Potential for recombinant ADAMTS13 as an effective therapy for acquired thrombotic thrombocytopenic purpura

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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 described.\(^1\)\(^-\)\(^3\)

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 Glycin, 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 described, 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 injected. 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 plasma. 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.

**Determination 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


