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BLOOD COAGULATION FACTOR XIII IN HUMAN TEARS

by Zsuzsanna Zita Orosz, M.D.

Supervisor:

Prof. László Muszbek, M.D., Ph.D., D.Sc., M.H.A.Sc.

UNIVERSITY OF DEBRECEN
Kálmán Laki Doktoral School

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Kálmán Laki Doctoral School of Thrombosis, Hemostasis, and Vascular Diseases, University of Debrecen

Head of the **Examination Committee**:   Prof. Dr. József Balla, M.D., Ph.D., D.Sc.
Members of the Examination Committee: Prof. Dr. Andrea Ludány, M.D., Ph.D., med.habil.
                                        Prof. Dr. János Németh, M.D., Ph.D., D.Sc.

The Examination takes place at the Library of the Clinical Research Center, Medical and Health Science Center, University of Debrecen
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Head of the **Defense Committee**:   Prof. Dr. József Balla, M.D., Ph.D., D.Sc.
Reviewers: Prof. Dr. János Kappelmayer, M.D., Ph.D., D.Sc.
                                        Dr. Illés Kovács, M.D., Ph.D.
Members of the Defense Committee: Prof. Dr. Andrea Ludány, M.D., Ph.D., med.habil.
                                        Prof. Dr. János Németh, M.D., Ph.D., D.Sc.

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BACKGROUND

Blood coagulation factor XIII (FXIII), also known as Laki-Lorand factor, is a clotting factor responsible for making mechanically stronger fibrin clots and is a key regulator of fibrinolysis. In the plasma it circulates as a tetrameric zymogen (pFXIII; FXIII-A_2B_2) that consists of two potentially active A subunits (FXIII-A) and two inhibitory/carrier B subunits (FXIII-B). FXIII-A dimer (FXIII-A_2) is also present in the cytoplasm of certain cells (cellular factor XIII; cFXIII). Platelets and megakaryocytes contain cFXIII in huge quantity; cFXIII is also present monocytes and in the bone marrow precursor of these cells. FXIII-A was also detected in a number of monocyte-derived macrophages including macrophages of different serous cavities, alveolar macrophages, tumor-associated macrophages, histiocytic and dendritic cells of lymph nodes, connective tissue histiocytes, perivascular dendritic macrophages, dermal dendocytes, etc. Recently, it was shown that in vitro a bronchial epithelial cell line is also able to synthesize FXIII-A that appears in the culture supernatant. FXIII-B is a glycoprotein, expressed in the liver and secreted by hepatocytes, its molecular mass is approximately 80 kDa. In normal conditions FXIII-B is in excess of FXIII-A in the plasma and about 50% of it circulates in free, non-complexed form. FXIII subunits derived from separate cellular sources form complex in the circulating blood. A reference interval of 14-28 mg/L has been established for FXIII-A_2B_2 in human plasma, where practically all FXIII molecules are bound to fibrinogen.

The activation of pFXIII occurs in the final phase of the clotting cascade by the concerted action of thrombin and Ca^{2+}, the reaction takes place on the surface of newly formed fibrin. In the extravascular environment cFXIII could be activated by thrombin and Ca^{2+} the same way as pFXIII, excluding the dissociation of FXIII-B. In the intracellular environment, during platelet activation, cFXIII does not need proteolytic cleavage for activation, the

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elevation of intracellular Ca^{2+} concentration seems sufficient to bring about the enzymatically active configuration.

Activated FXIII (FXIIIa) is a transglutaminase that can form $\varepsilon(\gamma$-glutamyl)lysyl cross-links between two polypeptide chains. The pimary physiological substrates of FXIIIa are fibrin and α_2-plasmin inhibitor (α_2-PI). By cross-linking fibrin chains, and α_2-PI to fibrin it makes a mechanically stronger fibrin clot, increases its resistance to shear stress, and provides protection against the prompt degradation by the fibrinolytic enzyme, plasmin.

Inherited FXIII deficiency, an autosomal recessive disorder, is a severe bleeding diathesis. It is frequently associated with intracranial, intramuscular and subcutaneous bleeding and impaired wound healing. As FXIII is essential for maintaining pregnancy, women with severe FXIII deficiency experience recurrent miscarriages.

