Fatal congenital thrombotic thrombocytopenic purpura with apparent ADAMTS-13 inhibitor: 
in vitro inhibition of ADAMTS-13 activity by hemoglobin

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Abstract

Severe ADAMTS-13 deficiency in thrombotic thrombocytopenic purpura (TTP) either is constitutional and caused by \textit{ADAMTS13} mutations, or acquired and most often due to ADAMTS-13 inhibitory autoantibodies. In strongly hemolytic serum of a pediatric patient, diagnosed with TTP postmortem, ADAMTS-13 activity was $<3\%$. Both parents had an ADAMTS-13 activity of $\sim50\%$. Sequencing of the \textit{ADAMTS13} gene revealed an intronic 687-2A→G substitution affecting exon 7, homozygous in the propositus and heterozygous in both parents, confirming constitutional ADAMTS-13 deficiency. ADAMTS-13 activity of normal plasma was inhibited by incubation with the propositus’ serum, suggesting alloantibody formation to ADAMTS-13. However, immunoglobulin purified from serum had no ADAMTS-13 inhibitory effect whereas the immunoglobulin-depleted hemolytic serum inhibited ADAMTS-13 activity of normal plasma, suggesting an inhibitory effect of hemolysis products. Incubation of hemoglobin, recombinant and from lysed erythrocytes, with normal plasma revealed an ADAMTS-13 inhibitory effect at hemoglobin concentrations of 2mg/mL or higher.
Introduction
Severe deficiency of ADAMTS-13 activity (<5% of normal plasma) is a strong risk factor for thrombotic thrombocytopenic purpura (TTP)\(^1\text{-}^3\). Two pathogenetic mechanisms were identified: homozygous or compound heterozygous mutations of the \textit{ADAMTS13} gene in congenital TTP\(^4\text{-}^10\), and inhibition of ADAMTS-13 by autoantibodies in acquired TTP\(^2\text{-}^3,^11\). Non-neutralizing autoantibodies\(^12\), possibly leading to increased clearance of ADAMTS-13 from the circulation, or interfering with its interaction with endothelial cells\(^13\) or the VWF A1/A3 domains\(^14\), may be additional mechanisms.

Investigation of premortem serum of a pediatric TTP patient revealed a severe ADAMTS-13 deficiency. Sequencing of the \textit{ADAMTS13} gene identified a homozygous mutation confirming constitutional ADAMTS-13 deficiency. Surprisingly, the patient's severely hemolytic serum had a pronounced ADAMTS-13 inhibitory effect which was not attributable to purified immunoglobulin (Ig) but caused by hemolysis products present in the sample. Subsequent experiments addressed an ADAMTS-13 inhibitory effect of hemoglobin.

Materials and methods

\textit{Patient}
The propositus, a Turkish boy\(^15\), had suffered from episodes of severe Coombs-negative hemolytic anemia and thrombocytopenia since birth. He died at the age of seven years from a severe attack of this disease until then classified as atypical Evans’ syndrome. Autopsy revealed microvascular occlusions by fibrin-poor platelet thrombi, suggesting the diagnosis of TTP. Strongly hemolytic serum from whole blood obtained before death and stored at 4°C was shipped frozen to our laboratory. The serum had a hemoglobin concentration of 17mg/mL (1.7g/dL), probably due to \textit{in vitro}-hemolysis. The study was conducted according to the guidelines on research on human subjects by Kantonale Ethikkommission, Bern. Informed consent for publication was obtained from the parents, the study was conducted according to the guidelines on research on human subjects of the responsible Ethics Committee (Kantonale Ethikkommission Bern).

\textit{ADAMTS-13 activity and routine inhibitor screening}
ADAMTS-13 activity was determined by immunoblotting of purified VWF substrate degraded by BaCl\(_2\)-activated ADAMTS-13 in serum or plasma. For inhibitor screening the residual ADAMTS-13 activity of pooled normal human plasma (NHP) was measured after 1:1 (vol:vol) incubation with patient serum/plasma. Assays were performed as described\(^2,^16\).
Inhibition of ADAMTS-13 activity by hemoglobin

Two solutions of concentrated hemoglobin were prepared. Solution 1 consisted of recombinant human hemoglobin (Baxter, Boulder, CO, USA) 50mg/mL in 0.15mol/L NaCl, 5mmol/L sodium phosphate, 0.03% TWEEN 80. Solution 2 was obtained from lysed washed erythrocytes. Citrated blood from a healthy donor was centrifuged for 10min at 4000g, and the supernatant plasma and buffy coat were discarded. The sediment was washed three times with physiologic saline, and incubated 1:3 (vol:vol) with distilled water for 10min at 37°C. After centrifugation for 10min at 4000g the supernatant solution contained 84mg/mL hemoglobin.

