Shigatoxin triggers thrombotic thrombocytopenic purpura in genetically susceptible ADAMTS13-deficient mice

David G. Motto,1 Anil K. Chauhan,2,3 Guojing Zhu,1 Jonathon Homeister,4 Colin B. Lamb,2 Karl C. Desch,1 Weirui Zhang,5 Han-Mou Tsai,6 Denisa D. Wagner,2,3 and David Ginsburg2,6,7,8,9

1Department of Pediatrics, University of Michigan, Ann Arbor, Michigan, USA. 2CBB Institute for Biomedical Research, Boston, Massachusetts, USA. 3Department of Pathology, Harvard Medical School, Boston, Massachusetts, USA. 4Department of Pathology and 5Department of Human Genetics, University of Michigan, Ann Arbor, Michigan, USA. 6Department of Internal Medicine, Albert Einstein College of Medicine, New York, New York, USA. 7Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan, USA. 8Howard Hughes Medical Institute, Chevy Chase, Maryland, USA. 9Life Sciences Institute, University of Michigan, Ann Arbor, Michigan, USA.

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening illness caused by deficiency of the vWF-cleaving protease ADAMTS13. Here we show that ADAMTS13-deficient mice are viable and exhibit normal survival, although vWF-mediated platelet-endothelial interactions are significantly prolonged. Introduction of the genetic background CASA/Rk (a mouse strain with elevated plasma vWF) resulted in the appearance of spontaneous thrombocytopenia in a subset of ADAMTS13-deficient mice and significantly decreased survival. Challenge of these mice with shigatoxin (derived from bacterial pathogens associated with the related human disease hemolytic uremic syndrome) resulted in a striking syndrome closely resembling human TTP. Surprisingly, no correlation was observed between plasma vWF level and severity of TTP, implying the existence of TTP-modifying genes distinct from vWF. These data suggest that microbe-derived toxins (or possibly other sources of endothelial injury), together with additional genetic susceptibility factors, are required to trigger TTP in the setting of ADAMTS13 deficiency.

Introduction

The life-threatening systemic illness thrombotic thrombocytopenic purpura (TTP) is characterized by sudden onset of the pentad of fever, hemolytic anemia, thrombocytopenia, neurological symptoms, and renal dysfunction (1). This clinical picture results from the abrupt and widespread deposition of platelet-rich thrombi in the small vessels of multiple organs, which is the pathologic hallmark of the disease (2). Although the incidence of TTP appears to be increasing, modern intensive therapy has decreased TTP mortality from 90% to under 20% (3). Morbidity also remains significant, as treatment carries considerable risks, including exposure to blood products obtained from multiple donors.

TTP results from loss of function of the recently described ADAMTS13 metalloprotease (1, 4). ADAMTS13 specifically cleaves vWF, an adhesive multimeric plasma glycoprotein that serves as the initial link for the attachment of circulating blood platelets to sites of vascular injury (1). The largest and most adhesive forms of vWF may contain up to 100 individual subunits and are termed ultra-large vWF (UL-vWF) (5). If not proteolytically processed by ADAMTS13 to smaller forms, circulating UL-vWF is thought to trigger widespread spontaneous platelet aggregation, ultimately leading to overt TTP.

Loss of ADAMTS13 function can be due to either genetic deficiency (familial TTP) or inhibitory autoantibodies to ADAMTS13 (acquired TTP) (6–8). Acquired TTP primarily affects individuals between 20 and 60 years of age (1). Plasma exchange is the treatment of choice, and approximately one-third of acquired TTP cases become chronic (9). Patients with the less-common familial form of TTP typically exhibit a chronic relapsing course and, unlike patients with acquired TTP, generally respond well to simple plasma infusion, usually without the need for plasma exchange (1).

Although familial TTP often manifests during infancy, about one-half of cases remains undiagnosed until mid-childhood or later, with rare ADAMTS13-deficient individuals still free of disease through the third decade of life (10). Family members with the same ADAMTS13 mutation(s) frequently demonstrate highly variable age of onset and disease severity (10), and both acquired and genetically deficient patients can be clinically recovered from an episode of TTP but still demonstrate UL-vWF multimers and severely decreased plasma ADAMTS13 activity (7, 11). These observations suggest that loss of ADAMTS13 activity and the associated increase in circulating UL-vWF may be necessary, but not sufficient, for the induction of a clinical episode of TTP, implying the existence of critical environmental triggers and/or genetic modifying factors for this disease.

