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An Acquired, Calcium-Dependent, Factor X Inhibitor

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Abstract

Acquired factor X (FX) deficiency unrelated to amyloidosis is a rare disorder in which an anti-FX antibody is infrequently detected. A patient with severe bleeding due to a calcium ion-dependent anti-FX IgG antibody is described. The FX affinity purified IgG bound the light chain of FX, but not FX lacking its γ -carboxyglutamic acid domain, and binding was enhanced >1000-fold in the presence calcium ions. The antibody also recognized prothrombin and factor VII with about 100-fold and 1000-fold lower affinity. Like a lupus anticoagulant, increasing concentrations of phospholipids in functional assays reduced the inhibitory activity of the antibody. The effect of these properties of the inhibitor on laboratory diagnostic studies is considered.

Keywords

Factor X; coagulation factor inhibitor

Introduction

Acquired, isolated factor X (FX) deficiency is an uncommon disorder and frequently associated with immunoglobulin light-chain (AL) amyloidosis [1]. Acquired FX deficiency unrelated to amyloidosis is rare with a recent review identifying 26 articles describing a total of 34 patients [2]. Although frequently suspected, the presence of an antibody, which enhances FX clearance or inhibits FX activity, was demonstrated in only 9 patients. Here a case of acquired FX deficiency due to a calcium-dependent anti-FX antibody is presented and the effect of the properties of the antibody upon laboratory diagnostic tests is discussed.

Case Report

A 72 year old gentleman presented to his local hospital with a one day history of progressive hemorrhagic symptoms, including blood streaked saliva, oozing from non-traumatic skin bruises and bloody urine, and was found to have markedly abnormal coagulation assays prompting his transfer to Barnes-Jewish Hospital in St. Louis. Four weeks prior to admission he had developed sinus and chest congestion associated with fever and cough that resolved over the following 2 weeks. He was a cattle rancher, but denied clear insect or tick bites and recent exposure to potential toxins or chemicals.

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Examination showed a sublingual hemorrhage with submandibular swelling of the neck and overlying ecchymosis, multiple bruises of the upper and lower extremities, a left thigh hematoma, and gross hematuria. Stool was brown, but guaiac positive. Initial laboratory studies showed a prolonged PT and aPTT, which partially corrected on mixing studies, and a normal thrombin time (Table 1). Tests of liver function were normal. He was treated with intravenous vitamin K and fresh frozen plasma while awaiting the results of specific coagulation factor assays and, due to progression of the sublingual and submandibular hemorrhage, a nasotracheal tube was placed for airway protection and he was transferred to the intensive care unit.

Coagulation factor assays returned showing isolated FX deficiency (13%) without an inhibitor pattern (Table 1). His history, physical examination, and only partial correction of the PT and aPTT in mixing studies were against FX deficiency due to amyloidosis and serum protein electrophoresis with immunofixation and serum free light chain tests ultimately returned normal results. With persistent gross hematuria and despite red blood cell transfusions (2 units), over the first 14 hours his hemoglobin decreased from 12.3 to 6.6 gm/dL and was associated with EKG and troponin signs of cardiac ischemia. An abdominal ultrasound examination demonstrated normal liver, spleen, and kidneys and an apparent small perisplenic hemorrhage. Bebulin (3 factor prothrombin complex concentrate) was administered. Direct ELISA testing of his serum against coagulation factors II, V, VII, IX, X, XI, and XII demonstrated the presence of a calcium-dependent IgG anti-FX antibody and the patient was treated with steroids, plasma exchange, intravenous immunoglobulin, and ultimately rituximab (Figure 1).

Although measured FX levels were greater than ~40% throughout the remainder of his course, hemorrhage due to continued gross hematuria required additional red blood cell transfusions (9 units) over the following 6 days. Inhibitory activity based on aPTT (Figure 1) and PT (not shown) mixing studies increased, peaking about day 6, with FX assays now showing an inhibitor pattern. Subsequently, the hemorrhagic symptoms improved, the endotracheal tube was removed on day 9 and the gross hematuria cleared by day 14. The patient was discharged on day 20 on tapering prednisone to complete a 4 week course of rituximab. Results of PT, aPTT, and FX assays, and complete blood counts at 45, 108, and 185 days following admission were normal.

Materials and Methods

Consent

The patient agreed to permit additional *in vitro* biochemical studies to further characterize his inhibitor and to the publication of his case.

