were shown.

Parallel plate flow chamber assay

Platelet thrombus formation in flowing blood on immobilized collagen was analyzed using a parallel plate flow chamber as described previously [17,21]. Acid-insoluble type I collagen-coated glass coverslips were placed in a flow chamber. The chamber was mounted on a fluorescence microscope (Axiovert 200M; Carl Zeiss, Oberkochen, Germany) equipped with a CCD camera system (DXC-390; Sony, Tokyo, Japan). Blood was collected into tubes containing argatroban (240 μM; Mitsubishi Chemical Corporation, Tokyo, Japan). The fluorescent dye mepacrine (10 μM; Sigma, St Louis, MO) was added to the blood. Whole blood samples were aspirated through the chamber and across the collagen-coated coverslip at a constant wall shear rate. To analyze the cumulative thrombus volume, image sets at 1.0-μm z-axis intervals within a defined area (156.4 μm x 119.6 μm) were captured using MetaMorph software (version 6.1.4; Universal Imaging, West Chester, PA). After blind deconvolution of image sets processed by AutoDeblur software package (version 8.0.2; AutoQuant Imaging, Troy, NY), 3D volumetric measurements of thrombi were accomplished using VoxBlast software (version 3.0; Vartek, Fairfield, IA).

Intravital microscopy

Intravital microscopy was performed as described previously [22,23]. Platelets were isolated from platelet-rich plasma and fluorescently labeled with calcein AM (2.5 μg/ml; Invitrogen). Recipient mice were anesthetized and labeled platelets were infused through retro-orbital plexus. The mesentery was gently exteriorized through a midline abdominal incision and arterioles of 100-150 μm diameters were visualized with a fluorescence microscope and a CCD camera system. The shear rate was calculated using an optical Doppler velocimeter as described [24]. Filter paper saturated with 10% ferric chloride was applied for 5 min on an arteriole by topical application. Thrombus formation in the arteriole was monitored for 40 min after injury or until complete occlusion occurred and lasted for more than 30 s. The following two parameters were evaluated: time to first thrombus formation, defined as the time required for formation of a thrombus larger than 30 μm; and occlusion time, defined as the time required for cessation of blood flow for at least 30 s.

Collagen plus epinephrine-induced thrombosis model

A mixture of 600 ng/g of collagen (Nycomed, Roskilde, Denmark) and 60 ng/g of epinephrine (Sigma) was infused into tail vein of mice [17,25]. Blood was collected 5 min after the infusion and platelet counts were determined.

Statistical Analysis

Statistical significance was assessed by the one-way analysis of variance followed by the Bonferroni's multiple comparison tests. Differences were considered to be significant at $P < 0.05$.

Results

Generation of *Adamts13*$^{S/S}$ mice

To address the functional implication of the distal C-terminal domains of

ADAMTS13 *in vivo*, we generated and characterized a congenic mouse model that has the C-terminally truncated form of ADAMTS13 on 129/Sv genetic background (Figure 1A). We confirmed the presence of IAP insertion in the *Adamts13* gene of the congenic (*Adamts13*$^{S/S}$) mice by PCR (data not shown) and detected an IAP chimeric transcript (~ 3.5 kb) by Northern blotting of RNA from liver (Figure 1B), primary site of synthesis [14]. An IAP-free ADAMTS13 mRNA (~ 5 kb) was detected in wild-type 129/Sv (*Adamts13*$^{L/L}$) mice and no ADAMTS13 mRNA was detected in ADAMTS13-deficient (*Adamts13*$^{-/-}$) mice on 129/Sv genetic background (Figure 1B). *Adamts13*$^{S/S}$ mouse plasma exhibited higher cleaving activity for both GST-mVWF73-H and FRETS-VWF73 than *Adamts13*$^{L/L}$ mouse plasma, whereas the activity in *Adamts13*$^{-/-}$ mouse plasma was below detection limits (Figure 1C,D). Therefore, the distal C-terminal domains of ADAMTS13 were not necessary for the cleavage of the VWF73-based peptide substrate as observed previously [8,14]. Platelet counts were not different among the genotypes (*Adamts13*$^{L/L}$, 74.4 ± 18.0 x 10^4/µL; *Adamts13*$^{S/S}$, 69.3 ± 4.4 x 10^4/µL; *Adamts13*$^{-/-}$, 67.2 ± 3.9 x 10^4/µL; mean ± SD, n = 8). Both *Adamts13*$^{S/S}$ mice and *Adamts13*$^{-/-}$ mice were viable and showed no TTP-like symptoms throughout the study.

