Brief report

Fatal congenital thrombotic thrombocytopenic purpura with apparent ADAMTS13 inhibitor: in vitro inhibition of ADAMTS13 activity by hemoglobin

Jan-Dirk Studt, Johanna A. Kremer Hovinga, Gerhard Antoine, Martin Hermann, Manfred Rieger, Friedrich Scheiflinger, and Bernhard Lämmle

Severe ADAMTS13 deficiency in thrombotic thrombocytopenic purpura (TTP) is either constitutional and caused by ADAMTS13 mutations, or acquired and most often due to ADAMTS13 inhibitory autoantibodies. In strongly hemolytic serum of a pediatric patient, diagnosed with TTP postmortem, ADAMTS13 activity was less than 3%. Both parents had an ADAMTS13 activity of approximately 50%. Sequencing of the ADAMTS13 gene revealed an intronic 687-2A>G substitution affecting exon 7, homozygous in the propositus' serum, suggesting constitutional ADAMTS13 deficiency. ADAMTS13 activity of normal plasma was inhibited by incubation with the propositus' serum, suggesting alloantibody formation to ADAMTS13. However, immunoglobulin purified from serum had no ADAMTS13 inhibitory effect, whereas the immunoglobulin-depleted hemolytic serum inhibited ADAMTS13 activity of normal plasma, suggesting an inhibitory effect of hemolysis products. Incubation of hemoglobin, recombinant and from lysed erythrocytes, with normal plasma revealed an ADAMTS13 inhibitory effect at hemoglobin concentrations of 2 g/L or higher.

Study design

Patient

The propositus, a Turkish boy, had suffered from episodes of severe Coombs-negative hemolytic anemia and thrombocytopenia since birth. He died at the age of 7 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 1.7 g/dL, probably due to in vitro hemolysis.

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 Ethikkommision, Bern).

ADAMTS13 activity and routine inhibitor screening

ADAMTS13 activity was determined by immunoblotting of purified VWF substrate degraded by BaCl2-activated ADAMTS13 or VWF A1/A3 domains. Hemolysis products were present in the sample. Subsequent experiments addressed an ADAMTS13 inhibitory effect of hemoglobin.

Inhibition of ADAMTS13 activity by hemoglobin

There were 2 solutions of concentrated hemoglobin prepared. Solution 1 consisted of recombinant human hemoglobin (Baxter, Boulder, CO) 50 g/L in 0.15 M NaCl, 5 mM sodium phosphate, 0.035% TWEEN 80. Solution 2 was obtained from lysed washed erythrocytes. Citrated blood from a healthy donor was centrifuged for 10 minutes at 4000 g, and the supernatant solution contained 84 g/L hemoglobin.

J.-D.S. and J.A.K.H. contributed equally to this work.

Reprints: Bernhard Lämmle, Department of Hematology and Central Hematology Laboratory, Inselspital, University Hospital, 3010 Bern, Switzerland; e-mail: bernhard.laemmle@insel.ch.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

© 2005 by The American Society of Hematology
Inhibition of ADAMTS13 activity by hemoglobin was assessed by routine ADAMTS13 inhibitor assay. NHP was incubated 1:1 (vol/vol) for 2 hours at 37°C with hemoglobin solution 1 or 2 prediluted in 0.01 M tris(hydroxymethyl)aminomethane, 0.15 M NaCl, pH 7.4 (TBS). Final hemoglobin concentrations after addition to NHP ranged from 0.5 to 40 g/L.

Preliminary experiments addressed a complexation of divalent metal ions required for ADAMTS13 activity, such as calcium or zinc, as a hypothetical mechanism by which hemoglobin might inhibit ADAMTS13. NHP was incubated 1:1 (vol/vol) with recombinant hemoglobin, 20 g/L, for 1 hour at 37°C. This mixture was diluted 1:10 with TBS and activated with increasing final concentrations of BaCl2 between 10 and 75 mM.

**Purification of total Ig**

Total Ig was purified from the propositus’ serum by adsorption to Ig-Therasorb (Miltenyi Biotech, Teterow, Germany; 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; molecular weight cut-off, 30 kDa).