Materials

Prothrombin, factor VII, factor IX, FX, FXa, Gla-domainless FX (GD-FX), factor XI, factor XII, protein C, and protein S were obtained from Enzyme Research Laboratories (South Bend, IN) and factor V from Haematologic Technologies (Essex Junction, VT). The chromogenic FXa substrate CS11(32) was from Hyphen/Aniara (West Chester, OH) and rabbit brain cephalin from Pentapharm/Centerchem (Norwalk, CT). FX deficient plasma was from George King Biomedical (Overland Park, Kansas) and other reagents were from Fisher Scientific (Pittsburg, PA) or Sigma (St. Louis, MO).

Inhibitor isolation

To isolate total IgG, plasma obtained prior to the plasma exchange on day 4 was applied to a protein A-agarose (CaptivA PriMAB, Repligen, Waltham, MA) column equilibrated in

phosphate buffered saline (PBS), the column washed with PBS, and the IgG eluted with 0.1 M glycine, pH 2.5, into fractions containing 1/10th volume of 1.0 M Tris, pH 8.3. Appropriate fractions were pooled and treated with 5 mM diisopropyl fluorophosphate (DFP) and dialyzed extensively against 0.1 M NaCl, 0.02 M Hepes, pH 7.4 (HS). Greater than 98% of the FX binding activity detected by direct ELISA assay was bound and eluted from the protein A column. IgG concentration was determined assuming an extinction coefficient (1%, 280 nm) of 14.0. Three mL of the isolated IgG (7.56 mg/mL) to which CaCl₂ (4 mM) had been added was applied to a 4 mL Affigel-15 (Bio-Rad, Hercules, CA) column containing 0.25 mg FX/mL of gel (linked following the manufacturer's instructions) equilibrated in HS with 4 mM CaCl₂ (Figure 2). The column was washed with start buffer and then eluted with HS with 5 mM ethylenediamine tetraacetic acid (EDTA) followed by glycine 0.1 M, pH 2.5. EDTA-eluted fractions containing FX binding activity by direct ELISA were pooled and dialyzed against HS.

Direct ELISA Assay

Wells of MaxiSorb microtiter plates (Nunc, Thermo Scientific, Rochester, NY) were coated overnight with proteins in HS (4 ug/mL, 50 uL) and washed with HS containing 20 mg/mL bovine serum albumin (HSA) with 4 mM CaCl₂ or 5 mM EDTA. Samples (100 uL) and mouse monoclonal anti-human γ -chain (100 uL, Sigma, A2064, St. Louis, MO; 1:25,000) diluted with HSA containing 4 mM CaCl₂ or 5 mM EDTA were incubated 90 and 60 min, respectively, with intervening washes with the appropriate HSA buffer. Substrate (200 uL, *p*-Nitrophenyl Phosphate Liquid Substrate System, Sigma, N7653) was then added to each well and the OD₄₀₅ determined at the specified time. Results are depicted as the mean of duplicate assays.

Effect of calcium chloride and sodium citrate on the anti-FX(a) activity of the affinity-purified antibody

Amidolytic activity—Reaction mixtures containing FXa (100 nM) and the IgG (200 nM) in HSA with CaCl₂ (5 mM) or sodium citrate (13 mM) were incubated 30 min at room temperature. Following 50-fold dilution into the appropriate CaCl₂ or sodium citrate buffer, samples (200 uL) were placed in wells of a microtiter plate, the FXa substrate CS-11(32) (4 mM, 50 uL) was added and the rate of A₄₀₅ generation was determined using a Spectromax 340 instrument (Molecular Devices, Sunnyvale, CA). Tests were done in triplicate and relative FXa activity was calculated using FXa standard curves.

Coagulant activity—Reaction mixtures (40 uL) containing FX (100 nM) and the IgG (200 nM) in HSA with CaCl₂ 2.5 mM were constructed at room temperature and then handled in two separate ways: 1) After 3 min, 10 uL of HS was added to the mixture and a sample removed immediately for assay; and 2) after 3 min, 10 uL sodium citrate (65 mM, final concentration 13 mM) was added and 5 min later a sample was removed for assay. CaCl₂ containing samples were assayed for FX activity by combining in sequence RecombiPlasTin 2G (120 uL, Instrumentation Laboratory, Bedford, MA), HSA (50 uL), samples (10 uL) at 37° and 30 sec later adding FX deficient plasma (60 uL). Citrate containing samples were assayed for FX activity by combining in sequence FX deficient plasma (60 uL), HSA (50 uL), 10 uL samples at 37° and 30 sec later adding RecombiPlasTin 2G (120 uL). Clotting times were determined with a fibrometer and relative FX activity was calculated based on standard curves constructed using each assay method. Results are reported as the mean of triplicate reactions.