The essential role of FXIII in the wound healing process was clearly proven in transgenic mice. In the FXIII-A deficient mice the healing of excisional wound was considerably delayed, incomplete re-epithelization, persisting necrotized fissure and abnormal scar formation was observed, while FXIII substitution restored the normal healing process. Beneficial effect of FXIII supplementation on the healing of surgical wounds in postoperative situations has also been demonstrated. Fibrin-gel formation, macrophage invasion, fibroblast migration and proliferation, production of extracellular matrix (ECM) and angiogenesis are the key events in wound healing. FXIII exerts its effect by cross-linking ECM proteins, by affecting endothelial cells, fibroblasts and macrophages. The latter cellular effects are related to the down-regulation of thrombospondin-1 (TSP-1) and to the up-regulation of c-Jun and Egr-1.

The role of FXIII in tissue repair and wound healing is, at least in part, mediated by its pro-angiogenic effect. Angiogenesis is involved in a wide range of physiopathological processes from wound healing to tumor growth. FXIII

seems to be capable of mediating endothelial cell-platelet interaction, its active form enhanced human umbilical endothelial cell (HUVEC) migration, inhibited their apoptosis. FXIIIa had no effect on the secretion of vascular endothelial growth factor (VEGF) into the medium, and neither VEGF protein level nor VEGF receptor-2 (VEGF-R2) messenger ribonucleinacid (mRNA) levels were affected by FXIIIa. In contrast, FXIIIa induced an almost complete disappearance of thrombospondin-1 (TSP-1) mRNA from the cells and markedly reduced the amount of TSP-1 in the medium. The role of FXIII in angiogenesis has also been verified in several in vivo animal models. In FXIII knockout mice the formation of new vessels dramatically decreased as compared to normal mice. Sub-epithelial injection of FXIIIa into the cornea induced the formation of a rich network of capillaries within 36 hours, at the same time the positive staining for TSP-1 in the fibers of the stroma and in keratocytes disappeared. Topical application of FXIII concentrate might be used to promote the angiogenesis in poorly healing wounds and the inhibition of FXIIIa induced angiogenesis might have a potential in antitumor therapy.

Not much is known about appearance of FXIII in body fluids other than blood. FXIII was measured in broncoalveolar lavage fluid in our laboratory. It was shown, that in normal conditions only cFXIII can be detected in alveolar lining fluid, while in inflammatory bronchoalveolar diseases pFXIII also appears, and the concentration of cFXIII becomes significantly elevated. The presence of FXIII in tears has not investigated. As fibrinolysis has a major role in maintaining corneal homeostasis, we assumed the presence of FXIII in tears and its involvement in the physiopathological processes concerning tears and the cornea.

In normal conditions the avascular corneal tissue is not in direct contact with blood, yet fibrinogen and components of the fibrinolytic system appear in tear fluid and their balance seems to be important in maintaining tear homeostasis. A major clinical symptom of plasminogen deficiency is ligneous

conjunctivitis, in which case fibrin rich pseudomebrane is formed on the tarsal conjunctiva that leads to ulceration and hyperplastic changes in the residual corneal epithelium. Mice, in which plasminogen gene was knocked out, developed conjunctival lesions indistinguishable from the human disease in both appearance and histology. It is very likely that the lack of plasminogen and consequently the lack of its active form, plasmin, the main fibrinolytic enzyme, results in impaired fibrin clearance leading to the formation of pseudomembrane. It is also known that plasminogen activators are synthesized in conjunctival and corneal epithelial cells and appear in tear fluid. Plasminogen activator inhibitor type 2 (PAI-2) was also found in normal human corneal epithelium and in normal tears. The impaired corneal wound healing in plasminogen deficient mice was complicated by severe inflammatory response, retrocorneal fibrin deposits, scar tissue formation and often stromal neovascularization. In mice with combined fibrinogen and plasminogen deficiency normal corneal wound healing was restored. This finding suggests that the balance of fibrin(ogen) deposition and the activation of fibrinolytic system in tears play an important role in corneal homeostasis. The regulatory effect of FXIII, a key regulator of fibrinolysis, in this mechanism is still to be explored.

AIM OF THE STUDY

1.	In addition to blood coagulation FXIII also exerts its effects in the extravascular compartment. It has an a well defined role in wound healing and angiogenesis. We were wondering if FXIII subunits appear in stimulated and non-stimulated tears and, if yes, in what concentration.

