ADAMTS13 (RHA) before LPS instillation could correct the VWF/ADAMTS13 imbalance during inflammation. It has been reported that human ADAMTS13 can cleave murine VWF as well as human VWF [27]. Our results showed a trend toward suppression of inflammation in the RHA-administered group as compared to the saline-administered group, particularly in terms of inflammatory cytokine and chemokine protein levels (Figure 4C, 4D). These results indicated that the VWF released in the LPS model can be cleaved by exogenous ADAMTS13, suggesting the applicability of ADAMTS13 as a therapeutic agent in VWF-mediated inflammatory conditions. Moreover, it has been reported that under inflammatory conditions, human neutrophil peptide inhibits VWF cleavage by competing with ADAMTS13 for binding to VWF [28]. It has also been reported that IL-6 inhibits the cleavage of UL-VWFM by ADAMTS13 under flow conditions [29] and that reactive oxygen species derived from activated leukocytes denature the ADAMTS13 recognition sites of VWF and the active center of ADAMTS13 [30,31]. Taken together, these reports supported our hypothesis that the functional VWF/ADAMTS13 balance in the lung is biased toward VWF under inflammatory conditions.

Treatment of wild-type mice with NMC/VW4, an antibody that selectively inhibits the VWF-A1 domain, ameliorated the LPS-induced lung inflammation, similar to the results observed using *Vwf* $^{-/-}$ mice (Figure 5A, 5B). Since the A1 domain of VWF interacts with GPIb on platelets and prevents the binding of PSGL1 to leukocytes, the experimental results suggested that VWF increases inflammation by enhancing platelet–leukocyte interactions. This interaction promoted leukocyte extravasation and inflammation. We attempted to determine the role of platelets in this LPS model by depleting them using an anti-mouse GPIb antibody.

Unfortunately, we observed severe pulmonary hemorrhage in platelet-depleted mice, which did not allow us to determine the role of platelet–leukocyte interactions (data not shown).

Based on our experimental results, we propose a putative mechanism for VWF-mediated inflammation in the lungs (Figure 6). When inflammatory signals are transmitted to ECs, strings of UL-VWFM in ECs are released and exposed to the blood. Circulating platelets bind to the A1 domain of VWF via GPIb, and then leukocytes bind to platelets, facilitating leukocyte extravasation. Finally, inflammation is promoted by activated leukocytes that migrate out of the blood vessels. In a VWF-deficient state, inflammation is suppressed because there is no VWF to provide footholds for leukocyte extravasation. This phenomenon can be mimicked by correction of the upregulated VWF/ADAMTS13 ratio using RHA or by inhibition of the interaction between UL-VWFM and blood cell components using VWF antibodies.

In this study, we analyzed direct binding between the VWF-A1 domain and blood cell components. However, the inflammatory pathophysiology is not only due to VWF but also due to complex interactions between P-selectin, other components stored in WPBs, and complement factors [32,33]. For example, P-selectin, which binds to leukocytes and plays an important role in leukocyte extravasation, is normally stored in WPBs; however, in the VWF-deficient state, WPBs are absent; thus, P-selectin is not stored in ECs [8]. Therefore, it is difficult to determine the relative contributions of VWF and P-selectin to leukocyte extravasation solely by experiments with *Vwf*$^{-/-}$ mice, and experiments using P-selectin-deficient mice are needed. NMC/VW4 treatment suppressed inflammation, but the level of suppression was clearly greater under VWF-deficient conditions (Figure 3C and 5A). These results indicated that there is not only a direct contribution of VWF to inflammation but also an indirect contribution by components within the WPBs or other factors.

In conclusion, by utilizing an LPS-induced lung inflammation model and agents that suppress VWF expression, we showed that VWF is involved in the progression of inflammation and that the induced inflammation can be alleviated by anti-VWF antibody and ADAMTS13 protein. These VWF-targeting agents may represent novel therapeutic options for the treatment of pulmonary inflammatory diseases. In a clinical point of view, the therapeutic potentials of anti-VWF antibody and ADAMTS13 protein after triggering lung inflammation should be investigated in a future study.