Effect of phospholipids on FXa inhibition by the antibody

Mixtures containing variable concentrations of the affinity purified IgG in HSA (50 uL), CaCl_2 25 mM (50 uL), cephalin (500, 150 or 50 uM; 50 uL), and FXa (10 nM; 50 uL) were incubated at 37° for 1 min, followed by the addition of FX deficient plasma (50 uL). Clotting times were determined with a fibrometer and relative FXa activity calculated using standard curves constructed at each cephalin concentration. Results are reported as the mean of duplicate reactions.

Western blotting

Proteins (3 ug), with or without reduction (5% 2-mercaptoethanol), were electrophoresed on 10-20% gradient SDS-PAGE gels (Novex, Carlsbad, CA) and transferred to nitrocellulose as previously described [3]. The membrane was blocked with HSA for 1 hr and then HSA with 5 mM CaCl_2 was used as the buffer for the following reactions: incubation with affinity purified IgG (2 ug/mL) for 90 min; washing; incubation with mouse monoclonal anti-human γ chain antibody (Sigma, A2064; 1:10,000) 60 min; and washing. The membrane was then developed (5 min for unreduced proteins, 20 min for reduced proteins) using BCIP/NBT-Blue Liquid Substrate System for Membranes (Sigma, B3804).

Results and Discussion

Initial evaluation of the patient's serum demonstrated a calcium-dependent anti-FX antibody by direct ELISA analysis. Therefore, additional plasma was obtained, total IgG isolated by protein A-agarose chromatography and the antibody purified by affinity chromatography on FX-Affigel (Figure 2). On ELISA the affinity purified antibody bound FX and FXa in a comparable fashion and the presence of calcium ions enhanced binding >1000-fold (Figure 3). Consistent with the calcium ion-dependence of its interaction with FX(a), western analysis showed the antibody bound the γ -carboxyglutamic acid (Gla) domain-containing light chain of FX (Figure 4) and did not interact with Gla-domainless FX on ELISA or western analysis (Figures 3 and 4). By ELISA the antibody appeared to also recognize prothrombin and factor VII about 100- and 1000-fold less well than FX, respectively, and its weak interaction with these proteins was confirmed by western blotting (not shown). ELISA testing also showed even lower reactivity of the antibody with protein S, and protein C, but western analysis suggested the bulk of this reactivity was due to trace contamination of these proteins with FX and/or prothrombin.

Early in his course the results of FX assays appeared inconsistent with the extent of the patient's hemorrhagic symptoms, presumably due to the plasma dilution involved in the procedure used for coagulation factor assays and the calcium-dependence of the antibody. FX activity determined following the incubation of FX (100 nM) with the affinity purified IgG antibody (200 nM) in the presence of calcium chloride (2.5 mM) was markedly increased following the addition of sodium citrate (13 mM) ($13.7 \pm 1.3\%$ versus $92.3 \pm 6.8\%$) (see Methods). The antibody had no effect on the amidolytic activity of FXa in the presence of calcium chloride or sodium citrate (not shown). The discrepancy between the substantial, but partial, correction of aPTT and PT mixing studies on admission and a FX assay that did not detect an inhibitor pattern (Table 1), is likely explained by initial low antibody levels which had reduced the plasma FX concentration by enhancing its clearance and the dilution of plasma required for the factor assay. With the subsequent apparent increase in antibody levels, inhibitory activity was discernible in FX assays by day 4 (Figure 1).

A specific FX inhibitor has been detected in only a minority of the previously described cases of acquired FX deficiency. When inhibitory activity was tested, however, the method typically involved the mixing of patient and normal citrated plasmas and the measurement of FX activity following dilution. This schema could miss a calcium-dependent antibody.

Calcium-dependence might also explain FX antibodies that have been described whose anti-FX activity was difficult to detect in citrate-based assays, but could be detected in continuous, time-dependent assays containing calcium ions (e.g. activation of FX by factor VIIa/tissue factor or Russell viper venom) [4].

Lupus anticoagulant assays were positive on days 1, 3, and 6 (negative on day 15), but were not felt to be interpretable in the face of a FX inhibitor. Testing of admission serum for anticardiolipin and anti- β_2 -glycoprotein I antibodies produced negative results. Subsequent studies showed that increasing phospholipid concentrations reduced the inhibitory effect of the affinity purified antibody, presumably reflecting competition between phospholipids and the antibody for Gla-domain binding sites (Figure 5). The results of lupus anticoagulant testing are not provided in the majority of the previous reported cases of acquired FX deficiency, but Ashrani et al. described two cases with transient lupus anticoagulants and FX deficiency [5]. They did not find specific FX inhibitory activity in mixing studies using citrated plasmas and a FX antibody was not detected on FX-crossed immunoelectrophoresis, though whether the latter test was performed in the presence of calcium ions was not reported. It is conceivable that these individuals had developed calcium-dependent anti-FX antibodies whose inhibitory activities were affected by phospholipid concentration in a fashion similar to the antibody in our patient.