2.	As the volume of collected tear samples is very limited and, according to our preliminary experiments, the conconcentration of FXIII subunits and complex in tears is much lower than in the plasma, the methods used for the determination of FXIII concentrations in plasma are not sensitive enough for their measurement in tears. For this reason our aim was to develop hypersensitive enzyme linked immunosorbent assays for the measurement of FXIII-A, FXIII-B and FXIII-A_2B_2 in human rears and to evaluate these methods according to international guidelines..

3.	Tear is unique among the extravascular fluid compartments in that respect that it nourishes and protects an avascular organ; the cornea and FXIII that appears in tears might have particular importance in local wound healing and angiogenesis. For this reason we aimed to investigate the changes of FXIII levels following penetrating keratoplasty (PKP) and its effect on the outcome of corneal transplantation, especially on the neovascularization of donor cornea.

PATIENTS, MATERIALS AND METHODS

Study population

The study population included 60 healthy volunteers without any eye disease (21 females, 39 males, median age: 28, interquartile range (IQR): 24-47). None of them used eye-drops or any other medication. Individuals with dry eye disease, allergy, ocular inflammation or contact lenses were excluded. Non-stimulated and stimulated tear samples were collected from these volunteers to determine FXIII-A, FXIII-B, $FXIII-A_2B_2$ and total protein concentrations. Protein concentration was determined from each sample, however, from a few samples, due to their small volume, only two FXIII parameters could be determined. Thus, the number of samples analyzed for FXIII-A, FXIII-B and $FXIII-A_2B_2$ were 52, 50 and 50, respectively. In these subgroups the female:male ratios were 19:33, 18:32 and 19:31, the median ages and the ranges were identical to those of the total study group.

31 patients (17 females and 14 males; median age: 64, inter-quartile range: 49-75) undergoing PKP, 11 among them had re-PKP, were also examined. The reasons for performing PKP were as follows: bullous keratopathy (17), corneal scar after injury (5), corneal dystrophies (5), corneal ulcer (3) and keratoconus (1). No early graft failure occurred in the first postoperative week during which all patients stayed in the hospital. We were able to follow twenty-three patients for 18 months at regular check-ups. Omitting unexpected events, the check-ups occurred 1 month and 3 months after surgery, then at every 3-month, and neovascularization of the donor cornea was registered.

The study protocol was approved by the Ethics Committee of the University of Debrecen. The research followed the tenets of the Declaration of Helsinki; informed consent was obtained from the patients after explanation of the nature and possible consequences of the study.

Tear sampling

Tear samples were non-traumatically collected into glass capillary tubes from the lateral inferior meniscus without topical anesthesia for one min between 7 and 8 a.m. According to our preliminary experiments 8.5 µL tears was sufficient for the measurement of FXIII-A, FXIII-B, FXIII-A_2B_2 and total protein concentrations. In tear samples from patients concentrations of FXIII subunits and complex were higher, in such cases 4 µL tears could be sufficient for the determinations.

From healthy volunteers samples were collected from both eyes, sampling was repeated after a half hour resting period. Tear samples were stored at +4 °C in LoBind tubes, determinations were performed within 5 days. In preliminary experiments it was shown that the concentration of these constituents remained unchanged for at least 10 days. If tear samples were stored undiluted at +4 °C without using any stabilizer FXIII-A, FXIII-B and FXIII-A_2B_2 concentrations after 10 days were 99.4%, 98.5% and 98,5%, respectively.

From healthy volunteers samples were collected before and after intranasal stimulation with a spray of 80% ethanol. As in tears from healthy individuals cellular elements were not detected the samples were analyzed without centrifugation.

From patients undergoing PKP tear collection was performed as described for healthy individuals, however in this case stimulation with ethanol was not applied. The determination of the above parameters was carried out before and 1, 2, 4, 7 days after surgery. Tear samples were collected before the application of the morning eye-drops (local corticosteroids and neomycin). Then, they were centrifuged to remove cellular elements and cell debris and the supernatant was used for analysis.

Laboratory methods

Measurement of total tear protein concentration was carried out from 1 µL 20-fold diluted tears by the bicinchoninic acid method (BCA Protein Assay Reagent, Thermo Scientific, Rockford, IL) using bovine serum albumin for calibration.

