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ADAMTS13 Autoantibodies Cloned from Patients with Acquired Thrombotic Thrombocytopenic Purpura: 2. Pathogenicity in an animal model

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Abstract

BACKGROUND—Acquired thrombotic thrombocytopenic purpura (TTP) is a potentially fatal disease in which ultra-large von Willebrand Factor (UL-VWF) multimers accumulate as a result of autoantibody inhibition of the VWF protease, ADAMTS13. Current treatment is not specifically directed at the responsible autoantibodies and in some cases is ineffective or of transient benefit. More rationale, reliable and durable therapies are needed, and a human autoantibody-mediated animal model would be useful for their development. Previously, TTP patient anti-ADAMTS13 variable region fragments (scFv's) were cloned that inhibited ADAMTS13 proteolytic activity *in vitro* and expressed features in common with inhibitory IgG in patient plasma. Here, pathogenicity of these scFv's is explored *in vivo* by transfecting mice with inhibitory antibody cDNA.

STUDY DESIGN AND METHODS—Hydrodynamic tail vein injection of naked DNA encoding human anti-ADAMTS13 scFv was used to create sustained ADAMTS13 inhibition in mice. Accumulation of UL-VWF multimers was measured and formation of platelet thrombi following focal or systemic vascular injury was examined.

RESULTS—Transfected mice expressed physiological plasma levels of human scFv and developed sustained ADAMTS13 inhibition and accumulation of unprocessed UL-VWF

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multimers. Induced focal endothelial injury generated platelet thrombi extending well beyond the site of initial injury, and systemic endothelial injury induced thrombocytopenia, schistocyte formation, platelet thrombi, and death.

CONCLUSIONS—These results demonstrate for the first time the ability of human recombinant monovalent anti-ADAMTS13 antibody fragments to recapitulate key pathologic features of untreated acquired TTP *in vivo*, validating their clinical significance and providing an animal model for testing novel targeted therapeutic approaches.

Keywords

Thrombotic thrombocytopenic purpura; ADAMTS13; von Willebrand Factor; microangiopathic hemolytic anemia; therapeutic plasma exchange; animal models

INTRODUCTION

Current therapeutic approaches to the treatment of antibody-mediated autoimmune disease are generally limited to the use of systemic immunosuppression with its attendant side effects rather than therapy that targets the pathogenic autoantibodies. To rectify this situation, there is a need to gain a better understanding of the repertoire of autoantibody expression within an individual patient and across multiple patients at a molecular level to delineate autoantibody clonality, epitope specificity, idiotypic relatedness, and functional significance *in vivo*. This knowledge may then lead to the design of animal models that can be used to test innovative therapies that specifically target pathogenic autoantibodies or the B-cells that produce them.

The acquired form of thrombotic thrombocytopenic purpura (TTP) is an example of an autoimmune disorder in which the majority of patients have reduced activity levels of the von Willebrand factor (VWF)-cleaving protease ADAMTS13 due to the development of autoantibodies that inhibit enzyme function.^{1–7} Decreased ADAMTS13 activity results in the accumulation of ultra-large VWF (UL-VWF) multimers that foster widespread platelet aggregation in the microcirculation when coincident with additional factors such as endothelial injury, and can lead to severe thrombocytopenia, microangiopathic hemolytic anemia, organ dysfunction, and death.^{8–10}

Therapeutic plasma exchange (TPE) is first-line therapy for TTP, reducing mortality from ~90% to ~15%, but is inherently “non-specific” i.e., independent of autoantibody specificity. Even with earlier recognition of disease and initiation of TPE, and the use of additional non-specific interventions (e.g., corticosteroids, cyclosporin, rituximab, splenectomy),^{11–13} the mortality for patients diagnosed with TTP has not changed significantly since the initial introduction of TPE over 25 years ago.¹⁴ Alternative therapeutic approaches for TTP have been proposed including the infusion of excess quantities of recombinant ADAMTS13 to override autoantibody inhibition,¹⁵ and agents that would target UL-VWF multimers either by reducing their size or by blocking their interactions with platelets.^{16–20} However, it is not clear that these approaches would obviate the need for TPE and/or use of immunosuppressive medications, since these interventions do not diminish the binding or production of pathogenic inhibitors.^{21,22} Moreover, interventions that affect pathogenesis

downstream of autoantibody-mediated ADAMTS13 inhibition have the potential downside of inhibiting normal hemostatic processes that are mediated through some of the same pathways.

