ADAMTS13 Endopeptidase Protects against Vascular Endothelial Growth Factor Inhibitor–Induced Thrombotic Microangiopathy

Luise Erpenbeck,*† Melanie Demers,*† Zsuzsanna K. Zsengellér,‡ Maureen Gallant,* Stephen M. Cifuni,* Isaac E. Stillman,§ S. Ananth Karumanchi,‡ and Denisa D. Wagner*†¶

*Program in Cellular and Molecular Medicine, Boston Children’s Hospital, Boston, Massachusetts; †Department of Pediatrics, Harvard Medical School, Boston, Massachusetts; ‡Departments of Medicine, Obstetrics and Gynecology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts; §Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts; and ¶Division of Hematology/Oncology, Boston Children’s Hospital, Boston, Massachusetts

ABSTRACT

Thrombotic microangiopathy (TMA) is a life-threatening condition that affects some, but not all, recipients of vascular endothelial growth factor (VEGF) inhibitors given as part of chemotherapy. TMA is also a complication of preeclampsia, a disease characterized by excess production of the VEGF-scavenging soluble VEGF receptor 1 (soluble fms-like tyrosine kinase 1; sFlt-1). Risk factors for VEGF inhibitor–related TMA remain unknown. We hypothesized that deficiency of the VWF-cleaving ADAMTS13 endopeptidase contributes to the development of VEGF inhibitor–related TMA. ADAMTS13−/− mice overexpressing sFlt-1 presented all hallmarks of TMA, including thrombocytopenia, schistocytosis, anemia, and VWF-positive microthrombi in multiple organs. Similar to VEGF inhibitor–related TMA in humans, these mice exhibited severely impaired kidney function and hypertension. In contrast, wild-type mice overexpressing sFlt-1 developed modest hypertension but no other features of TMA. Recombinant ADAMTS13 therapy ameliorated all symptoms of TMA in ADAMTS13−/− mice overexpressing sFlt-1 and normalized BP in wild-type mice. ADAMTS13 activity may thus be a critical determinant for the development of TMA secondary to VEGF inhibition. Administration of recombinant ADAMTS13 may serve as a therapeutic approach to treat or prevent thrombotic complications of VEGF inhibition.


Received December 1, 2014. Accepted March 20, 2015. Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Prof. Denisa D. Wagner, Division of Hematology/Oncology, Boston Children’s Hospital, Harvard Medical School, 3 Blackfan Circle, Third Floor, Boston, MA 02115. Email: Denisa.Wagner@childrens.harvard.edu

Copyright © 2016 by the American Society of Nephrology

Thrombotic microangiopathies (TMAs) are a heterogeneous group of life-threatening disorders characterized by thrombocytopenia, schistocytosis, hemolytic anemia, microvascular thrombosis and end-organ damage affecting the kidney and brain. Among the major subtypes of TMAs are thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome. TMAs may also be pregnancy-related: either as a facet of preeclampsia, characterized by hypertension and proteinuria, or as part of the Hemolysis, Elevated Liver enzymes and Low Platelet count (HELLP) syndrome. HELLP syndrome is a severe complication of preeclampsia, occurring in 0.5%–0.9% of all pregnancies. Acquired TMAs are also observed after solid organ transplants or related to certain drugs, advanced malignancies, severe hypertension or infections.

The pathologic mechanisms underlying TMAs are diverse. Typical hemolytic uremic syndrome is usually caused by an infection with shiga-like-
toxin–producing bacteria. TTP, on the other hand, is associated with a marked deficiency in the metalloprotease ADAMTS13 with under 5% of its normal activity. This deficiency can be caused by a genetic defect in the ADAMTS13 gene (hereditary TTP) or by autoantibodies against the enzyme (acquired TTP). In healthy individuals, ADAMTS13 is responsible for the cleavage of large multimers of von Willebrand Factor (VWF), which are released from endothelial Weibel–Palade bodies or from platelets upon stimulation. ADAMTS13 deficiency leads to an accumulation of highly thrombogenic ultra-large VWF that serves as a nidus for pathologic thrombus formation. Intriguingly, even patients with severe ADAMTS13 deficiency may remain asymptomatic for years, suggesting that a second hit is needed to provoke clinical manifestations. Known triggers of TTP are cancer, infection, pregnancy, and certain medications, such as some antineoplastic therapies. Both preeclampsia/HELLP syndrome and atypical hemolytic uremic syndrome can be associated with a decrease in ADAMTS13 concentration or activity, implying a more general role of this enzyme in TMA. Chemotherapies that inhibit vascular endothelial growth factor (VEGF) or VEGF signaling—e.g., bevacizumab, sunitinib, and afibercept—have been shown to induce TMA. While TMA is the most severe adverse consequence of anti-VEGF therapy, the spectrum of toxicities includes proteinuria and hypertension, issues that are believed to arise from renal microvascular injury.

