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ABSTRACT

Reportedly, complexes between factor XI and ADAMTS13 are detected with a commercial ADAMTS13/FXI ELISA kit in plasma and are decreased in thrombotic thrombocytopenic purpura (TTP). Using this kit, control and TTP patient plasma contained varying amounts of signal (25-670% of a reference plasma) but no signal was observed for mixtures of recombinant enzymes, suggesting little interaction. ADAMTS13/FXI complexes were undetectable by immunoprecipitation or gel filtration chromatography in control plasma or mixtures of recombinant proteins. These results suggest that ADAMTS13/FXI complexes are insignificant in plasma and unlikely to affect the function of either protein during normal hemostasis or in TTP.

Key words: von Willebrand Factor, factor XI, ADAMTS13.

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ADAMTS13 cleaves von Willebrand Factor (VWF) to produce the distribution of VWF multimer sizes characteristic of normal plasma.[1] Congenital or acquired autoimmune deficiency of ADAMTS13 is associated with the circulation of ultra-large VWF (ULVWF) multimers and thrombotic thrombocytopenic purpura (TTP).[1-3] Recent advances in clinical assay development have led to several new methods for measuring ADAMTS13 activity or ADAMTS13 antigen levels.[4-7] As more clinical assays are developed, the mechanisms by which ADAMTS13 cleaves VWF are becoming better understood. Recently, a commercially available kit has been used to demonstrate a complex between coagulation factor XI (FXI) and ADAMTS13, and since decreased levels of this complex are proposed as a biomarker for TTP, a physical interaction between the two proteinases must therefore occur. We have investigated the occurrence of this complex using normal human plasma, TTP patient plasma, FXI deficient plasma, and recombinant proteins. The results indicate that measuring FXI/ADAMTS13 complex is not useful for evaluating patients with TTP.

Design and Methods

Citrated normal human plasma was obtained from the American Red Cross, St. Louis, MO, USA or from healthy volunteers according to a protocol approved by the Institutional Review Board of Washington University in St. Louis. Polyclonal anti-ADAMTS13 was prepared in rabbits (Pocono Rabbit Farm and Laboratory, Canadensis, PA, USA) immunized with purified recombinant human ADAMTS13.[8] Recombinant human FXI and ADAMTS13 were prepared as previously described.[9,10] Single chain high molecular weight kininogen (HK) was purchased from Enzyme Research Labs. FXI deficient plasma was purchased from George King Biomedical (Lot 1122-N23e1, containing <0.1% of FXI by ELISA), and aPTT reagent containing Kaolin was from Helena Laboratories. IMUBIND ADAMTS13 (#813) and ADAMTS13/FXI ELISA (#811) kits were purchased from American Diagnostica.

Co-immunoprecipitation of FXI and ADAMTS13 was performed using either normal plasma or recombinant enzymes.