Animal models of acquired TTP that employ patient-derived recombinant anti-ADAMTS13 autoantibodies would facilitate the development and testing of novel therapies, but none have been described.²³ A number of currently available models for simulating acquired TTP in animals do not use antibodies at all, e.g. they trigger a TTP-like syndrome in mice by infusion of cleavage-resistant VWF²⁴ or by the infusion of UL-VWF multimers that overwhelm endogenous ADAMTS13 capacity.²⁵ Animal models of TTP that do employ antibodies to inhibit ADAMTS13 have been restricted to using xenoantibodies to ADAMTS13, e.g., polyclonal and polyspecific rabbit anti-ADAMTS13 antibodies in mice,^{26,27} or murine monoclonal antibodies in baboons.²⁸ Animal models employing xenoantibodies may not be appropriate for testing agents that block the idiotopes of human anti-ADAMTS13 autoantibodies or for evaluating engineered forms of ADAMTS13 designed to be uninhibitable by TTP patient autoantibodies^{29,30} since human ADAMTS13 autoepitopes could not be assumed to be the same as the epitopes to which rabbit and mouse immune systems respond. Furthermore, intravascular injections of anti-ADAMTS13 antibody, regardless of antibody source, result in only transient decreases in ADAMTS13 activity (~24 hours), which does not faithfully simulate the continuous inhibition of ADAMTS13 seen during the acute and chronic phases of the human disease.

In an accompanying manuscript in this series⁴⁴, we described the cloning and characterization of a large set of human monoclonal anti-ADAMTS13 antibody fragments from TTP patients, a subset of which inhibited ADAMTS13 proteolytic activity *in vitro* and expressed key features in common with inhibitory IgG in patient plasma. Here we test the clinical relevance of these antibodies by transfecting their cDNA into mouse hepatocytes by direct hydrodynamic tail vein plasmid gene transfer. We explore the effects of recombinant human autoantibodies expressed *in vivo* on murine ADAMTS13 activity, processing of murine UL-VWF multimers, and formation of murine platelet thrombi following focal or systemic vascular injury.

MATERIALS AND METHODS

Intraperitoneal injection of anti-ADAMTS13 scFv's in mice

Two- to 3-month-old mice (C57BL/6 or CAST/Ei, Jackson Laboratory, Bar Harbor, ME) were anesthetized with ketamine/xylazine, and blood samples (50 μ L) were obtained from jugular veins before and after intraperitoneal injection of 100 μ L PBS containing 30 μ g human anti-ADAMTS13 recombinant single chain variable region antibody fragments (scFv's). ScFv were produced in *Drosophila* S2 cells as described⁴⁴. Blood samples were taken at times indicated and anticoagulated with heparin. Plasma was separated from cells at 1500g for 15 min for ADAMTS13 activity assays.

Cloning scFv cDNA into pLIVE *in vivo* expression vector

The pLIVE plasmid expression vector (Mirus, Madison, WI), a vector driven by a liver-specific promoter composed of the minimal mouse albumin promoter and the mouse α -fetoprotein enhancer,³¹ was modified in its multiple cloning site to contain an immunoglobulin kappa-chain leader sequence upstream from Sfi I restriction sites and a V5-tag sequence to facilitate secretion of V5-tagged scFv antibody fragments from murine liver following hydrodynamic delivery. pLIVE vector was first digested with Nhe I and Xho I restriction enzymes and a double-stranded oligonucleotide formed from the annealing of “V5 FOR” (5’-

CTAGCACTAGTGGCCAGGCCGGCCAGTTCGAAGGTAAGCCTATCCCTAACCCCTC
TCCTCGGTCTCGATTCTACGCGTACCGGT *TAGC*-3’) and “V5 REV” (5’-

TCGAGCTAACCGGTACGCGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCT
TACCTTCGAACCTGGCCGGCCTGGCCACTAGTG-3’) was ligated into the cut and gel-

purified vector to introduce the 3’ Sfi I restriction site (bold), the V5 tag sequence (underlined), and a stop codon (italics). The resultant plasmid was digested with Nhe I and Sfi I and a second double-stranded oligonucleotide formed from the annealing of “LEAD FOR” (5’-

CTAGCATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGT
TCCACTGGTGACGGAGCTGCGGCC**AGGCCGCC**CCCATGGCCGGGGTACCTACT
AGT**GGCCAGGC**-3’) and “LEAD REV” (5’-

