**Long-Term Prevention of Congenital Thrombotic Thrombocytopenic Purpura in ADAMTS13 Knockout Mice by Sleeping Beauty Transposon-Mediated Gene Therapy**

Sebastien Verhenne, Nele Vandeputte, Inge Pareyn, Zsuzsanna Izsvák, Hanspeter Rottensteiner, Hans Deckmyn, Simon F. De Meyer, Karen Vanhoorelbeke

**Objective**—Severe deficiency in the von Willebrand factor–cleaving protease ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13) because of mutations in the ADAMTS13 gene can lead to acute episodes of congenital thrombotic thrombocytopenic purpura (TTP), requiring prompt treatment. Current treatment consists of therapeutic or prophylactic infusions of fresh frozen plasma. However, lifelong treatment with plasma products is a stressful therapy for TTP patients. Here, we describe the use of the nonviral sleeping beauty (SB) transposon system as a gene therapeutic approach to realize lifelong expression of ADAMTS13 and subsequent protection against congenital TTP.

**Approach and Results**—We demonstrated that hydrodynamic tail vein injection of the SB100X system expressing murine ADAMTS13 in Adams13−/− mice resulted in long-term expression of supraphysiological levels of transgene ADAMTS13 over a period of 25 weeks. Stably expressed ADAMTS13 efficiently removed the prothrombotic ultralarge von Willebrand factor multimers present in the circulation of Adams13−/− mice. Moreover, mice stably expressing ADAMTS13 were protected against TTP. The treated mice did not develop severe thrombocytopenia or did organ damage occur when triggered with recombinant von Willebrand factor, and this up to 20 weeks after gene transfer.

**Conclusions**—These data demonstrate the feasibility of using SB100X-mediated gene therapy to achieve sustained expression of transgene ADAMTS13 and long-term prophylaxis against TTP in Adams13−/− mice.

**Visual Overview**—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:836-844. DOI: 10.1161/ATVBAHA.116.308680.)

**Key Words:** ADAMTS13 • congenital TTP • gene therapy • sleeping beauty transposon system • von Willebrand factor

**Thrombotic thrombocytopenic purpura (TTP)** is a rare thrombotic disorder caused by deficiency in the von Willebrand factor (VWF)–cleaving protease ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13). In normal circulation, ADAMTS13 cleaves highly reactive ultralarge VWF (UL-VWF) multimers into smaller, less prothrombotic multimers preventing spontaneous platelet aggregation. Severe ADAMTS13 deficiency, caused by mutations in the ADAMTS13 gene (congenital TTP) or autoantibodies against ADAMTS13 (acquired TTP), can result in accumulation of UL-VWF multimers and intravascular deposition of VWF- and platelet-rich thrombi in the microcirculation.1–3 During an acute episode, TTP patients typically present with thrombocytopenia, hemolytic anemia, and organ injury including cerebral, renal, and cardiac manifestations.4,5

Acute TTP is a medical emergency with a mortality rate as high as 90% if left untreated. In patients with congenital TTP, infusion of fresh frozen plasma (10-15 mL/kg) replenishes the missing ADAMTS13 and ameliorates disease outcome.6,7 Congenital TTP patients with low residual plasma ADAMTS13 activity may have frequent recurrences.8 Knowing that each bout of TTP is life-threatening and recovery is associated with long-term deficits in health-related quality of life,9,10 a prophylactic regimen of fresh frozen plasma is initiated in patients with frequently recurring congenital TTP. Indeed, regular plasma infusions—of which the number depends on the patient’s phenotype—may prevent acute TTP episodes.11–13 Lifelong treatment with plasma, however, is inconvenient, and administration of plasma may result in fluid overload or elicit allergic reactions to plasma proteins. The risk of viral/prion transmission also remains a major concern. Administration of recombinant ADAMTS13 would be a promising strategy, and phase I clinical trials are underway to evaluate its safety and pharmacokinetics.14,15

© 2017 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.116.308680

836
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMTS13</td>
<td>a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>rVWF</td>
<td>recombinant VWF</td>
</tr>
<tr>
<td>SB</td>
<td>sleeping beauty</td>
</tr>
<tr>
<td>TTP</td>
<td>thrombotic thrombocytopenic purpura</td>
</tr>
<tr>
<td>UL-VWF</td>
<td>ultralarge VWF</td>
</tr>
<tr>
<td>VWF</td>
<td>von Willebrand factor</td>
</tr>
</tbody>
</table>

Unfortunately, this treatment is still prophylactic rather than curative.

