Potential for Recombinant ADAMTS13 as an Effective Therapy for Acquired Thrombotic Thrombocytopenic Purpura

Claudia Tersteeg, Alexandra Schiviz, Simon F. De Meyer, Barbara Plaimauer, Friedrich Scheiflinger, Hanspeter Rottensteiner, Karen Vanhoorelbeke

Objective—The metalloprotease ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) regulates the size of von Willebrand factor multimers. A deficiency in ADAMTS13 activity is associated with the life-threatening disease thrombotic thrombocytopenic purpura (TTP). The vast majority of patients have acquired TTP, where circulating anti-ADAMTS13 autoantibodies are causative for the decreased ADAMTS13 activity. Current treatment consists of plasma exchange, but improved therapies are highly warranted.

Approach and Results—We have developed a new rat model mimicking various aspects of acquired TTP to investigate the therapeutic efficacy of human recombinant ADAMTS13. A polyclonal antibody against ADAMTS13 completely blocked endogenous rat ADAMTS13 activity in Sprague–Dawley rats. When TTP was triggered using recombinant von Willebrand factor, the animals displayed severe TTP-like symptoms, such as thrombocytopenia, hemolytic anemia, and von Willebrand factor–rich thrombi in the kidneys and brain. Subsequent injection of 400, 800, or 1600 U/kg recombinant ADAMTS13 prevented full development of these symptoms. Analysis of plasma samples confirmed that recombinant ADAMTS13 was able to override circulating anti-ADAMTS13 inhibitory antibodies, resulting in restoration of ADAMTS13 activity and degradation of ultralarge von Willebrand factor multimers.

Conclusions—Recombinant ADAMTS13 was shown to be effective in averting severe acquired TTP-like symptoms in rats and holds promising value for the treatment of this severe and life-threatening disease in humans. (Arterioscler Thromb Vasc Biol. 2015;35:2336-2342. DOI: 10.1161/ATVBAHA.115.306014.)

Key Words: acquired TTP ■ ADAMTS13 ■ animal model ■ treatment ■ VWF

Von Willebrand factor (VWF) is a multimeric protein that is secreted via Weibel–Palade bodies in endothelial cells and α-granules in platelets into flowing blood. Secreted ultralarge VWF multimers can remain transiently bound to the endothelial cell surface or be released into the circulation. Unfolding of VWF by shear stress induces a conformational change, making the A2 domain available for proteolytic cleavage by ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). This proteolysis decreases the size of VWF, thereby reducing its thrombotic potential.1 A deficiency in ADAMTS13 activity is associated with thrombotic thrombocytopenic purpura (TTP), a life-threatening disease characterized by thrombocytopenia and hemolytic anemia.2 These conditions are a result of the formation of microthrombi in the smaller capillaries and lead to fever, neurological complications, renal impairment, and death through organ failure when left untreated.3

Decreased enzymatic activity may be caused by mutations in the ADAMTS13 gene and result in congenital TTP.4,5 However, the vast majority of patients have acquired TTP, where anti-ADAMTS13 autoantibodies either neutralize the activity of ADAMTS13 or enhance protein clearance.5,6 Standard of care treatment of acquired TTP patients consists of frequent plasma exchange with fresh frozen plasma and additional immunosuppressive treatment with corticosteroids or other agents, such as rituximab or cyclosporine.7,8 Plasma exchange is performed to remove inhibitory antibodies from the circulation and replenish ADAMTS13 enzyme activity. However, not all patients respond to this treatment method, and only 80% to 90% survive an acute episode.9 In addition, plasma exchange is time consuming and requires large amounts of plasma. Therefore, new therapies are highly warranted.10

Replenishment of plasma ADAMTS13 activity with recombinant ADAMTS13 (rADAMTS13) could significantly improve TTP therapy and reduce the mortality rate. rADAMTS13 therapy was in fact shown to result in decreased incidence and severity of TTP symptoms in a mouse model.
of congenital TTP, where ultralarge VWF multimers serve as trigger for inducing TTP-like symptoms. However, the expected efficacy of rADAMTS13 replacement therapy for acquired TTP is likely complicated by the presence of free circulating anti-ADAMTS13 autoantibodies that bind and neutralize infused rADAMTS13. Nevertheless, because the addition of increasing concentrations of rADAMTS13 to plasma of acquired TTP patients normalized ADAMTS13 activity in vitro by overriding the inhibitory antibodies, rADAMTS13 therapy seems to be a promising treatment opportunity for this patient group. In the presented study, we developed a rat model mimicking various aspects of acquired TTP, with which we further explored the feasibility of rADAMTS13 treatment in acquired TTP.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Establishing a Rat Model Resembling Acquired TTP