TGGCCACTAGTAGGTACCCCGGGCCATGGGGCCGCCTGGGCCGCAGCTCCGTCA
CCAGTGGAACCTGGAACCCAGAGCAGCAGTACCCATAGCAGGAGTGTGTCTGTCT
CCATG-3’) was ligated into the cut and gel-purified vector to introduce the immunoglobulin kappa-chain leader sequence (underline) and 5’ Sfi I restriction site (bold). To clone phage display-derived scFv cDNA, the desired scFv construct was removed from the pComb3X phagemid (Scripps Research Institute, La Jolla, CA) in which it had been originally isolated by phage display⁴⁴ by Sfi I digestion, gel purified, ligated into Sfi I-digested and gel-purified pLIVE vector, and amplified in the XL1-Blue strain of *E. coli* (Agilent Technologies, Santa Clara, CA). Plasmid DNA for injection was purified using an endotoxin-free plasmid purification kit (Qiagen Inc., Valencia, CA).

Hydrodynamic gene delivery of human anti-ADAMTS13 scFv cDNA

ScFv-containing pLIVE plasmids (30 μ g) were injected into tail veins of 2–3 month-old mice (C57BL/6 or CAST/Ei) following pLIVE manufacturer’s instructions. Briefly, mice were warmed by a heat lamp for several minutes and 30 μ g of pLIVE/scFv DNA diluted in 2 mL TransIT-QR hydrodynamic delivery solution were injected in the tail over 4–7 seconds by syringe with a 30-gauge needle. Prior to and after DNA delivery, blood samples (~100 μ L) were collected from the retro-orbital sinus under isofluorane anesthesia into heparinized capillary tubes and transferred to 10 μ L of 1.9% sodium citrate. *In vivo*-expressed scFv’s were detected using an immunoprecipitation protocol similar to that used for scFv ADAMTS13 epitope mapping⁴⁴ except that scFv’s were captured with anti-V5 agarose beads from 100 μ L of plasma and eluted into 40 μ L of PAGE reducing sample buffer, and 20 μ L of each sample were analyzed by SDS-PAGE (Novex NuPAGE, Life Technologies). Western blotting was performed with HRP-conjugated anti-V5 antibody (Invitrogen, Life

Technologies, Grand Island, NY) and chemiluminescent images (ECL, GE Healthcare Life Sciences) exposed on X-ray film were quantified using Image J software (<http://rsb.info.nih.gov/niH-image/>) normalizing to mouse plasma spiked with 20 ng of S2 cell-produced scFv 1–420. Quantification of *in vivo*-produced scFv 1–420 was performed by immunoprecipitation and Western blotting rather than Western blotting of plasma alone because the extraordinary high concentrations of albumin in mouse plasma distorted the electrophoretic mobility of plasma proteins including the scFv.

Arteriole laser injury in human scFv-expressing mice

Platelet thrombus formation in C57BL/6 mice injected >7 days earlier with scFv plasmids was imaged using an Olympus BX61WI microscope (Olympus, Center Valley, PA) with a 60×/0.9 numeric aperture water immersion objective and captured with a Cooke SensiCam CCD camera (Cooke, Auburn Hills, MI) coupled to a Lambda DG4 widefield excitation system (Sutter, Novato, CA)³². The microscope, camera, and DG4 were all controlled using Slidebook 5.0 software (Intelligent Imaging Innovations). F(ab')₂ fragments of rat anti-mouse CD41 (BD Pharmingen, San Diego, CA) were conjugated to Alexa⁴⁸⁸ according to the manufacturer's instructions (Life Technologies). Labelled F(ab')₂ fragments were infused via jugular vein (0.1 mg/kg) immediately prior to first injury. Vascular injury was induced with a pulsed nitrogen dye laser (SRS NL100, Photonic Instruments, St. Charles, IL) focused on the vessel wall of arterioles (20–40 µm diameter) through the microscope objective. Analysis of time-lapse videos (750 frames per injury) was performed using Slidebook 5.0 (Intelligent Imaging Innovations, Denver, CO). After background fluorescence was subtracted from all images in one injury video, the resulting thrombus fluorescence was analyzed in the software to calculate an X/Y aspect ratio. A median filter was applied to each injury before taking the average of all injuries. P value for differences between control and experimental injury X/Y aspect ratios was determined using the Mann Whitney test (GraphPad Prism software, La Jolla, CA).

