Ph.D. THESIS

NEW METHODS FOR THE DETERMINATION OF FACTOR XIII OF BLOOD COAGULATION USING MONOCLONAL ANTIBODIES

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Introduction

Blood coagulation FXIII is a protransglutaminase that exists in two forms. In the plasma it regularly circulates as a heterotetramer consisting of two potentially active A (FXIII-A) and two inhibitory B (FXIII-B) subunits (A_2B_2). FXIII-A is synthesised primarily in cells of bone marrow origin, while FXIII-B is synthesised in the liver. The formation of tetrameric complex very likely occurs in the plasma. FXIII-B subunits are in excess to FXIII-A and about 50 % of them circulates in free, non-complexed form. The cellular form of FXIII, a homodimer of FXIII-A (A_2), is expressed in platelets and in monocytes/macrophages in considerable amount.

The molecular mass of the FXIII-A subunit is ~83 kDa, non-glycosylated polypeptid. The primary structure of the A subunit of plasma FXIII appears to be identical to that of cellular FXIII. The active thiol group is in Cys-314, which is surrounded by a sequence (Tyr-Gly-Gln-Cys-Trp) common to transglutaminases of different sources. A number of polymorphisms have been identified in the amino acid sequence of XIII-A. The biochemical effect of these polymorphisms has not been explored. One of them, the Val34Leu polymorphism, occurs in the activation peptide, just 3 amino acid residues away from the thrombin activation site. A protective effect against myocardial infarction and thrombotic cerebral artery occlusion has been attributed to the Leu34 allele.

The molecular mass of the FXIII-B subunit is ~80 kDa, it contains 8,5% carbohydrate. FXIII-B is a typical mosaic pretein; it contains ten tandem repeats of approximatelz 60 amino acids each, the so called sushi-domains.

FXIII is a zymogen that is activated by the concerted action of thrombin and Ca^{2+} in the last stage of coagulation process. During its

activation an activation peptide of 37 amino acid residues is cleaved off from the N-terminus of FXIII-A by thrombin, then in the presence of Ca^{2+} the inhibitory/protective FXIII-B dissociates and the truncated FXIII-A becomes transformed into an active transglutaminase (FXIIIa).

In vitro cellular FXIII can be activated by thrombin and Ca^{2+} in the same way as plasma FXIII, with the exception of the dissociation of B subunits. In vivo in platelets the intracellular activation of FXIII does not involve proteolztic cleavage of the protein and during platelet activation, when intracellular Ca^{2+} concentration becomes elevated, the slow, progressive nonproteolztic activation of FXIIIA takes place.

Transglutaminases cross-link polypeptide chains through γ-glutamyl (ε-lysyl) isopeptide bonds. The main physiological task of activated FXIII in the plasma is to cross-link fibrin chains and covalently attach α_2-plasmin inhibitor to fibrin. These cross-linking reactions mechanically strengthen the fibrin clot and protect it from fibrinolysis. In addition to fibrin FXIIIa has several other protein substrates. Most of these protein substrates fall into three main categories: proteins of the coagulation and fibrinolytic system (factor V, plasminogen activator inhibitor-2), adhesive proteins (fibronectin, vitronectin, von Willebrand factor, thrombospondin), and contractile/cytoskeletal proteins. (actin, myosin). Cross-linking of these proteins, FXIII has involved in cell adhesion and migration, wound healing, tissue remodeling, maintaining pregnancy.

The function of cellular FXIII is far from being elucidated. Megakaryocytes, monocytes/macrophages can be considered as sites of synthesis for FXIII-A subunit of plasma FXIII, and platelets can be considered as carrier cells transporting FXIII-A to the site of their destruction. Besides being the precursor of the A subunit in the plasma, the function of platelet FXIII very likely covers other yet unexplored areas.

