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ORIGINAL ARTICLE

A novel polymorphism in the factor XIII B-subunit (His95Arg): relationship to subunit dissociation and venous thrombosis

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Summary. *Background:* Factor (F)XIII B-subunit, which plays a carrier role for zymogen FXIIIA, is highly polymorphic, but the molecular basis for these polymorphisms and their relationship to disease remains unknown. *Objectives:* To screen the *FXIIIB* gene coding region for common variation and analyze possible functional effects. *Methods and Results:* We examined the *FXIIIB* gene by PCR-SSCP and identified three common single nucleotide polymorphisms: A8259G, C29470T and A30899G. A8259G results in substitution of His95Arg in the second Sushi domain. An FXIII tetramer ELISA was developed to analyze B-subunit dissociation from A-subunit (leading to access to the catalytic site of FXIII). Increased subunit dissociation, 0.51 vs. 0.45 (fraction of total tetramer), was found in plasma from subjects possessing the Arg-allele. However, when the variants were purified to homogeneity and binding was analyzed by steady-state kinetics, no difference was observed. The relationship between His95Arg and venous thrombosis was investigated in 214 patients and 291 controls from Leeds. His/Arg + Arg/Arg genotypes were more frequent in patients than controls (22.4% vs. 15.1%). His95Arg was also investigated in the Leiden Thrombophilia Study, in which a similar difference was observed for 471 patients vs. 472 controls (18.5% vs. 14.0%), for a pooled odds ratio (OR) of 1.5 (CI95 1.1–2.0). *Conclusions:* We have identified three FXIIIB polymorphisms, one of which codes for substitution of His95Arg. The Arg95 variant associates with a moderately increased risk for venous thrombosis, and with increased dissociation of the FXIII subunits in plasma, although *in vitro* steady-state binding between purified subunits was not affected.

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Introduction

Factor XIII (FXIII) is a protransglutaminase that on activation by thrombin catalyses the formation of ε-(γ-glutamyl)-lysine bonds in polymerized fibrin. The cross-linking of fibrin renders the clot chemically and mechanically more stable as well as resistant to fibrinolysis [1,2]. In the circulation, FXIII appears as a heterotetramer of two A- and two B-subunits that are non-covalently associated, designated as A_2B_2 [1,2]. The active site of the enzyme is located in the A-subunit. FXIII is activated by cleavage of the peptide bond between Arg37 and Gly38 of the A-subunit followed by subsequent dissociation of the B-subunit from the A-subunit in the presence of Ca^{2+} [3,4]. Both steps are necessary to provide access of the substrate to the catalytic triad of FXIII and are enhanced by the presence of the main substrate polymerized fibrin. The B-subunit has no enzymatic activity and is involved in stabilization of the A-subunit in the aqueous environment of human plasma [5,6]. It is composed of 641 amino acids divided into 10 tandem repeats of about 60 amino acids each, which are called GPI structures or Sushi domains [7,8]. Each Sushi domain is encoded by a single exon [9]. The function of these domains has yet to be fully established although they are thought to serve mainly as protein-binding module [8].

The B-subunit has been shown to be highly polymorphic using protein phenotyping techniques such as isoelectric focusing and agarose gel-electrophoresis [10–17], but the molecular genetic basis of these polymorphisms and any relation to disease has not yet been reported. We therefore (i) screened the *FXIII B*-subunit gene exons coding for the Sushi domains for polymorphisms, (ii) examined functional effects of the polymorphism(s) using a novel FXIII A_2B_2 tetramer ELISA to study A- and B-subunit dissociation in both plasma and purified systems, and (iii) investigated associations with vascular risk in two case–control studies of venous thrombosis.

Materials, methods and subjects

Polymorphism identification

Standard polymerase chain reaction (PCR) was used to amplify DNA from 12 coding regions of the *FXIII B*-subunit gene. Oligonucleotides were designed based on the genomic FXIII DNA sequence [9] obtained from GenBank using Primer version 0.5 (Whitehead Institute for Biochemical Research, Cambridge, MA, USA) with the primer positioned outside the region of interest. Primers for exon I and XII also included the 5 and 3′ untranslated regions of the gene, respectively. Optimal PCR conditions for each pair of primer are available from the authors on request. PCR-single strand conformation polymorphism (SSCP) analysis [18] using MDE gel solution (Flowgen, Leicestershire, UK) was used to screen DNA for polymorphisms in each region of the *FXIIIB* gene. Four running conditions (with or without 10% glycerol; at 4 °C or room temperature) were carried out to maximize detection rate of SSCP. DNA sequencing was conducted to characterize the nucleotide substitution associated with the polymorphisms that had been detected by PCR-SSCP. The sequencing reactions were performed using ABI Prism™ BigDye terminator Cycle sequencing Ready Reaction kit and analyzed on an ABI 310 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The His95Arg genotype was further detected by restriction analysis using *Nsi* I (2 units at 37 °C, overnight incubation). The Arg allele is associated with the loss of the *Nsi* I restriction site. For the His allele, the 264 bp fragment was cut into 135 and 129 bp fragments. The difference of 6 bp between these two digested fragments was not resolved on a 1.5% agarose gel, but all the genotypes were readily distinguishable by identification of the 264 bp fragment and the 135/129 bp doublet.

FXIII A- and B-subunit antigen and FXIII activity assay

FXIII A- and B-subunit antigen levels were determined using subunit-specific ELISAs developed in-house [19]. FXIII activity was measured using a microtiter assay using fibrinogen and 5-(biotinamido)pentylamine as substrates as previously described [19].