First, rats were injected with polyclonal anti-ADAMTS13 antibodies (650 U/kg) to block endogenous rat ADAMTS13 activity and to establish a robust inhibitor titer of ~10 BU/mL. Then, animals were administered 2000 U/kg recombinant VWF (rVWF) to trigger TTP symptoms. Control animals received either control IgG instead of anti-ADAMTS13 antibodies or saline instead of rVWF. Animals were followed for 24 h, and blood samples were analyzed after 3 and 24 h. The normal platelet count at baseline was 650±177×10^3 platelets/μL blood (Figure 1A). Rats injected with anti-ADAMTS13 antibodies and triggered with rVWF developed thrombocytopenia, with a platelet count of 427±111×10^3/μL after 3 h and 218±40×10^3/μL after 24 h (Figure 1A). Neither injection of anti-ADAMTS13 antibodies without additional rVWF nor injection of control IgG with rVWF resulted in thrombocytopenia (652±146×10^3/μL and 766±63×10^3/μL after 24 h, respectively; Figure 1A). Hemoglobin levels also decreased significantly after 24 h when rats developed TTP (14.7±1.0 g/dL at baseline to 11.7±1.1 g/dL at 24 h versus 13.6±0.9 g/dL for anti-ADAMTS13+saline and 14.2±0.5 g/dL for control IgG+rVWF) as depicted in Figure 1B.

To further confirm the TTP phenotype, lactate dehydrogenase (LDH) activity, a marker for tissue damage, was measured in rats. In animals with TTP, a highly increased LDH activity was observed 3 h after disease induction (1289.5±63.3 mU/mL; Figure 1C). After 24 h, the LDH activity returned to normal levels (69.0±75.2 mU/mL; Figure 1C). Additionally, the percentage of schistocytes present in the blood was quantified, but no significant difference was observed between the groups. Next, rat brain and kidney sections were analyzed for the presence of microthrombi. In animals injected with anti-ADAMTS13 antibodies and triggered with saline, no microthrombi were observed (Figure 1D, left). However, rats having acquired TTP-like symptoms as a result of injection of anti-ADAMTS13 antibodies and rVWF showed microthrombi in the brain, as well as increased VWF staining in the capillaries around the glomeruli in kidney sections (Figure 1D, right). The combined results demonstrate that the chosen setup induces severe TTP symptoms in rats, comprising thrombocytopenia, hemolytic anemia, increased LDH activity, and the presence of VWF-rich microthrombi, and can thus be regarded as a valid animal model for key aspects of acute acquired TTP.

rADAMTS13 Prevents Thrombocytopenia and Hemolytic Anemia

To evaluate the potential efficacy of rADAMTS13 for acquired TTP in our rat model, TTP symptoms were triggered with anti-ADAMTS13 antibodies and rVWF. Fifteen minutes thereafter, animals were injected with vehicle or rADAMTS13 (400, 800, or 1600 U/kg). At this time point, the platelet count was already declined by 50%. Groups (n=6) were compared regarding the extent of TTP symptoms, including platelet counts, hemoglobin levels, and LDH activity after 3, 6, and 24 h. In the 800 U/kg dose group, 2 rats were excluded from analysis: one because of an imperfect rVWF injection and one because of death for unknown reasons during anesthesia at 3 h.

As expected from our previous results, thrombocytopenia was observed in animals treated with vehicle alone (0 U/kg rADAMTS13, Figure 2A), with a platelet count of 355±106×10^3/μL after 3 h, 231±102×10^3/μL after 6 h, and 299±107×10^3/μL after 24 h (Figure 2A). After treatment with 400, 800, or 1600 U/kg rADAMTS13, no thrombocytopenia was observed after 3, 6, and 24 h, demonstrated by comparable platelet counts to those measured at baseline (751±93×10^3 platelets/μL blood, Figure 2A). Hemoglobin levels also significantly decreased in rats treated with vehicle after 24 h as depicted in Figure 2B (from 13.1±1.2 g/dL at baseline to 10.9±1.3 g/dL at 24 h; Figure 2B). On treatment with rADAMTS13, hemoglobin concentration values remained stable over time (Figure 2B). LDH activity increased in rats treated with vehicle 3 and 6 h after TTP induction (1020±352 and 874±548 mU/mL; Figure 2C), with normal levels after 24 h (44.9±63.3 mU/mL). Injection of rADAMTS13 protected rats from cell and tissue damage because no increase in LDH activity was detected (Figure 2C). These data indicate that treatment with rADAMTS13 is able to prevent the development of severe TTP symptoms in our rat model mimicking acquired TTP.

rADAMTS13OverridesInhibitoryAntibodiesandRestoresADAMTS13Activity

The amount of rADAMTS13 administered in our rat model appeared sufficient to complex all polyclonal
anti-ADAMTS13 antibodies present in plasma samples and to provide enough free rADAMTS13 to digest UL-VWF (ultra-large von Willebrand factor). Immune complexes did in fact form between the polyclonal anti-ADAMTS13 antibodies and endogenous ADAMTS13 or rADAMTS13 in plasma from rats treated with 400, 800, or 1600 U/kg rADAMTS13 at 3 and 6 h after injection (significant for 1600 U/kg compared with other doses), but not in animals that received vehicle (0 U/kg rADAMTS13; Figure 3A). After 24 h, significant amounts of immune complexes were measured only in rats that received 1600 U/kg rADAMTS13.