The methods developed for the determination of FXIII subunits and complex in tears used the same antibodies that were used in the methods developed in our laboratory for the determination of the same FXIII species in the plasma. The assay for FXIII-A_2B_2 determination used biotinylated monoclonal capture antibody against FXIII-B and horseradish peroxidase (HRP) labeled monoclonal detection antibody against FXIII-A. Similarly, biotinylated and peroxidase labeled monoclonal antibodies were used for the measurement of FXIII-A or FXIII-B concentration. In the cases of FXIII-A and FXIII-B assays the capture and detection antibodies were directed against different epitopes of the same antigen molecules. The antibodies used in FXIII-A and FXIII-B assays reacted with free and complexed FXIII subunits to the same extent, i.e., total FXIII-A and FXIII-B concentrations were determined.

Due to the low concentration of FXIII in tears and to the very small volume of tears a chemiluminesce micro-ELISA using Lumigen PS-atto peroxidase substrate was designed and the reaction was carried out in 384 well streptavidin coated white microplate. The reaction scheme was the same for all three FXIII species. After preliminary experiments the following assay conditions were established: 50 µL of biotinylated capture antibody (1 µg/mL) in dilution buffer (0.5 mol/L NaCl, 5 mg/mL bovine serum albumin, 0.5 g/L Tween 20, in 15 mmol/L phosphate buffer, pH 7.2) was incubated for 1 hour at room temperature in streptavidin coated microplate. After thorough (5x) washing with washing buffer (0.5 mol/L NaCl, 0.5 g/L Tween 20, in 15 mmol/L phosphate buffer, pH 7.2) appropriate dilution of tear samples (in 50 µL final volume) were added and incubated for 1 hour. Following a new series of

washing 50 µL HRP-labeled capture antibody (0.5 µg/mL) in dilution buffer was added. After 1-hour incubation, followed by washing, 50 µL Lumigen PS-atto, prepared according to the instruction of the manufacturer, was added. Chemiluminescent signal was measured in Infinite M200 microplate reader within 5 min. Dilutions of REA-clot reference plasma N in dilution buffer were used for constructing calibration curve. Its assigned FXIII-A_2B_2 and FXIII-A values (20.84 mg/L and 10.63 mg/L) are based on calibration against WHO International Standard, Blood Coagulation Factor XIII, Plasma, Human, while its FXIII-B concentration (19.04 mg/L) was calibrated against highly purified FXIII-B prepared in our laboratory.

Method evaluation

Due to the small volume of tears, pooled tear samples could not be used for precision evaluation; instead standard plasma dilutions were used for the evaluation of precision performance of the chemiluminescent FXIII antigen assays. The evaluation was carried out according to the EP5-A2 guideline of The National Committee for Clinical Laboratory Standard (presently Clinical and Laboratory Standards Institute; CLSI) using single run per day with duplicate determinations for 20 days. The limits of detection and the limits of quantitation were determined according CLSI EP-17-A protocol. The partly nonparametric approach was selected for determination of limits of detection.

Recoveries were determined on tear samples spiked with various concentrations of highly purified FXIII-A_2, FXIII-B, FXIII-A_2B_2. Spiking solutions containing 90 µg/L or 400 µg/L FXIII-A_2, 210 µg/L or 820 µg/L FXIII-B and 150 µg/L or 730 µg/L FXIII-A_2B_2 were prepared in dilution buffer. These solutions were 10 times diluted in pooled tear. The control matrix contained 1.86 µg/L FXIII-A_2, 4.48 µg/L FXIII-B or 0.87 µg/L FXIII-A_2B_2. Recovery was expressed as the percentage of added FXIII.

Statistical analysis

The distribution of the results was analyzed by the Kolmogorov-Smirnov and by the Shapiro-Wilk tests. Statistical significance of the differences between males and females and between PKP patients with and without neovascularization was calculated by the Mann-Whitney U test. The differences in FXIII and protein levels in tears obtained from healthy individuals before and after stimulation were investigated by the Wilcoxon signed ranks test. The same test was used to calculate the statistical significance of the differences when the variables measured before and various days after PKP were compared. Age dependence of FXIII levels was tested by Pearson correlation test. Statistical analyses were performed by the Statistical Package for the Social Sciences (SPSS 13.0).