FXIII A_2B_2 tetramer antigen assay

An ELISA based on an anti-FXIIIB/anti-FXIIIA antibody sandwich principle was developed to measure the levels of A_2B_2 complex of FXIII. The sugar moiety of a polyclonal rabbit antihuman FXIII A antibody (Diagnostica Stago, Asnières, France) was labeled with biotin-ε-amino-caproyl hydrazide (BACH) according to the method of O'Shannessy [20]. Optimization of the ELISA was carried out by varying concentrations of the polyclonal rabbit antihuman FXIIIB antibody (Diagnostica Stago) used for coating the plates, the biotinylated anti-FXIIIA antibody for detection, the test plasma and the normal plasma for standard curve preparation, as well as streptavidin and bovine serum albumin (BSA) concentrations. Under optimal conditions, 96 well-plates (Nunc Maxisorp, Nunc A/S, Roskilde, Denmark) were coated with 1/600 final dilution of anti-FXIIIB antibody in 50 mmol L^{-1} sodium carbonate pH 9.6. Plates were washed five times with 200 μL 50 mmol L^{-1} Tris–HCl, 0.1 mol L^{-1} NaCl, pH 7.5 (Tris buffered saline or TBS) with 0.1% Tween-20, followed by blocking of non-specific-binding sites with 150 μL TBS containing 1% BSA. A range of dilutions of reference normal pooled plasma were made in TBS with 0.3% BSA. Test and control samples were diluted 1/400 and a total of 100 μL was loaded in duplicate. The plate was washed five times before applying 100 μL of biotinylated anti-FXIIIA antibody (1/500). Antibody–antigen sandwiches were detected by addition of 100 μL of 1/500 streptavidin conjugated with alkaline phosphatase (Sigma, St Louis, MO, USA). Bound conjugate was developed with 100 μL of *p*-nitrophenol phosphate (Sigma). The reaction was stopped with 100 μL 4 mol L^{-1} NaOH and absorbence was read at 405 with 550 nM as a reference filter. Levels of FXIII A_2B_2 complex were expressed in percentage of pooled normal plasma. Intra-assay CV was 5.2% ($n = 20$) and inter-assay CV was 6.8% ($n = 14$). Purified FXIII A- and B-subunits were obtained from Diagnostica Stago and analyzed for specificity of the assay.

FXIII A_2B_2 dissociation

Dissociation of FXIII A_2B_2 complex was determined by measuring levels of the complex before and after activation with a controlled amount of thrombin and calcium. Each test plasma sample was diluted 1/10 in TBS with 0.3% BSA containing 0.4% Gly-Pro-Arg-Pro-amide (Sigma) to prevent unwanted polymerization of fibrin. Five microliters of a mixture of 5 U mL^{-1} bovine thrombin (Sigma) and 0.5 mol L^{-1} $CaCl_2$ was added to 50 μL of diluted plasma. The mixtures were incubated at room temperature for 30 min and the dissociation reaction was stopped with 0.1 mol L^{-1} trisodium citrate. Samples were further diluted 1/4 and the remaining A_2B_2 complex was measured by ELISA as described above. Control experiments were performed as follows: (i) 0.5 mol L^{-1} $CaCl_2$ with TBS, (ii) 5 U mL^{-1} thrombin with TBS and (iii) TBS alone. The fraction of A_2B_2 complex that was dissociated upon limited thrombin/calcium activation was expressed as [A_2B_2 concentration before activation] − [A_2B_2 concentration after activation]/[A_2B_2 concentration before activation], the value of which is >0 and <1.

Purification of FXIII A-subunit

The FXIII A-subunit was purified from outdated platelet concentrates of mixed, unknown genotype obtained from the regional blood transfusion center. A quantity of 1 mmol L^{-1} EDTA was added to the platelet concentrates and platelets were further isolated by centrifugation at 3400 *g* for 20 min at 4 °C. Platelets were washed twice with Ca^{2+} free Tyrode's buffer containing 1 μg mL^{-1} prostacyclin, and snap-frozen in

liquid nitrogen until use. Frozen platelets were thawed, resuspended in 50 mmol L^{-1} Tris–HCl pH 7.5 containing 1 mmol L^{-1} EDTA, 2 mmol L^{-1} benzamidine and 1 μg mL^{-1} aprotinin, and were sonicated twice at maximum power for 15 s on ice. The lysate was centrifuged at 14 000 *g* for 2 h at 4 °C to eliminate particulates and was further dialyzed against equilibration buffer (20 mmol L^{-1} Tris–HCl, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} EGTA and 5 mmol L^{-1} 2-mercaptoethanol, pH 7.5), overnight with one change of buffer. The preparation was subjected to anion exchange chromatography (DEAE Sephacel, Sigma; 26 × 400 mm column) on a BioCAD SPRINT automated chromatography system (PerSeptive Biosystems, Framingham, MA, USA). FXIII A-subunit was eluted with a gradient from 0 to 0.5 mol L^{-1} NaCl in equilibration buffer. Fractions containing A-subunit were combined and concentrated in dialysis membranes on Aquacide II (Calbiochem, Merck Biosciences Ltd, Nottingham, UK), and subjected to gelfiltration on Sephadex G200 (Amersham Biosciences, Bucks, UK). Combined fractions containing A-subunit were concentrated and further purified using hydrophobic interaction chromatography (POROS 20HP2; 4.6 × 100 mm; PerSeptive Biosystems). After washing the column with equilibration buffer containing 0.5 mol L^{-1} NaCl, the A-subunit was eluted with a linear gradient of 0–60% ethylene glycol in equilibration buffer. Homogeneity of the A-subunit preparation was analyzed by SDS-PAGE. Protein concentration was measured at 280 nM using an extinction coefficient of $E^{1\,mg\,mL^{-1}}_{280\,nm} = 1.38$ [21].