Given the severity of the disease and the limitations of fresh frozen plasma prophylaxis, gene therapy for congenital TTP would offer an attractive alternative, as correction of the underlying genetic defect would result in lifelong expression of ADAMTS13 avoiding the need for plasma infusions. Gene therapeutic approaches have been viewed as highly promising for both rare genetic diseases and more common complex disorders. The therapeutic potential of this strategy has recently been demonstrated in clinical trials for hemophilia B,20 Leber’s congenital amaurosis,18 X-linked severe combined immunodeficiency disorder,18 and metachromatic leukodystrophy.19

In the TTP model, an initial preclinical study demonstrated that viral vector-mediated expression of a truncated form of ADAMTS13 protected Adams13−/− mice from developing TTP signs elicited by bacterial Shiga toxin.20 Although protection from TTP was only assessed 2 weeks after gene transfer, this study provided evidence that supports the validity of a gene therapeutic approach. However, to successfully translate gene therapy into a clinical setting, a long-term expression of the transposon (pT2/HCRHPi-ADAMTS13), but no transposase (control group). At 3 days post-injection, levels of transgene ADAMTS13 were remarkably high in both groups of mice (368±52% and 392±91% in the gene therapy group and in the control group, respectively; Figure 2A). Notably, long-term follow-up of transgene ADAMTS13 expression levels indicated that transgene expression was transient in the control group, as ADAMTS13 antigen levels rapidly decreased over time (Figure 2A). On the contrary, transgene ADAMTS13 levels remained high in the gene therapy group up to 25 weeks after gene transfer, the time point at which the experiment was ended (184±17% versus 10±2% in the gene therapy group versus the control group; Figure 2A).

Immunohistochemical analysis of liver sections confirmed the above results. Mice with long-term and high-level expression of transgene ADAMTS13 (gene therapy group) had a high number of ADAMTS13-expressing hepatocytes at 25 weeks post-injection (Figure 2B, right panel). In contrast, in mice with transient ADAMTS13 transgene expression (control group), only a few ADAMTS13-positive hepatocytes were found in liver sections at 25 weeks post-injection (Figure 2B, middle panel). Finally, we addressed whether the gene therapeutic approach induced an immune response against de novo expressed ADAMTS13. In both groups, no anti-ADAMTS13 antibodies were detected in plasma of the majority of mice (Figure 3). Although we observed absorbance values above background in mice M5 and M7 in the gene therapy group and in mice M5, M6, and M7 in the control group, only in mice M5 and M6 of the control group specific anti-ADAMTS13 antibodies were detected in plasma. Indeed, only in these 2 mice, preincubation of their plasma with recombinant ADAMTS13 removed anti-ADAMTS13 antibodies resulting in a reduction of the

Materials and Methods

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

Long-Term Expression of Supraphysiological Levels of Transgene Murine ADAMTS13 in Adams13−/− Mice

We aimed at obtaining long-term expression of transgene murine ADAMTS13 in Adams13−/− mice by using the SB100X transposon system (Figure 1). We cloned an expression cassette consisting of murine full-length ADAMTS13 cDNA under control of the liver-specific α1-antitrypsin promoter (HCRHPi)27 into the SB transposon vector to obtain pT2/HCRHPi-ADAMTS13. To integrate the ADAMTS13 transgene into the mouse genome, the plasmid containing the hyperactive variant of the original transposase under control of the ubiquitous CMV promoter was used (pCMV-SB100X).22 Plasmids were hydrodynamically injected in Adams13−/− mice resulting in in vivo transfection of murine hepatocytes.28–30

In the first group of Adams13−/− mice (n=7), both transposon (pT2/HCRHPi-ADAMTS13) and transposase (pCMV-SB100X) plasmids were hydrodynamically injected. In this group, the integration of the murine ADAMTS13 CDNA into the mouse genome was promoted by the transposase (gene therapy group). In the second group of mice, Adams13−/− (n=7) mice were injected with a solution containing only the transposon (pT2/HCRHPi-ADAMTS13), but no transposase (control group).
absorbance values to background level (data not shown). These results indicate that no profound antibody-mediated immune response against transgene ADAMTS13 was induced after gene transfer.

In conclusion, these data indicate that SB100X transposon-mediated gene delivery is a powerful technique to obtain long-term expression of supraphysiological levels of transgene ADAMTS13 in Adamts13−/− mice.

