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Regular Article

Persistent high factor VIII activity leading to increased thrombin generation – A prospective cohort study

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ABSTRACT

Introduction: A persistently elevated level of factor VIII (FVIII) is an independent risk factor for venous thromboembolism (VTE). Although the pathophysiology of VTE is unclear, the involvement of thrombin generation (TG) has been postulated. Consequently this study was designed to (i) investigate the relationships between FVIII, Thrombin generation test (TGT) parameters and D-dimer in VTE patients, (ii) determine whether elevated levels of FVIII and increased TG in these patients are transient or sustained.
Patients and Methods: After an initial period of anticoagulation had been completed 91 VTE patients and 52 healthy controls were recruited. FVIII levels were determined by one-stage clotting (FVIII:C) and chromogenic (FVIII:Ch) assays. The potential to generate thrombin was measured using the Calibrated Automated Thrombogram (CAT) and D-Dimer was by immuno-turbidometric assay.
Results: Patients' FVIII:C levels and FVIII:Ch, exhibited good agreement ($rs = 0.94$; $p < 0.0001$), although FVIII:C exhibited a mean bias of -6%. FVIII:Ch show a significant correlation with TGT Peak Thrombin ($rs = 0.30$; $p = 0.004$) and Peak Thrombin was found to be significantly higher ($p = 0.04$) in patients with FVIII>200 iu/dL. Furthermore elevated levels of FVIII and increased thrombin generation parameters appeared to be consistent over time.
Conclusion: Our data suggests that high FVIII leading to increased TG confers a significant risk of recurrent VTE and therefore we speculate that these patients may benefit from prolonged anticoagulation therapy.
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Introduction

A persistently elevated level of factor VIII (FVIII) activity is an important risk factor for both venous and arterial thrombosis [1]. Furthermore a FVIII level of >150 iu/dL is found in approximately 25% of patients with venous thrombosis [2–4], and a dose-response relationship appears to exist. Very high levels of FVIII (>200 iu/dL) increase the risk of recurrence of VTE [5]. Blood group and VWF levels are important determinants of the circulating FVIII level. Both VWF and FVIII levels increase the risk of thrombosis for non-O versus O blood groups; however, it is only FVIII that remains a strong risk factor once these 3 variables are adjusted for [2,3,6,7]. In one study exploring persistently elevated FVIII levels, only 50% of patients demonstrated any rise in VWF supporting the conclusion that increase in FVIII is not dependent on a primary elevation in VWF [7]. Lowe et al [8] demonstrated in a prospective study of post-operative DVT that the risk of thrombosis was associated with FVIII but not VWF. O'Donnell et al [7] conclude that increased FVIII concentration observed in patients with venous thromboembolic disease appears to be persistent and independent of the acute phase reaction. However, the cause of elevated FVIII in these patients is unclear; no link between FVIII concentrations and regulatory polymorphisms in the FVIII gene promoter have previously been found [9]. Although, more recently, a single nucleotide polymorphism (SNP) encoding a D1241E substitution within the B-domain of the FVIII gene has been identified and is significantly associated with FVIII level [10].

The pathophysiological mechanism by which FVIII exerts its thrombophilic effect is also unclear. It has been postulated that increased thrombin generation (TG) may be involved. Markers of *in vivo* thrombin generation such as thrombin-antithrombin complexes (TAT) and prothrombin fragment 1 + 2 (PF1 + 2) have been found to be significantly higher in 78-85% of patients with venous thromboembolism (VTE) and raised FVIII, compared to both normal controls and patients with VTE but no proven inherited or acquired thrombophilic state [11]. Kyrle et al [5] demonstrated that 20% of patients with no defined thrombophilic defect had at least one marker of TG (PF1 + 2) level elevated above the upper range of normal during a 12 month follow-up period. Mathematical models of the coagulation system involving pure coagulation factors, where TG was measured using a peptide substrate, have suggested that FVIII can influence the rate of TG [12]. Using an *in vitro* thrombin generation test (TGT) system, a more rapid onset of TG and higher level of peak thrombin

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has been shown when FVIII concentration was at the upper limit of normality [13].

More recently, increased *in vitro* TG has been associated with an increased risk of VTE recurrence: the AUREC study gives an adjusted hazard ratio for recurrence of 1.014 for each 1% increase when endogenous thrombin potential (ETP) is given as a percentage of normal ($p = 0.06$) [14]. Elevated ETP may also predict an increased risk of a first episode of deep vein thrombosis (DVT); however in the same study, increased ETP did not confer an increased risk of recurrence [15]. A scoring system taking into account raised ETP, Peak Thrombin and Lagtime has also recently been published. This study demonstrated that when all 3 parameters were abnormal, the hazard ratio of a recurrent thrombotic event was 6.27 versus 2.99 when only one of the TGT parameters was raised [16].

Several large prospective studies have shown that persistently raised D-dimer confers a higher risk of VTE recurrence [17–20]. The relationship between D-dimer and TGT parameters is however not well-defined. In one study, no correlation was shown between ETP and D-dimer; in multivariate analysis adjusted for ETP, sex and type of first event, D-dimer was also not significantly associated with the risk of recurrence [21]. However, an *in vitro* TGT may provide a further method for the assessment of patients at risk of thrombosis, and may be valuable in quantifying the risk of recurrence and whether continued anticoagulation therapy is indicated. Current British Committee for Standards in Haematology (BCSH) Guidelines recommend at least 3 months anticoagulation following a proximal DVT/PE, and at least 6 months in those with idiopathic VTE or permanent risk factors [22].