Inherited deficiencies of FXIII can be classified as FXIII-A and FXIII-B deficiencies. The former represents a rare, but usually very severe bleeding diathesis of autosomal recessive inheritance with low (in most cases <1% of average normal) FXIII activity and FXIII-A antigen in the plasma and in the platelets. In FXIII-A deficiencies the plasma level of FXIII-B is about 50% of normal average. In the even less frequent FXIII-B deficiency FXIII-B is hardly detectable in the plasma. In this case the bleeding symptoms are due to a considerable decrease of plasma FXIII-A in the absence of the protective FXIII-B. FXIII-A content of platelets is normal. Severe acquired plasma FXIII deficiency with intact platelet FXIII-A can be caused by antibodies against either of the FXIII subunits. Inherited FXIII-A deficiency results in severe bleeding disorder which frequently requires life-long substitution therapy.

Acquired FXIII deficiencies may develop in a number of diseases including different malignant haematological diseases, severe liver disease, acute stages of inflammatory gastrointestinal diseases and Henoch Schönlein purpura, conditions with consumption coagulopathy. In these cases FXIII level as well as the severity of bleeding diathesis vary in a rather broad range.

Increased plasma FXIII level has been reported in patients with obliterative atherosclerosis and diabetic angiopathy and in chronic leukemia patients with increased megakaryocytic activity.

The determination of FXIII plasma level can be based on functional or immunological methods. Functional methods based on two different principles. The UV spectrophotometric assays based on monitoring the release of ammonia in the transglutaminase reaction represent the most popular functional assays. However, the relatively low sensitivity of these assays do not allow to perform reproducible measurement of FXIII activity

below 5% of normal average. In such a low range of FXIII activity the traditional, rather cumbersome amine incorporation tests, measuring the incorporation of fluorescent, radiolabeled or biotinylated amine substrates into proteins are still to be performed.

For the adequate diagnosis and classification of FXIII deficiencies it is crucial to measure FXIII activity and the concentration of the complex FXIII and the individual FXIII subunits in the plasma. For classification it is also important to determine FXIII-A in platelets. For these purposes we need immunological methods using subunit specific antibodies. A few immunoassays have been developed for the measurement of FXIII subunits However, there is only a single report on the measurement of the concentration of tetrameric complex plasma FXIII. Yorifuji et al. described two sandwich ELISA methods for the measurement of plasma FXIII. In both methods polyclonal anti-FXIII-B antibody was used. The first method used the polyclonal anti-FXIII-B antibody for capture and an HRPO-labelled polyclonal anti-FXIII-A for the tag IgG. In the second method the polyclonal anti-FXIII-B antibody served as tag IgG and an anti-FXIII-B monoclonal antibody was used for capture. The latter antibody recognised FXIII-B primarily in the A_2B_2 complex.

Objectives

Diagnosis and classification of FXIII deficiencies require the use of both functional and immunoassays. For the adequate diagnosis and classification it is crucial to measure the FXIII concentration of the complex FXIII and also the individual FXIII subunits in the plasma. For classification it is also important to determine FXIII-A in platelets.

A highly specific, well reproducible immunoassay, which is available in almost unlimited quantities, is based on the use of monoclonal antibodies. Therefore we have decided:

1. to produce monoclonal antibodies after immunization with the isolated FXIII subunits and plasma FXIII
2. to test the specificity of monoclonal antibodies generated
3. to develop sandwich ELISA methods for the determination of the komplex and the isolated subunits
4. to test the specificity, precision and reproducibility of the developed methods
5. to establish the reference interval for plasma FXIII and cellular FXIII using the new assays
6. to compare the results obtained with the new methods with the results of activity measurement
7. detection of the different FXIII forms in the plasma, other body fluids and cell lysates using the developed methods

Materials and Methods

Purification of FXIII (A_2B_2), FXIII-A and FXIII-B

Plasma FXIII was prepared from outdated human plasma essentially as described by Lorand et al. To obtain highly purified B-subunits, the A subunits were removed from the tetrameric complex by repeated freezing and thawing followed by chromatography on organomercurial agarose. Crude FXIII-A was prepared from human placenta. The preparation was further purified by ion-exchange chromatography, gel filtration and finally by affinity chromatography.