Figure 1. Schematic representation of sleeping beauty (SB100X) transposon-mediated gene transfer. The SB100X transposon system is a 2-component system consisting of (1) a transposon plasmid that contains inverted terminal repeats (ITR, white arrow) flanking the gene of interest (GOI), and (2) a transposase plasmid encoding the hyperactive SB transposase (SB100X). On SB transposon-mediated gene transfer, the SB100X transposase (gray circles) will bind the ITR (white arrows), excise the engineered therapeutic gene (GOI) from the plasmid DNA, and integrate it into genomic TA dinucleotides of chromosomal DNA.
Long-Term Expression of Transgene ADAMTS13 Prevents the Accumulation of Prothrombotic UL-VWF Multimers in Adamts13−/− Mice

Because of the absence of ADAMTS13 activity, prothrombotic UL-VWF multimers accumulate in Adamts13−/− mice compared with wild-type littermates.31,32 Because these UL-VWF multimers are the major cause of TTP in humans, we assessed whether transgene ADAMTS13 was able to proteolyze UL-VWF multimers in Adamts13−/− mice (Figure 4).

At day 3 post-injection, when transgene ADAMTS13 levels were high in both groups, UL-VWF multimers were absent. The percentage of high molecular weight VWF multimers (including the UL-VWF multimers) in both the gene therapy group (29.9±0.9%; n=4) and control group (30.7±2.2%; n=5) was comparable to the percentage observed in Adamts13+/+ mice (36.0±1.6%; n=7), which do not have UL-VWF multimers,31 showing that UL-VWF multimers were efficiently proteolyzed by transgene ADAMTS13 (Figure 4A). At 25 weeks post-injection however, the percentage of high molecular weight VWF multimers in the control group (39.9±1.2%; n=5) increased again to values observed in Adamts13−/− mice (41.3±1.4%; n=8), which have UL-VWF multimers,31 indicating that the low levels of transgene ADAMTS13 were not sufficient to digest the UL-VWF multimers (Figure 4A and 4B). In contrast, the relative amount of high molecular weight VWF multimers was still decreased in the gene therapy group (31.7±1.3%; n=4) at 25 weeks post-injection, which is in line with the high levels of transgene Adamts13 present in these mice (Figure 4A and 4B).

In conclusion, SB transposon-mediated expression of the therapeutic ADAMTS13 gene leads to long-term normalization of the VWF multimer distribution in circulation and prevents accumulation of prothrombotic UL-VWF multimers in Adamts13−/− mice.

Sleeping Beauty Transposon-Mediated Gene Therapy Prevents the Onset of TTP Signs in Adamts13−/− Mice Up To 20 Weeks After Gene Transfer

It was previously shown that Adamts13−/− mice develop TTP signs when they are triggered with recombinant VWF (rVWF).14 We next investigated whether our SB100X-mediated gene therapeutic strategy could be an effective prophylactic approach to prevent acute TTP in Adamts13−/− mice.

We hydrodynamically injected 3 groups of Adamts13−/− mice with the SB100X transposon system (gene therapy...
Taken together, these results demonstrate that long-term SB within the normal range or did these mice lose weight. Let counts, plasma LDH, and hemoglobin levels remained.

To further emphasize the long-term prophylactic efficacy of our gene therapeutic approach, an additional gene therapy group was challenged with rVWF at 20 weeks after gene transfer (Figure 2A). To induce TTP signs, each group was challenged with a single dose of human rVWF (2000 VWF:RCoU/kg) at different time points (1, 4, or 10 weeks after gene transfer). In parallel, we hydrodynamically injected 3 groups of Adamts13−/− mice with a saline solution to serve as nonexpressing controls and also challenged these mice with rVWF at the 3 different time points (control groups). A single group of Adamts13−/− mice that was hydrodynamically injected with PBS and treated with PBS instead of challenged with rVWF was used as a healthy control group. Twenty-four hours after the challenge, platelet count, plasma lactate dehydrogenase (LDH) levels (marker for tissue damage), hemoglobin levels (marker for hemolytic anemia), and weight loss were determined in all groups to assess TTP signs (Figure 5). As expected, all mice in the control groups (no gene transfer) developed TTP at all time points of rVWF challenge: platelet counts were dramatically decreased (>80%; Figure 5A), plasma LDH levels were substantially elevated (Figure 5B), hemoglobin levels were decreased (Figure 5C), and a dramatic weight loss was observed (Figure 5D) when compared with values observed in healthy Adamts13−/− mice. Importantly, and in stark contrast, mice of the gene therapy groups did not develop TTP signs, regardless of the time point of challenge: they did not suffer from severe thrombocytopenia (Figure 5A), their plasma LDH and hemoglobin levels remained within the normal range (Figure 5B and 5C), and they did not lose weight on induction of TTP, reflecting a healthy state (Figure 5D).