Purification of FXIII B-subunit His95 and Arg95 variants

FXIII A_2B_2 tetramer was purified to homogeneity from single plasma donations of homozygous His95 (150 mL) and homozygous Arg95 (100 mL) genotypes using repeated ammonium sulfate precipitation and gelfiltration as previously described [22]. FXIII B-subunit was further isolated from these plasma FXIII preparations by anion exchange chromatography on a POROS 20 PI column (4.6 × 100 mM; PerSeptive Biosystems) connected to the BioCAD SPRINT. The preparations were dialyzed against 50 mmol L^{-1} Tris–HCl pH 7.5 containing 1 mmol L^{-1} EDTA and dissociation of B-subunit from the FXIII A_2B_2 complex was induced by incubation with 0.5 mol L^{-1} $CaCl_2$ for 30 min at 37 °C prior to application to the column. The non-bound fraction containing free B was washed from the column with 50 mmol L^{-1} Tris–HCl pH 7.5 containing 1 mmol L^{-1} EDTA and the bound fraction containing A_2 and remaining A_2B_2 was eluted (and discarded) with 10% acetic acid. Non-bound B-subunit fractions were pooled and dialyzed against 3L of TBS with 0.1% BSA with three changes of buffer to eliminate residual $CaCl_2$. Purity of the preparation was tested with SDS-PAGE and concentration was measured by absorbency at 280 nM using an extinction coefficient of $E^{1\,mg\,mL^{-1}}_{280\,nm} = 1.38$, although it was recognized that this extinction coefficient is specific for FXIII A_2B_2 tetramer, and that it may be different for isolated FXIII B-subunit.

FXIII A–B-binding analysis

Steady-state-binding kinetics between FXIII A- and B-subunit isolated from each His95 and Arg95 genotype was investigated by mixing 3 μg mL^{-1} of purified B-subunit variants with increasing amounts of purified A-subunit (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 9 μg mL^{-1}). The reaction mixture was incubated at room temperature for 30 min with gentle shaking. The mixture was diluted 1/5 in TBS with 0.3% BSA and the concentration of A_2B_2 complex that had formed was determined using the A_2B_2 ELISA. The amount of formed A_2B_2 (FXIII B_{bound}) at each concentration of A_2 was expressed as the percentage of the maximum concentration of the complex. Ligand–receptor kinetics was applied to determine the dissociation constant (K_D) from Lineweaver–Burk plots using EnzFitter for Windows version 2.0.15.0 (Biosoft, Cambridge, UK).

Subjects

DNA samples used for screening the *FXIII B*-subunit gene for polymorphisms by SSCP were obtained with informed consent from 104 healthy Caucasian subjects who attended the regional blood transfusion center. Samples were taken according to a protocol approved by the Leeds Teaching Hospitals (NHS) Trust Research Ethics Committee. Analysis of the relationship between His95Arg and FXIII measurements was performed in plasma samples from a study including 252 subjects with coronary artery disease and 192 healthy controls [23]. Two case–control groups of deep vein thrombosis from Leeds and Leiden (Leiden Thrombophilia Study or LETS) were included to analyze the relationship of His95Arg with disease. In the Leeds group, 214 Caucasian patients with a clinical diagnosis of venous thrombosis (deep vein thrombosis or pulmonary embolism) attending the local anticoagulant clinic in Leeds had been recruited as previously described [24]. Their mean age was 61 (SD 16.5), there were 92 (43.0%) females and 122 (57.0%) males. One hundred and sixteen (54.2%) patients had sustained DVT and 98 (45.8%) had a clinical diagnosis of pulmonary embolism (PE). Eighty-two cases of PE had no evidence of DVT and 16 had evidence of both DVT and PE. Previous episodes of venous thrombosis were reported in 62 (29%) of the patients. The thrombotic events were unprovoked in 150 (70.1%) of the patients. The remainder 64 (29.9%) patients had one or more risk factor (surgery, malignancy, immobilization, pregnancy, oral contraceptives or hormone replacement therapy). Two hundred and ninety one sex- and age-matched controls were recruited from the Local Family Health Service Authority (FHSA) General Practice Registers. All of them were clinically free from a personal and family history (in first-degree relatives) of vascular disease (arterial and venous). Mean age was 60 (SD 15.0), and gender distribution was 122 (41.9%) females and 169 (58.1%) males. Both patient and control groups were from the same geographical area. The study was approved by the United Leeds Teaching Hospitals Trust Research Ethics Committee. For the LETS study, a total of 474 consecutive outpatients with a first diagnosis of deep

vein thrombosis attending three anticoagulant clinics in the Netherlands – Leiden, Amsterdam and Rotterdam – were recruited as previously described [25]. All patients were younger than 70 years (mean age 45, SD 13.7) and free of known malignancy disease; there were 272 (57.4%) females and 202 (42.6%) males. Thrombotic events were unprovoked in 259 (54.6%) patients, whereas in 215 (45.4%) patients presence of one or more risk factor (surgery, puerperium, trauma, immobilization, pregnancy, or oral contraceptives) was recorded. Recurrency of thrombosis occurred in 90 (19.0%) patients over a mean follow-up of 7.3 (SD 2.7) years [26]. A total of 474 sex- and age-matched control subjects (mean age 45, SD 13.5 with identical gender distribution as the patients) who were friends or partners of the patients were included. None of the control subjects were biologically related to the patients, had a history of VT, or had any known malignancy disorder. The study was approved by the local medical ethics review board. For the purposes of the current study, 471 patients and 472 controls from LETS were available for FXIII His95Arg analysis.

Blood sample preparation

Venous blood was collected into 1.6 mg mL^{-1} EDTA for DNA extraction. DNA was extracted using a Nucleon extraction kit (Nucleon Biosciences, Coatbridge, Lanarkshire, UK) in Leeds, and by standard local salting out procedures in Leiden. For assays of FXIII activity and antigen levels, blood was taken into 0.1 mol L^{-1} trisodium citrate, separated by centrifugation at 2560 *g* for 20 min at room temperature, snap-frozen in aliquots in liquid nitrogen and stored at −40 °C until assay. Pooled normal plasma was obtained with informed consent from 47 healthy donors at the local blood transfusion center, according to a protocol approved by the Leeds Teaching Hospitals (NHS) Trust Research Ethics Committee, and used as reference plasma throughout the study.