Addendum

Conceptualization: Y. Onodera and K. Tatsumi; investigation: Y. Onodera, Y. Takabayashi, R. Kawasaki, R. Mori, C. Ohshima, and K. Tatsumi; formal analysis: Y. Onodera and K. Tatsumi; data curation: Y. Onodera and K. Tatsumi; writing – original draft: Y. Onodera; writing – review and editing: Y. Onodera, S. Mitani, C. Hosoda. Y. Takabayashi, A. Sakata, R. Kawasaki, R. Mori, C. Ohshima, K. Nishio, M. Sugimoto, K. Soejima, N. Mackman, M. Shima, K. Tatsumi

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Conflict of interest

AS, RK, KT and MS: members of Medicinal Biology of Thrombosis and Hemostasis established by Nara Medical University and Chugai Pharmaceutical Co., Ltd. RK: an employee of Chugai Pharmaceutical Co., Ltd. a stock owner of the company. KS: an employee of KM Biologics Co., Ltd. MS: patents for inventions relating to products of Chugai

Pharmaceutical Co., Ltd.; representative of Medicinal Biology of Thrombosis and Hemostasis collaborative research laboratory; research support from Chugai Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd. and CSL Behring.; honoraria or consultation fees from Chugai Pharmaceutical Co., Ltd.; speaker's bureau from Chugai Pharmaceutical Co., Ltd., CSL Behring, Sanofi, Bayer, Novo Nordisk Pharma, Takeda Pharmaceutical Co., Ltd., Pfizer and Fujimoto Seiyaku Corp. YO, SM, CH, YT, RM, CO, KN, MS, and NM: no conflict of financial interest.

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Figure legends

Figure 1. Mice model of LPS-induced lung injury.

(A) Gene expression of *Vwf* in different organs of untreated C57BL/6 mice was quantified by qPCR and normalized to *Rpl4* mRNA levels (n = 3). Data are shown as values relative to the liver. Br: brain, Lu: lung, He: heart, Li: liver, Ki, kidney, In: intestine, and Sp: spleen. **(B** and **C)** White blood cell counts in BALF and body weight were assessed before and 1, 2, 3, and 4 days after intratracheal instillation of LPS (2 mg/kg) (n = 3, each time point). **(D)** Experimental scheme of LPS-induced lung injury model. Mice were sacrificed 3 days after LPS instillation for BALF and lung collections. Data (mean ± standard deviation) were analyzed with one-way analysis of variance followed by Dunnett's post hoc test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus Li (A), or day 0 (B and C). WBCs, white blood cells; BW, body weight; BALF,

bronchoalveolar lavage fluid; LPS, lipopolysaccharide; IT, intratracheal instillation.

Figure 2. Variation of VWF expression following intratracheal instillation of LPS in wild-type mice.

(A) Gene expression of *Vwf* in the lung of PBS- or LPS-tread wild-type C57BL/6 mice was analyzed by qPCR and normalized to *Rpl4* mRNA levels. **(B and C)** VWF protein levels in BALF supernatant and plasma of PBS- or LPS-tread wild-type mice were quantified by ELISA. **(D)** VWF proteins were immune-stained using the lung sections of PBS- or LPS-tread wild-type mice. Scale bar: 100 μm. **(E)** The thickness of VWF-stained area of randomly selected large blood vessels in the lungs of PBS- or LPS-treated wild-type mice were quantified using Image J software (n = 15, each group). Data (mean ± standard deviation; n = 5−6) were analyzed with Student's *t*-test. **P < 0.01 and ***P < 0.001 between groups (A−C). PBS, phosphate-buffered saline; LPS, lipopolysaccharide; BALF, bronchoalveolar lavage fluid; VWF, von Willebrand factor.

Figure 3. Effects of genetic lack of VWF on LPS-induced lung injury.

(A) BALF gross appearance and RBC counts in BALF of PBS- or LPS-treated $Vwf^{+/+}$ and $Vwf^{-/-}$ mice were shown. **(B)** BALF gross appearance and RBC counts in BALF of PBS- or LPS-treated $mF3^{+/+}/hF3^{+/+}$ and $mF3^{-/-}/hF3^{+/+}$ mice are shown as a positive control of (A). **(C)** Gene expressions of inflammatory cytokines (*Il6*, *Tnf*, and *Il1b*) and chemokines (*Ccl2*, *Cxcl1*, and *Cxcl2*) in the lung of PBS- or LPS-treated $Vwf^{+/+}$ and $Vwf^{-/-}$ mice were quantified by qPCR and normalized to *Rpl4* mRNA levels. **(D)** Protein levels of inflammatory cytokines (IL6, TNFα, and IL1β) and chemokines (CCL2, CXCL1, and CXCL2) in the BALF supernatant of