To further emphasize the long-term prophylactic efficacy of our gene therapeutic approach, an additional gene therapy group was challenged with rVWF at 20 weeks after gene transfer (Figure 5). Again, mice did not develop TTP as platelet counts, plasma LDH, and hemoglobin levels remained within the normal range or did these mice lose weight. Taken together, these results demonstrate that long-term SB transposon-mediated expression of the ADAMTS13 therapeutic gene results in long-lasting prophylaxis against TTP in this animal model.

Discussion

In this study, we developed a novel integrating, nonviral gene therapy strategy to prevent congenital TTP in a mouse model. We demonstrated that SB100X-mediated gene transfer of murine Adamts13 cDNA in Adamts13−/− mice resulted in long-term and high-level expression of the therapeutic gene. Importantly, this gene therapeutic strategy was able to normalize the VWF multimer pattern and prevent onset of acute TTP disease up to 20 weeks after gene transfer.

The SB transposon-mediated technology allowed sustained expression of high levels of transgene ADAMTS13 (184±17%) in Adamts13−/− mice for 25 weeks post-injection (almost 6 months; the end of the study). Stable expression of high plasma ADAMTS13 antigen levels was associated with the presence of high number of Adamts13-expressing hepatocytes. When the SB100X transposase was absent, transgene expression rapidly dropped below WT levels within 3 weeks post-injection, resulting in a low number of Adamts13-expressing hepatocytes. Still, low-level transgene expression, associated with a low number of Adamts13-expressing hepatocytes, persisted for 25 weeks post-injection (10±2%), indicating that not all nonintegrated prokaryotic DNA was degraded or epigenetically silenced.27,33,34 De novo synthesis of Adamts13 did not elicit a profound antibody response in the majority of the mice. This observation is in agreement with the idea that Adamts13 is not immunogenic.35 Indeed, to date, alloantibody formation in congenital TTP patients treated with plasma infusions (containing exogenous Adamts13) has not yet been reported. Currently ongoing clinical trials using recombinant Adamts13 as an alternative treatment for congenital TTP will provide us with more information on the immunogenicity of Adamts13.
Notably, expressed ADAMTS13 was biologically active in vivo, as demonstrated by the normalization of the VWF multimer pattern in Adamts13−/− mice. Only mice expressing sustained high therapeutic ADAMTS13 levels were able to normalize the VWF multimer pattern for the whole 25-week period. These results indicate that the corresponding plasma ADAMTS13 activity was high enough to process newly secreted UL-VWF multimers. Furlan et al.36 showed that 5% plasma ADAMTS13 activity might be already sufficient to degrade the most adhesive VWF multimers into smaller forms that do not spontaneously recruit platelets in congenital TTP patients. In line with this observation, data obtained by Lotta et al.8 indicate that a small increase in residual plasma ADAMTS13 activity is sufficient to lower the risk of disease recurrence and the need for fresh frozen plasma prophylaxis in congenital TTP patients. In contrast, in the present study, we observed that the plasma ADAMTS13 activity associated with a mean antigen value of 10% (range, 2%–17%) was not yet sufficient to alter the VWF multimer distribution. Whether this can be explained by species-specific differences in threshold ADAMTS13 levels required to normalize the VWF multimer pattern or because of synthesis of the therapeutic enzyme in heterologous cells (hepatocytes) requires further investigation. Nevertheless, our study shows that the SB100X transposon system results in expression levels of ADAMTS13 of 184±17% in Adamts13−/− mice up to 6 months after gene transfer. Thus, it might be reasonable to assume that the SB100X system could be a proper vector able to provide stable therapeutic ADAMTS13 levels of >10% in congenital TTP patients. Most important, transgene ADAMTS13 is shown to prevent severe thrombocytopenia and extensive organ damage in a murine model of TTP. When challenged with a high dose of rVWF,14 Adamts13−/− mice expressing high levels of transgene ADAMTS13 did not develop TTP signs as no profound thrombocytopenia, elevated LDH levels, decreased hemoglobin levels, and increased weight loss were observed. Notably, this protective effect was sustained for up to 20 weeks after gene transfer. To our knowledge, this is the first gene therapy–based preclinical study demonstrating long-term prophylaxis for congenital TTP using a nonviral gene therapeutic approach.