Statistical analysis

Levels of FXIII A-subunit, B-subunit and A_2B_2 complex were not normally distributed and were transformed with logarithms to allow the use of parametric statistical testing. Data were expressed as geometric means with antilog 95% confidence intervals (CIs). FXIII activity and FXIII dissociated fraction were expressed as means with 95% CIs. A common polymorphism in the FXIII A-subunit activation peptide, close to the thrombin cleavage site, has been shown to affect rate of FXIII activation by thrombin [22]. Interactions between FXIIIB His95Arg, FXIIIA Val34Leu and levels of the dissociated fraction of FXIII were investigated by regression analysis. We calculated ORs as an estimate for relative risk, i.e., of the risk of venous thrombosis for those carrying the variant allele compared with the risk of homozygous wild-type carriers. A total of 95% CIs were based on a Poisson's distribution and calculated according to Woolf or derived from the models. A pooled OR from the two studies (Leeds and LETS) was derived by the Mantel–Haenszel method.

Results

Polymorphisms in the FXIIIB gene

All coding regions of the *FXIII B*-subunit gene were screened for polymorphisms in 104 apparently healthy Caucasian subjects. A total of three polymorphisms were detected in these subjects by PCR-SSCP in exon III, XI and XII. Figure 1 shows the different band patterns from each of three SSCP conditions used to identify the polymorphism in exon III. For each polymorphism identified, the sequence change was analyzed using two samples of each homozygote genotype and a pair of heterozygous samples. The results showed a substitution of A–G at nucleotide position 8259 (GenBank

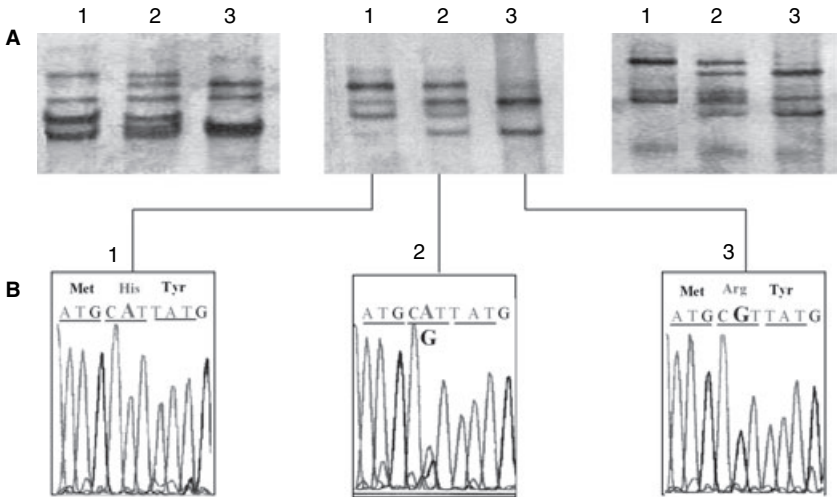

Fig. 1. SSCP gels (panel A) and sequence electropherograms (panel B) demonstrating the His95Arg polymorphism found in exon III of the FXIII B-subunit gene. Differences in band migrations were detected under three conditions: room temperature without glycerol (A-left), with glycerol (A-middle) and 4 °C with glycerol (A-right). A substitution of a nucleotide A (in bold) to G (in bold) at position 8259 caused a change of His to Arg at position 95 of mature protein (1 = homozygous His/His, 2 = heterozygous His/Arg and 3 = homozygous Arg/Arg).

locus HUMBFXIII) in exon III, a C to T transition at nucleotide position 29470 (GenBank locus HUMBFXIII) in exon XI and a substitution from A–G at nucleotide 30899 (GenBank locus HUMBFXIII) in exon XII. The nucleotide change in exon XI did not code for an amino acid change and the change in exon XII was in the 3′ UTR of the *FXIIIB* gene. The A–G transition at nucleotide 8259 of exon III resulted in a His-Arg change at codon 95 of the mature B-subunit protein.

Optimization of the FXIII A_2B_2 tetramer ELISA

An in-house ELISA was developed to measure the levels of FXIII A_2B_2 tetramer. Dilution of BACH-labeled-anti-FXIIIA antibody was chosen as 1/500 and higher maximum absorbency was obtained by increasing developing time from 10 to 15 min. Optimal dilutions of anti-FXIIIB antibody and streptavidin–alkaline–phosphatase were titrated at 1/600 and 1/500, respectively. To minimize background absorbency from 0.50 to 0.20, the concentration of BSA in the dilution buffer of the assay was increased from 0.1% to 0.3%. The dose–response curve of the A_2B_2 tetramer ELISA showed a sigmoidal shape typical for biological assays (Fig. 2A). The dilutions of pooled normal plasma used for standard curve preparation were chosen between 1/100 and 1/1600, within the linear part of the dose–response curve. A dilution of 1/400 was routinely employed for test and control plasma samples. Purified FXIII A-subunit, B-subunit and a combination of the two were used to test specificity of the assay. Recovery was $<1\%$ when incubating with A- or B-subunit alone. A 1:1 molar mixture of A- and B-subunits gave a recovery of approximately 100%.

FXIII A_2B_2 dissociation

Activation with thrombin and calcium significantly reduced FXIII A_2B_2 tetramer levels. Dissociation of the tetramer started at 0.1 U mL^{-1} and was practically complete at 10 U mL^{-1} thrombin (Fig. 2B). Addition of 0.5 U mL^{-1} thrombin led to approximately half pre-activation levels. A time-course of the dissociation with 0.5 U mL^{-1} thrombin showed a 50% reduction in levels when incubating for 30 min at room temperature (Fig. 2C). There was a similar increase in subunit dissociation rate with increasing calcium concentrations (data not shown). Control experiments showed that incubation with $CaCl_2$ alone up to 50 mmol L^{-1} or TBS alone for 30 min at room temperature did not induce dissociation of the A_2B_2. Some dissociation of the A_2B_2 complex could be induced by incubation with thrombin alone, or a high concentration of $CaCl_2$ alone (>500 mmol L^{-1}), but not to the same extent as in the presence of both thrombin and $CaCl_2$.