Production of Mouse Monoclonal Antibodies against FXIII Subunits

Balb/c mice were immunised with the purified plasma FXIII and FXIII subunits according to the protocol described in the I. attached paper. Immune spleen cells were fused with Sp-2/o myeloma cells. The culture supernatants were tested for reaction with FXIII-A, FXIII-B or with plasma FXIII (A_2B_2) in indirect ELISA systems. Hybridomas producing antibodies that reacted with the respective isolated subunits and the tetrameric complex were selected and cloned by limiting dilution technique. Antibodies were isolated from ascitic fluid by ammonium sulphate precipitation.

Selection of Monoclonal Antibodies

Hybridomas producing antibodies that reacted with the respective isolated subunit and the tetrameric complex equally well were selected by using indirect ELISA systems.

The question if the anti FXIII-A antibodies we intended to use in the FXIII-A ELISA reacted with different epitopes was addressed by the ELISA double antibody binding system and by competition direct ELISA.

Labeling of Monoclonal Antibodies

The selected antibodies were biotinylated via their sugar moieties or labelled with HRPO.

One step ELISA methods for the determination of plasma FXIII ($FXIII\text{-}A_2B_2$) and FXIII-A subunit concentration

Combinations of different anti-B and anti-A antibodies were tested in sandwich ELISA systems and three antibodies were selected for developing the one step assays. The anti-FXIII-B 1D2D6 and anti-FXIII-A 3A6H7 antibodies were biotinylated. The anti-FXIII-A 3B2H12 antibody was labelled with HRPO.

The Determination of Reference Interval for Plasma FXIII Concentration using the developed method

The guidelines of the National Committee for Clinical Laboratory Standards (NCCLS document C28-A, Villanova, PA) were followed. Blood samples were obtained from 102 females and 87 males. The apparently healthy consenting volunteers were members of the hospital staff and medical students (I. publication).

Preparation of platelet lysate

Blood samples were obtained from 41 apparently healthy volunteers and consenting patients. Washed platelet suspensions were prepared from

blood anticoagulated with acid-citrate-dextrose and were solubilised by 1% Triton X-100 and sonified (IV. publication).

Other Analytical Procedures

A spectrophotometric kinetic method, developed in our department was used for the determination of FXIII activity in the plasma (III. attached paper).

FXIII Val34Leu polymorphism was analyzed with a newly developed method based on the principle of amplification-created restriction site (II. publication).

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) were performed in 61 children: 33 children with recurrent bronchitis (25 boys, 8 girls, aged: 1-15 years, mean=2,7±3,2 years), 8 children with fibrosing alveolitis (5 boys, 3 girls, aged: 2-14 years). The control group consisted of 22 children (aged: 2-14 years, mean=6,8±5,2 years) with suspicious histories of foreign body aspiration but it was not confirmed by bronchoscopy and no sign of bronchial inflammation was seen. BAL was performed under general anesthesia with relaxation. The recovered fluid was filtered through sterile nylon gauze and centrifuged at 800 x g for 10 min. The cells were washed and resuspended in RPMI medium containing 0.2 % bovine serum albumin. Cell viability was assessed by trypan blue exclusion test. Differential cell count and numbers of injured macrophages were determined from smears stained with May-Grünwald-Giemsa. The albumin content of BAL fluid was measured by nephelometry (Behring Nephelometer Analyser-100).

Statistical methods

Four parameter logistic curve fitting and analysis of data were performed using Genesis-Lite software of Labsystems (Helsinki, Finland).

The distribution of FXIII concentration values in the reference sample group was investigated for normality by Lilliefors, Kolmogorov-Smirnov and Shapiro-Wilk tests. The reference interval was calculated by both parametric and non-parametric methods. The justification of partitioning the reference values according to gender was tested by the z-test and by comparing standard deviations of the male and female reference sample groups. Statistical significance was tested by Student's t-probe.

The correlation between two methods was analysed by Deming regression using Cbstat, a program written and kindly provided by Dr. K. Linnet. Error calculations were based on jack-knife principle. Statistical significance of the deviation of the intercept from zero was checked by Student t-test. Normalised values were also investigated with a Bland-Altman plot.

Mann-Whitney U test was used to compare the means of BALF FXIII concentration of control and patient groups. A value of $p < 0.05$ was considered statistically significant.