RESULTS

Evaluation of chemiluminescent FXIII ELISAs

The limit of detection was 0.014 µg/L, 0.019 µg/L and 0.016 µg/L for the FXIII-A_2, FXIII-B and FXIII-A_2B_2 assay, respectively. As at these levels the estimated total errors were below 25% (12.8%, 15.8% and 14.3% for FXIII-A, FXIII-B and FXIII-A_2B_2, respectively, the limits of quantitation were considered equal to the limits of detection for the three assays (EP17-A CLSI protocol).

The total (within laboratory) imprecision of the assays in the low concentration range did not exceed 12% in either of the FXIII antigen assays and in the high concentration range it was below 5.5 %. The FXIII antigen assays have a wide measuring range starting from the limit of quantitation, 0.014-2.0 µg/L for the FXIII-A_2 assay, 0.019-10.0 µg/L for the FXIII-B assay and 0.016-4.0 µg/L for the FXIII-A_2B_2 assay. The lower range is important for the measurement of low FXIII complex and FXIII subunit concentrations in normal tear samples, while the wide full range makes it possible to measure pathological samples with high FXIII concentrations from a single dilution. In the measuring ranges an excellent fitting was obtained with second order polynomial equations, which were used for calculating the results.

Recoveries of the respective FXIII species approximated 100% in all three assays. The specificity of the anti-FXIII-B, anti FXIII-A monoclonal antibody combination, used in FXIII-A_2B_2 assay, has been investigated earlier; no interference with free FXIII subunits was observed. Similarly, FXIII-A_2 ELISA was not influenced by the presence of FXIII-B and FXIII-A_2 did not interfere with the determination of FXIII-B. Plasma samples immunodepleted from FXIII-A, or FXIII-B did not give any reaction in the FXIII assays, which shows the specificity of the methods.

FXIII levels in tears from healthy individuals; changes upon the stimulation of tear production

FXIII-A, FXIII-B, FXIII-A$_2$B$_2$, total protein concentrations and tear flow in tears obtained from healthy individuals before and after stimulation of tear secretion was determined. Results were also expressed as µg FXIII or FXIII subunit/g tear protein. As demonstrated by Kolmogorov-Smirnov and the Shapiro-Wilk tests distribution of the values for all three parameters was non-Gaussian. The histograms of FXIII-A, FXIII-B and FXIII-A$_2$B$_2$ concentrations are considerably skewed toward the lower concentration range. Neither in non-stimulated nor in stimulated tears was there any gender difference in the concentrations of the three FXIII species and no significant age dependence could be observed. The low tear flow suggests that the procedure of tear sampling did not cause any significant stimulation of tear secretion.

In non-stimulated tears a low but consistent amount of FXIII-A and FXIII-B could be measured, 2.13 µg/L FXIII-A correspond to 2.66×10^{-11} mol/L and 7.22 µg/L FXIII-B correspond to 4.35×10^{-11} mol/L, respectively. The median FXIII-A$_2$B$_2$ level was very low and in 16 % of the samples FXIII-A$_2$B$_2$ concentration was below the limit of quantitation. Only a relatively small portion of FXIII-A and FXIII-B formed complex in tears; the median 0.67 µg/L corresponded to 0.21×10^{-11} mol/L FXIII-A$_2$B$_2$ that represents only 16% of total FXIII-A.

In tears obtained after stimulation the concentration of FXIII-A$_2$, FXIII-B and FXIII-A$_2$B$_2$ and total protein were lower than in non-stimulated tears, with the exception of FXIII-B the differences were statistically significant. However, when FXIII levels were normalized for protein concentration no significant difference between stimulated and non-stimulated tears could be observed.

FXIII levels in tears from patients undergoing penetrating keratoplasty

As compared to the pre-surgery level, 24 hours after surgery FXIII-A, FXIII-B and FXIII-A_2B_2 showed 10.4-fold, 9.4-fold and 20.5-fold elevation, respectively. The tear flow became elevated and the total protein concentration decreased, which is due to the increased production of more diluted tears. Consequently, the increases of FXIII levels normalized for protein concentration were even higher (16.9-fold, 17.7-fold and 25.7-fold). Following the first day, FXIII levels started to decrease gradually, but even 7 days after surgery they remained significantly elevated. The extent of the decrease in FXIII-A, FXIII-B and FXIII-A_2B_2 levels following the 1st postoperative day became statistically significant after different intervals, but on day 7 the levels of all three parameters were significantly lower than the respective 1st day values.