Figure 4. Long-term sleeping beauty (SB100X) transposon–mediated expression of high levels of transgene ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 motif, member 13) reduces von Willebrand factor (VWF) multimer size in Adamts13−/− mice. Adamts13−/− mice were hydrodynamically injected with 5 μg pT2-HCRHPi-ADAMTS13 with (+SB100X, gene therapy group) or without (−SB100X, control group) 0.2 μg pCMV-SB100x. A, The relative abundance of high molecular weight (HMW) VWF multimers, including the ultralarge (UL) VWF multimers, was determined by densitometric analysis. VWF multimer gel analysis was performed on plasma samples collected at 3 and 25 wk after gene transfer (GT). Plasma samples of nontreated Adamts13−/− and Adamts13+/+ mice were used as controls. B, Representative image of VWF multimer patterns and corresponding densitometric profiles of control Adamts13−/− mice (1; plasma ADAMTS13 antigen: 0% of NMP) and Adamts13−/− mice expressing low (2; plasma ADAMTS13 antigen: 9% of NMP) or high (3; plasma ADAMTS13 antigen: 224% of NMP) transgene ADAMTS13 antigen levels at 25 wk after GT. Distinguishable bands were divided into 3 subclasses: 1 to 5 dimers were designated as low molecular weight (LMW) VWF multimers, 6 to 10 dimers as medium molecular weight (MMW) VWF multimers, and >10 dimers as medium molecular weight (MMW) VWF multimers, >10 dimers as medium molecular weight (MMW) VWF multimers, and >10 dimers as medium molecular weight (MMW) VWF multimers. *P<0.05; **P<0.01; ***P<0.001 compared with Adamts13−/− mice. ns indicates not significant.
The use of the SB transposon technology holds high promise as it combines low cost and simplicity of naked DNA with the efficiency of gene transfer associated with retroviral vectors. SB has proven successful as a therapeutic vector in various preclinical studies demonstrating long-term gene expression in a wide range of tissues and primary cells. Moreover, SB technology reached clinical level as the first phase I clinical trials using SB transposons have recently been conducted, albeit in an ex vivo gene therapy setting. A second issue regarding the clinical use of the SB transposon system is the safety of SB-mediated integration. It has been shown that SB has no preference for integration into transcription units and transcriptional regulatory regions and that the majority of insertions that do occur in genes are located in introns. Its integration site preference in mammalian cells is highly favorable compared with other transposons and integrating viral vectors, which show preferential integration into transcriptionally active sites. Interestingly, genome-wide profiling studies have recently demonstrated that SB has the highest theoretical chance of inserting the gene of interest into a safe harbor locus. However, the genotoxic risk is not zero and insertional mutagenesis after SB-mediated gene therapy cannot be excluded. Therefore, attempts are made to minimize the risk of accidental transactivation of genes. Studies have shown that engineered fusion proteins comprising zinc finger proteins and the SB transposase can be used to effectively redirect transposon insertion to deliver the transgene into the liver.

A second issue regarding the clinical use of the SB transposon system is the safety of SB-mediated integration. It has been shown that SB has no preference for integration into transcription units and transcriptional regulatory regions and that the majority of insertions that do occur in genes are located in introns. Its integration site preference in mammalian cells is highly favorable compared with other transposons and integrating viral vectors, which show preferential integration into transcriptionally active sites. Interestingly, genome-wide profiling studies have recently demonstrated that SB has the highest theoretical chance of inserting the gene of interest into a safe harbor locus. However, the genotoxic risk is not zero and insertional mutagenesis after SB-mediated gene therapy cannot be excluded. Therefore, attempts are made to minimize the risk of accidental transactivation of genes. Studies have shown that engineered fusion proteins comprising zinc finger proteins and the SB transposase can be used to effectively redirect transposon insertion to deliver the transgene into the liver.21

Figure 5. Long-term sleeping beauty (SB100X) transposon-mediated expression of high levels of transgene ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13) prevents the onset of thrombotic thrombocytopenic purpura (TTP) signs in Adamts13−/− mice challenged with recombinant von Willebrand factor (rVWF). Adamts13−/− mice were injected with saline solution (control group, no gene transfer) or with plasmids coding for the SB100X system (gene therapy group) via hydrodynamic injection. Mice were subsequently challenged with a single dose of human rVWF (2000 VWF:RCoU/kg) at different wk post-hydrodynamic injection (wpi; control group and gene therapy group). Another control group (no gene transfer) was hydrodynamically injected with saline and subsequently injected with PBS instead of challenged with rVWF (non-rVWF challenged Adamts13−/− mice). Twenty-four hours after challenge with rVWF, blood was collected and analyzed.