Results

I. One step sandwich ELISA method for the determination of plasma factor XIII and the evaluation of the method

Generation of FXIII specific monoclonal antibodies.

After immunization with the isolated FXIII-A subunit 38 specific antibody producing hybridoma clones were selected. 23 of them were further selected according to the antibody affinity and cloned. Most of the antibodies reacted with the isolated A subunit and the tetrameric complex equally well. Three antibodies reacted exclusively with the free FXIII-A subunit.

After immunization with plasma FXIII 54 specific antibody producing hybridoma clones were selected. Most of the antibodies were specific to the B subunit and all reacted with the isolated B subunit and the tetrameric complex equally well. Six antibodies reacted with the FXIII-A subunit. 20 hybridomas producing FXIII B subunit specific antibody were further selected according to their antibody affinity and cloned.

One step sandwich ELISA method for the determination of plasma factor XIII concentration.

The antibodies that reacted with FXIII-B subunit were biotinylated via their sugar moieties. Anti-FXIII-A antibodies were labelled with HRPO. Combinations of different anti-B and anti-A antibodies were tested in sandwich ELISA system and the two antibodies the combination of which performed best, anti-FXIII-B 1D2D6 and anti-FXIII-A 3B2H12, were selected for developing the assay.

We developed a one-step sandwich ELISA using biotinylated anti-FXIII-B, HRPO-labelled anti-FXIII-A monoclonal antibodies and streptavidin-coated microplate. The reaction can be carried out in less than two hours.

The developed assay is specific to the complex plasma FXIII, no reaction with cellular FXIII (A_2) or with free B subunit of FXIII was observed even when the individual subunits were present in a molar concentration 4-fold higher than the linearity limit for FXIII tetramer. The addition of free FXIII-A or FXIII-B to the tetrameric A_2B_2 complex, even in 8-fold molar concentration, had no influence on the plasma FXIII concentration values measured by the assay. We demonstrated that neither fibrinogen nor other plasma components interfere with the FXIII-binding characteristics of the monoclonal antibodies used in the assay.

The assay is linear up-to 40 μg/L of FXIII in the assay mixture and extremely sensitive, its detection limit was found to be 0.95 μg/L in the diluted sample which corresponds to 0.05% of average normal plasma FXIII concentration. The assay has good reproducibility (the within-batch reproducibility (optimal condition variance) in the normal and low range was 2.0% and 3.3%, respectively, the day to day variation of the assay in the normal and low range and was 5.5% and 8.7% respectively.

Reference Interval of Plasma FXIII Concentration

FXIII determination was carried out on plasma samples from 189 apparently healthy reference individuals (102 females and 87 males). All three statistical methods used for calculation verified normal distribution of the reference values. Calculation of the reference interval by parametric and non-parametric method gave identical results and established a reference interval of 14.0-28.0 mg/L (67-133% of normal average) for plasma FXIII

mass concentration of the total population. Partitioning the reference values according to gender and calculating separate reference intervals was not justified.

Correlation of the FXIII concentrations measured by the ELISA with the results of the Spectrophotometric Functional Assay

Results obtained with ELISA and with the spectrophotometric functional assay on plasma samples from 141 healthy volunters and 200 patients were compared and a linear relationship was found with r = 0,893 correlation coefficient.

FXIII concentration in the plasma of persons with different FXIII Val34Leu genotype

Using the newly developed assay FXIII determination was carried out on plasma samples from 189 apparently healthy reference individuals. The normal frequency distribution of plasma FXIII concentration was proven. This finding suggested that the investigated controls represented a continuous single population not partitioned according to FXIII genotypes. In agreement with this finding, it was shown that there were no significant differences in the plasma FXIII concentrations of wild-type, heterozygous and homozygous Val34Leu groups.

II. One step sandwich ELISA method for the determination of FXIII-A and the evaluation of the method

Development and evaluation of the method

The same anti-FXIII-A antibody (3B2H12) that had been selected as tag-antibody in the ELISA developed for the determination of complex

plasma FXIII was used in the FXIII-A ELISA, as well. The antibody was labelled with HRPO. The 3A6H7 anti-FXIII-A monoclonal antibodiy was selected as capture antibody and biotinilated. Non-identity of the epitopes that reacted with the capture and detection antibodies was verified by two different methods.