Shigatoxin challenge in scFv-expressing mice

CAST/Ei mice were injected with scFv plasmids and challenged 10 days later with Shigatoxin-2 (50 pg/g weight, Toxin Technology, Sarasota, FL) via tail vein. CBCs, Wright-stained blood smears, and hematoxylin-eosin-stained tissues were prepared as described.³³

ADAMTS13 activity and VWF multimer analyses

Human and murine ADAMTS13 activities were measured using FRETs-VWF73 peptide as described⁴⁴. For measurement of murine ADAMTS13 activity, 8 µL mouse plasma was added to 42 µL of substrate buffer and 50 µL of diluted FRETs-VWF73 reagent. For measurement of murine ADAMTS13 activity in the presence of human scFv, 4 µL of scFv at twice final desired concentration were mixed with 4 µL mouse plasma, incubated at 37°C for 15 min and assayed for ADAMTS13 activity with FRETs-vWF73 reagent as above. For ADAMTS13 “inhibitor assay” with plasma from scFv-expressing mice, murine ADAMTS13 activity was measured by incubating 4 µL normal human plasma with 4 µL heat-inactivated mouse plasma drawn several days post-pLIVE injection, incubating at 37°C for 30 min, then adding to FRETs-vWF73 buffer and substrate. Murine VWF multimers were visualized by Western blot as described.³⁴

Study approval

Animal studies were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee.

RESULTS

Human anti-ADAMTS13 monoclonal antibodies inhibit murine ADAMTS13

In the accompanying manuscript in this series⁴⁴, we described a set of human monoclonal anti-ADAMTS13 single chain antibody fragments (scFv's) cloned from four unrelated TTP patients that displayed characteristics *in vitro* one would expect from disease-related pathogenic antibodies, e.g., ability to inhibit ADAMTS13 enzymatic activity, epitope specificities shared with patient plasma IgG, etc. To test the clinical relevance of these recombinant antibodies and to provide an *in vivo* system for studying the pathophysiology of TTP, we extended these *in vitro* findings to the development of a murine model of acquired TTP.

We first screened for the ability of our human scFv antibody fragments to inhibit the activity of murine plasma ADAMTS13 *in vitro* and found that the majority of scFv's that inhibited human ADAMTS13 activity also inhibited murine ADAMTS13 (data not shown). Of the human scFv's that crossreacted with murine ADAMTS13, we chose antibody clones 1–416, 1–420, 1–428, and 1–431 to pursue because characterization of these antibodies *in vitro* showed potent inhibition of ADAMTS13 activity, binding to human ADAMTS13 epitopes most commonly targeted by patient plasma IgG, and idiotypes shared by patient plasma IgG⁴⁴.

Dose response inhibition curves with mouse plasma revealed 1–420 to be most potent (Fig. 1A) so it was chosen for evaluation *in vivo*. Mice were given intraperitoneal injections of 30 µg 1–420 and mouse plasma showed rapid inhibition of ADAMTS13 resulting in <10% pre-injection activity within ~2 hours (Figure 1B) which persisted for ~24 hours (Figure 1B, inset).

Transfection of mice with scFv cDNA leads to prolonged ADAMTS13 deficiency

We next wanted to create a state of sustained antibody-mediated ADAMTS13 deficiency to simulate TTP disease and observe the effect of such deficiency on thrombus formation in the settings of focal and systemic vascular injury. To accomplish this, the cDNA for scFv 1–420 was cloned into the pLIVE plasmid vector, a vector designed for the hydrodynamic gene transfer of naked DNA.³¹ We modified the vector to facilitate the insertion of phage display-derived scFv antibody fragment cDNA's upstream of a liver-specific promoter composed of the minimal mouse albumin promoter and the mouse α -fetoprotein enhancer (Fig. 2). Tail vein injection of a 2-mL solution of pLIVE plasmid over 4 – 7 seconds delivers the scFv cDNA to mouse liver by hydrostatic force. Both control scFv cDNA and 1–420-containing plasmids initially led to a drop in ADAMTS13 activity *in vivo* for several days that was then followed by recovery of ADAMTS13 in the control group after the expected physiological effects of rapid hydrodynamic injection of a one blood volume-equivalent through the portal

circulation to the liver resolved.³¹ Inhibition of ADAMTS13 activity by *in vivo*-expressed 1–420 persisted for over 2 weeks (Fig. 3A) and for as long as 3 months (data not shown).