The donor cornea showed neovascularization in 10 cases out of 23, which occurred between months 3-12 after PKP. The FXIII levels before and during the first week after PKP were analyzed retrospectively in the patient groups with and without neovascularization of transplanted graft. The pre-operative FXIII levels were higher in patients whose cornea demonstrated neovascularization during the follow-up period. Due to the relatively small number of patients, the level of statistical significance was reached only in the case of FXIII complex. Similarly, there was significantly higher elevation of post-surgery FXIII subunit and FXIII complex levels in the neovascularized group as compared to the patients with non-neovascularized cornea.

DISCUSSION

Due to the low volume of tears and to the low concentration of FXIII complex and FXIII subunits in this body fluid, the sensitivity of one-step quick sandwich ELISAs, originally designed for the determination of FXIII-A$_2$B$_2$ and FXIII-A in the plasma was insufficient to measure these FXIII species in tears. Besides, there was no well-established method for the measurement of FXIII-B. To be able to develop highly sensitive, well reproducible, relative robust assays for the determination of FXIII-A, FXIII-B and FXIII-A$_2$B$_2$ complex in tears the following modifications were introduced:

1. The use of chemiluminescent substrate, Lumigen PS-atto, for the measurement of peroxidase activity, instead of colorimetric substrate, highly increased the sensitivity of the reaction.

2. White, non-transparent microplate was used instead of transparent ones for the evaluation of chemiluminescent reaction, to eliminate light scattering.

3. In the plasma assays the sandwich, consisting of the two antibodies and the antigen, was formed in solution in a one hour incubation period. The one-step reaction was replaced by multi-step reaction in which after each one hour incubation step, i.e., after the binding of capture antibody, FXIII antigen and detection antibody, the plate was extensively washed and the sandwich was formed on the surface of streptavidin coated wells. This modification significantly decreased the background and, by allowing full complex formation between the antibodies and the antigen, increased the sensitivity.

4. To adapt to the low available volume of tears, the reaction was carried out in 384 well streptavidin coated microplate rather than in the regular 96 well microplate.

As compared to the plasma assays, these modifications decreased the detection/quantitation limits of FXIII-A$_2$B$_2$ and FXIII-A assays by 59-fold and

25-fold, respectively. A similar increase of sensitivity was achieved for the FXIII-B assay.

The high sensitivity of the assays made it possible to determine FXIII levels in tiny volumes of tears that can be obtained from healthy individuals without the stimulation of tear secretion. The concentration of FXIII-A and FXIII-B in tears is much lower than in the circulation. According to our estimation, FXIII-A concentration in non-stimulated tears is approximately 5,200-fold less than in plasma (11 mg/L). FXIII-B concentration in the plasma is 21 mg/L, i.e. the tear:plasma ratio for FXIII-B is 1:2,900. The molar ratio of FXIII-B:FXIII-A in tears is 3.5, i.e., it is almost double of their ratio in plasma (2:1). In tears only 16% of FXIII-A formed complex with FXIII-B. This is in sharp contrast to human plasma, where FXIII subunits are in more than thousand-fold higher concentration than in tears and $FXIII-A_2$ is practically fully complexed. The median FXIII-A and FXIII-B concentrations for non-stimulated tears correspond to 1.33×10^{-11} mol/L $FXIII-A_2$ and to 4.35×10^{-11} $FXIII-B_2$, respectively. Using these data and the K_d of 0.16×10^{-9} mol/L for complex formation, established in our laboratory, 0.27×10^{-11} mol/L $FXIII-A_2B_2$ was calculated so theoretically 20% of FXIII-A is complexed. This value is close to 0.21×10^{-11} mol/L (0.67 µg/L), the median of measured FXIII complex concentration, and represents 16% of FXIII-A. Practically 100% of FXIII-A was calculated to be in complex when plasma concentrations of FXIII subunits were used for the calculation. These findings suggest, that the difference between plasma and tears is simply due to the low FXIII subunit concentrations (below the K_d of complex formation) in tears and not to an inhibitor of complex formation.

FXIII is in considerable excess in the plasma; around 5% of its normal plasma level is sufficient to maintain hemostasis in normal conditions. The average half-life of FXIII FXIII in the plasma is 9-12 days, while the turnover

rate of non-stimulated tear is 8-16% per min. Thus, the small corneal surface is continuously washed with freshly produced tear containing free FXIII-A.