To determine whether sFlt-1 overexpression can induce TMA in mice lacking ADAMTS13, ADAMTS13−/− mice were injected with Ad-sFlt-1. Overexpression of sFlt-1 has been widely used in both mice and rats as a model of preeclampsia, as high levels of sFlt-1 can induce the classic symptoms of hypertension and proteinuria in a dose-dependent manner in rodents. Expression of sFlt-1 levels in the plasma reaches a maximum 7 days after injection of the Ad-sFlt-1 virus and subsequently sFlt-1 expression declines. As controls, wild-type (WT) mice were treated with identical amounts of Ad-sFlt-1 as the ADAMTS13−/− Ad-sFlt-1 mice, or ADAMTS13−/− received equivalent amounts of Ad-null virus. The mice were then monitored for pathologic and clinical signs of TMA.

Table 1. sFlt-1 plasma levels in WT and ADAMTS13−/− mice at days 7 and 10 after adeno-virus injection

<table>
<thead>
<tr>
<th>Day/Group</th>
<th>WT Ad-sFlt-1</th>
<th>ADAMTS13−/− Ad-null</th>
<th>ADAMTS13−/− Ad-sFlt-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7</td>
<td>6345.91±576.13 ng/ml n.s. to ADAMTS13−/− Ad-sFlt-1 day 7</td>
<td>0.63±0.15 ng/ml a to ADAMTS13−/− Ad-sFlt-1 day 10</td>
<td>7177.26±286.91 ng/ml</td>
</tr>
<tr>
<td></td>
<td>n.s. to WT Ad-sFlt-1 day 10 (P=0.08)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 10</td>
<td>4995.59±388.71 ng/ml n.s. to ADAMTS13−/− Ad-sFlt-1 day 10</td>
<td>0.69±0.12 ng/ml</td>
<td>4813.54±307.73 ng/ml</td>
</tr>
</tbody>
</table>

Values are average ± SEM, n=7–13. There was no significant difference between sFlt-1 plasma levels of WT and ADAMTS13−/− Ad-sFlt-1 mice on day 7 or day 10, respectively. From day 7 to day 10, sFlt-1 levels declined significantly in the ADAMTS13−/− Ad-sFlt-1 mice and showed a trend toward lower sFlt-1 levels in the WT Ad-sFlt-1 mice. n.s., not significant.

sFlt-1 Overexpression Induces Thrombocytopenia and Schistocytosis in ADAMTS13−/− Mice

As one of the principal symptoms of TMA is thrombocytopenia, platelet counts were analyzed on days 4, 7, and 10 (Figure 1A). The Ad-sFlt-1 WT mice showed a minor but significant drop in platelet count on day 4 from which they recovered by day 7. The ADAMTS13−/− Ad-null mice had no significant alterations in their platelet counts throughout the observation period. On the other hand, ADAMTS13−/−
Ad-sFlt-1 mice experienced a significant drop in platelet counts as early as day 4, which became even more pronounced by day 7. On day 10, platelet counts of ADAMTS13<sup>−/−</sup> Ad-sFlt-1 mice were no longer significantly different from untreated ADAMTS13<sup>−/−</sup> mice or the control groups. Alternative platelet measurements by flow cytometry were initially also used to exclude artifacts such as changes in platelet size that might lead to erroneous results in Hemavet platelet count determination (Supplementary Figure 1).