His95Arg and A_2B_2 dissociation in plasma

Of the three polymorphisms found in the *FXIIIB* gene, His95Arg causes a change in the amino acid sequence and was therefore studied further for functional effects on subunit dissociation. FXIII activity, A_2B_2 antigen, subunit antigen levels and dissociated fraction were examined in relation to each FXIIIB His95Arg genotype. There were no differences in FXIII activity, subunit antigen levels and A_2B_2 levels prior to thrombin activation in relation to genotype. Following activation with thrombin, a genotype-specific response was observed

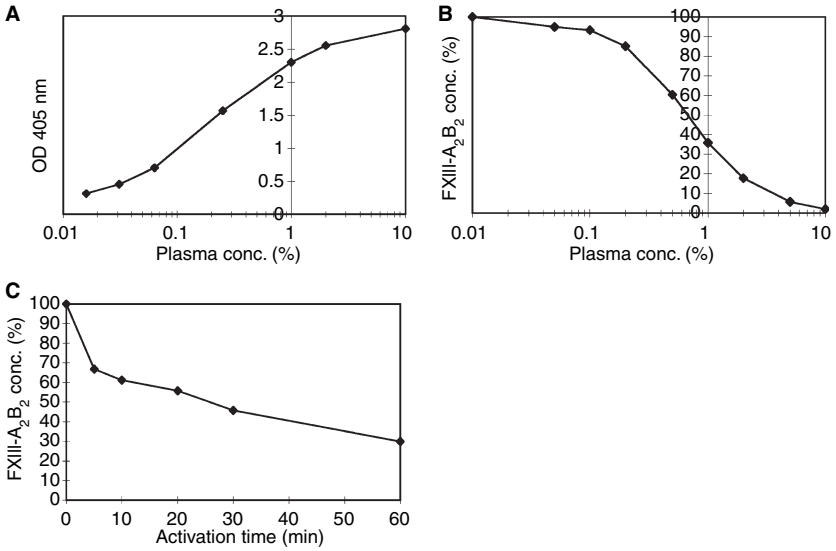

Fig. 2. Development of a FXIII A_2B_2 tetramer ELISA. Panel A shows a typical dose–response curve of the assay. Panel B shows a dose–response activation and dissociation of the FXIII subunits and hence disappearance of the A_2B_2 tetramer as analyzed by the ELISA after incubation with thrombin and calcium. By increasing thrombin concentrations from 0.01 U mL^{-1}, approximately half-normal levels of FXIII A_2B_2 were found at the concentration of thrombin at 0.5 U mL^{-1}. Panel C shows a time course of A_2B_2 complex dissociation by 0.5 U mL^{-1} thrombin. A mixture of diluted plasma, thrombin and calcium was incubated at room temperature for 5, 10, 20, 30, 40, 50 and 60 min. The complex concentration was reduced to about 50% at the incubation time of 30 min.

Table 1 Levels of FXIII subunit antigen, activity, A_2B_2 complex and dissociated fraction in relation to FXIIIB His95Arg

	His/His ($n = 361$)	His/Arg ($n = 80$)	Arg/Arg ($n = 3$)
FXIII A-subunit antigen	107.1 (103.8, 110.4)	104.8 (99.2, 110.7)	106.9 (66.0, 139.0)
FXIII B-subunit antigen	106.3 (103.8, 108.8)	106.8 (101.7, 112.0)	98.4 (81.0, 125.0)
FXIII activity	99.1 (95.3, 102.8)	103.0 (95.4, 110.5)	98.7 (64.9, 132.5)
FXIII A_2B_2 complex	86.4 (83.7, 89.2)	85.9 (80.7, 91.4)	104.4 (87.7, 130.8)
Dissociated fraction	0.45 (0.43, 0.47)	0.50 (0.47, 0.54)	0.52 (0.39, 0.60)

Assays were performed in a total of 444 subjects (252 patients with vascular disease and 192 controls). Levels of FXIII-A, B-subunit, activity and A2B2 complex are expressed as percentage of normal pooled plasma. FXIII-A and B-subunit antigen data are presented as geometric mean (95% confident interval; CI). Activity, A2B2 complex and dissociated fraction data are presented as mean (95%CI). Data for the Arg/Arg genotype are reported as mean or geometric mean (range).
Dissociated fraction $= \frac{[A_2B_2]b-[A_2B_2]a}{[A_2B_2]b}$, whereby b = before; a = after activation.

with a stepwise increase of the fraction of A_2B_2 that dissociated with the number of Arg alleles (Table 1). This increase in dissociated fraction with the number of Arg alleles was also present after stratification by FXIIIA Val34Leu polymorphism.

Binding studies with purified proteins

FXIII A-subunit was purified to homogeneity from platelets (Fig. 3). The preparation demonstrated a single band on SDS-PAGE corresponding to that of plasma FXIII A-subunit (Fig. 3B). His95 and Arg95 B-subunits were purified from plasma FXIII by incubation with calcium followed by anion exchange chromatography (Fig. 4). Figure 4B shows homogeneity of the B-subunit preparation by SDS-PAGE. The steady-state binding between purified A-subunit and B-His95 and B-Arg95 subunits was investigated using a receptor–ligand model of binding kinetics (Fig. 5). Dissociation constants (K_D) for the two forms of the FXIIIB His95Arg polymorphism were calculated from the double reciprocal plots between the concentrations of A_2 and bound B_2 (insets of Fig. 5). The K_D