Multiple ADAMTS13 mutations have been identified in familial TTP patients (4, 12, 13). However, the absence of cases with clear null mutations has led to the hypothesis that complete deficiency of ADAMTS13 may be lethal. Consistent with this notion, individuals with familial TTP usually exhibit low levels of residual ADAMTS13 activity, potentially accounting for some of the phenotypic variability observed among patients with this disorder (4). However, differences in residual ADAMTS13 activity are unlikely to account for the observed variation among individuals within a given family, as these persons are expected to harbor the same ADAMTS13 mutations. To address the
requirement for ADAMTS13 in normal hemostasis, and to further explore the pathogenesis of TTP in vivo, we generated ADAMTS13-deficient mice by gene targeting.

**Results**

ADAMTS13-deficient mice exhibit normal development and survival. The murine Adamts13 gene was disrupted by replacing exons 1–6 of murine Adamts13 with a neomycin resistance cassette (Figure 1A). Exons 1–6 contain the translation start site, signal peptide, propeptide, and most of the protease domain (including the active site cysteine residue). Thus, removal of these sequences should result in a nonfunctional protein. Disruption of Adamts13 was confirmed by PCR and Southern blot analysis, and transcripts containing exons 1–6 were not detectable by RT-PCR of liver mRNA (Figure 1B and C).

Intercross of Adamts13+/– mice yielded the expected Mendelian distribution of Adamts13 genotypes (Table 1), demonstrating that ADAMTS13 is not required for murine embryonic development or perinatal survival. Although pregnancy has been associated with the onset of TTP in humans (1), both male and female Adamts13+/– mice are fertile, and female Adamts13+/– mice experienced uncomplicated pregnancies with grossly normal litter sizes. Survival of Adamts13+/–, Adamts13+/–, and Adamts13+/– mice followed for more than 2 years was also identical (Figure 2A).

We also generated a second independently targeted ADAMTS13-deficient mouse line with exon 3 of Adamts13 deleted (Adamts13ex3–/– [see Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI26007DS1]). Intercross of Adamts13ex3–/– mice also resulted in the expected Mendelian distribution of progeny, and on analyses indicate that these mice possess a phenotype indistinguishable from that of the mice deficient in exons 1–6 of Adamts13, with which all subsequent experiments were performed.

ADAMTS13-deficient mice demonstrate loss of vWF-cleaving activity with an unchanged vWF multimer distribution. In humans, ADAMTS13 regulates the size of vWF multimers in the circulation by cleaving vWF subunits between tyrosine 1605 and methionine 1606 (14). Cleavage of murine vWF at this same site by murine ADAMTS13 has recently been demonstrated in vitro (15). Absence of ADAMTS13 activity in familial or acquired TTP results in the appearance of UL-vWF (8, 16–18). Despite the complete loss of vWF-cleaving activity (Figure 3, A and B), no difference was evident among the vWF multimer patterns of Adamts13+/– and Adamts13+/– mice (Figure 3C) on a mixed-strain C57BL/6J and 129X1/SvJ genetic background (Adamts13B/CN2). Furthermore, vWF multimers from both genotypes appeared similar in size to those from patients with familial TTP (data not shown). Thus, mice of this genetic background appear to exhibit UL-vWF regardless of Adamts13 expression status.

In contrast to the striking thrombotic microangiopathy seen in humans with TTP, peripheral blood smears prepared from healthy Adamts13B/CN2–/– mice were indistinguishable from those of their wild-type littermates (Figure 4, A and B) without evidence of schis-
tocytoses, rbc fragments, or thrombocytopenia. Automated peripheral blood analysis revealed no significant difference in rbc, wbc, or platelets (Table 2). Finally, histopathological survey revealed that tissues prepared from Adamts13B/129–/– mice were indistinguishable from their wild-type littermates controls (Figure 4, C–H). Specifically, the typical pink-staining “hyaline thrombi” seen in affected organs from TTP patients was not observed in Adamts13B/129–/– mice, nor were any other vascular or developmental abnormalities detected.