As pronounced thrombocytopenia noted with sFlt-1 overexpression in the absence of ADAMTS13 suggested an induction of TMA, we went on to analyze the number of schistocytes in blood smears, an important characteristic of TMAs (Figure 1B). At day 4, schistocyte counts did not differ from those of untreated mice in any group. However, by day 7, ADAMTS13<sup>−/−</sup> mice overexpressing sFlt-1 showed an almost 2-fold increase in schistocytes, together with anisocytosis (strong variation in the size of red blood cells) and polychromasia (variations in the staining behavior of erythrocytes; Figure 1, B and D). Schistocytosis still persisted at day 10 in the ADAMTS13<sup>−/−</sup> Ad-sFlt-1 group. As a severe destruction of red blood cells would be expected to lead to a compensatory rise in reticulocyte counts, reticulocyte numbers were likewise assessed in the blood smears (Figure 1, C and D). At day 7, reticulocyte counts started to increase in the ADAMTS13<sup>−/−</sup> Ad-sFlt-1 group and were significantly higher compared with the ADAMTS13<sup>−/−</sup> Ad-null mice. At day 10, this increase became even more pronounced. As the most severe destruction of red blood cells was observed on day 7 in the ADAMTS13<sup>−/−</sup> Ad-sFlt-1 mice, these results indicate a compensatory upregulation of red blood cell formation and thus reticulocytosis. Interestingly, WT Ad-sFlt-1 mice also showed a notable increase in their reticulocyte counts by day 10, indicating that low-grade red blood cell destruction might also have been taking place in those mice, although schistocyte counts remained normal in the majority of the mice throughout the study. There were no major differences in phenotypes between untreated WT mice and WT mice receiving Ad-null therapy (Supplementary Table 1).
Patients suffering from TMA characteristically have hemolytic anemia, resulting from the severe destruction of red blood cells. Hemoglobin values were assessed as a measure for the severity of the resulting anemia (Figure 1E). In the WT Ad-sFlt-1 mice, two mice were anemic by day 4, although overall hemoglobin was not significantly reduced at that point. At days 7 and 10, WT mice had slightly elevated levels of hemoglobin, in line with the previously proposed notion of a compensatory upregulation of erythrocyte production. In the ADAMTS13<sup>−/−</sup> mice, none of the Ad-null-treated mice, but five of 13 ADAMTS13<sup>−/−</sup> Ad-sFlt-1 mice developed anemia with values <11 g/dl (hemoglobin 3.2–10.7 g/dl) by day 7. Average hemoglobin in the ADAMTS13<sup>−/−</sup> Ad-sFlt-1 was also significantly lower compared with untreated ADAMTS13<sup>−/−</sup> mice, corroborating our observation that the development of TMA is strongly enhanced in ADAMTS13 deficiency.

**sFlt-1 Causes VWF Release and Leads to VWF-Rich Microthrombi in Numerous Organs of ADAMTS13<sup>−/−</sup> Mice**

In TTP, schistocytes have been proposed to be a result of mechanical slicing of red blood cells by strands of VWF spanning across blood vessels.7–26 Because of the significant increase in schistocytes in the ADAMTS13<sup>−/−</sup> Ad-sFlt-1 mice, we hypothesized that sFlt-1 leads to an increased release of stored VWF into the blood. We therefore measured plasma VWF in WT and ADAMTS13<sup>−/−</sup> mice, injected with Ad-null or Ad-sFlt-1 (Figure 1F). We found a strong increase in plasma VWF of the WT Ad-sFlt-1 mice. However, VWF levels were not elevated in the ADAMTS13<sup>−/−</sup> Ad-sFlt-1 mice (Figure 1F). A plausible explanation for this observation would be the consumption of the uncleaved VWF strands into thrombi within the vasculature in the ADAMTS13<sup>−/−</sup> Ad-sFlt-1 mice, reflected by the thrombocytopenia seen in this group (Figure 1A). Microthrombi are a pathologic characteristic of TMA and have been observed in murine models of TTP.27,28 For that reason, we performed immunohistochemical staining for VWF-rich thrombi in livers, kidneys, lungs, and hearts of WT and ADAMTS13<sup>−/−</sup> mice treated with Ad-sFlt-1 and in Ad-null ADAMTS13<sup>−/−</sup> mice at day 7 after virus injection. VWF-positive thrombi could be found in all four organ groups in the ADAMTS13<sup>−/−</sup> mice that had received Ad-sFlt-1 (Figure 2, Supplementary Figure 2, Table 2), but were extremely rare in the control mice. Particularly, the kidneys of ADAMTS13<sup>−/−</sup> Ad-sFlt-1 mice appeared to be very prone to microthrombosis, which is in line with the current knowledge of renal injury secondary to VEGF inhibitors.17

**ADAMTS13<sup>−/−</sup> Mice Overexpressing sFlt-1 Are Susceptible to Proteinuria and Elevated BP**

In patients treated with VEGF inhibitors, proteinuria and hypertension are common and by far the most prevalent side effect of the treatment.19,18 Similarly, preeclampsia is defined as new-onset hypertension and proteinuria during pregnancy, and both features are thought to originate at least in part from high levels of the VEGF scavenger sFlt-1.21 Therefore, we next investigated whether the lack of ADAMTS13 exacerbates the onset of these two prominent symptoms. Indeed, ADAMTS13<sup>−/−</sup> mice that had received Ad-sFlt-1 quickly developed pronounced proteinuria, determined as albumin-creatinine ratio21 (Figure 3A). In spite of an overall low albumin-creatinine ratio, WT mice still showed a significant increase by day 7, from 9.3±3.67 mg/g to 16±7.9 mg/g, indicating that sFlt-1 induces modest proteinuria in mice with ADAMTS13.