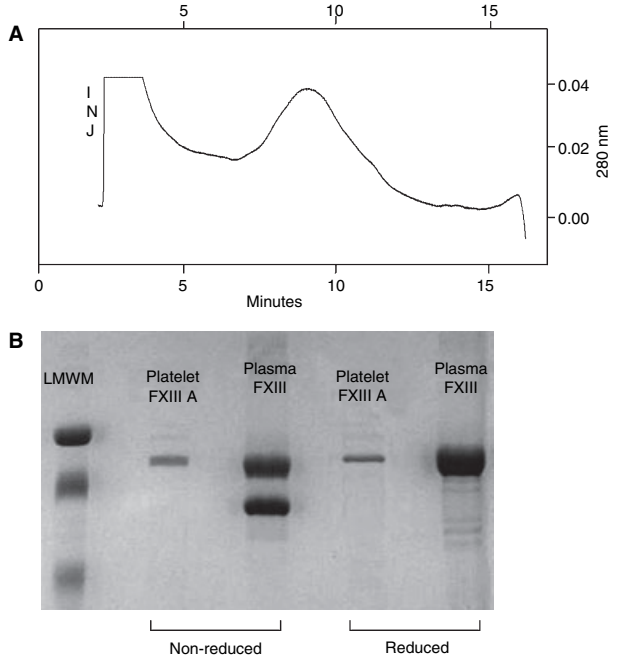

Fig. 3. Purification of the FXIII A-subunit from human platelets. Panel A shows a chromatogram from the hydrophobic interaction chromatography (final step in the purification procedure) and panel B shows SDS-PAGE of protein preparations. The FXIII A-subunit eluted from the hydrophobic interaction column between 7 and 12 min. Platelet FXIII A was purified to homogeneity as judged by SDS-PAGE. LMWM: low molecular weight marker. Right-hand side of the gel shows samples reduced with 0.1 M DTT and left-hand side non-reduced samples. Plasma FXIII produces two bands of which the top one corresponds with the A-subunit and the bottom one with the B-subunit. Plasma FXIII A- and B-subunit bands migrate at similar position under reducing conditions.

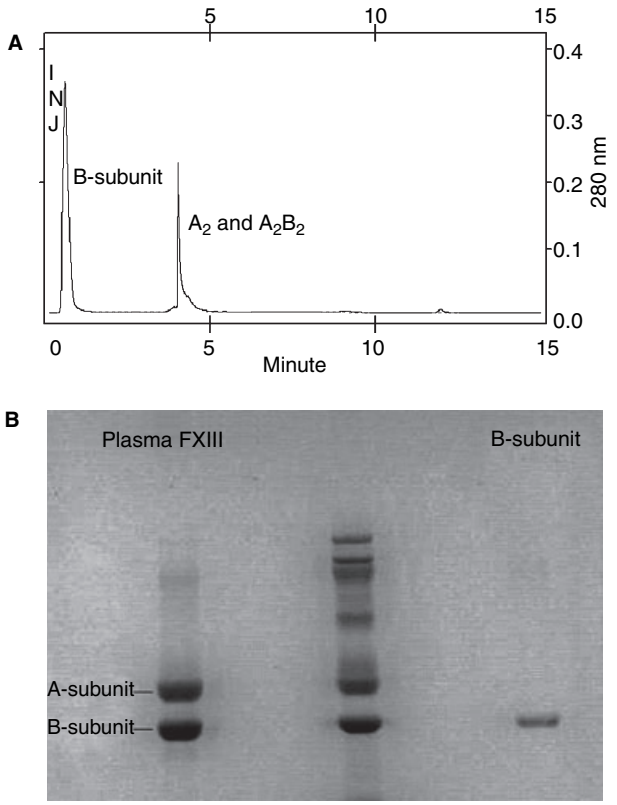

Fig. 4. Purification of FXIII B-subunit from human plasma. Panel A shows a chromatogram of anion exchange chromatography and panel B shows SDS-PAGE patterns on an 8% polyacrylamide gel of purified B-subunit of FXIII. FXIII B-subunit eluted in the flow-through fraction of the anion exchange chromatography (Panel A). The preparation showed one band on SDS-PAGE, corresponding with the B-subunit of plasma FXIII (Panel B). The middle lane in Panel B shows plasma FXIII of intermediate purity.

