Defective $\alpha_2$antiplasmin cross-linking and thrombus stability in a case of acquired factor XIII deficiency

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Abstract

Acquired factor XIII (FXIII) deficiency is a rare and life-threatening condition which is often misdiagnosed or missed completely. A 72 year old woman presented with symptoms of major unprovoked bleeding but routine coagulation screening tests and platelet count were normal. Low FXIIIa activity levels and abnormal urea clot stability led to diagnosis of acquired FXIII deficiency. A modified Bethesda inhibitor titre of 1.6 BU/ml indicated the presence of a FXIII inhibitor. Bleeding responded to a single dose of FXIII concentrate and immunosuppression with prednisolone induced remission. A subsequent relapse was treated with combined prednisolone and Rituximab resulting in a prolonged, ongoing remission. Here we analyse the mechanisms underlying this idiopathic case of acquired FXIII deficiency.

Prospective analysis of patient plasma revealed minimal FXIIIa activity and antigen in presentation and relapse samples. Thrombi formed from these samples lysed rapidly and showed an absence of cross-linked α_2AP. Western blotting revealed the presence of FXIII-B, indicating only FXIII-A and FXIII-A_2B_2 were affected. FXIII activity and antigen levels normalised on remission. Our data suggest the presence of inhibitor induced clearance of FXIII from plasma. As a consequence reduced thrombus stability was evident due to defective α_2AP cross-linking, thereby explaining symptoms of excessive bleeding.

Introduction

Plasma factor XIII (FXIII) circulates as a heterodimer, A_2B_2, and is activated by thrombin and calcium to become a transglutaminase (TG) enzyme (Lorand and Konishi 1964, Schwartz*, et al* 1973). FXIII functions in haemostasis by cross-linking the α- and γ- chains (Gladner and Nossal 1983) of fibrin in order to increase the mechanical stability of clots and confers fibrinolytic resistance by cross-linking fibrinolysis inhibitors, such as α_2-antiplasmin (α_2AP), to fibrin (Sakata and Aoki 1980).

The importance of FXIII in maintaining haemostasis is made evident in clinical cases of its severe deficiency. Congenital FXIII deficiency is a rare autosomal recessive disorder characterised by a severe haemorrhagic diathesis, including unprovoked umbilical stump and intracranial bleeding (Aziz and Siddigui 1972, Losowsky*, et al* 1965), abnormal wound healing (Aziz and Siddigui 1972, Fisher*, et al* 1966) and spontaneous miscarriage in women (Fisher*, et al* 1966). Cases of mild and moderate deficiency of FXIII resulting from reduced synthesis or increased consumption are described in association with several medical conditions including Crohn's disease, ulcerative colitis, liver disease, sepsis and disseminated intravascular coagulation. In general, however, in these cases the patients have a moderate reduction in FXIII-A subunit levels and rarely present with unprovoked bleeding (Kohler*, et al* 2011). On the other hand, severe acquired FXIII deficiency is an exceptionally rare disorder with less than 100 cases described (Souri*, et al* 2015). The most common conditions associated with antibody mediated acquired FXIII deficiency are connective tissue disorders and malignancy but around half of the cases reported have no clear associated disorder (Boggio and Green 2001). Treatment with FXIII concentrate to arrest bleeding and immunosuppressive therapy to eliminate autoantibodies is required.

Unlike most single coagulation factor deficiencies, FXIII deficiency cannot be detected using routine clot based coagulation tests. This often leads to delayed or misdiagnosis of this condition which delays necessary treatment. Here we describe the clinical and laboratory characteristics of a patient that presented with idiopathic acquired FXIII deficiency. We have sequentially analysed the effects of the autoantibody on FXIII activity and in fibrinolytic assays.

Methods

Patient plasma samples

Samples of patient plasma from the time of initial presentation, relapse and remission were stored at -70°C until the time of analysis.

Collection of blood and preparation of plasma

Pooled normal plasma (PNP) that was essentially free of platelets was prepared from 20 normal donors as previously described (Booth*, et al* 1988).