In line with the previously reported cases of acquired, non-amyloidosis-related FX-deficiency, bleeding in our patient was preceded by a respiratory illness (38%) and hematuria (39%) was a major hemorrhagic symptom [2]. Prothrombin complex concentrate (Bebulin) was used sparingly due to evident cardiac ischemia and the potential risk of thromboembolic complications [6] and treatment with high dose steroids, intravenous immunoglobulin, and daily plasmapheresis as originally described by Smith et al. was instituted [4]. Acquired FX deficiency is usually [2], though not always [7,8], transient in nature, but progressive hemorrhage and increasing plasma levels of inhibitory activity based on aPTT (and PT) mixing studies (Figure 1) despite ongoing therapy led to the early addition of rituximab treatment.

Acknowledgments

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Figure 1. Clinical Course

The figure shows FX levels (•), aPTT (○) and aPTT 50/50 mixing (□) results. The dotted lines enclose the aPTT normal range. Therapeutics are also listed: Medrol- methyl prednisolone 125 mg IV Q12H; Pred- prednisone 100 mg p.o. QD; FFP- fresh frozen plasma (○); PCC- Bebulin 25 U/kg IV (◇); IVIG- intravenous immunoglobulin 1.0 gm/kg IV (□); Ritux- rituximab 375 mg/M² IV (○); and PEX- 1.0 volume plasma exchange (■).

Figure 2. Affinity purification of the IgG antibody

Three mL of protein A-agarose isolated IgG (7.56 mg/mL) was applied to a 4 mL column of FX-Affigel in HS with 4 mM CaCl_2 and eluted with HS with 5 mM EDTA and 0.1 M glycine, pH 2.5. Flow rate was 7.5 mL/hr and fraction size 1 mL. Fractions were tested for OD₂₈₀ (○) and anti-FX activity by direct ELISA (●). For the latter, samples were diluted 1000-fold and the OD₄₀₅ at 5 min is depicted.

Figure 3. Direct ELISA reactivity of the affinity purified IgG

Various concentrations of the antibody were tested by direct ELISA for reactivity with coagulation-related proteins: FX (●), FXa (○), prothrombin (■), factor VII (◆) and Gla-domainless FX (□) in CaCl_2 (4 mM)-containing buffer; FX (◇) in EDTA (5 mM)-containing buffer. The ELISA was developed for 60 min.

Figure 4. Western reactivity of the affinity purified IgG

Western blots were performed as described in Methods. Top, Coomassie stained gels of unreduced (left) and reduced (right) proteins; bottom, western blots of the same proteins; middle, MW of protein standards in kDa; X- FX; GD-X- Gla-domainless FX.

Figure 5. Effect of phospholipid concentration on affinity purified IgG activity
Antibody inhibition of FXa was determined by coagulation assay at cephalin concentrations of 10 (◇), 30 (□) and 100 (○) uM. See Methods.

Table 1**Laboratory values**

Coagulation			
PT	43.2 s	(11.5-15.7)*	
50/50 Mix	18.2 s		
PTT	77.8 s	(25.0-35.0)	
50/50 Mix	41.7 s		
Thrombin time	15.6 s	(15.0-22.0)	
Factor II	105%		
Factor V	109%		
Factor VII	142%		
Factor VIII	198%		
Factor IX	109%		
Factor XI	92%		
Factor X			
1:10 dilution	13%		
1:20 dilution	12%		
1:40 dilution	13%		
vWF antigen	107%		
vWF activity	135%		
Lupus Anticoagulant			
DRVVT	positive		
StaClot	positive		
Anticoagulant poisoning screen	negative		
Other			
Hemoglobin	12.3 gm/dL	(13.8-17.2)	
White blood cells	10.6 K/mm ³	(3.8-9.8)	
Platelets	202 K/mm ³	(140-440)	
Urea nitrogen	31 mg/dL	(8-25)	
Creatinine	0.95 mg/dL	(0.70-1.30)	

* Normal range in parenthesis; vWF- von Willebrand factor; DRVVT- dilute Russell viper venom time (American Diagnostica, Greenwich, CT); StaClot- correction with hexagonal phase phospholipids (Stago, Parsippany, NJ).