***Adamts13*$^{S/S}$ mice have normal VWF multimers**

As previously reported [17], UL-VWF multimers persisted in plasma of *Adamts13*$^{-/-}$ mice on 129/Sv-genetic background (Figure 2). Thus, ADAMTS13 activity is important for the size regulation of VWF multimers in mice at least on this genetic background. However, the VWF multimer patterns in *Adamts13*$^{S/S}$ mice were indistinguishable from those in *Adamts13*$^{L/L}$ mice (Figure 2). These results suggest that the distal C-terminally truncated form of mouse ADAMTS13 exhibits VWF-cleaving activity sufficient for maintenance of normal size distribution of plasma VWF multimers under steady state *in vivo*.

***In vitro* thrombogenesis is increased in *Adamts13*$^{S/S}$ mice only at a high shear rate**

When whole blood was perfused over a collagen-coated surface in a parallel plate flow chamber at a shear rate of 1000 s^{-1}, platelet thrombus formation was significantly promoted in *Adamts13*$^{-/-}$ mice (Figure 3A) compared with *Adamts13*$^{L/L}$ mice, consistent with the presence of UL-VWF multimers in plasma of *Adamts13*$^{-/-}$ mice. However, whole blood thrombus formation at 1000 s^{-1} was not significantly different between *Adamts13*$^{S/S}$ mice and *Adamts13*$^{L/L}$ mice (Figure 3A), indicating that the distal C-terminally truncated form of mouse ADAMTS13 does not completely lose the activity.

As fluid shear rate increases progressively, the interaction between VWF and platelet GPIbα becomes more important in platelet thrombus formation [26]. It has been reported that thrombus formation in mouse blood on collagen surface is completely dependent on the VWF-GPIbα interaction above a threshold shear rate between 2000 s^{-1} and 5000 s^{-1} [27]. In addition, ADAMTS13 cleaves VWF and down-regulates thrombus formation in shear rate-dependent manner [28]. Based on these observations, we further examined thrombus formation at a higher shear rate of 5000 s^{-1}. As expected, thrombus formation in *Adamts13*$^{-/-}$ mice was significantly elevated compared to *Adamts13*$^{L/L}$ mice at 5000 s^{-1} (Figure 3B). In addition, we found accelerated thrombus formation in *Adamts13*$^{S/S}$ mice compared to *Adamts13*$^{L/L}$ mice at this higher shear rate (Figure 3B). These results suggest that the distal C-terminally truncated form of mouse ADAMTS13 has reduced activity compared with the full-length form and does not sufficiently limit thrombus formation under high shear rate *in vitro*.