Despite the formation of immune complexes, sufficient levels of free rADAMTS13 were present in the circulation after injection of 400, 800, or 1600 U/kg rADAMTS13. ADAMTS13 activity of 68.4%±21.9%, 101.2%±22.7%, and 714.1%±817.9%, respectively, was detected in rat plasma after 24 h (Figure 3B). Plasma from animals that received vehicle (0 U/kg rADAMTS13) showed no ADAMTS13 activity at 3

**Figure 1.** Recombinant von Willebrand factor (rVWF) is able to trigger thrombotic thrombocytopenic purpura (TTP) symptoms in rats with anti-ADAMTS13 antibodies. Animals were administered 650 U/kg polyclonal anti-ADAMTS13 or IgG control antibodies (at 0 minutes) and 2000 U/kg rVWF or saline (at 15 minutes). A and B, Platelet count and hemoglobin levels were measured in EDTA anticoagulated blood at baseline and 3 and 24 h after injections. C, Tissue damage was determined by measuring lactate dehydrogenase (LDH) activity in EDTA plasma. Graphs show mean±SD. *P<0.05; **P<0.01. Control IgG+rVWF, n=4; anti-ADAMTS13+saline, n=9; and anti-ADAMTS13+rVWF, n=7. D, After 24 h, animals were euthanized and tissues were removed for histological analysis. Sections were stained for VWF (brown staining). Top, Brain sections from control animals (left) and animals with acquired TTP (right). Scale bars indicate 20 μm. Bottom, Kidney sections from control animals (left) and animals with acquired TTP (right). Scale bars indicate 50 μm. ADAMTS13 indicates a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13.

**Figure 2.** Recombinant ADAMTS13 (rADAMTS13) treatment prevents severe thrombotic thrombocytopenic purpura (TTP) symptoms in rats with acquired TTP. Animals with acquired TTP were administered 0, 400, 800, or 1600 U/kg rADAMTS13 at 30 minutes. A and B, Platelet count and hemoglobin levels were measured in EDTA anticoagulated blood at baseline and 3, 6, and 24 h after injections. C, Tissue damage was determined by measuring lactate dehydrogenase (LDH) activity in EDTA plasma. 0, 400, and 1600 U/kg, n=6; 800 U/kg, n=4. Graphs demonstrate mean±SD. *P<0.05; **P<0.01. ADAMTS13 indicates a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13.
and 6 h, but partly regained activity after 24 h (43.9%±33.5%; Figure 3B). In conclusion, despite the formation of immune complexes, sufficient free rADAMTS13 was present in the circulation of all acquired TTP rats treated with the enzyme.

rADAMTS13 Treatment Reduces High Molecular Weight VWF Multimer Size

As expected, levels of rVWF, measured using an antigen ELISA specific for human VWF, were high in rat plasma samples 3 h after rVWF injection and gradually decreased after 6 and 24 h, reflecting the clearance of the protein over time (Figure 4A). Similar VWF levels were detected at all 3 time points irrespective of rADAMTS13 dose, indicating that increased VWF proteolysis by rADAMTS13 did not influence VWF clearance, in line with previous observations.13

To confirm that successful treatment of acquired TTP-like symptoms in rats with rADAMTS13 was linked to proteolytic degradation of rVWF, the VWF multimer pattern was analyzed in plasma from all treated rats. To allow detection of rVWF but not endogenous rat VWF, a plasma volume was loaded at which a multimer pattern was only discernible for samples from rats treated with rVWF (Figure 4B). At this plasma dilution, rVWF multimers were observed after 3 and 6 h, but at an intensity that fell below the detection limit at 24 h. Using densitometry, the multimer pattern was analyzed, and the percentage of high molecular weight (HMW) multimers was calculated (Figure 4C).