These results demonstrate that ADAMTS13 deficiency is not sufficient for the development of frequent spontaneous TTP in Adamts13B/129–/– mice. In contrast, at least half of humans with familial TTP present at birth or in the neonatal period (10). Hemostatic and other differences between humans and mice could account for the disparate phenotypes of these 2 species. For example, mice could possess an alternate protease whose function partially compensates for ADAMTS13 deficiency.

Figure 2

Figure 3
Plasma vWF-cleaving activity and vWF multimer size distribution for ADAMTS13-deficient mice and littermate controls. (A) vWF-cleaving activity in plasma from Adamts13B/129+/+, Adamts13B/129+/–, and Adamts13B/129–/– mice measured using purified human full-length vWF as a substrate. Activity was determined by analysis of the specific vWF-cleavage product indicated by the arrow, and comparison to the pattern in the control lane with ADAMTS13 activity completed inhibited by EDTA, as indicated. Specific ADAMTS13 activity was seen in plasma from Adamts13B/129+/+, and Adamts13B/129+/– mice but not Adamts13B/129–/– mice. (B) vWF-cleaving activity in plasma from Adamts13B/129+/+, Adamts13B/129+/–, and Adamts13B/129–/– mice determined using recombinant murine GST/vWF A2 domain as a substrate, which contains the previously identified ADAMTS13 cleavage site. Activity was demonstrated by appearance of the expected cleavage product indicated by the arrow. Specific ADAMTS13 activity was seen in plasma from Adamts13B/129+/+, and Adamts13B/129+/– mice but not Adamts13B/129–/– mice. Serial dilution of Adamts13B/129+/+ plasma demonstrated sensitivity to less than 1.5% of control plasma activity. (C) The vWF multimer distribution in plasma from Adamts13B/129+/+ and Adamts13B/129–/– mice. (D) The vWF multimer distribution in plasma from Adamts13B/CN2+/+ and Adamts13B/CN2–/– mice. The approximate range of UL-vWF is indicated by the brackets.
Figure 4
Lack of TTP pathology in Adamts13+/- mice. (A and B) Peripheral blood smears from Adamts13+/- (A) and Adamts13+/+ (B) mice appear identical, without evidence of schistocytes, rbc fragments, thrombocytopenia, or nucleated rbc. (C–H) H&E–stained tissue sections from Adamts13+/- (C, E, and G) and Adamts13+/+ (D, F, and H) are indistinguishable, without evidence of the pink-staining "hyaline thrombi" typically seen in affected organs from humans with TTP. Representative sections are shown from kidney (C and D), brain (E and F), and heart (G and H). Magnification, ×1,000 (A and B); ×400 (C and D); ×100 (E and F); and ×200 (G and H).

Introduction of the CASA/Rk genetic background alters the vWF multimer distribution, decreases survival, and results in spontaneous TTP. To examine the effect of genetic background on the ADAMTS13 deficiency phenotype, we crossed the Adamts13 mutation onto the CASA/Rk strain. We chose CASA/Rk because of its relatively large genetic distance from C57BL/6J and 129X1/SvJ and our previous observation that mice of this strain exhibit vWF levels approximately 5- to 10-fold higher than C57BL/6J mice (19). We reasoned that in the setting of ADAMTS13 deficiency, an increase in plasma vWF level may lead to increased susceptibility to TTP. After 1 (Adamts13/C) and 2 (Adamts13/C) generations of backcross to CASA/Rk, mean plasma vWF level increased by 40% and 50%, respectively (Figure 5A). This increase in vWF did not affect viability, as the numbers of Adamts13/+/+ and Adamts13+/+ mice observed at weaning were consistent with the expected Mendelian distributions (Table 1).