PBS- or LPS-treated *Vwf* $^{+/+}$ and *Vwf* $^{-/-}$ mice were quantified by enzyme-linked immunosorbent assay. **(E)** The ratio of the wet weight to the dry weight of the lungs of PBS- or LPS-treated *Vwf* $^{+/+}$ and *Vwf* $^{-/-}$ mice were calculated. **(F)** FITC-dextran concentration in the BALF supernatant extravasated out of blood vessels were evaluated by fluorescence detection in PBS- or LPS-treated *Vwf* $^{+/+}$ and *Vwf* $^{-/-}$ mice. The data were normalized to those in plasma. **(G)** Extravascular infiltration of leukocytes was assessed by hematoxylin–eosin staining of the lung sections of PBS- or LPS-treated *Vwf* $^{+/+}$ and *Vwf* $^{-/-}$ mice. Scale bar: 100 μm. **(H)** The numbers of mononuclear cells (MNCs) extravasated into lung parenchyma in the lung sections of PBS- or LPS-treated *Vwf* $^{+/+}$ and *Vwf* $^{-/-}$ mice were counted using the Hybrid Cell Count module built into the Keyence microscope system. Images were randomly selected and analyzed. Data (mean ± standard deviation; n = 3−6 for (A), n = 3 for (B), n = 5−6 for (C), n = 4−6 for (D), n = 7−10 for (E), n = 8−11 for (F), n = 15 for (H), each group) were analyzed with two-way ANOVA followed by Bonferroni post hoc test. *P < 0.05, **P < 0.01, and ***P < 0.001 between groups, or $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, and $^{\#\#\#}$P < 0.001 versus PBS-treated controls of the respective genotypes (A−F, and H). Data are shown relative to the value of the PBS-treated *Vwf* $^{+/+}$ group (C and F). PBS, phosphate-buffered saline; LPS, lipopolysaccharide; RBCs, red blood cells; BALF, bronchoalveolar lavage fluid.

Supplementary Figure 1. LPS-induced lung injury in *Vwf* $^{+/-}$ mice.

(A) Gene expressions of inflammatory cytokines (*Il6*, *Tnf*, and *Il1b*) and chemokine (*Ccl2*) in the lung of LPS-treated *Vwf* $^{+/+}$, *Vwf* $^{+/-}$, and *Vwf* $^{-/-}$ mice were quantified by qPCR and normalized to *Rpl4* mRNA levels. Data are shown relative to the value of the *Vwf* $^{+/+}$ group. **(B)** Protein levels of inflammatory cytokines (IL6, TNFα, and IL1β) and chemokine (CCL2)

in the BALF supernatant of LPS-treated *Vwf*$^{+/+}$, *Vwf*$^{+/-}$, and *Vwf*$^{-/-}$ mice were quantified by ELISA. Data (mean ± standard deviation; n = 7−9 for (A) and n = 8−9 for (B)) were analyzed with one-way ANOVA followed by Bonferroni post hoc test. *P <0.05 and **P <0.01 between the groups. BALF, bronchoalveolar lavage fluid.

Figure 4. The effects of genetic lack of ADAMTS13 on LPS-induced lung injury.

(A) Gene expression of inflammatory cytokines (*Il6*, *Tnf*, and *Il1b*) and chemokines (*Ccl2*, *Cxcl1*, and *Cxcl2*) in the lungs of PBS- or LPS-treated *Adamts13*$^{+/+}$ and *Adamts13*$^{-/-}$ mice were quantified by qPCR 3 days after instillation and normalized to *Rpl4* mRNA levels. Data are shown relative to the value of the PBS-treated *Adamts13*$^{+/+}$ group. **(B)** Protein levels of inflammatory cytokines (IL6, TNFα, and IL1β) and chemokines (CCL2, CXCL1, and CXCL2) in the BALF supernatant of PBS- or LPS-treated *Adamts13*$^{+/+}$ and *Adamts13*$^{-/-}$ mice were quantified by ELISA. **(C)** Gene expression of inflammatory cytokines (*Il6*, *Tnf*, and *Il1b*) and chemokines (*Ccl2*, *Cxcl1*, and *Cxcl2*) in the lung of saline- or recombinant human ADAMTS13 (RHA, 80 μg/kg)-administered PBS- or LPS-treated wild-type mice were quantified by qPCR. Data are shown relative to the value of the saline-administered PBS-treated wild-type group. **(D)** Protein levels of inflammatory cytokines (IL6, TNFα, and IL1β) and chemokines (CCL2, CXCL1, and CXCL2) in the BALF supernatant of saline- or RHA-administered PBS- or LPS-treated wild-type mice were quantified by ELISA. Data (mean ± standard deviation; n = 5−7 for (A), n = 6−8 for (B), and n = 4−6 for (C) and (D), were analyzed with two-way ANOVA followed by Bonferroni post hoc test. *P <0.05 and **P < 0.01 between groups, or $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, and $^{\#\#\#}$P < 0.001 versus PBS-treated *Adamts13*$^{+/+}$ groups (A and B) and PBS-treated controls of the respective reagent-administered groups (C and D). PBS, phosphate-buffered saline; LPS, lipopolysaccharide; BALF, bronchoalveolar lavage fluid: RHA, recombinant