Before injection, rVWF contained 47.9%±2.7% HMW multimers. After injection of 0 U/kg rADAMTS13 into rats, this value decreased to 36.4%±2.9% at 3 h and 30.3%±1.1% at 6 h. Administration of 400 U/kg rADAMTS13 did not alter the percentage of HMW multimers. A significantly lower percentage of HMW VWF multimers, however, was observed in rats that received 800 (32.2%±2.5% and 26.8%±1.6%) and 1600 (28.8%±4.9% and 24.2%±5.5%) U/kg rADAMTS13. Thus, increasing concentrations of rADAMTS13 resulted in cleavage of HMW multimers into smaller, less-thrombogenic multimers.

rADAMTS13 Treatment Diminishes the Presence of VWF-Rich Microthrombi

Brain, kidney, liver, and lung sections were analyzed for the presence of microthrombi in untreated and treated rats. VWF-specific brown staining was observed for all tissues in endothelial cells lining the arteries. No microthrombi were observed in liver and lung sections. VWF-rich microthrombi were

Figure 3. Recombinant ADAMTS13 (rADAMTS13) overrides inhibitory antibodies and restores ADAMTS13 activity. A, Anti-ADAMTS13 antibodies bound to endogenous or recombinant ADAMTS13 were measured as circulating immune complexes in citrated plasma using ELISA. B, ADAMTS13 activity was measured using FRETS-VWF73. Citrated plasma obtained at baseline and 3, 6, and 24 h after injections was used undiluted (0, 400, and 800 U/kg groups) or diluted in heat-inactivated rat plasma (1600 U/kg group). 100% activity corresponded with ADAMTS13 activity in normal rat plasma. 0, 400, and 1600 U/kg, n=6; 800 U/kg, n=4. Graphs demonstrate mean±SD. *P<0.05; **P<0.01. ADAMTS13 indicates a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; and VWF, von Willebrand factor.

Figure 4. Recombinant ADAMTS13 (rADAMTS13) treatment reduces high molecular weight (HMW) von Willebrand factor (VWF) multimer size. A, Human VWF antigen levels were determined in citrated plasma at baseline and 3, 6, and 24 h after injections using ELISA. B, VWF multimer analysis was performed on citrated plasma samples obtained from rats 6 h after injection of 0 or 1600 U/kg rADAMTS13. VWF was separated on SDS-agarose gels, resulting in separation of the different sized multimers. C, Densitometry analysis was performed on the VWF multimer gels. The total number of distinguishable bands was counted, and the relative abundance HMW multimers (>10-mer) was calculated relative to the complete multimer. 0, 400, and 1600 U/kg, n=6; 800 U/kg, n=4. Graphs show mean±SD. *P<0.05. ADAMTS13 indicates a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; and rVWF, recombinant von Willebrand factor.
observed in brain sections of rats that received 0 U/kg rADAMTS13 (Figure 5A, left), but not in those administered 400, 800, or 1600 U/kg rADAMTS13 (Figure 5B). Microthrombi present in rats treated with vehicle were negative for fibrin (Figure 5A, right). In kidney sections, increased VWF staining but no fibrin staining was observed in the capillaries around the glomeruli of rats that did not receive rADAMTS13 compared with rADAMTS13-treated rats (Figure 5C). These results demonstrate that rats with acquired TTP-like symptoms formed VWF-rich, fibrin-poor microthrombi in the brain and kidney and that treatment with rADAMTS13 reduced this microthrombi formation and, thus, also TTP symptoms.

Discussion

We have developed a rat model resembling acquired TTP, in which rADAMTS13 was demonstrated to avert several severe symptoms typical of this disease, including thrombocytopenia, hemolytic anemia, increased LDH activity, and VWF-rich microthrombi in the small arterioles.

Several novel therapeutic opportunities have already been demonstrated for TTP. In a shigatoxin-induced TTP mouse model, ADAMTS13 gene transfer showed promising results for the treatment of congenital TTP. For acquired TTP, this approach seems prohibited because of the presence of autoantibodies against ADAMTS13, unless an eradication of antibodies can be achieved on constant expression of the transgene. Recently, however, transgenic ADAMTS13 expressed in platelets was demonstrated to prevent both congenital and acquired TTP. Although a truly elegant approach, packing ADAMTS13 into platelets is not directly translatable toward patients because this technique is challenging if not impossible at the moment. Plasminogen activation has been suggested as a possible bypass for ADAMTS13 deficiency because plasmin cleavage of VWF reduced the formation of VWF-rich microthrombi in Adams13−/− mice in which TTP-like symptoms had been induced with rVWF. A recent case report described the successful treatment of an acquired TTP patient who was unresponsive to plasma exchange with N-acetylcysteine. This compound, which is clinically used for chronic obstructive lung disease, reduces the size of VWF multimers by selective breakage of intermolecular disulfide bonds. Furthermore, inhibition of the VWF–platelet glycoprotein Ib interaction using inhibitory anti-VWF antibodies was successful in preventing and treating acquired TTP in baboons. These treatment strategies hold great promise in decreasing the number and frequency of plasma exchange cycles required to achieve remission. They will, however, probably not be able to replace such therapy because they neither replenish ADAMTS13 activity nor remove circulating antibodies in these patients. By contrast, therapy with rADAMTS13 may overcome these limitations and obviate the risky procedure of plasma exchange. Administration of rADAMTS13 has already proved successful in treating congenital TTP-like symptoms in Adams13−/− mice triggered with rVWF.