Plasma samples were diluted in buffer (50 mM Hepes, pH 7.4, 0.15 M NaCl), and human serum albumin was precleared with mouse anti-HSA (1:500 of ascites, Sigma) and protein G-Sepharose (1:10, GE Healthcare). Immunoglobulins were further removed by incubation with protein G-Sepharose (1:10). FXI and ADAMTS13 were immunoprecipitated from the pre-cleared samples using either monoclonal anti-human FXI (1G5.12, 1.6 μg/mL, final concentration)[10] or monoclonal anti-human ADAMTS13 (5C11, 4.6 μg/mL, final concentration)[11] and protein G-Sepharose (1:10). Recombinant FXI, ADAMTS13, or mixtures of the proteins, were immunoprecipitated similarly but without pre-clearing of albumin or immunoglobulins. Protein G-immunoprecipitates were washed three times and eluted by boiling in reducing Laemmli buffer for 5 min.[12] Samples were electrophoresed by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. FXI was detected with horseradish peroxidase (HRP) conjugated goat anti-human FXI (0.8 μg/mL in casein blocking buffer, Enzyme Research Labs). ADAMTS13 was detected with rabbit anti-human ADAMTS13 (1:2,500 of ascites in casein blocking buffer) followed by HRP-conjugated swine anti-rabbit immunoglobulins (0.06 μg/mL in casein blocking buffer, DAKO). Western blots were developed using ECL plus reagent (GE Healthcare). Chemifluorescence was detected using a STORM imager and quantitated using ImageQuantTL software.[9] Gel filtration chromatography was performed using three tandemly linked Bio-Sil SEC 250 columns (7.5 X 200 cm) attached to a Varian ProStar HPLC and equilibrated in buffer A (50 mM Hepes, pH 7.4, 0.3 M NaCl), at 0.5 mL/min with 0.5 mL fractions collected. Columns were calibrated with standards ranging from 1.35 to 670 kDa (BioRad), and supplemented with catalase (232 kDa, GE Healthcare). Chromatography was performed by loading ADAMTS13, FXI, or a combination of the two proteins at the same concentration. ADAMTS13 and FXI activities were determined for each fraction to establish retention volumes. ADAMTS13 activity was detected using a fluorogenic peptide substrate, FRETS-VWF72, consisting of the VWF sequence Arg^{1597}-Arg^{1668} with the mutation N1610C. The amino-terminus was labeled with QSY 21 succinimidyl ester and the mutated Cys^{1610} was labeled with Alexa Fluor 594 C^5 maleimide (Molecular Probes). Fluorescence intensities were measured as previously described[9] on a Perkin Elmer Victor2V using a 590 nm excitation filter (7 nm band pass) and a 615 nm emission filter (10 nm band pass). Concentrations of ADAMTS13 were determined by comparison of the measured initial rate to the initial rate for known concentrations of ADAMTS13. FXI activity was detected using the aPTT assay as described,[10] except that 75 μL of each reagent was used. Clotting times were compared to a standard curve prepared using dilutions of pooled normal plasma (American Red Cross).

Results and Discussion

The IMUBIND ADAMTS13/FXI ELISA detected a varying amount of apparent ADAMTS13/FXI complex in normal plasma from different sources. Compared to the manufacturer's standard plasma supplied with the kit (defined as 100%), American Red Cross plasma (3 units pooled) contained 560-670% of ADAMTS13/FXI complex, and citrated plasma pooled from six volunteers contained 36-110% of ADAMTS13/FXI complex. Three idiopathic TTP patient plasmas (<5% ADAMTS13 activity)[13] showed different amounts of apparent ADAMTS13/FXI complex (25%, 31%, and 79% ADAMTS13/FXI complex) when compared with the manufacturer's standard. In addition, a FXI deficient plasma yielded a value of 13% ADAMTS13/FXI complex. In normal plasma, the ADAMTS13 antigen concentration determined with the IMUBIND ADAMTS13 antigen assay (0.9-1 μg/mL) was similar to values previously reported.[14, 15] The TTP patient plasmas varied in their ADAMTS13 antigen levels using the IMUBIND ADAMTS13 antigen kit (0.4, 0.5, and 0.8 μg/mL)[13], and the FXI deficient plasma contained 0.5 μg/mL of ADAMTS13. The results suggest a high degree of variability in the concentration of ADAMTS13/FXI complex detected using the IMUBIND ADAMTS13/FXI ELISA kit in both normal and TTP patient plasma. Congenitally-deficient FXI plasma cannot contain any ADAMTS13/FXI complex but gave a substantial signal in the IMUBIND ADAMTS13/FXI ELISA, indicating that the assay method may not be specific for the ADAMTS13/FXI complex.

ADAMTS13/FXI complex occurrence was further investigated by immunoprecipitation with well-characterized monoclonal antibodies. When ADAMTS13 was immunoprecipitated from normal plasma with anti-ADAMTS13, co-immunoprecipitation of FXI was essentially undetectable (Figure 1A, lanes 9-12). Conversely, immunoprecipitation of FXI with anti-FXI did not co-immunoprecipitate ADAMTS13 (Figure 1A, lanes 1-4). Control experiments showed that each antibody recognized only the corresponding recombinant protein (Figure 1A) confirming the specificity of the antibodies.