To evaluate the influence of ADAMTS13 deficiency on the development of hypertension in mice overexpressing sFlt-1, systolic BP was measured in a set of mice before virus injection, on days 7 and 10 (Figure 3B). In line with the previous reports of BP increase after sFlt-1 overexpression,21,29 the WT Ad-sFlt-1 mice showed a significant elevation in their BP by day 7, which became more pronounced at day 10. ADAMTS13<sup>−/−</sup> mice were not significantly elevated in the WT Ad-sFlt-1 mice, at day 7 after Ad-sFlt-1 or Ad-null injection of rhADAMTS13 or PBS (as vehicle) (Table 2). In the WT Ad-sFlt-1 mice, at day 7 after Ad-sFlt-1 or Ad-null injection of rhADAMTS13 or PBS (as vehicle) (Table 2). In the WT Ad-sFlt-1 mice, at day 7 after Ad-sFlt-1 or Ad-null injection of rhADAMTS13 or PBS (as vehicle) (Table 2). In the WT Ad-sFlt-1 mice, at day 7 after Ad-sFlt-1 or Ad-null injection of rhADAMTS13 or PBS (as vehicle) (Table 2). In the WT Ad-sFlt-1 mice, at day 7 after Ad-sFlt-1 or Ad-null injection of rhADAMTS13 or PBS (as vehicle) (Table 2). In the WT Ad-sFlt-1 mice, at day 7 after Ad-sFlt-1 or Ad-null injection of rhADAMTS13 or PBS (as vehicle) (Table 2).

**Table 2. Organs positive for VWF-rich thrombi in WT and ADAMTS13<sup>−/−</sup> mice, at day 7 after Ad-sFlt-1 or Ad-null injection and treatment with rhADAMTS13 or PBS (as vehicle)**

<table>
<thead>
<tr>
<th>Group/Organ</th>
<th>Liver</th>
<th>Kidney</th>
<th>Lung</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Ad-sFlt-1</td>
<td>0/6 (0%)</td>
<td>1/6 (16%)</td>
<td>1/6 (16%)</td>
<td>0/6 (0%)</td>
</tr>
<tr>
<td>ADAMTS13&lt;sup&gt;−/−&lt;/sup&gt; Ad-null</td>
<td>0/6 (0%)</td>
<td>0/6 (0%)</td>
<td>0/6 (0%)</td>
<td>1/6 (16%)</td>
</tr>
<tr>
<td>ADAMTS13&lt;sup&gt;−/−&lt;/sup&gt; Ad-sFlt-1</td>
<td>5/6 (83%)</td>
<td>6/6 (100%)</td>
<td>4/6 (66%)</td>
<td>5/6 (83%)</td>
</tr>
<tr>
<td>b to WT Ad-sFlt-1</td>
<td>c to ADAMTS13&lt;sup&gt;−/−&lt;/sup&gt; Ad-sFlt-1</td>
<td>n.s. to WT Ad-sFlt-1</td>
<td>b to WT Ad-sFlt-1</td>
<td></td>
</tr>
<tr>
<td>ADAMTS13&lt;sup&gt;−/−&lt;/sup&gt; Ad-sFlt-1+PBS</td>
<td>4/5 (80%)</td>
<td>5/5 (100%)</td>
<td>2/5 (40%)</td>
<td>4/5 (80%)</td>
</tr>
<tr>
<td>ADAMTS13&lt;sup&gt;−/−&lt;/sup&gt; Ad-sFlt-1+rhADAMTS13</td>
<td>3/9 (33%)</td>
<td>2/9 (22%)</td>
<td>1/9 (11%)</td>
<td>3/9 (33%)</td>
</tr>
<tr>
<td>n.s. to ADAMTS13&lt;sup&gt;−/−&lt;/sup&gt; Ad-sFlt-1+PBS (P=0.09)</td>
<td>b to ADAMTS13&lt;sup&gt;−/−&lt;/sup&gt; Ad-sFlt-1+PBS</td>
<td>n.s. to ADAMTS13&lt;sup&gt;−/−&lt;/sup&gt; Ad-sFlt-1+PBS (P=0.21)</td>
<td>n.s. to ADAMTS13&lt;sup&gt;−/−&lt;/sup&gt; Ad-sFlt-1+PBS (P=0.09)</td>
<td></td>
</tr>
</tbody>
</table>

Last two rows: treatment of ADAMTS13<sup>−/−</sup> Ad-sFlt-1 mice with PBS (as vehicle) or rhADAMTS13 was carried out from day 4 to day 7 after Ad-sFlt-1 or Ad-null injection. Statistical comparison between the groups was performed by chi-squared test between the indicated groups. n.s., not significant.

*P<0.05.

**P<0.01.

**P<0.001. 

www.jasn.org  BASIC RESEARCH


123
mice that had received Ad-null virus did not show alterations in their BP throughout the observation period. In contrast, in ADAMTS13−/− mice, BP increased by approximately 25 mmHg 10 days after Ad-sFlt-1 injection, a more substantial increase than that observed in the WT Ad-sFlt-1 mice. In conclusion, ADAMTS13−/− mice overexpressing sFlt-1 not only displayed pathophysiological parameters of TMA, but also an exacerbation of clinical symptoms typical of some patients exposed to VEGF inhibitors.