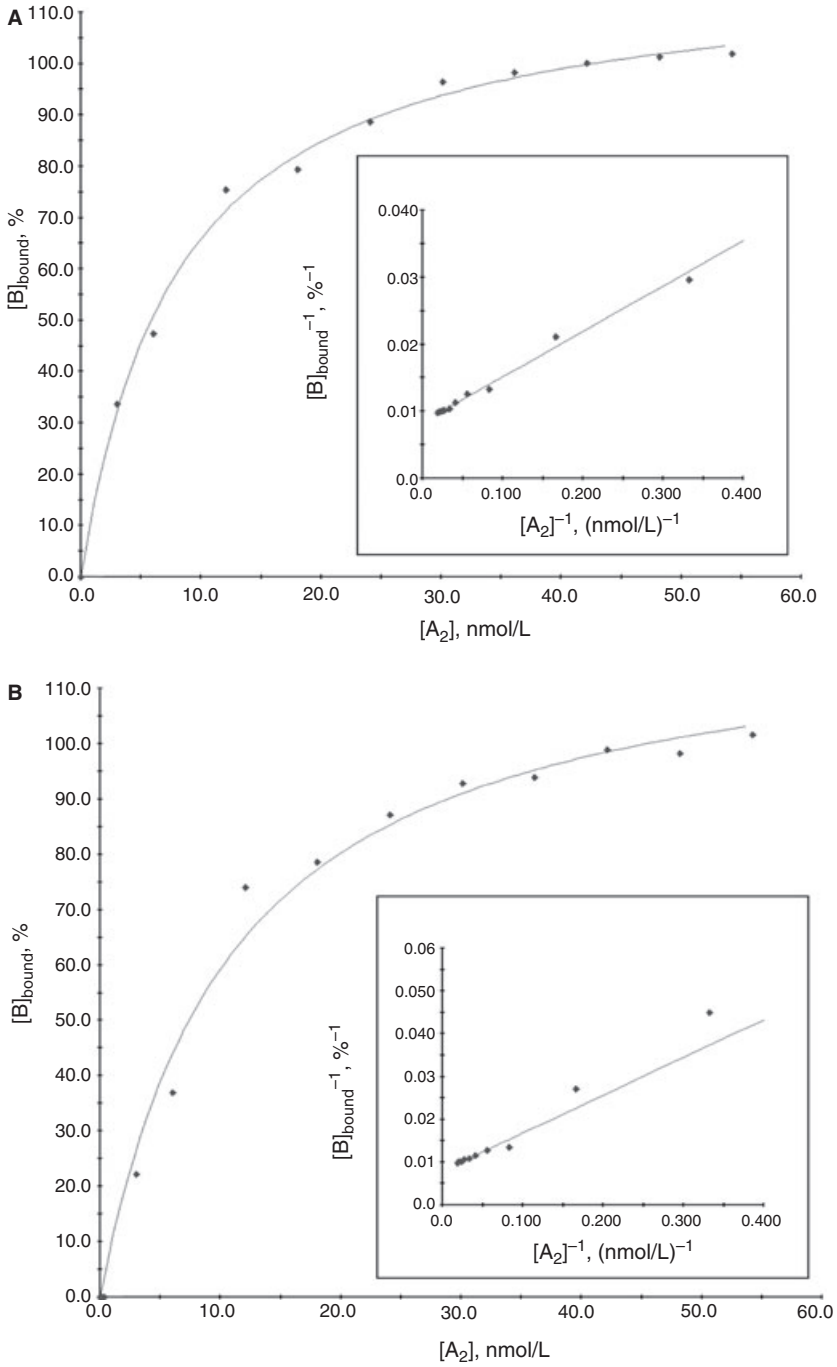

Fig. 5. Steady-state-binding kinetics of purified FXIII B-subunit variants and A-subunit. Increasing concentrations (0–54 nmol L^{-1}) of purified FXIII A-subunit [A_2] was incubated with 38 nmol L^{-1} purified FXIII His95 (Panel A) or Arg95 (Panel B) B-subunit and the amount of bound B-subunit was measured by A_2B_2 ELISA. Data were analyzed by double-reciprocal Lineweaver–Burk plots (insets) and fitted to a receptor–ligand model of binding kinetics to calculate dissociation constants. No difference in dissociation constant was observed (K_D 8.06×10^{-8} vs. 8.13×10^{-8} mol L^{-1} for His95 and Arg95, respectively).

of A–B interaction was not different for the His95 or Arg95 variants of B-subunit (8.06×10^{-8} vs. 8.13×10^{-8} mol L^{-1}, respectively).

His95Arg and venous thrombosis

The relationship between FXIIIB His95Arg and venous thrombosis was first investigated in a relatively small case–control study of 214 patients with venous thrombosis and 291 control subjects from Leeds. Frequencies of the His/Arg and Arg/Arg genotypes were greater in patients with venous thrombosis (21.5 and 0.9%) than in controls (14.4 and 0.7%, respectively; Table 2). The genotype distributions of His95Arg were also investigated in a larger case–control study of patients with venous thrombosis from Leiden (LETS). Again, the His/Arg and Arg/Arg genotypes were more frequent among the

Table 2 Genotype distribution of FXIIIB His95Arg in patients with venous thrombosis and control subjects

	Leeds			LETS		
	VT	Controls	OR (95% CI)	VT	Controls	OR (95% CI)
All subjects	214	291		471	472	
His/His	166 (77.6)	247 (84.9)	1	384 (81.5)	406 (86.0)	1
His/Arg	46 (21.5)	42 (14.4)	1.6 (1.0–2.6)	81 (17.2)	62 (13.1)	1.4 (1.0–2.0)
Arg/Arg	2 (0.9)	2 (0.7)	1.5 (0.2–10.7)	6 (1.3)	4 (0.8)	1.6 (0.4–5.7)

Genotypes expressed as number of subjects (percentage of group total).
VT, venous thrombosis; Leeds, Leeds study; LETS, Leiden thrombophilia study.

patients (17.2% and 1.3%) than controls (13.1% and 0.8%; Table 1). When we analyzed the effect of heterozygous His/Arg genotype on risk, the OR was 1.6 (CI95 1.0–2.6) for the Leeds study, and 1.4 (CI95 1.0–2.0) in the Leiden study. Similar ORs were observed for the homozygous Arg/Arg genotype in both the Leeds and LETS studies (1.5 and 1.6, respectively), although the CIs for this genotype were large because of the small number of homozygous subjects in each group. The pooled estimate of both studies for His/Arg genotype and venous thrombosis led to a Mantel–Haenszel OR of 1.5 (CI95 1.1–2.0).

Discussion

We have comprehensively investigated the coding regions of the *FXIII B*-subunit gene for common polymorphisms in a population of healthy Caucasian subjects. Three polymorphisms were found, of which one is a novel polymorphism that codes for a His to Arg substitution at amino acid residue 95 in the second Sushi domain of the B-subunit. The FXIIIB Arg95 allele associated with an approximately 50% increased vascular risk in two clinical studies of venous thrombosis, Leeds and LETS. Increased FXIII subunit dissociation rates were observed for the Arg95 variant under controlled limited activation of with thrombin and calcium in plasma, although no differences were found in steady-state-binding kinetic analysis using purified A- and variant B-subunits.