In the present study, we established a small animal model for acquired TTP to test whether the recombinant protease is proficient in treating TTP-like symptoms also in the presence of circulating inhibitory antibodies. Such a setup reflects the situation in acquired TTP patients, where circulating anti-ADAMTS13 antibodies would require high doses of rADAMTS13 to override the inhibitor before a surplus of active ADAMTS13 could cleave accumulated ultralarge VWF multimers, thereby preventing thrombotic complications typical for TTP. To trigger TTP-like symptoms in our rat model, 2 pharmacological interventions were needed. Polyclonal anti-ADAMTS13 antibodies served to inhibit endogenous rat ADAMTS13 and establish a defined level of circulating antibodies and a high concentration of rVWF to cause spontaneous platelet aggregation and thus thrombocytopenia, hemolytic anemia, and VWF-rich thrombi in the kidney and brain. Administration of rVWF alone was inadequate to cause TTP-like symptoms, probably because it was sufficiently processed by endogenous ADAMTS13. Likewise, ADAMTS13 deficiency induced by the anti-ADAMTS13 antibody was not associated with any clinical symptoms, comparable to the situation in ADAMTS13-deficient mice.

In the absence of inhibitory antibodies, the dose required to establish an ADAMTS13 activity in rat plasma of 1 U/mL can be estimated to be ≈50 U/kg, taking into account
rADAMTS13’s in vivo recovery of 60% in rats and that a rat contains 31 mL plasma per kg body weight. Eight-, 16-, and 32-times higher doses of rADAMTS13 were used to treat acute TTP-like symptoms (400, 800, and 1600 U/kg); these doses were based on a pharmacokinetic study showing that 400 U/kg is just sufficient to neutralize an inhibitor titer of ≤10 BU/mL. Although these high doses could have been expected to cause side effects, no adverse events, such as an increased bleeding tendency, were observed even with 1600 U/kg rADAMTS13. This observation is concordant with a mouse study where a dose of 3460 U/kg rADAMTS13 did not induce any adverse events.

According to the measured ADAMTS13 activity levels in rat plasma, 800 U/kg rADAMTS13 best hit the target of 1 U/mL. This dose was efficacious in averting acquired TTP because it prevented development of all symptoms of the disease observed in untreated animals. Efficacy was also demonstrated for the lowest dose tested (400 U/kg), even though ADAMTS13 activity only reached endogenous rat ADAMTS13 baseline levels (≈0.6 U/mL) and no relevant HMW multimer cleavage of rVWF was noted. These observations clearly suggest that lower than physiological concentrations of ADAMTS13 might suffice to ameliorate the condition of patients with acute TTP.

When rADAMTS13 would be administered to patients, potential long-term consequences of immune complexes formed between rADAMTS13 and the patient anti-ADAMTS13 autoantibodies need to be also considered. Circulating immune complexes between plasma ADAMTS13 and anti-ADAMTS13 autoantibodies have been demonstrated in acquired TTP patients during the acute and the remission phase. These complexes are suspected to also deposit in tissues where they could contribute to the pathogenesis of acquired TTP, as demonstrated for other autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis. Future studies should therefore address whether ADAMTS13-specific immune complexes provoke an immune response (eg, complement and leukocyte activation) and associate with the disease’s pathogenicity.

In our rat model, we administered rADAMTS13 15 minutes after the rVWF trigger, a time point where rats were suspected to already show TTP-like symptoms. Indeed, we previously demonstrated that thrombocytopenia developed within minutes after rVWF injection and concomitant with the occurrence of VWF-rich microthrombi. Hence, our model allowed studying treatment in the early stage of TTP. The rat model did not allow rADAMTS13 treatment after a substantially longer time window after TTP onset because a recovery of TTP symptoms already began after 24 h, as demonstrated by a slight increase in platelet counts and decreased LDH activity levels, and because of the reversible nature of the effects triggered by the combination of inhibitor and rVWF. To study the effect of rADAMTS13 treatment during deep thrombocytopenia, the baboon model of acquired TTP seems to be better suited because repeated injections of an inhibitory monoclonal anti-ADAMTS13 antibody induced TTP symptoms without the need for any trigger that lasted ≤11 days. Another possible limitation of this study is that our rat model does not reflect the continuous antibody production as in acquired TTP patients. However, as with plasma exchange, treatment with rADAMTS13 will probably also need supplemental administration of immunosuppressive drugs, such as rituximab, to decrease the continuous production of new autoantibodies, thereby preventing them from overruling rADAMTS13 therapy.