**Figure 2.** ADAMTS13−/− mice show VWF-rich thrombi in multiple organs after sFlt-1 overexpression. Organs were collected from WT and ADAMTS13−/− mice 7 days after injection of Ad-sFlt-1 or Ad-null and immunohistochemical staining for VWF (brown) was performed. Representative photographs of liver, kidney, lung, and heart sections are shown. Arrowheads indicate VWF-rich thrombi. Scale bar, 100 μm.

ADAMTS13−/− Mice Injected with Ad-sFlt-1 Are More Prone to Glomerular Endotheliosis than WT Mice

Endothelial swelling, resulting in enlarged glomeruli, and capillary loop occlusion are histologic indicators of kidney damage due to VEGF inhibition. Therefore, hematoxylin and eosin (H&E) micrographs (Figure 3C, upper panel) of kidneys from the WT Ad-sFlt-1, the ADATMS13−/− Ad-null, and the ADAMTS13−/− Ad-sFlt-1 mice were analyzed for percentage of open capillary volume (Figure 3D) and average glomerular area (Figure 3E). In the kidneys of ADAMTS13−/− Ad-sFlt-1 mice, the open capillary lumen was dramatically reduced and glomeruli enlarged, consistent with the strong proteinuria in these animals. Electron microscopy was performed to further evaluate glomerular structure (Figure 3C, lower panel). It revealed swollen endothelial cells and obliterated capillary lumens in the ADAMTS13−/− Ad-sFlt-1 mice, while both WT sFlt-1 mice as well as ADAMTS13−/− Ad-null mice showed open capillary loops. WT Ad-sFlt-1 kidney tissue had modest endothelial damage, suggesting a certain amount of structural damage even without ADAMTS13-deficiency.

**Treatment with recombinant human ADAMTS13 Rescues ADAMTS13−/− Ad-sFlt-1 Mice from the Development of TMA**

To test whether insufficient cleavage of VWF through ADAMTS13 was directly responsible for the observed phenotype in the ADAMTS13−/− Ad-sFlt-1 mice, we attempted to rescue these mice by administering recombinant human ADAMTS13 (rhADAMTS13) every 24 hours, from day 4 to day 7 after virus injection. Seven days after receiving Ad-sFlt-1, ADAMTS13−/− mice that received rhADAMTS13 did not experience a drop in platelet count (Figure 4A). Blood smears normalized and did not show schistocytosis (Figure 4, B and F). Reticulocytes were significantly lowered by rhADAMTS13 treatment (Figure 4, C and F) and none of the rhADAMTS13-treated mice developed anemia (Figure 4D). To exclude that these differences were due to changes in the levels of sFlt-1 expression, we measured sFlt-1 levels in ADAMTS13−/− mice injected with Ad-sFlt-1 and then treated with rhADAMTS13 or PBS. The values between these two groups were not significantly different, with a mean of 4835±240.2 ng/ml for the ADAMTS13−/− Ad-sFlt-1+PBS mice compared with 4258.1±319.7 ng/ml for the ADAMTS13−/− Ad-sFlt-1+rhADAMTS13 mice (n=4, P=0.20).

Interestingly, in rhADAMTS13-treated ADAMTS13−/− mice after Ad-sFlt-1 challenge, an almost 2-fold increase of plasma VWF was observed compared with vehicle (PBS)-treated ADAMTS13−/− mice by day 7 (Figure 4E), similar to the WT mice after Ad-sFlt-1 injection (Figure 1F). This implies that rhADAMTS13 did not prevent the release of VWF after sFlt-1-induced endothelial damage, but that released VWF could now be cleaved and was no longer recruited into thrombi. Indeed, at day 7 after Ad-sFlt-1 injection, the
formation of microthrombi was reduced by more than 50% in all organ types (Figure 4G, Table 2, last 2 rows) after rhADAMTS13 treatment in ADAMTS13−/− mice, with the kidneys being the most strongly protected. The rhADAMTS13-treated mice also fared better in terms of kidney histology (Figure 5A), with a significantly higher open capillary volume (Figure 5B) and smaller glomeruli (Figure 5C). Kidney function of ADAMTS13−/− Ad-sFlt-1 mice became comparable to that of control mice upon treatment with rhADAMTS13 (Figure 5D), and BP in the ADAMTS13−/− Ad-sFlt-1 group normalized (Figure 5E).