In vivo thrombus growth is accelerated in *Adamts13*$^{S/S}$ mice

To examine whether the truncation of the distal C-terminal domains in ADAMTS13 affects thrombus formation *in vivo*, we carried out intravital microscopy experiments in a model of experimental arteriolar thrombosis. In this model, vascular injury was induced by topical application of ferric chloride on a mesenteric arteriole, which provoked the generation of free radicals leading to the endothelial disruption [23]. The diameter and shear rate of studied arterioles were 118.0 ± 13.1 μm and 1362 ± 219 s^{-1} (mean ± SD, n = 16) for *Adamts13*$^{L/L}$ mice, 122.8 ± 11.1 μm and 1394 ± 136 s^{-1} (n = 16) for *Adamts13*$^{S/S}$ mice, and 115.6 ± 10.8 μm and 1405 ± 225 s^{-1} (n = 12) for *Adamts13*$^{-/-}$ mice and not significantly different among the groups. Both time to first thrombus (Figure 4A) and occlusion time (Figure 4B) following injury in *Adamts13*$^{-/-}$ mice (time to first thrombus = 5.1 ± 1.9 min, occlusion time = 9.2 ± 1.6 min; mean ± SD) were significantly decreased compared with *Adamts13*$^{L/L}$ mice (time to first thrombus = 7.8 ± 1.3 min, occlusion time = 15.3 ± 3.6 min), indicating that ADAMTS13 contributes to down-regulation of thrombogenesis at the site of arteriolar injury in 129/Sv mice. In the case of *Adamts13*$^{S/S}$ mice, time to first thrombus after injury (7.6 ± 1.2 min) was not different from *Adamts13*$^{L/L}$ mice. However, the initial thrombi grew rapidly to occlusive size in *Adamts13*$^{S/S}$ mice and occlusion time was significantly shorter in *Adamts13*$^{S/S}$ mice (12.5 ± 1.9 min) compared with *Adamts13*$^{L/L}$ mice (Figure 4B). These results suggest that the distal C-terminally truncated form of mouse ADAMTS13 is less active in down-regulating thrombus growth *in vivo* compared to full-length ADAMTS13.

To elucidate the consequences of the lack of the distal C-terminal domains in ADAMTS13 on systemic thrombosis, we performed collagen plus epinephrine infusion model experiments. In this model, widespread intravascular thrombosis was induced by intravenous infusion of collagen fibrils in combination with epinephrine, and the incorporation of platelets into thrombi was monitored by the reduction in circulating platelet counts [29]. Consistent with our previous observation [17], platelet counts following the infusion were significantly lower in *Adamts13*$^{-/-}$ mice (2.8 ± 0.8 x 10^4/µl, mean ± SD) than in *Adamts13*$^{L/L}$ mice (8.5 ± 3.3 x 10^4/µl), suggesting that ADAMTS13 contributes to inhibition of platelet aggregation in this experimental system (Figure 5). Platelet counts after the infusion in *Adamts13*$^{S/S}$ mice (5.6 ± 2.4 x 10^4/µl) were significantly higher than in *Adamts13*$^{-/-}$ mice and lower than in *Adamts13*$^{L/L}$ mice (Figure 5), whereas platelet counts of untreated mice were not different among the groups (*Adamts13*$^{L/L}$, 66.6 ± 4.4 x 10^4/µL; *Adamts13*$^{S/S}$, 77.0 ± 6.5 x 10^4/µL; *Adamts13*$^{-/-}$, 71.0 ± 4.9 x 10^4/µL, mean ± SD of four mice). These findings complement accelerated thrombus growth in *Adamts13*$^{S/S}$ mice compared to *Adamts13*$^{L/L}$ mice, indicating that the distal C-terminally truncated form of mouse ADAMTS13 has significantly reduced activity *in vivo*.

Discussion

It is now evident that genetic background is an important phenotypic determinant in mutant mice with hemostatic defects. For instance, mice carrying the factor V Leiden (R504Q) mutation have shown increased perinatal thrombotic mortality on the mixed 129/Sv and C57BL/6J background relative to C57BL/6J background [30]. Similar effects of genetic backgrounds on phenotypes have been observed in other mutants such as the thrombomodulin G404P-mutant mice [31], the fibrinogen-deficient mice [32] and the tissue factor-deficient mice [33]. In ADAMTS13-deficient mice, genetic backgrounds have also been shown to significantly affect their thrombotic phenotypes [34]. Thus, phenotypes of ADAMTS13 mutant mice should be compared to control mice on the appropriate and uniform strain background. We have previously demonstrated that ADAMTS13 deficiency in mice results in a prothrombotic state with accumulation of UL-VWF multimers on 129/Sv background [17]. Therefore in this study, we applied a spontaneous mutation in the *Adamts13* gene of C57BL/6 mice onto 129/Sv mice by 10-generation backcrossing, and obtained the congenic mice that were expected to have 99.9% 129/Sv genome and primarily expressed the distal C-terminally truncated ADAMTS13. Then, we compared their phenotypes with positive and negative control animals: the wild-type 129/Sv mice and the 129/Sv-background ADAMTS13-deficient mice. By this approach, we minimized the background effects and defined the significance of the distal C-terminal domains of ADAMTS13 in mice.