Acquired TTP patients have a heterogeneous antibody profile, and inhibitory titers may differ not only from patient to patient but also from day to day for a single patient. Potential treatment with rADAMTS13 would therefore benefit from a dosing strategy that is tailored to the actual inhibitor titer of these patients. This parameter proved crucial for primary dose calculation in vitro and was also predictive for calculating the amounts of rADAMTS13 required to override inhibitors in rats. In the clinical setting, this strategy would require measuring the inhibitory titer before administering rADAMTS13, which, although conceptually attractive, may be challenging considering the patients’ critical state. Nonetheless, such a strategy would allow more informed medical decisions, a higher probability of the desired outcome, a reduction in adverse reactions, and reduced healthcare costs because of targeted therapy.

A phase I clinical study has recently been initiated to assess rADAMTS13 in the treatment and prophylaxis of congenital TTP (ClinicalTrials.gov: NCT02216084). Our study provides evidence that rADAMTS13 may also be effective in treating acquired TTP because it was able to overcome circulating inhibitors and reconstitute ADAMTS13 activity in a rat model mimicking acute acquired TTP. Therefore, rADAMTS13 holds promise for the treatment of this severe and life-threatening disease in humans.

Acknowledgments

Special thanks go to Karima Benamara for her excellent editing of the article.

Sources of Funding

C. Tersteeg is a Postdoctoral Fellow supported by the Research Foundation–Flanders (FWO), Belgium (12N0715N). This study was funded by the FWO G.0584.11 N, the KU Leuven OT grant O1/14/71, and KU Leuven program financing IF/10/014.

Disclosures

A. Schiviz, B. Plaimauer, F. Scheiflinger, and H. Rottensteiner are employees of Baxalta Innovations GmbH, Vienna, Austria. The other authors report no conflicts.

References

binant ADAMTS13 thus holds promising value for the treatment of this severe and life-threatening disease in humans.

antibodies, resulting in prevention of severe thrombotic thrombocytopenic purpura symptoms and restoration of ADAMTS13 activity. Recom-
thrombotic thrombocytopenic purpura. With this model, we demonstrated that recombinant ADAMTS13 has the ability to override inhibitory
episode. Therefore, new treatment strategies are highly warranted. In the present article, we developed a rat model mimicking acquired
ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). For the vast majority of patients, this is

purpura patients currently consists of plasma exchange using fresh frozen plasma, but still 10% to 20% of the patients die during an acute

factor-cleaving activity in plasma of acquired TTP patients by overrid-
Scheiflinger F. Recombinant ADAMTS13 normalizes von Willebrand


De Meyer SF, Savenchenko AS, Haas MS, Schatzberg D, Carroll MC, Schiz V, Dietrich B, Rottensteiner H, Scheiflinger F. Wagner DD. Protective anti-inflammatory effect of ADAMTS13 on myocardial isch-


Luken BM, Turenhouot EA, Hulstein JJ, Van Mourik JA, Fijnheer R, Voorberg J. The spacer domain of ADAMTS13 contains a major binding site for antibodies in patients with thrombotic thromboem-


Zheng XL, Wu HM, Shang D, Falls E, Skipwith CG, Cataland SR, Bennett CL, Kwaan HC. Multiple domains of ADAMTS13 are targeted by autoan-
tibodies against ADAMTS13 in patients with acquired idiopathic throm-

Significance

The life-threatening disease thrombotic thrombocytopenic purpura is associated with a deficiency in von Willebrand factor–cleaving protease ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). For the vast majority of patients, this is caused by an acquired formation of autoantibodies inhibiting the activity of ADAMTS13. Treatment of acquired thrombotic thrombocytopenic purpura patients currently consists of plasma exchange using fresh frozen plasma, but still 10% to 20% of the patients die during an acute episode. Therefore, new treatment strategies are highly warranted. In the present article, we developed a rat model mimicking acquired thrombotic thrombocytopenic purpura with this model, we demonstrated that recombinant ADAMTS13 has the ability to override inhibitory antibodies, resulting in prevention of severe thrombotic thrombocytopenic purpura symptoms and restoration of ADAMTS13 activity. Recom-
binant ADAMTS13 thus holds promising value for the treatment of this severe and life-threatening disease in humans.
Potential for Recombinant ADAMTS13 as an Effective Therapy for Acquired Thrombotic Thrombocytopenic Purpura
Claudia Tersteeg, Alexandra Schiviz, Simon F. De Meyer, Barbara Plaimauer, Friedrich Scheiflinger, Hanspeter Rottensteiner and Karen Vanhoorelbeke

Arterioscler Thromb Vasc Biol. 2015;35:2336-2342; originally published online September 3, 2015;
doi: 10.1161/ATVBAHA.115.306014
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/35/11/2336

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2015/09/03/ATVBAHA.115.306014.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Potential for recombinant ADAMTS13 as an effective therapy for acquired thrombotic thrombocytopenic purpura

Claudia Tersteeg ¹, Alexandra Schiviz ², Simon F De Meyer ¹, Barbara Plaimauer ², Friedrich Scheiflinger ², Hanspeter Rottensteiner ², Karen Vanhoorelbeke ¹

¹ Laboratory for Thrombosis Research, IRF Life Sciences, KU Leuven Kulak, Kortrijk, Belgium
² Baxalta Innovations GmbH, Vienna, Austria
Materials and Methods

Anti-ADAMTS13 IgG, human recombinant VWF and rADAMTS13
Preparation of anti-ADAMTS13 IgG, human recombinant (r)VWF and rADAMTS13 has been previously described1-3.