Factor XI circulates in plasma as a complex with HK,[16] suggesting that HK could be involved in binding to ADAMTS13. Further studies using recombinant ADAMTS13 and FXI in the presence and absence of HK demonstrated no co-immunoprecipitation with either antibody (Figure 1B). This suggests that little, if any, interaction occurs between ADAMTS13, FXI, or HK.

Interaction between ADAMTS13 and FXI might not be detected by immunoprecipitation if complex formation interfered with monoclonal antibody binding or vice versa. Therefore, the interaction was further investigated using gel filtration chromatography.

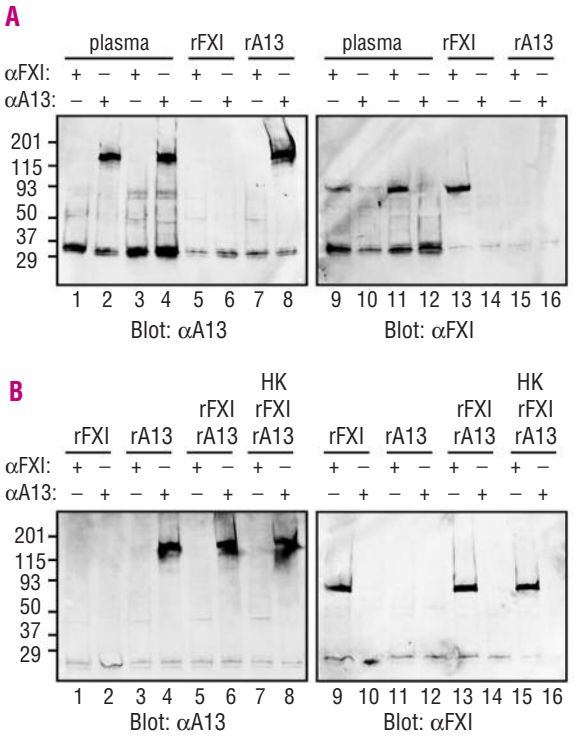

Figure 1. Immunoprecipitation of ADAMTS13 and FXI. (A) Plasma diluted 1:10 (v/v) (*lanes 1, 2, 9, and 10*) or 1:5 (v/v) (*lanes 3, 4, 11 and 12*) was incubated with anti-FXI (*lanes 1, 3, 9, and 11*) or anti-ADAMTS13 (*lanes 2, 4, 10 and 12*) and antibody:antigen complexes were precipitated using protein G-Sepharose. Similarly, recombinant FXI (5.6 nM) (*lanes 5, 6, 13, and 14*) or recombinant ADAMTS13 (5 nM) (*lanes 7, 8, 15 and 16*) were incubated with anti-FXI (*lanes 5, 7, 13, and 15*) or anti-ADAMTS13 (*lanes 6, 8,14, and 16*) and immunoprecipitated using protein G-Sepharose. (B) Recombinant FXI (5.6 nM) (*lanes 1, 2, 9 and 10*) and ADAMTS13 (5 nM) (*lanes 3, 4, 11, and 12*) alone were immunoprecipitated with either anti-FXI (*lanes 1, 3, 9, and 11*) or anti-ADAMTS13 (*lanes 2, 4, 10, and 12*). Combinations of recombinant FXI and ADAMTS13 in the absence (*lanes 5, 6 13, and 14*) or presence (*lanes 7, 8, 15, and 16*) of HK (8 nM) were immunoprecipitated with either anti-FXI (*lanes 5, 7, 13 and 15*) or anti-ADAMTS13 (*lanes 6, 8, 14, and 16*). In each panel, the left panel was probed with anti-ADAMTS13, while the right panel was probed with anti-FXI. The band that appears at ~30 kDA is the reduced form of the IgG light chain.