Plasma of the congenic mice exhibited higher cleaving activity against GST-mVWF73-H and FRETS-VWF73 compared to plasma of the wild-type mice. We previously observed that the recombinant distal C-terminally truncated mouse ADAMTS13 cleaves GST-mVWF73-H to the similar extent as compared to the full-length form [14]. The other group reported that the recombinant distal C-terminally truncated mouse ADAMTS13 is slightly less active in cleaving GST-mVWF73-H than the full-length form [15]. These findings suggest that the distal C-terminally truncated ADAMTS13 in mouse plasma has equivalent or slightly lower specific activity against VWF73-based substrates compared to the full-length ADAMTS13. Thus, the data in the present study imply that the distal C-terminally truncated ADAMTS13 is more abundant than the full-length form in circulating blood in 129/Sv mice. Preferential expression of the distal C-terminally truncated mouse ADAMTS13 has also been found in HeLa cells [14] and HEK 293T cells [15]. Unfortunately, since we have failed to determine the ADAMTS13 antigen levels in mouse plasma, it remains unclear whether the distal C-terminal truncation of ADAMTS13 actually increases plasma levels of the enzyme. Despite the congenic mice had the higher *in vitro* ADAMTS13 activity in plasma, they showed prothrombotic phenotypes, suggesting the importance of the distal C-terminal domains in ADAMTS13 activity *in vivo*.

We reconfirmed that ADAMTS13 deficiency in 129/Sv mice allowed the accumulation of UL-VWF multimers in plasma (Figure 2); therefore, promising the essential contribution of ADAMTS13 on preventing the accumulation of UL-VWF multimers in 129/Sv mice. Under these situations, lack of the distal C-terminal domains of ADAMTS13 in 129/Sv mice did not increase plasma VWF multimer sizes (Figure 2), showing that the distal C-terminally truncated ADAMTS13 maintained the VWF-cleaving activity *in vivo*. Although the distal C-terminally truncated form of mouse ADAMTS13 was reported to show considerably lower activity than the full-length form for purified human VWF multimers under *in vitro* static conditions [15], our results show that the distal C-terminal truncation of mouse ADAMTS13 allows retention of normal size distribution of plasma VWF multimers *in vivo* at least under steady state.

In the parallel-plate flow chamber experiments, ADAMTS13 deficiency in 129/Sv mice markedly enhanced thrombogenic responses (Figure 3), indicating that ADAMTS13 is critical for limiting platelet thrombus formation under whole blood flow conditions. In the same experimental conditions, the distal C-terminal truncation of ADAMTS13 in 129/Sv mice did not promote thrombogenesis at 1000 s^{-1} (Figure 3A) but significantly promoted thrombogenesis at 5000 s^{-1} (Figure 3B). It is conceivable that the distal C-terminally truncated ADAMTS13 is active but not fully competent to cleave VWF within growing thrombus under flow. Because both the interaction of GPIbα-VWF and the cleavage of VWF by ADAMTS13 are facilitated by increasing fluid shear rate, the function of the distal C-terminal domains may become vital to down-regulate thrombogenesis under high shear conditions. Actually, similar results were obtained in the *in vivo* arteriolar injury model experiments (Figure 4). The distal C-terminal truncation of ADAMTS13 in 129/Sv mice did not affect the time to first thrombus formation in the arterioles where fluid shear rates were around 1500 s^{-1} (Figure 4A). However, when thrombus grew to a larger size, the arteriolar lumen was narrowed, which resulted in increase in shear rates [23]. Then, the distal C-terminal truncation of ADAMTS13 significantly reduced the occlusion time compared to full-length ADAMTS13 (Figure 4B). Therefore, the distal C-terminal domains are important for ADAMTS13 to sufficiently down-regulate thrombogenesis under high shear rate *in vivo* as well as *in vitro*. After the induction of systemic platelet aggregation by challenge with a mixture of collagen and epinephrine, consumptive thrombocytopenia was also enhanced by the distal C-terminal truncation of ADAMTS13 in 129/Sv mice (Figure 5), supporting the idea that the distal C-terminal domains are required for optimal down-regulation of platelet aggregation *in vivo*. The complete deficiency of ADAMTS13 in 129/Sv significantly accelerated thrombus growth to injured vessel wall and systemic thrombi compared to 129/Sv mice with truncation of the distal C-terminal domains in ADAMTS13 (Figures 4, 5). Therefore, we can conclude that the distal C-terminally truncated ADAMTS13 has significantly decreased activity in limiting thrombosis *in vivo*.