A. Thrombocytopenia was assessed by measuring platelet counts in EDTA-treated blood using an automated cell counter.
B. Tissue damage was determined by measuring lactate dehydrogenase (LDH) activity in citrated plasma using a colorimetric assay kit.
C. Hemoglobin (Hb) levels were measured in EDTA-treated blood using an automated cell counter.
D. Weight loss was determined by calculating the difference in weight before and 24 h after challenge with rVWF. Mean ADAMTS13 antigen levels at the indicated time points are shown. *P<0.05; **P<0.01; ***P<0.0001. ns indicates not significant.
into specific sequences in the genome.\textsuperscript{46,47} To further reduce the genotoxic risk, one could explore the use of gene transfer strategies that retain plasmid DNA in an episomal state, such as minicircles or inclusion of “scaffold/matrix attachment regions” DNA sequences.\textsuperscript{48,49}

In conclusion, we successfully achieved long-term therapeutic expression of full-length ADAMTS13 in Adamts13\textsuperscript{−/−} mice using the SB transposon system. This study is the first report demonstrating that a single administration of naked plasmid DNA can result in long-term correction of ADAMTS13 deficiency and prophylaxis of TTP in Adamts13\textsuperscript{−/−} mice. Considering the rate of progress in areas of vector improvement and administration, we think that the present study provides a proof of concept supporting the further development of a nonviral SB transposon–mediated gene therapy for congenital TTP in humans.

Acknowledgments

The authors thank L. Deforche for help with preparing the figures. S. Verhenne and K. Vanhoorelbeke designed research and wrote the article. S. Verhenne, N. Vandeputte, I. Pareyn, and K. Vanhoorelbeke performed experiments. S. Verhenne, S.F. De Meyer, and K. Vanhoorelbeke analyzed data and interpreted results. Z. Izsák and H. Rottensteiner provided essential materials. Z. Izsák, H. Rottensteiner, H. Deckmyn, and S.F. De Meyer critically reviewed the article.

Sources of Funding

This work was supported by Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO) G.0584.11, G.0D13.15; N. KU Leuven grant OT/14/071 and Answering TTP foundation grant RXO-D0264.

Disclosures

H. Rottensteiner is an employee of Baxalta Innovations GmbH, Vienna, Austria. The other authors report no conflicts.

References


ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13) gene transfer by the nonviral sleeping beauty transposon system results in long-term stable expression (25 weeks) of supraphysiological plasma ADAMTS13 antigen levels in Adamts13−/− mice.

Sleeping beauty transposon–mediated gene transfer of Adams13 cDNA leads to long-term prophylaxis (up to 20 weeks) of thrombotic thrombocytopenic purpura in Adamts13−/− mice.

Our findings support the development of a nonviral sleeping beauty transposon–mediated gene therapeutic approach for treatment of congenital thrombotic thrombocytopenic purpura in humans.
Long-Term Prevention of Congenital Thrombotic Thrombocytopenic Purpura in ADAMTS13 Knockout Mice by Sleeping Beauty Transposon-Mediated Gene Therapy
Sebastien Verhene, Nele Vandeputte, Inge Pareyn, Zsuzsanna Izsvák, Hanspeter Rottensteiner, Hans Deckmyn, Simon F. De Meyer and Karen Vanhoorelbeke

Arterioscler Thromb Vasc Biol. 2017;37:836-844; originally published online March 2, 2017; doi: 10.1161/ATVBAHA.116.308680
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2017 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/37/5/836

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2017/03/01/ATVBAHA.116.308680.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/
Materials and Methods

Plasmids
The plasmid expressing the transposase, pCMV(CAT)T7-SB100 (referred as pCMV-SB100x in the text), contains the hyperactive transposase (SB100x) cDNA of which expression is driven from a cytomegalovirus (CMV) enhancer/promoter.\(^1\) The transposon plasmid, pT2/HCRPi-ADAMTS13, was constructed using the In-Fusion® HD Cloning Kit (Clontech Laboratories, Mountain View, CA). The expression cassette containing a liver-specific promoter (HCRPi: apolipoprotein hepatic control locus region (ApoE-HCR) linked to a human α1-anti-trypsin promoter and a truncated 1.4 kb human factor IX) coupled to murine full-length ADAMTS13 cDNA was amplified from pBS-II-SK-HCRPi-muADAMTS13\(^2\) using Phusion® High Fidelity DNA polymerase (New England Laboratories, Ipswich, MA) and primers flanking the desired region. In parallel, pT2/CAGGS-GFP\(^1\) was digested with ClaI/NotI-HF (New England Laboratories) thereby excising the CAGGS-GFP expression cassette. Subsequently, the HCRPi-ADAMTS13 polymerase chain reaction product was ligated into the linearized pT2-vector backbone according to the In-Fusion cloning procedure for spin-column purified PCR fragments and the resulting plasmid was sequenced (GATC Biotech AG, Konstanz, Germany). Plasmid DNA used for hydrodynamic tail vein injection was prepared using the Endofree® Plasmid Mega Kit (Qiagen, Venlo, The Netherlands).