As WT mice had shown a modest rise in their albumin/creatinine values as well as an increase in BP after Ad-sFlt-1 injection, WT Ad-sFlt-1 mice were also given rhADAMTS13. Albumin/creatinine values decreased after rhADAMTS13 treatment, although this did not reach significance (Figure 5D). Astonishingly, BP in WT Ad-sFlt-1 mice was completely normalized by rhADAMTS13, implying that additional rhADAMTS13 can be helpful even in the presence of endogenous ADAMTS13 (Figure 5E).

**DISCUSSION**

TMA associated with VEGF inhibitors can occur in cancer therapies or through high levels of endogenous circulating sFlt-1 as in severe preeclampsia/HELLP syndrome. In both cases, TMA confers significant morbidity and mortality to patients.3,17,33,34 However, the mechanisms underlying VEGF-related TMA are still poorly understood and prognostic criteria to identify patients at risk are lacking. Here, we generated an animal model of VEGF-related TMA in ADAMTS13−/− mice and found that replenishment of ADAMTS13 rescues the classic features of TMAs, including thrombocytopenia, schistocytosis, hemolytic anemia, and microthrombosis in multiple organs. These findings may have direct implications for the prediction and treatment of VEGF-related TMA or thrombosis arising in humans.

The development of animal models for TMAs is valuable both for understanding fundamental disease mechanisms and for pursuing new treatment strategies. Complete VEGF deletion from kidney podocytes is sufficient to induce renal TMA,17 a finding that may account for the prominent renal manifestations of clinical VEGF inhibitor toxicity.35,36 In our model of systemic VEGF inhibition, renal microthrombosis and proteinuria were also among the most prominent...
findings in the ADAMTS13−/− mice. We speculate that VEGF-inhibitor–related TMA is predominantly noted in the vascular bed of the kidneys, because VEGF is constitutively expressed in the adult glomerular podocytes. However, using a systemic agent, we most likely did not reach the same local level of VEGF inhibition in the podocytes, which would explain the less-severe phenotype in our WT mice. Furthermore, our treatment course was fairly short (7–10 days), in contrast to the clinical scenarios where patients are exposed to VEGF inhibitors for several months to years.

In addition to the above-mentioned model of renal TMA, a limited number of mouse models for congenital TTP and a baboon model for acquired TTP have been established. Interestingly, ADAMTS13−/− mice, similar to humans deficient in the enzyme, do not readily develop TTP, but require additional triggers to provoke the onset of the disease. Such triggers include shiga toxin injection, administration of recombinant human VWF containing ultra-large VWF multimers, or by genetically rendering the VWF molecule resistant to cleavage by ADAMTS13. Thus, in the present work, VEGF inhibition may be viewed as the second hit, which then triggers TTP-like symptoms in ADAMTS13−/− mice. Indeed, pregnancy—a known trigger of TTP for patients with ADAMTS13 deficiency—also leads to a physiologic increase of plasma sFlt-1 (without reaching the very high levels found in preeclamptic women), giving more weight to the putative connection between low ADAMTS13, sFlt-1 overexpression, and TMA.

In analogy to the model using recombinant human VWF injection, our model increased VWF concentration in the blood of anti-VEGF–treated mice. Inhibitors of VEGF function such as sFlt-1 are known to cause endothelial activation and release of VWF. In the ADAMTS13−/− Ad sFlt-1 mice, the released hyperactive VWF cannot be cleaved readily, leading to fully developed TMA. In the WT group, plasma VWF also increased after sFlt-1 overexpression; however, the mice only showed minor symptoms, including increased BP, a mild drop in platelet count, and an increase in reticulocyte numbers and hemoglobin. Most likely the latter two can be seen as a compensation for a low-grade destruction of red blood cells which was too minor to be detected. It is conceivable that in WT animals, an initial, overwhelming release of large numbers of Weibel–Palade bodies from the vasculature leads to transient consumption of ADAMTS13, a drop in platelets and
initiation of a thrombotic reaction, which is abrogated as the released VWF is cleaved into smaller, less active multimers. If patients have compromised ADAMTS13 activity or levels, release of ultra-large VWF with consumption of existing ADAMTS13 in microthrombosis could start a vicious circle leading to severe disease. Additionally, VWF directly influences inflammation by promoting the extravasation of leukocytes and the destabilization of the endothelial barrier. ADAMTS13−/− mice display increased leukocyte rolling and enhanced extravasation of neutrophils in thiglycollate-induced peritonitis. Moreover, treatment with rhADAMTS13 has a strong anti-inflammatory effect in models of ischemic brain injury, atherosclerosis, and myocardial infarction. Thus, the interplay between ADAMTS13 and VWF exerts a role in more than the regulation of thrombosis.