That the recombinant human antibodies were expressed in mouse plasma was confirmed by immunoprecipitation of scFv 7 days after scFv DNA vector injection (Fig. 4B). To estimate plasma levels of scFv *in vivo*, an aliquot of normal mouse plasma was spiked with 20 ng of recombinant 1–420, and immunoprecipitation was performed in parallel. Quantification using the spiked sample as a reference led to estimates of 0.4 – 0.8 µg/ml mouse plasma for scFv's from 3 control mice and 0.4 – 10 µg/ml mouse plasma for 1–420 from 3 experimental mice. If the concentration of mouse plasma ADAMTS13 is similar to that in humans (~1 µg/ml),^{35,36} the stoichiometric ratio of scFv to ADAMTS13 was ~3 to ~5 except for one of the 1–420 mice in which it was ~10-fold higher. Excess unbound plasma scFv was affirmed by mixing plasma derived from cDNA-transfected mice 1:1 with normal human plasma, analogous to the “inhibitor assay” used clinically to diagnose patients with antibody-mediated ADAMTS13 deficiency (Figure 4C).³

Laser-induced vascular injury in scFv-transfected mice leads to elongated thrombi

The above data show that DNA transfection of human anti-ADAMTS13 antibodies using hydrodynamic delivery leads to rapid and stable ADAMTS13 inhibition *in vivo*. We next examined the effects of ADAMTS13 inhibition on the temporal and spatial aspects of platelet thrombus formation in these mice using the cremaster arteriole laser injury model (Figure 4A, Supplementary Videos 1 and 2).³² Thrombi in control mice enlarged spherically around the ~1 µm injury site to a diameter of ~30 µm and then remained relatively constant in size during the 3-minute observation period due to frequent embolization. In clear contrast, thrombi in ADAMTS13-inhibited mice grew in an asymmetrically elongating fashion to a length >80 µm beyond the injury site ($P < 0.0001$) consistent with the presence of strings of unprocessed UL-VWF multimers, although a contribution from adhesion of plasma VWF multimers to the damaged vasculature cannot be excluded. Length/width aspect ratio measurements of thrombi quantified these differences (Figure 4B). Agarose gel electrophoresis of plasma confirmed the presence of UL-VWF multimers in mice expressing 1–420 (Figure 4B, inset) relating the findings to underlying mechanism.

Shigatoxin challenge induces TTP phenotype in scFv-transfected mice

Though pathogenesis of TTP is linked to ADAMTS13 deficiency, the natural history of the disease suggests that additional genetic or environmental factors are required for disease onset.¹⁰ In murine models of congenital TTP, the administration of the bacterial agent Shigatoxin-2 was found to precipitate disease phenotype.^{33,37} To further test the clinical relevance of our cloned human scFv's, we challenged mice expressing human anti-ADAMTS13 inhibitory antibodies by intravenous infusion of Shigatoxin-2. CAST/Ei strain mice, which have 5 times more circulating VWF and are sensitive to the development of TTP in an ADAMTS13 knock-out background,¹⁰ were injected with 1–420 or control antibody plasmid, given a sublethal dose of Shigatoxin-2 10 days later, and followed for 10 days. As shown in Figure 5A, 6 of 10 control mice survived and had normal platelet counts throughout the post-Shigatoxin-2 period. However, all (10/10) mice rendered ADAMTS13 deficient by 1–420 died by day 4 post-Shigatoxin-2 (half within 24–48 hours of injection,

Figure 5B) and each suffered a fall in platelet count to less than one-third its starting value. Schistocytes were seen on peripheral blood smear, and thrombi were readily detected in brain, heart, and kidney, but not in control animals that had died concurrently or were sacrificed after Shigatoxin-2 challenge (Figure 5C). These experiments demonstrate that the *in vivo* expression of just the variable region of a single human anti-ADAMTS13 antibody suffices to mediate the most salient features of TTP in an animal model.