human ADAMTS13 protein; ELISA, enzyme-linked immunosorbent assay.

Supplementary Figure 2. Kinetics of plasma ADAMTS13 levels after recombinant human ADAMTS13 administration in *Adamts13*$^{-/-}$ mice

Recombinant human ADAMTS13 (RHA, 80 μg/kg) were intravenously administered to *Adamts13* -/- mice (n = 5). Partial blood drawings with citrate were serially conducted before administration, 15 minutes, 3, 6, 12, 24, 48, and 72 hours after administration. Plasma ADAMTS13 levels were measured using chromogenic ADAMTS13-act–enzyme-linked immunosorbent assay (ELISA). Data were represented as mean ± standard deviation.

Figure 5. Suppression of LPS-induced lung injury by VWF antibody administration

(A) Gene expression of inflammatory cytokines (*Il6*, *Tnf*, and *Il1b*) and chemokines (*Ccl2*, *Cxcl1*, and *Cxcl2*) in the lungs of PBS- or NMC/VWF4 (0.4 mg/kg)-administered PBS- or LPS-treated wild-type mice were quantified by qPCR and normalized to *Rpl4* mRNA levels. Data are shown relative to the value of the PBS-administered PBS-treated wild-type group. **(B)** Protein levels of inflammatory cytokines (IL6, TNFα, and IL1β) and chemokines (CCL2, CXCL1, and CXCL2) in the BALF supernatant of PBS- or NMC/VWF4-administered PBS- or LPS-treated wild-type were quantified by enzyme-linked immunosorbent assay. Data (mean ± standard deviation; n = 5−7 for (A) and n = 6−8 for (B)) were analyzed with two-way ANOVA followed by Bonferroni post hoc test. *P < 0.05 and **P < 0.01 between groups, or $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, and $^{\#\#\#}$P < 0.001 versus PBS-treated controls of the respective reagent-administered groups. PBS, phosphate-buffered saline; LPS, lipopolysaccharide; BALF, bronchoalveolar lavage fluid.

Supplementary Figure 3. The effect of IgG on LPS-induced lung injury

Gene expression of inflammatory cytokines (*Il6*, *Tnf*, and *Il1b*) and chemokines (*Ccl2*, *Cxcl1*, and *Cxcl2*) in the lungs of PBS- or normal mouse IgG (0.4 mg/kg)-administered LPS-treated wild-type mice were quantified by qPCR and normalized to *Rpl4* mRNA levels. Lungs were collected 3 days after administration. Data are shown relative to the value of the PBS-administered LPS-treated wild-type group. Data (mean ± standard deviation; n = 4, each group) were analyzed with Student's *t*-test. PBS, phosphate-buffered saline; LPS, lipopolysaccharide.

Figure 6. Regulation of von Willebrand factor by ADAMTS13 ameliorates LPS-induced lung injury in mice

Inflammatory stimuli transmitted to vascular endothelial cells by LPS infiltration of the trachea result in the release of UL-VWFM from vascular endothelial cells. Although UL-VWFM can be cleaved by circulating ADAMTS13, the ratio of VWF is considered to be locally higher around the site of inflammation. After platelet adhesion to the highly active UL-VWFM, WBCs were exuded outside the vessel after leukocyte rolling. The activation of WBCs outside the blood vessels is expected to promote inflammation. In aVWF-deficient state, inflammation is suppressed because there is no VWF to provide footholds for leukocyte extravasation. Administration of RHA or NMC/VW4 inhibits these reactions and reduces inflammation through less leukocyte extravasation.

VWF, von Willebrand factor; WBC, white blood cell; EC, endothelial cell; RHA, recombinant human ADAMTS13; NMC/VW4; anti-VWF A1 domain antibody.