Clinical manifestations of ADAMTS13 deficiency are relatively rare in the general population. However, autoantibodies against the enzyme are present in up to 5% of healthy individuals and might become problematic under VEGF inhibition. In addition, certain patient groups such as those suffering from immunemediated diseases like SLE and the antiphospholipid antibody syndrome can be far more susceptible to the development of inhibitory autoantibodies. Finally, the two patient groups most likely to be exposed to high levels of VEGF-inhibitors—cancer patients and preeclamptic women—are known to have significantly decreased levels of ADAMTS13 activity or antigen. In patients with metastatic disease, ADAMTS13 activity can be severely reduced by 50%–95%. Likewise, small studies have reported a decrease in ADAMTS13 activities in patients with severe preeclampsia, with ADAMTS13 activity being associated with early-onset preeclampsia, independent of VWF-Ag levels. However, it is not known if these alterations antedate clinical symptoms. Future studies should evaluate whether low ADAMTS13 activity is a determinant of HELLP syndrome among patients with preeclampsia who have high sFlt-1 levels.

Autoantibodies against the enzyme have been described in isolated cancer cases, yet in the majority, reduced production through liver dysfunction and/or increased clearance (possibly through consumption in a prothrombotic state) has been suggested as the main cause. However, our results suggest that low ADAMTS13 enzyme activity can lead to severe clinical consequences when exposed to anti-VEGF state. Thus, determining the activity of ADAMTS13 before
treatment with a VEGF inhibitor and possibly monitoring it during treatment could be an important step to assess the susceptibility of the patient to TMA.

To date, the only specific therapy for TMAs resulting from VEGF inhibition is the removal of the inhibitory agent,\textsuperscript{4,19,57} interrupting either the cancer treatment, or, in the case of preeclampsia/HELLP patients, terminating pregnancy and delivering the placenta. Because of the great need for novel therapeutic approaches to prevent TMA, we tested whether ADAMTS13\textsuperscript{−/−} mice could be cured of TMA by administering rhADAMTS13. Indeed, the ADAMTS13\textsuperscript{−/−} mice treated with rhADAMTS13 were, for the most part, protected from TMA under VEGF inhibition. Thrombus formation was still visible in a low number of mice, but overall thrombotic events were greatly reduced. As this treatment was started on day 4 after virus injection, when the onset of TMA had already occurred, it did not so much prevent as most likely halt and/or reverse existing symptoms. In the WT Ad-sFlt-1 mice, treatment with rhADAMTS13 also normalized BP. It is conceivable that the above-mentioned beginning of a thrombotic reaction in the WT Ad-sFlt-1 mice was sufficient to cause some amount of kidney damage and a subsequent rise in BP, which was impeded by rhADAMTS13 treatment.

Our findings that ADAMTS13\textsuperscript{−/−} mice are more prone to BP elevation upon challenge with sFlt-1 are interesting and need to be studied further. We speculate that enhanced renal damage may contribute to hypertension; however, experiments with angiotensin II or salt infusions and vascular reactivity studies are needed to reveal the underlying mechanisms.

Our data thus suggest that rhADAMTS13 may constitute an effective treatment option in patients suffering from anti-VEGF treatment–associated TMA with reduced ADAMTS13 activity and perhaps even with normal activity, as supernormal ADAMTS13 levels appear to be beneficial during anti-VEGF therapy. rhADAMTS13 is currently being evaluated as a therapeutic for TTP patients. So far, tests in animal models have revealed very good tolerance with no toxicity even at the highest dose levels.\textsuperscript{58,59} Other possible treatments may include antibodies or aptamers which directly target the A1-domain of VWF that promotes platelet binding and which are also being developed for use in TTP patients.\textsuperscript{60,61} Finally, N-acetylcysteine was shown to reduce the size and activity of VWF in human and mouse plasma,\textsuperscript{62} and has been successfully used in refractory TTP in one patient.\textsuperscript{63}

In conclusion, we have uncovered an important role for ADAMTS13 in preventing TMA following systemic VEGF inhibition. Therefore, we suggest the evaluation of ADAMTS13 activity as a predictor for the development of TMA under antiangiogenic therapy and of pregnancy-related TMA. Furthermore, we propose that treatment with rhADAMTS13 could be a promising new therapeutic tool in the care of cancer patients that need anti-VEGF treatment, as it can reduce thrombotic complications and halt or even reverse the development of TMA symptoms.

**CONCISE METHODS**

**Animals**

ADAMTS13\textsuperscript{−/−}\textsuperscript{64} and WT mice were on a C57BL/6J background. Animals were 11–12 weeks old and sex- and weight-matched. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Boston Children’s Hospital (protocol no. 13–06–2427).