Inhibition of ADAMTS-13 activity by hemoglobin was assessed by routine ADAMTS-13 inhibitor assay 16. NHP was incubated 1:1 (vol:vol) for 2h at 37°C with hemoglobin solution 1 or 2 prediluted in 0.01mol/L Tris(hydroxymethyl)aminomethane, 0.15mol/L NaCl, pH 7.4 (TBS). Final hemoglobin concentrations after addition to NHP ranged from 0.5-40mg/mL.

Preliminary experiments addressed a complexation of divalent metal ions required for ADAMTS-13 activity, such as calcium or zinc 17,18, as a hypothetical mechanism by which hemoglobin might inhibit ADAMTS-13. NHP was incubated 1:1 (vol:vol) with recombinant hemoglobin, 20mg/mL for 1h at 37°C. This mixture was diluted 1:10 with TBS and activated with increasing final concentrations of BaCl₂ between 10-75mmol/L.

Purification of total Ig

Total Ig were purified from the propositus' serum by adsorption to Ig-Therasorb (Sepharose CL-4B coupled with polyclonal sheep antibodies to human Ig). The purified Ig and the Ig-depleted serum were concentrated to original serum levels by centrifugation through filter membranes (Biomax, Millipore, Bedford, MA, USA; molecular weight cut-off, 30kDa).

Investigation of the ADAMTS13 gene

Amplification and sequencing of PCR products were performed as described 6. SpliceView program (Institute of Biomedical Technologies, Segrate, Italy) was used for predicting alternative splice sites resulting from the detected mutation.
Results and discussion

ADAMTS-13 activity was <3% in premortem serum of the propositus, and ~50% in plasma of both parents (Fig. 1A). Sequencing of all 29 exons of the ADAMTS13 gene including their intron/exon boundaries revealed a point mutation (687-2A→G) at the 3' splice acceptor site of intron 6 affecting exon 7, between amino acids 228-229, homozygous in the propositus and heterozygous in both parents. We hypothesize that this mutation could result in two alternative splice sites, causing either a frame shift and premature termination of the polypeptide, or an in-frame insertion of eight amino acids. These findings confirmed constitutional ADAMTS-13 deficiency with hereditary TTP in the propositus, misdiagnosed as atypical Evans’ syndrome until autopsy. His fatal course illustrates the importance of rapid and comprehensive diagnostics in patients suffering from congenital thrombotic microangiopathies including determination of ADAMTS-13 activity and ADAMTS-13 inhibitors because plasma infusion is a highly effective treatment 19-21.

Pronounced inhibition of the ADAMTS-13 activity of NHP was observed after incubation with an equal volume of the propositus’ hemolytic serum, corresponding to a titer of 1-2BU/mL and suggested alloantibody formation to ADAMTS-13 triggered by repeated transfusions of blood products containing ADAMTS-13. However, total Ig purified from the propositus’ serum had no ADAMTS-13 inhibitory effect (Fig. 1B). In contrast, the Ig-depleted hemolytic serum inhibited ADAMTS-13 activity of NHP to a similar extent as the original serum. This indicated that the observed inhibition was not caused by antibodies to ADAMTS-13 but was related to other factors present in the sample, possibly originating from lysed erythrocytes.

Subsequent experiments addressed a possible ADAMTS-13 inhibitory capacity of hemoglobin. NHP was incubated with hemoglobin, either recombinant or purified from lysed erythrocytes at various concentrations. Slight inhibition of the ADAMTS-13 activity of NHP (≤1BU/mL) was observed after incubation with hemoglobin at a final concentration of 2mg/mL (Fig. 1C). Moderate inhibition (1-2BU/mL) occurred at 5mg/mL, and strong inhibition (≥2BU/mL) at 10mg/mL or higher. The ADAMTS-13 inhibitory effect was similar for recombinant and erythrocyte-derived hemoglobin, indicating that it can be linked specifically to the hemoglobin molecule.