Table 2
Hematologic data of Adamts13+/+, Adamts13/C, and Adamts13+/+ mice

<table>
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<tr>
<th></th>
<th>wbc (×10^3 cells/μl)</th>
<th>HGB (g/dl)</th>
<th>HCT (%)</th>
<th>PLT (×10^3 cells/μl)</th>
<th>MCV (fl)</th>
<th>RDW (%)</th>
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<tr>
<td>Adamts13+/+</td>
<td>+/+</td>
<td>9.5 ± 2.6</td>
<td>16 ± 1.4</td>
<td>57 ± 2.7</td>
<td>1,460 ± 202</td>
<td>11.9 ± 0.6</td>
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<tr>
<td></td>
<td>+/-</td>
<td>10.0 ± 2.3</td>
<td>17 ± 1.3</td>
<td>59 ± 3.0</td>
<td>1,360 ± 167</td>
<td>11.5 ± 0.8</td>
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<tr>
<td></td>
<td>+/-</td>
<td>10.7 ± 2.4</td>
<td>16 ± 0.8</td>
<td>57 ± 2.5</td>
<td>1,350 ± 252</td>
<td>11.6 ± 0.7</td>
</tr>
<tr>
<td>Adamts13/C</td>
<td>+/+</td>
<td>10.3 ± 4.2</td>
<td>17 ± 1.0</td>
<td>61 ± 3.3</td>
<td>1,270 ± 211</td>
<td>13.1 ± 1.8</td>
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<tr>
<td></td>
<td>+/-</td>
<td>8.4 ± 3.4</td>
<td>17 ± 0.7</td>
<td>61 ± 1.9</td>
<td>1,370 ± 155</td>
<td>12.3 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>10.5 ± 3.5</td>
<td>17 ± 0.8</td>
<td>59 ± 2.5</td>
<td>1,193 ± 274</td>
<td>12.7 ± 1.2</td>
</tr>
<tr>
<td>Adamts13/C</td>
<td>+/+</td>
<td>11.1 ± 4.5</td>
<td>17 ± 1.2</td>
<td>59 ± 3.6</td>
<td>1,170 ± 152</td>
<td>14.6 ± 2.8</td>
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<tr>
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<td>+/-</td>
<td>9.2 ± 2.6</td>
<td>16 ± 1.2</td>
<td>59 ± 2.8</td>
<td>1,260 ± 294</td>
<td>15.5 ± 1.7</td>
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<tr>
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<td>17 ± 1.0</td>
<td>60 ± 2.9</td>
<td>954 ± 417</td>
<td>13.9 ± 1.8</td>
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Values are mean ± SD. HCT, hematocrit; HGB, hemoglobin; MCV, mean corpuscular volume; PLT, platelet; RDW, red cell distribution width.
ADAMTS13 deficiency prolongs vWF-mediated platelet-endothelial interactions in vivo. Following release of Weibel-Palade bodies, platelets transiently adhere to the stimulated murine endothelium in vivo, in a glycoprotein Ib– (on platelets) and vWF- (on the endothelium) dependent manner (20, 21). ADAMTS13 has been shown to cleave newly released UL-vWF multimers that are present on the surface of endothelial cells following stimulation in vitro (22). These observations support the hypothesis that ADAMTS13 regulates vWF function by cleaving newly released UL-vWF from the surface of endothelial cells, thus reducing platelet adhesion and spontaneous aggregation that would ultimately lead to thrombosis and development of TTP.

We investigated platelet-endothelial interactions in the animal model of ADAMTS13 deficiency (23). Following release of Weibel-Palade bodies, platelets transiently adhere to the stimulated murine endothelium in vivo, in a glycoprotein Ib– (on platelets) and vWF- (on the endothelium) dependent manner (20, 21). ADAMTS13 has been shown to cleave newly released UL-vWF multimers that are present on the surface of endothelial cells following stimulation in vitro (22). These observations support the hypothesis that ADAMTS13 regulates vWF function by cleaving newly released UL-vWF from the surface of endothelial cells, thus reducing platelet adhesion and spontaneous aggregation that would ultimately lead to thrombosis and development of TTP.

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Figure 5
Baseline vWF level and platelet counts of mice used in this study. (A) vWF levels in plasma from Adamts13B129/129, Adamts13B129/129, Adamts13B129/C129, and wild-type CASA/Rk mice. vWF levels were measured by ELISA and expressed in units/milliliter, with the vWF level in pooled C57BL/6J plasma set to 10 U/ml as a standard. The mean values for each mouse strain and genotype combination are indicated by horizontal bars. (B) Baseline platelet counts in healthy-appearing Adamts13B129/129, Adamts13B129/C129, and Adamts13B129/C129 mice displayed as ADAMTS13 positive (+/+ and +/-) and ADAMTS13 negative (–/–). The mean values for each group are indicated by horizontal bars. Baseline thrombocytopenia was observed in 0 of 33 Adamts13B129/129 mice (0%), 2 of 48 Adamts13B129–/– mice (4%), and 5 of 24 Adamts13B129–/– mice (21%). If more than 1 baseline platelet measurement for an individual mouse was available, the lowest value was used. Statistical significance was assessed by Student’s t test.