Wild-type ADAMTS13 was produced in stably transfected Chinese hamster ovary (CHO) cells under serum- and protein-free conditions and rADAMTS13 was purified from large-scale culture harvests by a conventional multistep chromatography procedure. The purified rADAMTS13 was formulated in a physiological protein-free buffer and consisted of 0.865 mg/mL ADAMTS13 antigen and 1426 U/mL ADAMTS13 activity.

Von Willebrand factor was produced in CHO cells in co-expression with Factor VIII and purified from the flow-through of the FVIII capture step including a furin-mediated maturation process to yield fully-processed rVWF. Purified rVWF was formulated in a protein-free buffer and consisted of 166 U/mL VWF antigen and 205 U/mL VWF activity.

A polyclonal anti-ADAMTS13 antibody preparation was generated in goats using purified full-length human rADAMTS13 as antigen. Goat IgG was purified from citrated plasma using Protein G sepharose 4 FF (GE Healthcare Life Science) and dissolved in 94 mM Glyc in, 60 mM Tris, pH 5.0. The purified preparation had an inhibitory activity of about 735 BU/mL against human ADAMTS13.

VWF activity was determined using immunoturbidimetric VWF activity (VWF:Ac) assay (INNOVANCE® VWF Ac.; BCS system; Siemens, Marburg, Germany). ADAMTS13 activity was determined with a fluorescence resonance energy transfer (FRET)-based assay essentially as described4, using the synthetic fluorogenic FRETs-VWF73 minimal peptide as substrate (Peptide Institute, Inc., Japan). For reference, pooled normal human plasma (NHP; George King Biomedical Inc., Overland Park, USA) was assigned an ADAMTS13 activity concentration of 1 U/mL.

Rat model of acquired TTP
Male Sprague-Dawley rats (9-12 weeks old) were used. For intravenous injection, a 24G butterfly needle was inserted into the lateral tail vein of anesthetized animals (isoflurane). Goat-anti-ADAMTS13 IgG antibodies (250 µL) were injected at a dose of 650 U/kg body weight. Fifteen minutes later, 2000 VWF:RCoU/kg rVWF (3.5 mL) was injected5. Another 15 min later, rats were treated with 400, 800, or 1600 U/kg rADAMTS13 (400 µL). The butterfly needles were removed and the rats allowed to recover from anesthesia. Animal studies were approved by the Institutional Animal Care and Use Committee of KU Leuven (Belgium).

Blood collection
Blood collection was performed at baseline (7 days before the start of the experiment) and 3, 6 and 24 h after injections. Blood was collected on citrate (7:1 vol/vol of blood:3.8% sodium citrate) or EDTA (15:1 vol/vol of blood:0.5 M EDTA) via retro-orbital venipuncture. Total blood cell counts were obtained from blood collected on EDTA using the Hemavet 950 (Drew Scientific, Dallas, USA). A blood smear was made from citrated whole blood and stained with May-Grünwald-Giemsa. Four pictures were made per rat per time point, and the percentage of schistocytes (red blood cell fragments) over the total number of red blood cells was quantified. Plasma was obtained from blood collected on sodium citrate or EDTA by centrifugation at 2500xg for 6 min and stored at -80°C.

Determination of ADAMTS13 activity and LDH levels
ADAMTS13 activity in rat plasma was evaluated using a fluorogenic VWF substrate (FRETs-VWF73, Peptide International, Louisville, USA) essentially as described for murine plasma5. Citrated plasma samples (undiluted or diluted in heat inactivated normal rat plasma) were added to Hepes buffered saline (pH 7.4) with 1 mg/ml BSA, followed by addition of the FRETs-VWF73 substrate. FRETs-VWF73 was excited at 355 nm and emission measured at 460 nm every 3 min for 3 h. Fluorescence intensities were depicted as a function of time and
the slope of the resulting curve was calculated and compared with a standard curve derived from slopes of serial dilutions of normal rat plasma set at 100% ADAMTS13 activity. ADAMTS13 activity in the presence of 5 mM EDTA was used as a negative control. Lactate dehydrogenase (LDH) activity was measured in EDTA plasma using an LDH activity colorimetric assay kit (Biovision, Milpitas, USA) according to the manufacturer’s instructions.