The binding of platelets to VWF is reported to accelerate the cleavage of VWF by ADAMTS13 under static [35] and flow [36] conditions *in vitro*. It has also been shown that ADAMTS13 can cleave platelet-bound VWF multimers [37] and limit thrombus formation through the cleavage of VWF at the surface of forming thrombi [28] in *in-vitro* flow chamber systems. Therefore, in our experimental settings, ADAMTS13 attenuates thrombus growth, possibly through the cleavage of VWF multimers bound on the surface of platelet-rich thrombi under high shear rate. The distal C-terminal domains may be necessary for ADAMTS13 to efficiently recognize and cleave platelet-bound VWF multimers on a growing thrombus. Conceivably, the distal C-terminal domains may contribute to the interaction with unidentified ADAMTS13-binding co-factors localized on the surface of platelets or subendothelium, and this interaction may be necessary for ADAMTS13 to control VWF-mediated thrombus formation. However, we cannot rule out the possibility that the distal C-terminal domains of ADAMTS13 contribute to the prevention of thrombosis independent from the VWF-cleaving activity of ADAMTS13, nevertheless VWF has been suggested as the only relevant substrate for ADAMTS13 [38] and functions of ADAMTS13 other than its VWF-cleaving activity have yet to be reported.

The distal C-terminally truncated ADAMTS13 is expressed in a lot of mouse strains including the BALB/c, C3H/He, C57BL/6 and DBA/2 strains as substitute for the full-length form [14,15]. Our present results suggest that thrombotic response in these strains would be increased, at least partially, by their incomplete ADAMTS13 activity. This should be taken into account when studying genetically modified mice with heterogeneous genetic background.

In summary, our results define the role of the distal C-terminal domains in ADAMTS13 *in vivo*. Deletion of the C-terminal two Tsp1 and two CUB domains permits normal size distribution of plasma VWF multimers under steady state, but exacerbates platelet thrombosis after thrombogenic stimulation in mice. Thus, the distal C-terminally truncated ADAMTS13 is not fully active *in vivo*. These distal C-terminal domains of ADAMTS13 may play a role in the efficient processing of VWF multimers during platelet thrombus growth, and thus their functions may become increasingly important when vascular damage is induced.

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Authorship

F.B.: designed research, performed experiments, analyzed data and wrote the paper

A.K.C.: performed experiments, contributed vital analytical tools and analyzed the data

K.K.: designed research, performed experiments and wrote the paper

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S.M.: contributed vital analytical tools and interpreted the data

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The authors do not declare any conflict of interest.