Figure 6
Spontaneous TTP in Adamts13B129/129 mice. (A) Peripheral blood smear showing thrombocytopenia, schistocytes, and fragmented rbc. (B–J) Tissue sections showing vWF-rich and fibrin-poor thrombi in small blood vessels from brain (B–D), heart (E–G), and kidney (H–J). Sections were stained with H&E (B, E, and H), anti-vWF (C, F, and I), or anti-fibrinogen (D, G, and J). Difficult-to-visualize thrombi are indicated with arrows. Magnification, ×1,000 (A); and ×200 (B–J).
Adamts13B/129+/+ and Adamts13B/129–/– mice injected with 200–250 pg/g of Stx i.v. exhibited no consistent change in rbc or platelet counts (Figure 8, A–D), with similar mortality in both groups (∼20%). However, striking differences were observed between ADAMTS13-deficient and control mice in the Adamts13B/CN2 genetic background (Figure 8, E–H). At 125 pg/g Stx, 6 of 13 Adamts13B/CN2–/– mice died by day 10, compared with 1 of 9 control Adamts13B/CN2+/+ mice (P < 0.01). Stx also induced profound thrombocytopenia in the Adamts13B/CN2–/– mice, with 12 of 13 animals demonstrating an at least 50% decrease in platelet count, and 8 of 13 mice showing a greater than 80% reduction (Figure 8G), compared with only modest decreases in Adamts13B/CN2+/+ controls (Figure 8E). Five of 13 Adamts13B/CN2+/+ mice (Figure 8H) also developed severe anemia compared with 0 of 9 controls (Figure 8F). In addition, examination of peripheral blood and tissue histology from Adamts13B/CN2–/– mice revealed the classic TTP findings of microangiopathic hemolytic anemia and widespread vWF-rich and fibrin-poor hyaline thrombi in the small vessels of multiple organs (Figure 9). These results demonstrate that Stx infusion into Adamts13B/CN2–/– mice results in a syndrome closely resembling human TTP.

Plasma vWF level is not a risk factor for TTP in mice. Plasma vWF levels in the Mus castaneus strain CASA/Rk are known to be 5- to 10-fold higher than in the more commonly studied Mus musculus strain C57BL/6J (19). Could these elevated vWF levels account for the increased mortality and baseline thrombocytopenia as well as the enhanced susceptibility to Stx-induced TTP observed in Adamts13B/CN2–/– mice compared with littermate +/+ controls? As human plasma vWF levels are also highly variable (25), could genetic and other factors modifying vWF levels also serve as key risk factors for TTP in humans? Surprisingly, we observed no correlation between elevated plasma vWF level and mortality (Figure 10A), baseline platelet count (Figure 10B), or degree of thrombocytopenia (Figure 10C). These data demonstrate that elevated plasma vWF level is not a TTP risk factor in mice and suggest that a genetic modifying factor (or factors) other than vWF level introduced by the CASA/Rk cross plays a key role in TTP pathogenesis.

Discussion

Our results demonstrate that complete ADAMTS13 deficiency is not lethal in mice and in fact is surprisingly well tolerated on some genetic backgrounds. These observations may reflect the
situation in humans, where many familial TTP cases are not diagnosed until early adulthood, with rare individuals carrying ADAMTS13 disease-causing mutations reported to remain free of TTP through the third decade of life (10). Taken together, these observations in humans and mice suggest that loss of ADAMTS13 activity, with the associated increase in circulating UL-vWF multimers, may be necessary, but not sufficient, for the induction of clinical TTP and imply the existence of other key genetic modifying factors and/or environmental triggers.