The lower limit of detection of the assay was estimated to be 0.35 ng/ml. A measuring range of 0.4-40 ng/ml was selected from the calibration curve. Using 1,000-fold sample dilution, as recommended for standard procedure in plasma and platelet lysate, this range corresponds to 0.4-40 µg/ml FXIII-A concentration. In the case of a 10-fold dilution of plasma samples FXIII-A concentration as low as 4 ng/ml could be measured.

The absorbance measured with FXIII-A was not influenced by the addition of equimolar FXIII-B, i.e. the formation of tetrameric complex (A_2B_2) did not influence the accessibility of anti-FXIII-A-1 and anti-FXIII-A-2 antibodies to the respective epitopes. FXIII-B alone showed no reaction in the assay.

Comparison with FXIII A_2B_2 ELISA and with FXIII activity measurement

The results of FXIII-A and FXIII A_2B_2 ELISA were compared on 214 plasma samples including samples with FXIII concentration below, within and above the reference interval. The molecular mass of FXIII-A and FXIII-B is 83 kD and 80 kD, respectively. As in healthy individuals and in patients without FXIII-B deficiency FXIII-A is fully complexed with FXIII-B the mass concentration of FXIII-A was expected to be 51% of the concentration of FXIII A_2B_2 complex. In close agreement with this calculation the slope of the regression line was 0.52, the intercept did not deviate significantly from zero and FXIII-A and FXIII A_2B_2 concentrations showed good correlation

(r= 0.965). These findings support the results obtained with purified proteins and clearly indicate that complexing with FXIII-B and binding to fibrinogen do not influence the determination of FXIII-A and the assay reliably measures FXIII-A concentration in plasma.

FXIII-A concentrations were also compared with the results of FXIII activity measurement. Although the two methods are based on different principles the results correlated fairly well (r= 0.870).

FXIII-A in the platelets

Washed platelets were prepared from the blood of 41 healthy individuals and lysed by 1% Triton X-100. FXIII-A and protein concentration in the lysate was determined. The average FXIII-A content of a single platelet was estimated to be 60 ± 10 fg. According to our calculation FXIII-A represented about 3 % of total platelet proteins.

FXIII concentration in the bronchoalveolar lavage fluid (BALF)

Using the developed two methods we investigated FXIII in the bronchoalveolar lavage fluid of 66 children (20 control, 33 with chronic brochitis and 11 with alveolitis. In control BALF FXIII A_2 was present in small but well detectable concentration while no plasma FXIII (A_2B_2) could be measured. In patients with severe chronic bronchitis and alveolitis the concentration of free FXIII-A significantly increased and plasma FXIII was also present without bronchoscopically observed bleeding.

Discussion

The determination of plasma FXIII is indicated in the following clinical conditions:

1/ Diagnosis and classification of inherited deficiencies of either of the FXIII subunits,

2/ Diagnosis and monitoring of acquired FXIII deficiencies,

3/ Monitoring replacement therapy,

4/ As FXIII has been implicated in the risk for arterial vascular disease and venous thrombosis the assessment of FXIII concentration might be important in this respect, as well.

As it has been mentioned in the introduction basically there are two ways for the determination of FXIII: FXIII activity measurement and the determination of FXIII antigen concentration by immunological methods. The simplicity of the UV spectrophotometric test based on monitoring ammonia released by activated FXIII and the ease of its automatisation makes the test suitable for being the front-line functional assay in detecting FXIII deficiencies and it is used in clinical laboratories with increasing frequency. However, this assay is relatively insensitive and below 5% of FXIII activity neither the precision nor the accuracy of the assay is satisfactory. Once the diagnosis of severe FXIII deficiency is established the quantitative measurement of FXIII activity in such low range requires the use of one of the cumbersome, time-consuming and not well standardisable amine incorporation assays.