**sFlt-1 Overexpression**

Mice received lateral tail vein injections with 2.2×10\textsuperscript{9} plaque-forming units of adenovirus encoding sFlt-1 (Ad-sFlt-1) or empty CMV (Ad-null) at equivalent doses. The details of the virus construction are described elsewhere and were amplified at the Vector Laboratories.\textsuperscript{21,25,29}

**Recombinant ADAMTS13 (rhADAMTS13) Treatment**

rhADAMTS13 was kindly provided by F. Scheiffinger and H. Rottensteiner, Baxter Bioscience. Treatment with rhADAMTS13 (or PBS vehicle) by retro-orbital intravenous injections was started at day 4 after virus injection at a dose of 3460 U/kg every 24 hours\textsuperscript{32} until day 7 after virus injection.

**Analysis of Peripheral Blood**

Blood was collected via the retro-orbital sinus into EDTA-coated capillary tubes and was analyzed by a Hemavet 950FS (Drew Scientific) for complete blood counts. Peripheral blood smears were prepared by standard procedures and fixed in methanol. Blood smears were stained with Wright–Giemsa stain (Sigma-Aldrich) and schistocytes and reticulocytes were counted in a blinded manner as percent of total cells, according to the recommendations of the International Council for Standardization in Haematology.\textsuperscript{65}

**ELISAs**

Plasma sFlt-1 levels were measured using the Mouse sVEGF R1/Flt-1 Quantikine ELISA Kit (R&D Systems) in mouse plasma at days 7 and 10 after virus injection. Plasma VWF levels were quantified by ELISA on day 7,\textsuperscript{27,64} VWF levels in plasma of WT or ADAMTS13\textsuperscript{−/−} mice were calculated and shown as fold increase over pooled plasma VWF levels of untreated WT or untreated ADAMTS13\textsuperscript{−/−} mice, respectively.

**Tissue Preparation and Analysis**

Mice were anesthetized with Avertin (tribromoethanol). Perfusion was performed with 15 ml of PBS through the heart via the left ventricle. Organs were harvested and fixed in zinc fixative (100 mM Tris–HCl containing 37 mM zinc chloride, 23 mM zinc acetate, and 3.2 mM calcium acetate). Paraffin-embedded sections were stained with H&E. Glomerular area and open capillary volume was analyzed using ImageJ (National Institutes of Health, Bethesda, MD) on at least 20 H&E-stained glomeruli per mouse, as described previously.\textsuperscript{29} VWF immunohistochemistry staining was performed.\textsuperscript{27} Slides were evaluated by light microscopy using a Carl Zeiss Axioplan microscope and Axiovision software.
Urine Analysis
Urine was collected before Ad-null or Ad-sFlt-1 injection and after injection on days 4, 7, and 10. Albumin was measured using the Albuwell M kit (Exocell) according to the manufacturer’s instructions. Creatinine was determined using the Creatinine (urinary) Colorimetric Assay Kit (Cayman Chemical) and the ratio between albumin and creatinine was calculated.

BP Measurements
Systolic BP was measured before Ad-null or Ad-sFlt-1 injection and after on days 7 and 10, using an IITC 12M22931 noninvasive blood pressure system (IITC Life Science), a validated method that has been compared with both telemetry and direct BP measurements.

Mice were trained twice several days before the onset of measurements to get them accustomed to the procedure. For this, mice were placed in restrainers and allowed to settle down for 10 min in the tail-cuff chamber at 34°C. The noninvasive BP methodology employs a tail-cuff placed on the proximal part of the tail to occlude the blood flow. Upon deflation, the system uses a highly sensitive photoelectric sensor for detection of BP pulses. BP was measured five times and the average value is presented.

Electron Microscopy
Harvested kidneys were fixed in glutaraldehyde and embedded in araldite-epon mixture; 1 μm sections were cut and stained with Toluidine blue. Electron microscopy images were acquired using a Hamamatsu Orca-HR Digital Camera and Advanced Microscopy Techniques (AMT) Corp. image capture system.

Statistical Analysis
Data are presented as mean ± SEM. For statistical tests, a two-sided Student’s t test was used when two groups were compared. For comparison of more than two groups, the ANOVA test with Bonferroni adjustment was applied. The chi-squared test was employed for analysis of Table 2.

ACKNOWLEDGMENTS
The authors thank L. Cowan for help with the preparation of the manuscript and K. Martinod and S.L. Wong for critical input and discussions. This research was supported by the National Heart, Lung, and Blood Institute of the National Institutes of Health grant R01-HL102101 (to D.D.W.). L.E. is a recipient of a fellowship of the Deutsche Akademie der Naturforscher Leopoldina (Nationale Akademie der Wissenschaften). S.A.K. is an investigator of the Howard Hughes Medical Institute.

DISCLOSURES
S.A.K. has financial interest in Aggamin Therapeutics and is a consultant to Siemens. S.A.K. is a co-inventor on patents related to angiogenic biomarkers in preeclampsia that are held by the Beth Israel Deaconess Medical Center. The other authors have no conflicting financial interests.

REFERENCES


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2014121165/-/DCSupplemental