Of note, C57BL/6J mice primarily express a form of ADAMTS13 truncated after the sixth thrombospondin type-1 motif (termed ADAMTS13S), resulting from insertion of a retrovirus-like element during strain derivation and therefore truncated; and the wild-type allele of all B/CN1 mice was always CASA/Rk derived and therefore truncated; and the wild-type allele of all B/129 mice was always C57BL/6J derived and therefore truncated; and the wild-type allele of all B/CN1 and B/CN2 mice was always CASA/Rk derived and therefore nontruncated (data not shown). ADAMTS13S lacks the 2 C-terminal CUB domains but retains grossly normal vWF-cleaving activity in vitro. However, several missense and truncation mutations distal to TSP1-6 have been found in human familial TTP patients, suggesting that these sequences may be required for full ADAMTS13 function in vivo. Similarly, truncated C57BL/6J ADAMTS13 may also be hypomorphic in vivo, potentially accounting for the UL-vWF multimers observed in mice homozygous for this allele. The absence of thrombosis or other manifestations of TTP in wild-type C57BL/6J mice suggests that these mice may harbor 1 or more “balancing mutations” selected during strain derivation (27, 28), compensating for the relative ADAMTS13 deficiency. Loss of these balancing mutation(s) with the genetic cross onto the CASA/Rk strain might account for the emergence of the more overt TTP phenotype seen in the Adamts13<sup>13<sub>B/CN2</sub>/−</sup> mice.

Interestingly, a common ADAMTS13 variant (P475S) recently was identified in the Japanese population that demonstrates negligible vWF-cleaving activity in vitro (12). With an allele frequency of 5.1%, approximately 10% of the Japanese population may be heterozygous for P475S (resulting in Adamts13 activity of ~50%), with up to 1% possessing severely reduced ADAMTS13 activity secondary to P475S homozygosity. However, the frequency of TTP among the Japanese is not reported to be higher than in other populations (29), suggesting sufficient residual activity from the P475S allele or the influence of other genetic or environmental factors. These observations of hypomorphic ADAMTS13 alleles in both humans and mice suggest the possibility that moderately reduced ADAMTS13 activity may be underbalancing selection, similar to hemoglobin mutations in sickle cell anemia and α-thalassemia (30, 31) and cystic fibrosis (32, 33).

The high degree of population variability for plasma vWF levels in humans and mice, together with its function as the only known substrate for ADAMTS13, suggested genetic factors controlling vWF level as leading candidates for modifiers of TTP susceptibility. Indeed, the 25–30% reduction in plasma vWF seen in individuals with blood type O might be expected to confer protection against TTP. Surprisingly, our analyses demonstrated no correlation between elevated plasma vWF level in the mouse and the manifestations of TTP. Though this observation remains to be confirmed in humans, these findings suggest that the critical determinants of TTP susceptibility may lie in other genetic factors, perhaps associated with variations in platelet or vessel wall function. However, we cannot exclude a subtle qualitative difference in vWF not reflected in the antigen level measurement.

Future characterization of these murine strain–specific modifiers of TTP susceptibility and pathologically distinct, likely indicating divergent pathological mechanisms following this disease-initiating event. These data also suggest that Stx, or other similar microbially derived toxins, may be key environmental triggers for TTP. These observations might also explain the frequent association of an infectious prodrome with acute episodes of both acquired and familial TTP (1, 10).

Finally, it is important to note that C57BL/6J has become the standard “control” inbred mouse strain for gene targeting experiments, particularly in cardiovascular research (34). Our findings suggest that an endogenous mutation in the C57BL/6J Adamts13 allele, along
with balancing mutations at one or more additional loci, could complicate the analysis of vascular and hemostatic function in this specific genetic background. This concern may require reinterpretation of a considerable body of previous work addressing atherosclerosis, inflammation, and clot formation in gene-targeted mice.

**Methods**

**Mouse strains.** Mice of strain C57BL/6J and CASA/Rk were obtained from The Jackson Laboratory. Mice were maintained in a specific pathogen–free facility at the University of Michigan. All procedures were approved by the University Care and Use Committee on Animals.