Mice
ADAMTS13 knockout (Adamts13\(^{-/-}\)) and wild-type (Adamts13\(^{+/+}\)) mice were bred from heterozygous Adamts13\(^{+/-}\) mice with a mixed C57BL/6J-129X1/SvJ-CASA/Rk background (kind gift of D. Ginsburg, University of Michigan, MI).\(^3\) All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the KU Leuven (P163/2015, Belgium).

Hydrodynamic gene transfer
Five to 7-week old Adamts13\(^{-/-}\) mice (weighing 16-21 g) were injected with 5 µg transposon plasmid DNA and 0.2 µg transposase plasmid DNA via hydrodynamic tail vein injection.\(^4\)\(^5\) Plasmid DNA was diluted in a saline solution (0.9% NaCl) equivalent to 10% of the body weight and administered via the lateral tail vein in 5-7 seconds. Adamts13\(^{-/-}\) mice that were hydrodynamically injected with only transposon plasmid DNA (no transposase) or with saline solution were used as controls.

Blood collection
Animals were anesthetized using 5% isoflurane (Nicholas Piramal Limited, London, UK) in 100% O\(_2\). Whole blood was drawn from the retro-orbital plexus and collected on 0.5 M EDTA (1 volume to 40 volumes of blood) or 3.8% sodium citrate (1 volume to 7 volumes of blood). Platelet-poor plasma was prepared from citrated blood by centrifugation at 4300 g for 6 minutes at room temperature and stored at -80°C for further analysis.

Induction of TTP
To induce TTP signs, saline- or SB-treated Adamts13\(^{-/-}\) mice were challenged with a single intravenous bolus injection of recombinant human VWF (rVWF; 2000 VWF:RCoU/kg body weight) in the lateral tail vein as described.\(^5\) Twenty-four hours
after challenge, blood was collected to monitor TTP signs. Platelet count and hemoglobin levels were determined in EDTA-anticoagulated blood using the Hemavet 950FS Multi-species Hematology system (Drew Scientific Inc., Dallas, TX). Lactate dehydrogenase (LDH) activity was measured in citrated plasma using an LDH activity colorimetric assay kit (Biovision, Milpitas, CA) according to the manufacturer’s instructions. Body weight loss was determined by calculating the difference in body weight assessed before and 24 hours after challenge with rVWF.

**ADAMTS13 antigen analysis**
Transgene ADAMTS13 antigen levels in plasma were determined via an in-house developed enzyme-linked immunsorbent assay (ELISA) as described, with some minor modifications.² A 96-well microtiter plate was coated overnight at 4°C with our anti-mouse ADAMTS13 monoclonal antibody 14H7B8 (5 µg/mL in PBS). After blocking with a 3% skimmed milk solution, plasma samples (diluted in PBS containing 0.3% (m/v) skimmed milk) were added and incubated for 90 minutes at 37°C. Captured ADAMTS13 was detected via our in-house developed polyclonal rabbit anti-mouse ADAMTS13 antibodies (5 µg/ml in PBS containing 0.3% skimmed milk) and polyclonal goat anti-rabbit antibodies labeled with horseradish peroxidase (HRP; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) (1/10,000 in PBS containing 0.3% skimmed milk). The colouring reaction was initiated after addition of ortho-phenylenediamine (Sigma-Aldrich, St Louis, MO) and H₂O₂, and stopped with 4 M H₂SO₄. The ADAMTS13 antigen level in pooled plasma of more than 20 *Adamts13*+/+ mice was used as a reference (100%, normal murine plasma, NMP).

**Immunohistochemistry**
After sacrificing the animal, the left liver lobe was removed, embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands), snap-frozen in liquid nitrogen and stored at -80°C. Liver lobes were cut in 10 µm thick cryosections using a Leica CM1950 Cryostat (Leica Biosystems, Richmond, IL) and stored at -20°C. Before use, sections were air-dried and fixed in 4% paraformaldehyde. Sections were then blocked and permeabilized with 5% normal goat serum in PBS containing 0.1% Triton X-100, 1% BSA, and 0.1% Tween20 for 1 hour at room temperature followed by overnight incubation with our polyclonal rabbit anti-mouse ADAMTS13 antibodies² (10 µg/ml in PBS containing 1% BSA and 0.1% Tween20) at 4°C. After washing in PBS, endogenous peroxidase activity was blocked using 0.3% H₂O₂ in PBS for 15 minutes at room temperature. Subsequently, sections were washed and incubated with HRP-labeled goat anti-rabbit antibodies (1/500 in PBS containing 1% BSA and 0.1% Tween20; Jackson ImmunoResearch Laboratories Inc.) for 45 minutes at room temperature. After another washing step, ADAMTS13-positive liver cells were visualized with diaminobenzidine (DAB; Dako, Glostrup, Denmark) followed by counterstaining with hematoxylin (Sigma-Aldrich) for 2 minutes. Sections were dehydrated and mounted in DPX (Fluka, Bornem, Belgium). Pictures were taken at 10x magnification using a Zeiss Primo Star microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