FXIII activity assay

FXIII activity was quantified using an in-house activity assay as previously described (Mitchell*, et al* 2014).

Chandler model thrombus lysis

Chandler model thrombus lysis was performed as previously described (Mutch*, et al* 2010). Patient plasma samples, PNP or commercial FXIII-depleted plasma (Affinity Biologicals, Ancaster, ON, Canada) containing fluorescein isothiocyanate (FITC)-labelled fibrinogen (43.5 μg/ml), with a FITC/fibrinogen ratio of approximately 6:1, were recalcified (10.9 mM $CaCl_2$). The loops were rotated for 90 min at 30 r.p.m. Formed thrombi were washed in 0.9% (w/v) NaCl and bathed at 37°C in 10 mM Tris, 0.01% Tween 20, pH 7.4 containing 1 μg/ml single-chain tissue-type plasminogen activator (tPA; Genetech, San Francisco, CA, USA). Samples were taken at 30 min intervals for 4 h. Fluorescence was read, excitation 485 nm and emission 528 nm, on a Biotek Instruments Fluorometer. After 4 h of lysis, thrombi were dissolved in 8 M urea, 0.2 M Tris (pH 8), 40 mM dithiothreitol and 4 % sodium dodecyl sulphate (SDS) at 37°C for 12 h for Western blotting.

Western blotting

Patient plasma samples or dissolved thrombi were run on 4–12% polyacrylamide bis–Tris NuPAGE gels with MOPS running buffer (Life Sciences, Paisley, UK) under reducing conditions. Fibrogammin P® (20 μg/ml; CSL Behring, Sussex, UK) was

included as a positive control for the FXIII-B_2 subunit. Proteins were transferred to nitrocellulose and immunoblotted with a polyclonal antibody for FXIII-B (200 ng/ml; sc-18015 Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a polyclonal antibody to α_2AP (2.5 μg/ml; SA2AP-IG, Affinity Biologicals). Appropriate horseradish-peroxidase (HRP)-conjugated secondary antibodies, were applied and proteins were detected using ECL (Thermo Fisher-Scientific, Leicestershire, UK) with a UVP Biospectrum 810 system and analysed with UVP visionWorks LS Image Acquisition and Analysis software.

FXIII ELISA

FXIII-A antigen was measured using a ZYMUTEST FXIII-A ELISA kit (Hyphen Biomed, # RK034A; Quadratech Diagnostics LTD, Surrey, UK). Patient plasma samples, normal pooled plasma (PNP) or FXIII depleted plasma were diluted 1:50 and added to plates pre-coated with a sheep polyclonal antibody specific for human FXIII-A and incubated for 1 h in the presence of Anti-(h)-FXIII-HRP immunoconjugate. Wells were then washed 5 times and colour was developed using a 3,3',5,5' – Tetramethylbenzidine containing hydrogen peroxide and stopped after 5 min with 0.45 M sulphuric acid. The absorbance was measured at 405 nm using a Labsystems iEMS plate reader.

Clinical Case

A 72 year old woman presented with extensive, unprovoked soft tissue bleeding on the limbs and trunk (Figure 1A.), and a large splenic haematoma was confirmed by CT scan. There was no previous personal or family history of pathological bleeding. The patient had a history of uncomplicated, significant previous haemostatic challenges including dental surgery and parturition. She was not taking any medication. Routine laboratory investigations showed anaemia with an otherwise normal full blood count. PT, aPTT and fibrinogen levels by Clauss assay were normal.

Further investigation revealed abnormal urea clot stability tests which did not fully correct upon addition of normal plasma. FXIII activity, as measured by the Dade Behring Berichrom® FXIII activity kit, was significantly reduced at 0.09 U/ml (Normal Range: 0.59-1.63; Figure 1B.). A modified Bethesda inhibitor titre of 1.6 BU/ml was measured, indicating the presence of a FXIII inhibitor.