To exclude an artifact related to our assay, NHP was incubated 1:1 (vol:vol) with hemoglobin solution 1 and 2 (final hemoglobin concentrations, 10mg/mL and 25mg/mL each) for 2h at 37°C. Identical aliquots were investigated for their ADAMTS-13 activity by three other laboratories using different assays 22-24. All laboratories found a severe or borderline severe ADAMTS-13 deficiency (<5% or 5-9% of the normal), instead of an activity of 50% corresponding to the proportion of NHP in the mixture.
The ADAMTS-13 inhibitory capacity of hemoglobin depended upon incubation temperature and time. ADAMTS-13 activity of NHP was completely inhibited after incubation with 30mg/ml hemoglobin for 3h at 37°C but only reduced to 25% at 4°C. Incubation for 5min-2h revealed mild inhibition (≤1BU/mL) after 5min, moderate inhibition (1-2BU/mL) after 15-30min, and strong inhibition (≥2BU/mL) after 1-2h (not shown).

ADAMTS-13 inhibition by hemoglobin was attenuated, but not completely reverted by the addition of increasing BaCl₂ concentrations upto 40mmol/L.

Inhibition of ADAMTS-13 activity occurred at hemoglobin concentrations potentially found in strong intravascular hemolysis, as observed in incompatible erythrocyte transfusion, immune-mediated hemolytic anemias, or others. These conditions are not usually associated with TTP, and it is unclear whether short-lived elevation of free hemoglobin in plasma will lead to a clinically relevant ADAMTS-13 inhibition. In our case, the serum hemoglobin concentration of 17mg/mL probably resulted from in vitro-hemolysis in stored native whole blood. Nevertheless, some practical conclusions can be drawn from our observations. Marked hemolysis and the presence of hemoglobin apparently influence most of the current ADAMTS-13 assays. Lowering of ADAMTS-13 activity values should be expected under these circumstances and inhibitor screening may lead to the erroneous assumption of ADAMTS-13 inhibitory antibodies. Whether the ADAMTS-13 inhibitory capacity of hemoglobin is of any physiological or pathophysiological relevance, e.g. in severe intravascular hemolysis, will need further study.

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Legend to figure 1


**A** ADAMTS-13 activity in 1:20 diluted patient serum or plasma.

Lanes 1-6, assay calibration by normal plasma (NHP) dilutions of 1:20 (100% activity), 1:40 (50%), 1:80 (25%), 1:320 (6.25%), 1:640 (3%), and buffer control (0%).
Lane 7, propositus (premortem serum); lane 8, father (plasma); lane 9, mother (plasma).

**B** ADAMTS-13 inhibition by purified immunoglobulin (Ig) and Ig-depleted hemolytic serum.

Residual ADAMTS-13 activity of NHP incubated 1:1 (vol:vol) with concentrated total Ig purified from the propositus' serum, or with concentrated Ig-depleted hemolytic serum (final NHP dilution, 1:20).

Lanes 1-4, assay calibration by NHP dilutions of 1:20 (100% activity), 1:40 (50%, equivalent to an inhibitor titer of 1BU/mL), 1:80 (25%), and buffer control (0%).
Lanes 5-6, NHP 1:1 (vol:vol) with purified Ig (duplicates); lanes 7-8, NHP 1:1 (vol:vol) with Ig-depleted serum (duplicates).

**C** Inhibition of ADAMTS-13 activity by hemoglobin.

Residual ADAMTS-13 activity of NHP incubated 1:1 (vol:vol) with recombinant or erythrocyte-derived human hemoglobin (final NHP dilution, 1:20).

Lanes 1-4, assay calibration by NHP dilutions of 1:20 (100% activity), 1:40 (50%, equivalent to an inhibitor titer of 1BU/mL), 1:80 (25%), and buffer control (0%).
Lanes 5-10, NHP incubated 1:1 (vol:vol) with recombinant hemoglobin; lanes 11-16, NHP incubated 1:1 (vol:vol) with purified hemoglobin from lysed erythrocytes. Final hemoglobin concentrations are 20mg/mL (lanes 5, 11), 10mg/mL (lanes 6, 12), 5mg/mL (lanes 7, 13), 2mg/mL (lanes 8, 14), 1mg/mL (lanes 9, 15), and 0.5mg/mL (lanes 10, 16).
Figure 1

A

B

C
Fatal congenital thrombotic thrombocytopenic purpura with apparent ADAMTS-13 inhibitor: \textit{in-vitro} inhibition of ADAMTS-13 activity by hemoglobin

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