We hypothesised that high FVIII and increased TG (*in vivo* and or *in vitro*) are correlated, and may provide a method for the prediction of continuing VTE risk. The aims of our cohort study therefore were to: (i) investigate the relationships between elevated FVIII, TGT and D-dimer in patients with a history of previous VTE, after an initial period of anticoagulation had been completed; (ii) investigate whether increased FVIII levels and elevated TGT parameters are sustained or transient.

Samples, materials and methods

Patients and healthy volunteers

This study was approved by the local ethics committee and Research & Development at University College London Hospitals and St George's Hospital NHS Trust. The criteria for participation in the study were that patients had to be over 18 years of age with a prior history of spontaneous VTE, and had completed their course of anticoagulation therapy at least 4 weeks previously. Prospective thrombophilia screening was not undertaken; however all patients' hospital records were reviewed in order to ascertain whether individuals were known to have either a heritable thrombophilia (protein C, protein S or antithrombin deficiency, factor V Leiden or prothrombin 20210 gene mutation) or anti-phospholipid antibodies.

The control group of healthy volunteers composed of friends or non-blood relatives of patients; they were age-matched to within 10 years of patients and sex-matched. Volunteers on medications including combined oral contraceptives, hormone replacement therapies, aspirin or clopidogrel were excluded, as well as those with microangiopathic haemolytic anaemia, malignancy, superficial thrombophlebitis, haemorrhagic stroke, age <18 years, antiphospholipid antibody positive, anticoagulation stopped less than 4 weeks prior to sample collection.

In order to investigate whether high FVIII levels and/or increased *in vitro* TGT parameters were sustained over time, a subset of 15 patients were tested on more than one occasion. These patients were selected at random and recalled over a 6 to 24 month period after first sampling had occurred. For the remaining patients who did not have FVIII testing repeated, historical FVIII levels were reviewed. These values were for use in the assessment of the continuity of factor VIII levels (not for correlation with subsequent TGT parameters); and were obtained from the patient hospital notes, the assays having been performed after the acute phase of VTE and completion of anticoagulation therapy.

Blood sampling and plasma preparation

After written informed consent, venous blood was taken by clean venepuncture with minimal stasis, via a 19G butterfly needle, into a four citrated (0.105 mol/L), an EDTA, and a plain glass Vacutainer® (Becton Dickinson, Oxford, UK). Immediately after venesection, corn trypsin inhibitor (CTI. Cambridge Bioscience, Cambridge, UK) at a final whole blood concentration of 18.3 μg/mL [23] was added to two of the citrated tubes. Citrated platelet poor plasma was prepared by double centrifugation at 2000 ×*g* for 15 minutes at room temperature and stored in 1.0 mL aliquots at −80 °C until testing. EDTA blood was used for full blood count and serum, for biochemical analyses, was prepared from clotted blood in plain sample tubes by centrifugation at 2000 ×*g* for 10 minutes.

Assays

The same batches of reference preparation and reagents were used throughout this study in order to reduce the effect of inter-assay variation.

In vitro thrombin generation was assessed using the calibrated automated thrombogram (CAT) system [24] in conjunction with the manufacturer's PPP-Low reagent, which gives a reaction concentrations of 1pmol/L tissue factor (TF) / 4 μmol/L phospholipid (PL) (Thrombinoscope BV, Maastricht, The Netherlands). Thrombin generation and sample specific calibrators were tested in triplicate for each sample; the fluorescence signal was measured at 20 s intervals over a period of 60 minutes. Results were calculated using Thrombinoscope software version 3.0. The TGT provides information relating to the dynamics of thrombin generation, the TG curve is described in terms of: the lag-time; the time to peak; peak thrombin; and the area under the TG curve, which is also known as the endogenous thrombin potential (ETP).

Within each TGT run 2 quality control plasmas were also tested, a frozen pooled normal plasma (PNP) to permit normalisation of results (results were expressed as a ratio to PNP e.g. patient ETP result/PNP ETP result = nETP [25]) and a commercial lyophilised normal plasma (Coagulation Reference Plasma, Technoclone Ltd, Surrey, UK). Preliminary studies to assess the sensitivity of TGT parameters to variation in levels of FVIII were undertaken using an *in vitro* spiking model. Pooled normal plasma (not taken into CTI) was spiked with a commercial FVIII concentrate (Haemofil M, Baxter Healthcare, Berkshire, UK). The Haemofil M was reconstituted according to the manufacturer's instructions; subsequently, dilutions were made in Owren's veronal buffer with 1% bovine serum albumin, pH 7.35, and then a common volume was added to PNP to give FVIII levels of approximately 150%, 200%, 300% and 400%. These levels were verified by assay of FVIII procoagulant activity.

For all other haemostatic tests, reagents were from Siemens Healthcare Diagnostics (Marburg, Germany) and assays were performed on a CA-1500 automated coagulation analyser (Sysmex UK Ltd, Milton Keynes, UK). Innovin and Actin FS were used in prothrombin time (PT) and activated partial thromboplastin time (APTT) respectively; fibrinogen (Fg) was measured by the Clauss technique. D-Dimer was assayed using an immuno-turbidometric method (Innovance D-Dimer kit). Factor VIII was assayed using both a one-stage clotting technique (FVIII:C) with samples tested at three dilutions and a chromogenic method (FVIII:Ch). The amidolytic assay of factor VIII utilised single dilution of samples with an automatic

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