DISCUSSION

The goals of this study were to test the clinical relevance of antibodies cloned and characterized *in vitro* in the accompanying manuscript in this series⁴⁴ and to develop an animal model of acquired TTP that could be useful for exploring the pathophysiology of disease. To our knowledge, this report is the first to describe human anti-ADAMTS13 autoantibodies that function in an animal model. Whether by injection of scFv protein (Fig. 1) or by *in vivo* expression of antibody mediated by hydrodynamic gene transfer of scFv DNA-containing plasmids (Figs. 3 and 4), murine ADAMTS13 proteolytic activity was inhibited, resulting in the accumulation of UL-VWF multimers. After triggering endothelial injury with Shigatoxin-2, key pathologic features of TTP were observed including thrombocytopenia, microangiopathic hemolytic anemia, formation of platelet thrombi in vital organs, and death (Fig. 5). As in patients with antibody-mediated ADAMTS13 deficiency, these mice exhibited sustained inhibition of ADAMTS13 (<5%) and “positive inhibitor assays”, i.e. their plasmas inhibited ADAMTS13 activity when mixed with normal human plasma (Figure 4C). The altered clot morphology revealed by focal arteriole injury via laser and intravital video-microscopy^{26,38,39} in the setting of prolonged autoantibody-mediated ADAMTS13 inhibition (Fig. 4) may illustrate the process by which clots extend linearly and cause the blockage of microvessels in patients with acquired TTP. We recognize that the role of platelets and other aspects of the pathophysiology of thrombosis in the laser injury model may differ in important ways from the sequence of events that lead to disseminated microvascular thrombi in TTP, and additional studies will be needed to identify the differences should they exist. Our observation that inhibition of ADAMTS13 function and the subsequent pathology that develops can be mediated by a monomeric antibody fragment lacking a constant region domain suggests that antibody-mediated clearance of ADAMTS13 or other effector functions conferred by IgG Fc domains may not be necessary for the expression of disease in patients.

To date, animal models of acquired TTP have been limited to the use of rabbit or mouse antibodies to human ADAMTS13 that produce transient enzyme inhibition.^{26–28} Such xenoantibodies would not be expected to necessarily primarily target human autoepitopes and, if so, would therefore not be helpful for testing novel therapies such as altered forms of recombinant ADAMTS13 that are engineered to be unrecognizable by human pathogenic autoantibodies.^{29,30} In point of fact, we would not expect that antibodies to human ADAMTS13 generated by mice would bind to the same epitopes as our clones 1–416, 1–420, 1–428, and 1–431 because these four human antibodies also crossreact with murine ADAMTS13 (Fig. 1). Tolerance mechanisms in healthy murine immune systems would not permit such antibodies to be made because they would be autoreactive.

Of the 51 anti-ADAMTS13 scFv antibodies previously described⁴⁴, 1–416, 1–420, 1–428, and 1–431 were initially selected for evaluation here not only because of their ability to inhibit ADAMTS13 *in vitro* but because rabbit anti-idiotypic antisera raised to each of these scFv demonstrated the presence of crossreactive idiotypes in a number of unrelated patient plasma samples. Of the four antibodies, scFv 1–420 was then chosen to pursue in our animal model because it appeared most potent (Fig. 1). However, a recent study from our laboratory suggests that scFv 1–420 may in fact be the ideal example of a prototypical human TTP pathogenic antibody for use in an animal model because of its ability to significantly reduce or even largely eliminate the binding of polyclonal patient IgG to ADAMTS13 from 20 of 23 unrelated patients with acquired TTP.⁴⁰ In addition, in this study, we used hydrogen-deuterium exchange mass spectrometry, the epitope for 1–420 was determined to near single amino acid resolution, and it was found that 1–420 bound to the same ADAMTS13 spacer domain loop segments that are required for binding of ADAMTS13 to VWF. This suggests that inhibitory antibody 1–420 functions by blocking enzyme/substrate engagement thus supporting its use in an animal model for guiding the design of altered ADAMTS13 molecules that would resist antibody binding but still allow VWF engagement and proteolysis.