Repeated clinical examination, chest X-rays (smoker), whole body CT scan and autoantibody studies have continued to date (3 years from presentation) and show no evidence of underlying malignancy or connective tissue disease. The patient was treated with a single dose of FXIII concentrate (Fibrogammin P®) and was commenced on immunosuppression with prednisolone 1 mg/kg. Following treatment there were no further bleeding episodes and serial scans showed progressive improvement in the splenic haematoma. High dose steroid treatment was continued until normalisation of the urea clot stability tests, which coincided with normalisation of the quantitative FXIII assay.

Seven weeks after stopping steroids, there was laboratory evidence of relapse with abnormal urea clot stability test but no associated clinical symptoms of bleeding.. This was confirmed by repeat measurements using the Dade Behring Berichrom® FXIII activity assay. The patient was recommenced on steroids and was given rituximab 375 mg/ m^2 weekly for four weeks. She showed a positive response to treatment as evidenced by normalisation of the urea clot stability test and FXIII assay. The patient has now been off all immunosuppressive treatment for around 2 years with no evidence of an emerging underlying disorder.

Characterisation of FXIII activity and expression

Samples of the patient's blood were taken and stored throughout the treatment time from initial presentation to remission and plasma samples were subsequently analysed in order to characterize the mechanisms underlying the clinical progression.

FXIIIa activity and antigen are depleted in patient plasma

Patient plasma was assessed for both FXIII activity and FXIII antigen levels in order to determine whether deficiency resulted from the absence of FXIII or the presence of non-functional protein.

FXIIIa activity was quantified in patient plasma using an in-house activity assay (Mitchell, *et al* 2014) and FXIII-A antigen was measured using a commercial ELISA (ZYMUTEST). FXIIIa activity levels and the FXIII-A antigen in the initial presentation patient plasma sample were below the limits of detection in both assays. In the relapse

samples low levels of activity (0.1 IU/ml) and antigen (39%) were detected (Figure 2A-B.). The values were substantially lower than those obtained with a normal plasma control (0.43 IU/ml and 111% respectively).

Western blotting using an antibody specific to the FXIII-B subunit in patient plasma samples revealed FXIII-B antigen in the initial presentation, relapse and remission samples. This suggests that only FXIII-A and FXIII-A_2B_2 have been cleared from plasma by the inhibitor, leaving circulating levels of FXIII-B. (Figure 2C.)

The ability of the inhibitor in the patient's plasma to neutralize the activity of purified FXIII (Fibrogammin P®) was further examined using an in-house activity assay. Purified FXIII (final concentration - 0.5 U/ml) was mixed with increasing percentages of patient plasma from initial presentation. Interestingly, there were no differences in FXIII activity levels from 20% to 80% patient plasma (Fig 5.2D) These findings contrast with those from the modified Bethesda assay performed on the initial and relapse samples and point towards a time and temperature dependent inhibition of FXIII-A.

Patient model thrombi are susceptible to fibrinolysis due to a lack of cross-linked α_2AP
Model thrombi formed from patient plasma taken at the time of initial presentation (194.6 FU/min^{-1}) and relapse (183.5 FU/min^{-1}) lysed rapidly relative to normal plasma (17.7 FU/min^{-1}), with lysis rates similar to that of commercial FXIII-depleted plasma (186.6 FU/min^{-1}) (Figure 3A.). FXIII exerts antifibrinolytic activity by cross-linking α_2AP to fibrin, thereby stabilizing clots against fibrinolytic degradation. Western blotting of dissolved patient thrombi revealed free α_2AP, but a clear absence of cross-linked α_2AP in thrombi formed from initial presentation and relapse patient plasma samples compared to the normal plasma control samples (Figure 3B.).

Discussion

Here we report a case of acquired FXIII deficiency due to a plasma based anti-FXIII-A inhibitor which demonstrates time and temperature dependant characteristics (Kohler, *et al* 2011). Idiopathic acquired FXIII deficiency is rare, can be difficult to treat and is associated with significant mortality and morbidity. It can be caused by the

development of an inhibitor to FXIII or by disruption to the production and activity of the FXIII molecule.