Determinaton of rVWF antigen levels
Human VWF antigen present in the plasma samples of rats injected with rVWF was quantified by ELISA. An in-house developed monoclonal antibody against human VWF (6D1) was used to specifically capture rVWF from rat plasma and a polyclonal rabbit anti-human VWF was conjugated with horse radish peroxidase (HRP; Dako, Everlee, Belgium) to detect bound VWF. A normal human plasma (NHP) pool from 20 individuals was used to set up a calibration curve and undiluted NHP was assigned a VWF antigen concentration of 100%.

Determination of circulating immune-complexes
Circulating immune-complexes of goat anti-ADAMTS13 antibodies bound to ADAMTS13 were measured by ELISA, using an in-house rabbit polyclonal ADAMTS13-specific capture antibody (K1-4) and a biotinylated polyclonal rabbit anti-goat IgG as detection antibody (Vector Laboratories, Inc., Burlingame, USA) in combination with horseradish peroxidase-conjugated avidin (Invitrogen Life Technologies, Gent, Belgium) and TMB as substrate (Thermo Scientific, Inc., Rockford, USA). The final immune-complex titer was determined as the highest dilution of the test sample with an OD above negative control plasma, prepared from rat blood taken 3 h after a single intravenous administration of normal goat IgG and rADAMTS13.

VWF multimer analysis
VWF multimer analysis was performed as described. Briefly, VWF was separated on sodium dodecyl sulphate (SDS) 1.5% iso electric focusing (IEF) agarose gels. The gels were fixed on Gelbond (Cambrex Bio Science Rockland Inc., Rockland, USA) and VWF was detected using anti-human VWF-Ig labeled with alkaline phosphatase and a substrate kit (BioRad, Hercules, USA). Densitometric analysis was performed using ImageJ software (version 1.47, National Institute of Health, Bethesda, USA). For each lane, the complete multimer was selected and the density was graphed. The lowest 5 (1-5 mer), the intermediate (6-10 mer), and high molecular weight (HMW; >10 mer) multimers were selected and the density of the HMW multimers relative to the complete multimer was calculated as a percentage.

Histology
After 24 h, rats were sacrificed by cervical dislocation under isoflurane anesthesia. Following exsanguination, heart, kidney, liver, brain and lung tissues were collected and fixed in 4% paraformaldehyde. Tissues were embedded in paraffin and 5 µm thick sections were cut. Slides were stained with Haematoxylin & Eosin (Sigma-Aldrich) for general histologic analysis. Martinus, Scarlet and Blue (MSB) staining was performed to visualize fibrin (red) and collagen (blue). Sections were placed into Bouin’s 2000 fixative (American MasterTech, Lodi, USA) at 56°C for 1 h, followed by a 0.5% naphthol yellow S solution (Santa Cruz, Heidelberg, Germany), a 1% crystal ponceau 6R solution (Santa Cruz), a 1% phosphotungstic acid solution (Sigma-Aldrich), and a 0.5% methyl blue solution (Sigma-Aldrich). VWF was stained using a polyclonal rabbit anti-human VWF antibody (Dako), followed by biotinylated swine anti-rabbit F(Ab)2 (Dako) and the Vectastain ABC kit (Vector Laboratories, Burlingame, USA). VWF staining was visualized using DAB (Dako) and counterstained using Haematoxylin to visualize nuclei.
Statistics
All data are presented as mean ± standard deviation. Statistical comparisons between two
groups of samples were performed by Mann-Whitney U testing using GraphPad Prism 5
(GraphPad Software, Inc., La Jolla, USA). A p-value of less than 0.05 was considered
significant and is indicated with an asterisk (*). A p-value < 0.01 is indicated with two
asterisks (**).

References
M, Gerritsen H, Lammle B, Schwarz HP, Scheiflinger F. Cloning, expression, and
functional characterization of the von Willebrand factor-cleaving protease (ADAMTS13).
Blood. 2002;100:3626–3632.

2. Plaimauer B, Kremer Hovinga JA, Juno C, Wolfsegger MJ, Skalicky S, Schmidt M,
ADAMTS13 normalizes von Willebrand factor-cleaving activity in plasma of acquired

UB, Ehrlich HJ, Schwarz HP. Structure and function of a recombinant von Willebrand


Scheiflinger F, Schwarz HP, Muchitsch E-M. A new mouse model mimicking thrombotic
thrombocytopenic purpura: correction of symptoms by recombinant human ADAMTS13.

N, Vandenbulcke A, Deckmyn H, Rottensteiner H, De Maeyer M, de Meyer SF,
and secretion and contributes to thrombotic thrombocytopenic purpura in mice. J

7. Ruggeri ZM, Zimmerman TS. The complex multimeric composition of factor VIII/von

8. De Meyer SF, Vandeputte N, Pareyn I, Petrus I, Lenting PJ, Chuah MKL,
VandenDriessche T, Deckmyn H, Vanhoorelbeke K. Restoration of plasma von
Willebrand factor deficiency is sufficient to correct thrombus formation after gene