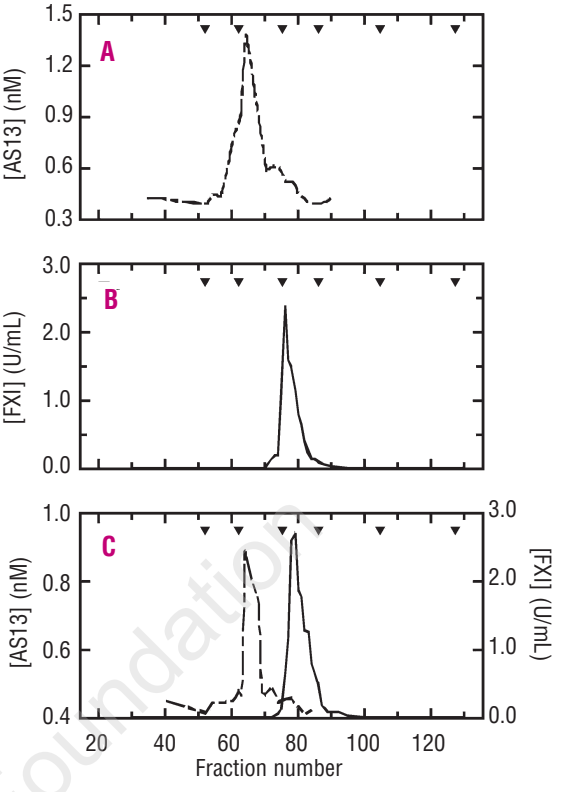

Figure 2. Gel Filtration Chromatography of ADAMTS13 and FXI. ADAMTS13 (0.1 mg, dashed line) (A), FXI (0.1 mg, solid line) (B), and combination of the two proteins (C) were chromatographed on Bio-Sil SEC 250. Molecular weight standards, indicated by the arrow heads (▼), eluted in the following order: thyroglobulin (670 kDa), catalase (232 kDa), IgG (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B_{12} (1.35 kDa).

Recombinant ADAMTS13 alone eluted near catalase (232 kDa, Figure 2A). The relatively early elution of ADAMTS13 upon gel filtration chromatography indicates that ADAMTS13 is asymmetric in solution under these conditions.[17] FXI eluted slightly earlier than IgG (158 kDa, Figure 2B), similar to previous results.[18] When ADAMTS13 and FXI were chromatographed together their elution positions were unchanged (Figure 2C). The absence of a new peak with a shorter retention time confirms that ADAMTS13 and FXI do not bind significantly to each other, even when mixed at concentrations of ~1.2 μM which is much higher than the normal plasma concentration of ADAMTS13 (~5 nM)[14,15] and FXI (~30 nM).[16]

The variability in the results obtained with different sourced normal plasmas, as well as TTP patient and the congenitally-deficient FXI plasmas, suggests that, in general, the ADAMTS13/FXI ELISA will not be useful for evaluating patients with thrombotic microangiopathy. Specific binding between ADAMTS13 and FXI was not detectable by several other methods, with or without HK, which indicates that the signals observed in the ADAMTS13/FXI ELISA may represent a small amount of non-specific binding that is brought out by an especially sensitive ELISA design. Alternatively, the ADAMTS13/FXI ELISA may possibly detect an unidentified plasma component other than HK that simultaneously binds small amounts of ADAMTS13 and FXI bound together in a larger complex. However, failure to observe significant binding between ADAMTS13 and FXI suggests that a complex between these two proteins is unlikely to play a role in normal hemostasis or in the pathophysiology of TTP.

Authors' Contribution

PJA, DG and JES conceptualized and designed the experiments, analyzed the data and contributed to manuscript preparation. HBF, WG, EMM, and KV contributed critical reagents, and revisions of the final version of the manuscript.

Conflicts of Interest

PJA' spouse is an employee of Affymetrix, Inc.; JES is a consultant for Baxter Healthcare.

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