The use of rapid, large volume intravenous injection of plasmid DNA for transfer of exogenous genes into mice is a much simpler approach than those employing viral vectors for transfection and avoids the laborious steps necessary for virus preparation and purification as well as safety concerns associated with systemic administration of recombinant virus to animals. There are numerous examples in the literature of hydrodynamic-based transfections of plasmid DNA in animals used to study the effects of *in vivo*-expressed transgenes such as those encoding recombinant enzymes, hormones, cytokines and other proteins^{41,42} but, to our knowledge, never antibody fragments. The approach we describe here could prove useful for exploring the pathophysiological effects of autoantibodies in other disorders where the antibodies, as in acquired TTP, may not require the expression of full-length IgG for bivalency or Fc domains for effector function. In addition to its utility for the study of acquired TTP, we anticipate that the sustained inhibition of ADAMTS13 mediated by anti-ADAMTS13 DNA transfection may prove useful in murine models of other disease states such as ischemic stroke, myocardial infarction, atherosclerosis, malignant (cerebral) malaria, and pre-eclampsia where perturbations in ADAMTS13 is believed to play a role in disease pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Inhibition of murine ADAMTS13 by human anti-ADAMTS13. (A) Dose response curve showing residual murine ADAMTS13 activity when normal mouse plasma was mixed *in vitro* with increasing amounts of human ADAMTS13 inhibitory scFv's 1-416, 1-420, 1-428, 1-431 or irrelevant anti-Rh(D) scFv negative control E1M2.⁴³ (B) Time course of murine ADAMTS13 inhibition *in vivo* following intraperitoneal injection of scFv 1-420 (inset: over 24 hour period).

Fig. 2. Schematic diagram of pLIVE plasmid vector modified for *in vivo* scFv expression. Shown are positions of Ig-kappa leader sequence, Sfi I restriction sites for inserting pComb3X-derived scFv construct, and V5-tag sequence.

Fig. 3. Inhibition of murine ADAMTS13 by *in vivo*-expressed human anti-ADAMTS13 scFv. (A) Time course of murine ADAMTS13 inhibition *in vivo* following hydrodynamic tail vein delivery into 2 pairs of mice with pLIVE plasmid containing scFv 1–420 cDNA (triangles and circles) or negative control human platelet factor 4 scFv X24-3 cDNA (diamonds and squares). (B) Western blot of plasma immunoprecipitated *in vivo*-expressed scFv drawn 7 days post scFv cDNA injection. Lanes 1–3 show negative control scFv X24-3 for 3 mice; lanes 4–6 show scFv 1–420 for 3 mice; lane 7 represents the amount of scFv 1–420 immunoprecipitated from an equivalent volume of mouse plasma spiked with 20 ng purified scFv 1–420 protein. (C) ADAMTS13 “inhibitor assay” performed on a 1:1 mix of normal human plasma and heat-inactivated murine plasma (56°C for 30 min to destroy endogenous mouse ADAMTS13) derived from 2 untransfected mice (left-hand set of bars), 2 mice transfected with control scFv plasmid (middle set of bars), and 5 mice transfected with plasmid containing scFv 1–420 cDNA (right-hand set of bars).

Fig. 4. Platelet thrombus formation after cremaster arteriole injury in mice transfected with human anti-ADAMTS13 cDNA. (A) Intravital microscopy and time-lapse video illustrating formation of platelet thrombus over a 180-second period post laser injury. Platelets were labeled with Alexa⁴⁸⁸-conjugated F(ab')₂ fragments of anti-mouse CD41 in control scFv-transfected animal (normal ADAMTS13 activity, left panel) and scFv 1-420-transfected animal (<10% normal ADAMTS13 activity, right panel). Blood flow in direction of arrows. Bar represents 30 μ m in all video frames. (Original videos in Supplementary Data.) (B)

Length/width aspect ratios for platelet thrombi were averaged for 28 injuries in 5 control mice and 33 injuries in 7 scFv 1–420-transfected mice and plotted $\pm$ SEM as a function of time. Arterioles of 20–40 μ m diameter were selected. To the right of each tracing, VWF multimer analyses of plasma from representative pair of control and ADAMTS13-inhibited mice shows accumulation of UL-VWF in scFv 1–420-transfected mouse.

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Fig. 5. Thrombocytopenia, thrombus formation, and death after injection of Shigatoxin-2 in mice transfected with human anti-ADAMTS13 cDNA. (A) Platelet counts in mice expressing control scFv (upper panel) and 1-420 scFv (lower panel) antibodies following Shigatoxin-2 (Stx-2) injection on Day 0. ADAMTS13 activities prior to Stx-2 injections are listed on the right-hand side of each panel. (B) Kaplan-Meier survival curves for mice expressing control scFv and scFv 1-420. P value determined using GraphPad Prism software (La Jolla, CA) and the log-rank Mantel-Cox test. (C) Peripheral blood smears (panels 1, 2) and organ histology (panels 3 through 8) in representative control scFv (left-hand panel set) and scFv 1-420 (right-hand panel set) expressing mice. Arrows in panel 2 point to schistocytes. Original magnifications, $\times 100$; insets, $\times 500$.