Polymorphisms of the FXIII B-subunit were first described by Board in 1980 [10]. Since that initial report, several other groups investigated phenotypic variation in FXIIIB from serum and plasma using agarose electrophoresis and isoelectric focussing [11–17]. Three common alleles of the *FXIIIB* gene – FXIIIB*1, FXIIIB*2 and FXIIIB*3 – have been described. Determination of the primary structure and nucleotide sequence of the gene for FXIII B-subunit [7,9] has made it possible to characterise polymorphisms of this protein at the DNA level. In the present study, we have systematically screened the coding region and adjacent 3 and 5′ untranslated regions of the *FXIII B*-subunit gene for polymorphisms and found three common single nucleotide substitutions. The first polymorphism concerns a substitution of C with T at nucleotide position 29470 (GenBank locus HUMBFXIII), which causes no change in the amino acid sequence of the protein and has previously been reported by Bottenus *et al.* [9]. The second polymorphism occurs at nucleotide position 30899 (an A–G transition), which is located in the 3′ untranslated region and has also been previously described [27]. The 3′ untranslated region plays a role in determining mRNA stability [28] and there is the possibility that a polymorphism occurring in this area such as A30899G may influence concentration of the messenger and protein expression. Whether A30899G indeed has any effect on mRNA stability is an area for further investigation. The third polymorphism that we found is novel, involves an A–G transition at position 8259 and results in an amino acid change from His to Arg at codon 95 of the mature protein (FXIIIB His95Arg). This polymorphism is relatively common with an approximate allele frequency of 15–25% in our subjects from the North–West of Europe.

The substitution of His95 with Arg occurs in the second Sushi domain of the FXIII B-subunit. As this domain may be involved in the binding between the A- and B-subunit, we investigated the effect of the polymorphism on A–B-subunit interactions and binding kinetics in plasma and purified systems. An ELISA based on anti-FXIIIB/anti-FXIIIA antibody sandwich principle was developed to test this hypothesis. The assay specifically detects the A_2B_2 complex but is unable to detect either the A- or B-subunit alone after dissociation has taken place. The use of standard doses of thrombin to induce dissociation of the A_2B_2 complex could therefore be used to give a rough indication of the effects of the His95Arg polymorphism on this process. Levels of FXIII A-subunit antigen, B-subunit antigen and A_2B_2 complex before thrombin activation were not different in plasma samples from subjects possessing the Arg95 allele compared with His95, demonstrating that the substitution of His95 with Arg does not affect protein expression and/or secretion. However, levels of A_2B_2 tetramer after limited thrombin activation were significantly decreased and the calculated dissociated fraction increased in plasma from subjects possessing the Arg95 variant of FXIII B-subunit.

The decrease of A_2B_2 levels after limited thrombin activation with the corresponding increase in dissociation indicated that the substitution of His95 with Arg in the FXIII B-subunit weakens the heterologous association with the A-subunit. Additionally, as this polymorphism was found in exon III of FXIIIB, which codes for the second Sushi domain, these results may implicate a role for this domain in the binding of the catalytic A-subunit to the carrier B-subunit. To investigate this further, we purified the His95 and Arg95 variants of the FXIII

B-subunit from human plasma, and analyzed binding kinetics of its association with A-subunit purified from platelets. Using receptor–ligand kinetics, we analyzed K_D for A–B interaction, which overall was similar to that described by Radek *et al.* [29]. However, we found no difference in the K_D for the two B-subunit variants, indicating that the substitution of His95 with Arg has no direct effect on steady-state binding to A-subunit. The reason for this apparent discrepancy between our results using purified proteins and those obtained from plasma, in which dissociation of the subunits was found to be increased in subjects possessing the Arg allele compared with subjects possessing the His allele, is not entirely clear. There are several hypotheses that could provide explanation for these apparently inconsistent findings: (i) there are no differences in binding between the His95 and Arg95 variants and the plasma results are a chance finding, (ii) an effect of His95Arg shows up only in combination with other genetic or environmental factors as present in plasma but not in a purified system, or (iii) there are no differences in steady-state binding but in other aspects of the dissociation reaction of the A- and B-subunits that occur when FXIII is activated. This later hypothesis may require further investigation using different methodologies to investigate protein interaction/dissociation.

As conditions in a purified *in vitro* study are different from those of *ex vivo* dissociation experiments, plasma factors other than FXIII and fibrinogen may be involved in regulating the dissociation of the complex in plasma. Alternatively, other polymorphisms of the *FXIIIA* gene may interact with the FXIIIB His95Arg polymorphism and contribute to alterations in the dissociation of the subunits. After adjustment for the Val34Leu polymorphism in the FXIII A-subunit, however, subunit dissociation in plasma samples containing the Arg95 variant of B-subunit remained increased. This indicates that His95Arg and Val34Leu have separate and possibly additive effects on the activation rate of FXIII. Interactions between FXIIIB His95Arg and other common polymorphisms of the A-subunit may also be possible. It has been reported that Leu564 and Tyr204 variants of the FXIII A-subunit for example are associated with altered cross-linking activity of the enzyme [30]. However, the relative contribution of these latter polymorphisms to vascular risk [31] and FXIII activation rates by linkage analysis in twins (de Lange *et al.*, in prep.) is negligible. Hence, although a combinatory effect with other FXIII polymorphisms on subunit dissociation cannot be excluded from our findings, they are unlikely to be major.

In two groups of patients with venous thrombosis from Leiden and Leeds, similar differences in genotype distribution of the FXIIIB His95Arg polymorphism were found, consistent with a 50% increased risk of venous thrombosis. With an overall frequency of the Arg95 that was slightly less in LETS than in Leeds, there was a similar over-representation of the allele in patients with venous thrombosis, leading to a pooled risk estimate for venous thrombosis of 1.5 in subjects carrying the Arg95 allele. Together, our data show the identification of three polymorphisms in the gene for FXIIIB, of which one leads to an amino acid change in the second Sushi domain of FXIII B-subunit of His95 to Arg. The substitution of His95 with Arg increases FXIII subunit dissociation under limited, controlled activation conditions in plasma and associates with a moderately increased risk for venous thrombosis.

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