**Generation of ADAMTS13-deficient mice.** Gene targeting was performed essentially as previously described (35). Briefly, a targeting vector was designed to replace exons 1–6 of murine Adamts13 with a neomycin-resistance cassette (Figure 1A). With the assistance of the Transgenic Animal Facility at the University of Michigan, this vector was electroporated into murine ES cells from strain 129X1/Sv, and neomycin-resistant clones were selected, verified, expanded, and subsequently injected into C57BL/6J blastocysts, which were then implanted into pseudopregnant C57BL/6J females. High-level male agouti chimeric progeny were bred with C57BL/6J females, and germine transmission of the disrupted Adamts13 allele was verified by Southern blotting of Spl-digested DNA with cDNA probes both 5’ and 3’ outside of the targeting arms and EcoRI-digested DNA with cDNA probes specific for Adamts13 exons 4–6 and 7–8 (data not shown). Resulting F1, Adamts13+/− heterozygotes were intercrossed to obtain F2 mice (designated Adamts13B129+/−, Adamts13129+/−, and Adamts13129+/−). F2 Adamts13129+/− mice were also backcrossed to wild-type CASA/Rk mice, and the resulting Adamts13 heterozygotes were either intercrossed to obtain Adamts13B129+/−, Adamts13B129+/−, and Adamts13B129+/− mice or were backcrossed again to wild-type CASA/Rk mice. The resulting Adamts13 heterozygotes from this second backcross were intercrossed to obtain Adamts13B129+/−, Adamts13B129+/−, and Adamts13B129+/− mice. Littermate controls were used for all experiments.

**PCR and RT-PCR.** The locations of primers used for PCR genotyping are shown in Figure 1A. Tail genomic DNA was extracted and purified as described previously (35). PCR and agarose gel electrophoresis were performed by standard techniques. For RT-PCR, liver mRNA was obtained from Adamts13129+/−, Adamts13129+/−, and Adamts13129+/− mice using the Oligotex mRNA Midi Kit (Qiagen). RT-PCR was performed with the SuperScript One-Step RT-PCR Kit (Invitrogen Corp.), using oligo-dT for first-strand synthesis, and primers specific to Adamts13 exons 1–6 for the subsequent PCR. The control primers were for murine vWF.

**Analysis of peripheral blood.** Blood was collected into 20-µl EDTA-coated capillary tubes (Drew Scientific) by retro-orbital bleeding under isoflurane anesthesia. Analysis was performed with a Bayer ADVIA 120 whole blood analyzer (Bayer HealthCare) using the murine C57BL/6 algorithm. Peripheral blood smears were prepared by standard techniques and were fixed and stained with the HEMA 3 system (Fisher Scientific).

**Tissue preparation and analysis.** Anesthetized mice were exsanguinated by cardiac puncture and subsequently flushed via the left ventricle with 3 ml PBS (pH 7.4) followed by 3 ml Z-fix (Anatech Ltd.). Organs were removed and incubated for 24–48 hours in Z-fix and subsequently embedded in paraffin, sectioned, and stained with H&E by standard techniques. Immunohistochemistry to identify vWF or fibrin was performed using the ABC method (Vector Laboratories) and anti-vWF A0082 (DakoCytomation) or anti-fibrinogen YNGMFbg7S (Accurate Chemical & Scientific Corp.) antibodies.

**vWF cleavage assays.** Whole blood was obtained from the indicated anesthetized mice by cardiac puncture (with 0.1 M sodium citrate as an anti-coagulant), and plasma was subsequently prepared by centrifugation. Cleavage of glutathione-S-transferase/vWF A2 (GST/vWF A2) was performed as described previously (36). Briefly, 5 µl of plasma was incubated with 100 ng of fusion protein in reaction buffer (5 mM Tris, 10 mM BaCl2, 1 mM PMSF, pH 8.0) for 1 hour at 37°C. Cleavage products were resolved by SDS–14% PAGE and transferred to nitrocellulose. Proteins...
Male and female mice were studied at age 4–8 weeks, at a weight of 14–21 g. Platelets for infusion were isolated from experimental procedures. Blood was harvested from the retro-orbital venous plexus by puncture and collected in 1-ml polypropylene tubes containing 300 µl of heparin (30 U/ml), and platelet-rich plasma (PRP) was obtained by centrifugation. The PRP was transferred to fresh tubes containing 2 ml of prostaglandin I2 (PGI2) (2 mg/ml) and incubated at 37°C for 5 minutes. The washing step was performed essentially as described previously (37). Briefly, 0.75% SeaKem HGT (Cambrex) × 14 cm gels were prepared in electrophoresis buffer (40 mM Tris-acetate, pH 7.8, 0.1% SDS, 1 mM EDTA) and cooled to 4°C. Whole blood was drawn into Na+-heparin–coated glass capillary tubes, and plasma was separated by centrifugation and diluted 1:10 in sample buffer (0.1 M Na2PO4, pH 7.0, 1.0% SDS). Prior to electrophoresis, 1:10 volume loading buffer was added (50% glycerol, 1.0% bromophenol blue). One to 10 µl of diluted plasma was used per well. Gels were electrophoresed at 4°C until the bromophenol blue had migrated approximately 10 cm. Proteins were then transferred to nitrocellulose electrophoretically and were subsequently immunoblotted with anti-vWF antisera A0082 (DakoCytomation), followed by a HRP-conjugated goat anti-rabbit sera (Bio-Rad Laboratories) as the secondary antibody. Detection was with ECL (Amersham Biosciences). Plasma vWF levels were quantified by ELISA as described previously (38), using anti-vWF antisera A0082 as the primary antibody and HRP-conjugated vWF antisera (DakoCytomation) as the secondary antibody. Detection was with 1-step Ultra TMB (Pierce Biotechnology Inc.).