**Analysis of murine plasma VWF multimers**
Citrated plasma samples were 1/5 diluted in sample buffer (8 M urea, 5% (m/v) sodium dodecyl sulphate (SDS), 10 mM Tris, 1 mM EDTA, and 0.3% bromophenol
blue; pH 8.0) and incubated at 60°C for 30 minutes. VWF multimers were separated on a low-resolution (1.2%) SDS isoelectric focusing agarose gel (GE Healthcare Europe GmbH, Diegem, Belgium), fixed on a GelBond® film (Lonza, San Diego, CA), by electrophoresis at 10-15 mA in a multiphor II apparatus (GE Healthcare).

Following electrophoresis, the gel was rinsed in distilled water, dried under cooled air and blocked in 5% skimmed milk in TBS containing 0.05% Tween 20. After overnight incubation with polyclonal anti-human VWF antibodies (1/750 diluted in TBS; Dako), in-house conjugated with alkaline phosphatase, and subsequent rinsing with TBS containing 0.05% Tween 20, VWF multimers were visualized using an Alkaline Phosphate conjugate substrate kit (BioRad, Hercules, CA). Densitometric analysis was performed using Image J software (version 1.47, National Institute of Health, Bethesda, MD). The density profile of each VWF multimer pattern was graphed. Distinguishable bands were divided into 3 subclasses: (1) 1-5 dimers were designated as low molecular weight VWF multimers (LMW), (2) 6-10 dimers as medium molecular weight VWF multimers (MMW), and (3) >10-dimers as high molecular weight VWF multimers (HMW; including UL-VWF multimers). UL-VWF multimers did not resolve in single bands but appeared as a smear that was included in the HMW subclass. The total density of each subclass was divided by the density of all multimer bands to calculate the relative abundance of all subclasses.

**Anti-ADAMTS13 antibody ELISA**

The presence of anti-ADAMTS13 antibodies in murine plasma was determined via ELISA. A 96-well microtiter plate was coated overnight at 4°C with our polyclonal rabbit anti-mouse ADAMTS13 antibodies (5 µg/mL in PBS). After blocking with a 1% BSA solution, expression medium containing recombinant murine ADAMTS13 was added (1/2 diluted in PBS containing 0.5% (v/v) BSA). Next, a dilution series of murine plasma samples was added (1/20 diluted in PBS, 0.5% BSA) and incubated for 60 minutes at 37°C. Captured anti-ADAMTS13 antibodies were detected with goat anti-mouse antibodies labeled with HRP (Jackson ImmunoResearch Laboratories Inc.) (1/20,000 in PBS containing 0.5% BSA). The colouring reaction was done as described above. NMP of Adamt13+/+ mice was used as a negative control. A dilution series of NMP of Adamt13−/− mice spiked with 1 µg/ml of the anti-mADAMTS13 monoclonal antibodies 14C1 and 19H2 was used as a positive control. The background cut-off value was calculated as the mean negative control value plus two times the standard deviation of the negative control values.

To further confirm the presence of anti-ADAMTS13 antibodies in murine plasma, microtiter plates were again coated with polyclonal rabbit anti-mouse ADAMTS13 antibodies and expression medium containing recombinant murine ADAMTS13 was added. Next, plasma samples (1/20 dilution) were added after preincubation with either recombinant ADAMTS13 (100 µg/ml) or buffer for 1 hour at 37°C. Finally, goat anti-mouse antibodies labeled with HRP were added. NMP of Adamt13+/+ mice and NMP of Adamt13−/− mice spiked with anti-mADAMTS13 monoclonal antibodies 14C1 and 19H2 (1 µg/ml of each), both preincubated with either recombinant ADAMTS13 or buffer, were used as a negative and positive control, respectively. The background cut-off value was calculated as the mean negative control value plus two times the standard deviation of the negative control values.

**Statistical analysis**

Data are represented as mean ± SEM. For statistical analysis, Prism Version 6.0 software (GraphPad Software, La Jolla, CA) was used. One-way analysis of variance...
followed by Dunnett’s multiple comparisons post hoc test was conducted to assess the variance of the relative amounts of HMW multimers between Adamts13−/− mice and the different experimental groups. Unpaired t testing was used to compare saline- and SB-treated Adamts13−/− mice challenged with rVWF at different time points. A probability value < 0.05 was considered as statistically significant.
References