The determination of mass concentration of plasma FXIII by an immunoassay is useful in measuring low level of FXIII quantitatively, in monitoring substitution therapy and it is essential for the classification of congenital FXIII deficiencies. Several immunoassays for the measurement

of FXIII have been reported. All, but one measured the concentration of A or B subunit of FXIII and not that of the tetrameric A_2B_2 complex.

The concentration of the individual subunits does not necessarily relate to the concentration of plasma FXIII. In FXIII-B deficiency, for instance, no A_2B_2 complex is present while FXIII-A is detectable, although in low concentration. In normal conditions, about 50% the B subunits in the plasma exists in free form and its concentration can change, at least to a certain limit, independently from the concentration of A_2B_2 complex. A further problem with the assays mentioned above was that the equal reactivity of complexed and free subunits with the antibodies was not proven. Finally, the assays were not calibrated against purified FXIII and therefore, the results could be expressed only as percentage of normal and not as mass concentration. Yorifuji et al. described two sandwich ELISA methods for the measurement of plasma FXIII (16). In both methods polyclonal anti-FXIII-B antibody was used. The first method used the polyclonal anti-FXIII-B antibody for capture and an HRPO-labelled polyclonal anti-FXIII-A for the tag IgG. In the second method the polyclonal anti-FXIII-B antibody served as tag IgG and an anti-FXIII-B monoclonal antibody was used for capture. The latter antibody recognised FXIII-B primarily in the A_2B_2 complex. The interference of fibrinogen with the two assays was not investigated. However, in separate experiments it was shown that at lower plasma dilutions fibrinogen interfered with the binding of polyclonal anti-FXIII-A antibody to the antigen. In the second assay the free B subunit gave some interference with the determination of the tetrameric complex which might explain why the assay measured an unusually high, 0.9±0.2 mg/L (~5%), A_2B_2 concentration in 11 patients with homozygous FXIII deficiency.

We developed one-step new sandwich ELISA assays for the determination of FXIII A_2B_2 and FXIII A_2 using biotinylated anti-FXIII-B, biotinylated anti-FXIII-A and HRPO-labelled anti-FXIII-A monoclonal antibodies. We used the same HRPO-labelled anti-FXIII-A antibody in the two assay. In a streptavidin-coated microplate the reaction can be carried out in less than two hours. Free FXIII subunits did not interfere with the assays. As in the plasma FXIII, due to its low dissociation constant (K_d ~10^{-8} M), is fully associated with fibrinogenit was important to exclude the possible influence of fibrinogen on the assays. The presence of fibrinogen had no effect on the measurement of purified FXIII. Furthermore, the recovery of FXIII added to normal or FXIII deficient plasma was close to 100%. This facts made it possible to calibrate the assays for purified FXIII preparation and express the results as mass concentration. The results obtained with the ELISA technique and with a kinetic spectrophotometric assay were comparable.

The high sensitivity and good reproducibility of the assays allow the quantitative measurement of traces of FXIII present in the plasma of some patients with homozygous FXIII deficiency (~0,05 %). As relatively low amount of FXIII is required for maintaining sufficient haemostasis and pregnancy the knowledge of exact FXIII level at low FXIII concentrations might be of predictive value on the phenotypic appearance of a FXIII deficiency and on the requirement of substitution therapy. It was also demonstrated that the assays are suitable for monitoring the decay of FXIII following substitution therapy.and the detection of wery low level (ng/mL) of FXIII in different body fluids.

Using the developed methods we demonstrated that in the bronchoalveolar lavage fluid of patients with chronic inflammatory diseases

plasma FXIII can be detected and the amount of cellular FXIII released from damaged or activated macrophages significantly increased.

A reference interval of 14.0-28.0 mg/L for plasma FXIII mass concentration was established. The distribution of FXIII levels measured in 189 healthy volunteers was Gaussian and no gender difference was observed. It is interesting that in the single previous report, in which plasma FXIII mass concentration was determined, an average of 21.6 mg/L plasma FXIII was measured on 13 healthy controls. This value is almost identical with the normal average (21.0 mg/L) calculated in our study from a much higher number of individual results. Plasma FXIII concemtration was not influenced by the Val34Leu polymorphism of plasma FXIII.

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