The clinical features observed in this case are characteristic of acquired FXIII. The response to treatment with FXIII concentrate (Fibrogammin P®) to control bleeding was brisk, although this is not always the case (Gregory and Cooper 2006, Hayashi, *et al* 2012, Ichinose, *et al* 2015) The early response of the inhibitor to prednisolone therapy was however short-lived and the response to the combination of prednisolone combined with rituximab has been much more persistent.

In this case, FXIII antigen and activity levels in the patient's plasma obtained at initial presentation were below the lower limit of detection of both assays, suggesting complete clearance from the patient's circulation. Thrombi formed from initial presentation samples lysed at a similarly rapid rate to commercial FXIII-depleted plasma and contained no cross-linked α_2AP. At the time the laboratory detected relapse, the activity and antigenic levels were 0.1IU/mL and 39% respectively, there was no evidence of renewed bleeding in the patient, however thrombi formed from the relapse samples lysed rapidly and revealed an absence of cross-linked α_2AP, similar to the initial presentation samples. This suggests that the low activity levels of FXIII (0.1 IU/mL) were not sufficient to facilitate significant α_2AP cross-linking. This observation is in line with our previous report which demonstrated that levels of 50% plasma FXIII are necessary to achieve adequate α_2AP cross-linking (Fraser, *et al* 2011). Fibrin-fibrin cross-links induced by FXIIIa confer mechanical stability to thrombi (Mockros, *et al* 1974, Shen and Lorand 1983) and could partially account for the bleeding complications observed in the patient. Thrombi formed in mice in the absence of FXIII are smaller in size due to lack of retention of red blood cells (Aleman, *et al* 2014). This unique mechanism, involving an interaction between red blood cells and fibrinogen, could account for some of the clinical features observed in the acquired FXIII deficient patient.

The BCSH guidelines recommend diagnosis of FXIII deficiency using either ammonia release or amine incorporation assays for FXIII activity and immunoassays for both A and B subunits to detect FXIII antigen. Recommended diagnosis of acquired FXIII deficiency is through the use of mixing studies with normal plasma and analysis of FXIII activity in platelet lysates, which under these circumstances, should be normal

(Mumford*, et al* 2014). The urea clot solubility test is not currently recommended but we have shown it can be a useful additional test in the diagnosis and monitoring of relapse, particularly in situations in which activity tests are not readily available or reliable.

The Bethesda inhibitor titre of 1.6 BU/ml indicated the presence of a plasma based FXIII inhibitor, however, no change was detected in the activity of purified FXIII (Fibrogammin P®) when mixed with increasing concentrations of patient plasma from the initial presentation (Figure 2D). This in-house activity assay is performed immediately post-mixing of the sample. The different set up of these assays, one performed immediately at room temperature (in-house FXIIIa activity assay) while the other following a 2 h pre-incubation at 37°C (Bethesda test) indicate that the acquired inhibitor may neutralise FXIII in a time and temperature dependent manner. Indeed, this form of progressive inhibitor is well described for factor VIII which, in contrast to fast acting acquired inhibitors to factor IX, requires prolonged incubation with plasma to exert inhibitory function.

FXIII autoantibodies have been reported in a rising number of case studies in recent years, particularly in Japan (Ichinose*, et al* 2015). A recent study investigating autoantibody mediated acquired FXIII deficiency describe three types of inhibitor: Type Aa, which inhibits FXIII by blocking the assembly of the A and B subunits, and also dissociates the A subunit from the B subunit. Type Ab antibodies inhibit the action of activated FXIII and type B accelerate the clearance of FXIII-A_2B_2 from the circulation (Ichinose*, et al* 2015). This patient shows no detectable FXIII-A_2 or FXIII-A_2B_2 and low levels of the B-subunit present, presumed to be free circulating FXIII-B. The presence of FXIII-B in plasma suggests that the inhibitory antibody is targeted towards the FXIII-A subunit.