**Intravital microscopy.** Male and female mice were studied at age 4–8 weeks, at a weight of 14–21 g. Platelets for infusion were isolated from mice of the same genotype at age 4–6 months. The Animal Care and Use Committee of the CBR Institute for Biomedical Research approved all experimental procedures. Blood was harvested from the retro-orbital venous plexus by puncture and collected in 1-ml polypropylene tubes containing 300 µl of heparin (30 U/ml), and platelet-rich plasma (PRP) was obtained by centrifugation. The PRP was transferred to fresh tubes containing 2 ml of prostaglandin I2 (PGI2) (2 mg/ml) and incubated at 37°C for 5 minutes. After centrifugation at 1,500 g, pellets were resuspended in 1 ml modified Tyrode-HEPES buffer (137 mM NaCl, 0.3 mM Na2HPO4, 2 mM KCl, 12 mM NaHCO3, 5 mM HEPES, 5 mM glucose, 0.35% BSA) containing 2 ml PGI2, incubated at 37°C for 5 minutes, and subsequently centrifuged at 1,500 g for 5 minutes. The washing step was repeated twice to remove PGI2. Platelets were then fluorescently labeled with 0.25 mg/ml calcein AM (Invitrogen Corp.) for 10 minutes at room temperature. Fluorescent platelets (1.25 × 108 platelets/kg) were infused through the retro-orbital plexus.

**PLATELET MORPHOMETRY**

**Figure 10**

Plasma vWF level is not a risk factor for thrombocytopenia or Stx-induced TTP in Adamts13BCND+ mice. No correlation was observed between plasma vWF level and Stx-induced mortality (A), baseline platelet count (B), Stx-induced thrombocytopenia (C), or Stx-induced anemia (data not shown). The P value in A was calculated by Student’s 2-tailed t test, and the r values in B and C are indicated. Open diamonds indicate Adamts13BCND+ and Adamts13BCND− mice, and filled squares indicate Adamts13BCND− mice.

Intravital microscopy was as described previously with slight modification (21). Briefly, mice were anesthetized with 2.5% tribromoethanol (0.15 ml/10 g), and fluorescent platelets were infused after 5 minutes. An incision was made through the abdominal wall to expose the mesentery and mesenteric venules of 200- to 300-µm diameter. One venule per animal was filmed for 3 minutes for baseline determination before A23187 superfusion (30 µl of a 10 µmol/l solution), and filming continued until after the platelet sticking and rolling decreased to baseline. Adherent fluorescent platelets were quantified over 250-µm-long and 100- to 120-µm-wide venular segments visible on a given frame lasting 0.2 seconds and subsequently translated to the number of fluorescent platelets adhering/mm²/frame. Results are reported as the mean ± SEM. The statistical significance of the difference between means was assessed by the 2-tailed Student’s t test. P < 0.05 was considered statistically significant.

Stx challenge. Stx-2 (Sigma-Aldrich) was diluted to a final concentration of 25 pg/µl in normal saline. Anesthetized mice were injected i.v. with the indicated dose of Stx. Blood for complete blood count analysis and peripheral blood smears was obtained by retro-orbital bleeding prior to injection and subsequently on a daily basis.

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Address correspondence to: David Ginsburg, University of Michigan, 5028 Life Sciences Institute, 210 Washtenaw Avenue, Ann Arbor, Michigan 48109, USA. Phone: (734) 647-4808; Fax: (734) 936-2888; E-mail: ginsburg@umich.edu.