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Figure Legends

Figure 1. Generation of *Adamts13*$^{S/S}$ mice with 129/Sv-genetic background. (A) Gene and protein structure of ADAMTS13 in the wild-type (*Adamts13*$^{L/L}$) 129/Sv mice, the *Adamts13*$^{S/S}$ mice on 129/Sv genetic background, and the *Adamts13*$^{-/-}$ mice on 129/Sv genetic background. An intracisternal A-particle (*IAP*) insertion into intron 23 creates a pseudo-exon 24 including a premature stop codon. ADAMTS13 with a truncated C-terminus is mainly expressed in *Adamts13*$^{S/S}$ mice. *S*, signal peptide; *P*, propeptide; *MP*, metalloprotease domain; *Dis*, disintegrin-like domain; *T* (*numbered 1-8*), thrombospondin type 1 motif domain; *Cys*, cysteine-rich domain; *Sp*, spacer domain; *CUB*, complement components C1r/C1s, urchin epidermal growth factor, and bone morphogenic protein-1 domain. (B) Expression of *Adamts13* mRNA in liver. Poly(A)$^+$ RNA isolated from liver of indicated mice was probed with a 1.3-kb Adamts13 cDNA corresponding to exons 3-13. (C) GST-mVWF73-H assay. Plasma ADAMTS13 activity of indicated mice was measured using a recombinant mouse VWF73 peptide, GST-mVWF73-H. Results from 6 mice for each genotype are shown. Standard reactions using graded amounts of pooled plasma from 10 *Adamts13*$^{L/L}$ mice were performed simultaneously. (D) FRETS-VWF73 assay. Plasma ADAMTS13 activity in indicated mice was determined using a fluorogenic human VWF73 peptide, FRETS-VWF73. Data are mean ± SD of 6 mice for each genotype. The average activity measured in *Adamts13*$^{L/L}$ mice was arbitrarily defined as 100%.

Figure 2. Plasma VWF multimers. (A) VWF multimer patterns. Plasma samples (1 μL/lane) from *Adamts13*$^{L/L}$, *Adamts13*$^{S/S}$, and *Adamts13*$^{-/-}$ mice were electrophoresed on SDS-agarose

gels and transferred to nitrocellulose membranes. VWF multimers were detected with anti-VWF antibodies. (B) Relative intensities of plasma VWF multimers. The chemiluminescent intensities of the VWF multimer patterns (*A*) were scanned using image analysis software. An average of multiple lanes from 4 mice for each genotype are shown. *HMW*, high molecular weight; *LMW*, low molecular weight.

Figure 3. In vitro thrombogenesis on collagen surface under flow. (A) Thrombus formation at 1000 s^{-1}. Whole blood from *Adamts13*$^{L/L}$, *Adamts13*$^{S/S}$, or *Adamts13*$^{-/-}$ mice containing mepacrine-labeled platelets was perfused over an acid-insoluble type I collagen-coated surface at a wall shear rate of 1000 s^{-1}. The cumulative thrombus volume, analyzed using a multi-dimensional imaging system, was measured every 0.5 min until 4 min. Data are the mean ± SEM of 25 mice for each genotype. (B) Thrombus formation at 5000 s^{-1}. Whole blood samples from indicated mice were perfused over an acid-insoluble type I collagen-coated surface at a wall shear rate of 5000 s^{-1}. The cumulative thrombus volume was measured every 20 s until 80 s. Blood from 2 mice was pooled and used for experiments. Data are the mean ± SEM of 15 samples for each genotype. *Asterisks* indicate significant differences at $P < 0.05$ in comparison to *Adamts13*$^{L/L}$ mice.

Figure 4. In vivo thrombogenesis in ferric chloride-injured mesenteric arterioles. (A) Time to first thrombus formation. Calcein AM-labeled platelets representing approximately 2.5% of total platelets were observed in mesenteric arterioles of live mice after injury with 10% ferric chloride. The time required for formation of a thrombus > 30 μm was measured. (B) Occlusion time. The time required for a complete stop of blood flow was measured after injury

with 10% ferric chloride. *Symbols* represent data from a single mouse. *Bars* represent the mean values of groups (n = 16 for *Adamts13*$^{L/L}$ mice, n = 16 for *Adamts13*$^{S/S}$ mice and n = 12 for *Adamts13*$^{-/-}$ mice).

Figure 5. Platelet counts after collagen plus epinephrine infusion. Mice were injected with 600 ng/g of collagen plus 60 ng/g of epinephrine via tail vein and platelet counts were measured 5 min after injection. *Symbols* represent platelet counts from a single mouse. *Bars* represent the mean values of 25 mice in each group. Platelet counts of untreated mice were not different among the groups.