Unfortunately, further studies on the mechanism of action of this inhibitor were prohibited due to a lack of sample. A recent publication (Souri, *et al* 2015*)* reports an autoantibody with the ability to both block the assembly of FXIII-A_2B_2 and also 'steal' FXIII-A that is already in complex with FXIII-B. Patients with this type of antibody had reduced FXIII-A antigen and activity, similar to what we have reported. The characterised antibodies also blocked activation of FXIII, but interestingly had no inhibitory activity towards activated FXIII-A *(Souri, et al 2015)*. Further study would

have been necessary to examine the inhibitory capacity of the autoantibody we have reported towards pre-activated FXIIIa in order to determine whether our data fits with the profile of this antibody type.

In summary, we have reported a case of autoimmune acquired FXIII deficiency that was successfully treated with Rituximab therapy. Further analysis of patient plasma showed a dramatic reduction in FXIII-A antigen and activity, suggesting the presence of a strong inhibitor to FXIII-A. The presence led to defective α_2AP cross-linking and reduced thrombus stability, manifesting in excessive bleeding in the patient. This report, in line with others, describing cases of acquired FXIII inhibitors, highlights the requirement for standardised screening and increased awareness of bleeding disorders which present with normal platelet counts and coagulation screening tests. Such improvements would prevent the failure to detect potentially fatal disorders such as FXIII deficiency.

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Authorship Contributions

J.L.M. performed the research, analyzed the data and wrote the manuscript; S.W. and S.K. performed the research and analyzed data; H.G.W and N.J.M. supervised the research, analyzed the data and wrote the manuscript.

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Legends

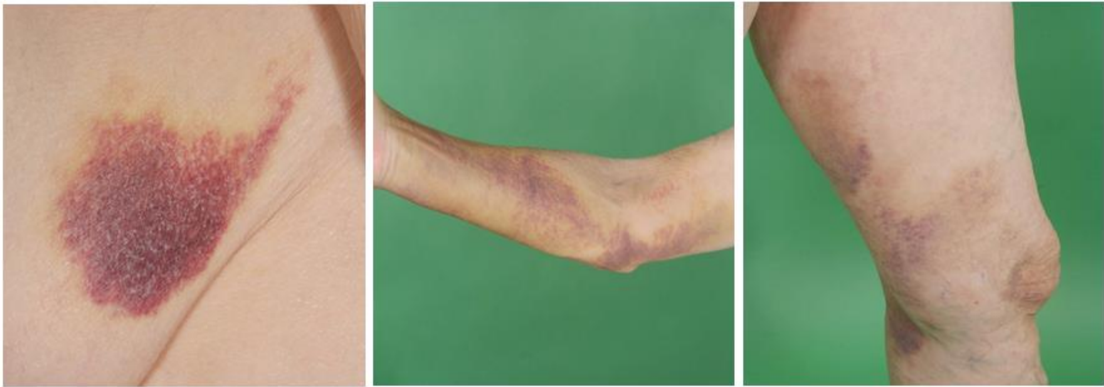
Figure 1: *Clinical presentation and measurements.* (A) Photographs taken at the time of initial presentation illustrating extensive bruising to limbs and torso and leg swelling. (B) Clinical measurements of FXIII activity using a Dade Behring Berichrom® FXIII activity kit taken at various time points throughout case presentation.

Figure 2: *FXIII-A antigen and activity are absent from patient plasma.* (A) FXIII activity (IU/ml) (A) and FXIII-A antigen (B) was measured in pooled normal plasma, FXIII-depleted plasma and patient plasma samples by in-house activity assay and ELISA respectively. Antigen values are expressed as % FXIII-A determined by calibrated control standard plasma. (C) Pooled normal plasma, FXIII-depleted plasma and patient plasma were separated under reducing conditions on NuPAGE 4-12% gels before transferring to nitrocellulose and probing with a specific antibody to the FXIII-B subunit. (D) FXIII activity (IU/ml) was measured in FXIII-depleted plasma and the initial presentation (IP) plasma with and without the addition of 0.5 U/ml purified FXIII (final concentration) by in-house activity assay.