**Investigation of the ADAMTS13 gene**

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

**Results and discussion**

ADAMTS13 activity was less than 3% in premortem serum of the propositus, and approximately 50% in plasma of both parents (Figure 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 to 229, homozygous in the propositus and heterozygous in both parents. We hypothesize that this mutation could result in 2 alternative splice sites, causing either a frameshift and premature termination of the polypeptide, or an in-frame insertion of 8 amino acids. These findings confirmed constitutional ADAMTS13 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 ADAMTS13 activity and ADAMTS13 inhibitors because plasma infusion is a highly effective treatment.

Pronounced inhibition of the ADAMTS13 activity of NHP was observed after incubation with an equal volume of the propositus’ hemolytic serum, corresponding to a titer of 1 to 2 Bethesda units (BU)/mL and suggested alloantibody formation to ADAMTS13 triggered by repeated transfusions of blood products containing ADAMTS13. However, total Ig purified from the propositus’ serum had no ADAMTS13 inhibitory effect (Figure 1B). In contrast, the Ig-depleted hemolytic serum inhibited ADAMTS13 activity of NHP to a similar extent as the original serum. This indicated that the observed inhibition was not caused by antibodies to ADAMTS13 but was related to other factors present in the sample, possibly originating from lysed erythrocytes.

Subsequent experiments addressed a possible ADAMTS13 inhibitory capacity of hemoglobin. NHP was incubated with hemoglobin, either recombinant or purified from lysed erythrocytes at various concentrations. Slight inhibition of the ADAMTS13 activity of NHP (≤ 1 BU/mL) was observed after incubation with hemoglobin at a final concentration of 2 g/L (Figure 1C). Moderate inhibition (1-2 BU/mL) occurred at 5 g/L, and strong inhibition (≥ 2 BU/mL) occurred at 10 g/L or higher. The ADAMTS13 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 solutions 1 and 2 (final hemoglobin concentrations, 10 g/L and 25 g/L each) for 2 hours at 37°C. Identical aliquots were investigated for their ADAMTS13 activity by 3 other laboratories using different assays. All laboratories found a severe or borderline severe ADAMTS13 deficiency (< 5%...
or 5%-9% of the normal), instead of an activity of 50% corresponding to the proportion of NHP in the mixture.

The ADAMTS13 inhibitory capacity of hemoglobin depended upon incubation temperature and time. ADAMTS13 activity of NHP was completely inhibited after incubation with 30 g/L hemoglobin for 3 hours at 37°C but only reduced to 25% at 4°C. Incubation for 5 minutes to 2 hours revealed mild inhibition (≤ 1 BU/mL) after 5 minutes, moderate inhibition (1-2 BU/mL) after 15 to 30 minutes, and strong inhibition (≥ 2 BU/mL) after 1 to 2 hours (not shown).

ADAMTS13 inhibition by hemoglobin was attenuated, but not completely reverted by the addition of increasing BaCl₂ concentrations up to 40 mM.

Inhibition of ADAMTS13 activity occurred at hemoglobin concentrations potentially found in strong intravascular hemolysis, as observed in incompatible erythrocyte transfusion, immunemediated 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 ADAMTS13 inhibition. In our case, the serum hemoglobin concentration of 17 g/L 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 ADAMTS13 assays.2,22-24 Lowering of ADAMTS13 activity values should be expected under these circumstances and inhibitor screening may lead to the erroneous assumption of ADAMTS13 inhibitory antibodies. Whether the ADAMTS13 inhibitory capacity of hemoglobin is of any physiologic or pathophysiologic relevance (eg, in severe intravascular hemolysis) will need further study.

Acknowledgments

We thank Dr W. Böhö (Milenyi Biotech, Teterow, Germany) for providing Ig-Therasorb columns, and Drs U. Budde (Laboratory Keeser and Arntz, Hamburg, Germany), M. Böhö (University Hospital Frankfurt, Germany), and J.-P. Girma (INSERM U.143, Le Kremlin-Bicêtre, France) for performing confirmatory determinations of ADAMTS13 activity.

References

Fatal congenital thrombotic thrombocytopenic purpura with apparent ADAMTS13 inhibitor: in vitro inhibition of ADAMTS13 activity by hemoglobin

Jan-Dirk Studt, Johanna A. Kremer Hovinga, Gerhard Antoine, Martin Hermann, Manfred Rieger, Friedrich Scheiflinger and Bernhard Lämmle