Figure 3: *Patient model thrombi are highly susceptible to fibrinolysis.* (A) Model thrombi were prepared under flow from either FXIII-depleted plasma (Δ); pooled normal plasma (▲); initial presentation patient plasma (■) or relapse patient plasma (●) with incorporated fluorescently-labelled fibrinogen. Thrombi were lysed in a bathing buffer containing 1 μg/ml single-chain tissue-type plasminogen activator (t-PA) and samples were taken every 30 min. Fluorescence release from thrombi is directly proportional to fibrinolysis (B) Model thrombi were dissolved and separated under reducing conditions on NuPAGE 4-12% gels before transferring to PVDF and probing with a specific antibody to α_2AP. Shown is free α_2AP (70 kDa) and cross-linked α_2AP (160 kDa) and above.

Figure 1

A



B

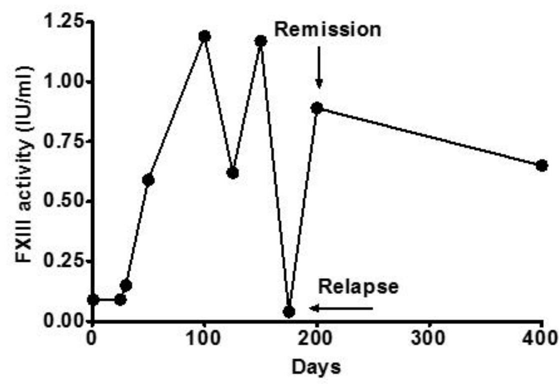


Figure 2

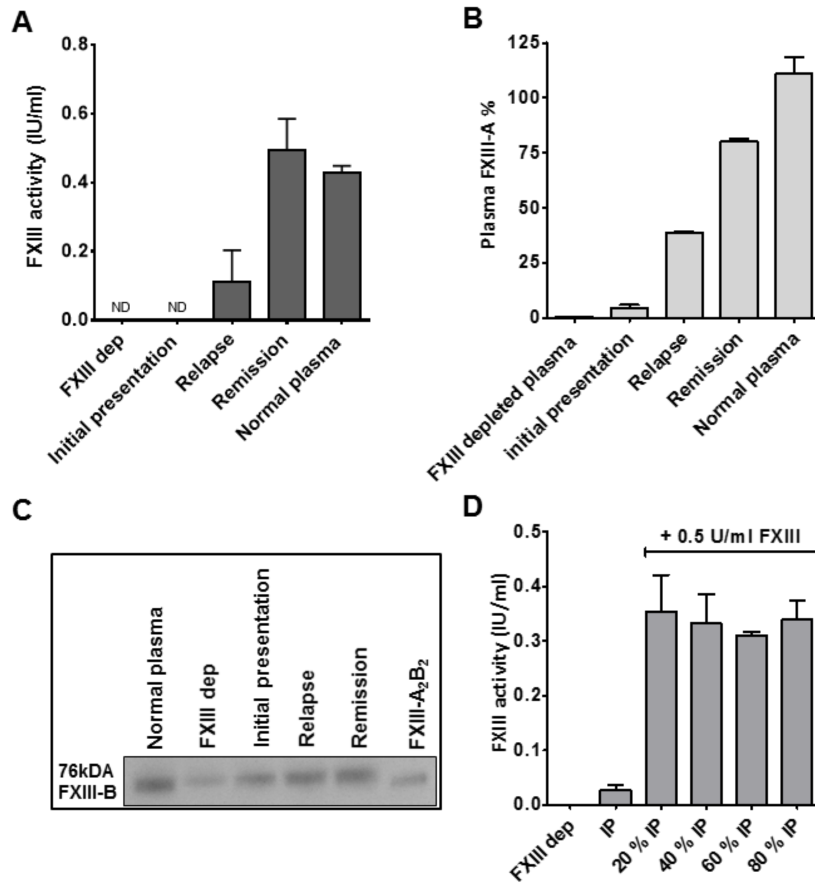


Figure 3