The source of FXIII subunits present in tears was not explored in the present study and only speculations can be offered. The tear film consists of three layers: the gelatinous mucus in contact with the epithelial surface, the aqueous solution of proteins and other water-soluble molecules that make up the bulk of the thickness, and the surface layer consisting of lipids, that protects the tear film from evaporation. Most of the water, salts and proteins are secreted by the lacrimal gland and the accessory lacrimal tissue, although minor components may be secreted by the conjunctival epithelium or may leak out from conjunctival blood vessels. The main source of the lipids is the Meibomian gland. FXIII-A has been demonstrated in monocytes and various macrophages including histiocytes and dendritic cells; macrophages of the conjunctival tissue might be candidates for the site of tear FXIII-A production. Recently it was shown that in vitro a bronchial epithelial cell line is able to synthesize FXIIII-A that appears in the culture supernatant. Corneal epithelial cells should also be tested for FXIII-A production. FXIII-A as a constituent of FXIII-A_2B_2 complex might also leak out from the plasma and dissociate in tear conditions. The main site of FXIII-B synthesis has been localized to hepatocytes, which secrete it into the circulation. Transudation of free FXIII-B, and to a lesser extent FXIII-A_2B_2, from conjunctival vessels seems to be the most likely source of FXIII-B in tears.

As it has been reported, nasal stimulation of tear secretion, in parallel with the increased flow rate, decreased total protein concentration; a similar decrease was observed in the concentration of FXIII species. This finding suggests that the dilution effect caused by increased tear fluid production is responsible for the decreased FXIII levels. After stimulation the tear flow rate is much higher, in our experiments, 7-times higher than the basal flow rate. Thus, in spite of the decreased concentration following stimulation, the total amounts of FXIII-A and FXIII-B that appear in tear fluid within one min is 4.5-5-times higher than in

non-stimulated condition, i.e., after stimulation more FXIII gets into contact with the corneal and conjunctival epithelium.

Following PKP a tremendous elevation of FXIII-A, FXIII-B and FXIII-A_2B_2 levels was observed which peaked at 24 hours after the surgical intervention, and after a gradual decrease still remained significantly elevated a week later. It is to be emphasized that during this period, in contrast to the FXIII levels, the total protein concentration significantly decreased. Even the peak FXIII-A and FXIII-B levels measured after PKP corresponded only to 0.5% and 1.5% of the respective plasma levels. The turnover rate of tears is considerably increased after PKP, in our case the increase by day one was 12-fold and on day 7 still 6-fold. It seems that the surgical wound is continuously washed with tears of considerable FXIII content.

It has been shown that Ca^{2+}, at concentration in the millimolar range, can activate FXIII-A_2, but not FXIII-A_2B_2, without proteolysis. The reference interval for Ca concentration in tears was estimated 0.36-0.79 mmol/L. Thus, part of FXIII-A_2 might go through non-proteolytic activation. In inflammatory conditions, when other components of the coagulation system might also leak out from the vessels, thrombin may form and activate FXIII. Elastase secreted by inflammatory granulocytes may also induce proteolytic activation of FXIII. As activated FXIII easily binds to cells, the actual local FXIII-A_2 concentration might be significantly higher than that measured in the tear fluid.

As FXIIIa in the extracellular compartment promotes wound healing, the occurrence of FXIII and its subunits in body fluid other than blood could be of high importance concerning local tissue repair. FXIII in tears might act as a topical "hormone" and through a direct cellular effect could promote repair of minor injuries and corneal wound healing. In normal conditions the injury of avascular corneal tissue heals without neovascularization, however in certain pathological cases neovascularization of injured cornea is a major, sight-threatening complication of surgical interventions. FXIII at higher

concentrations, due to its proangiogenic effect and to the inhibition of normal fibrin turnover by fibrinolysis, might be harmful concerning the final outcome. In a rabbit cornea model, injection of high amount of FXIIIa caused neovascularization associated with almost complete disappearance of thrombospondin-1 from the cornea. Our study patients who developed neovascularization of donor cornea had considerably higher pre and post-surgery FXIII levels in tears than patients with non-neovascularized cornea. The significant time-gap between the measurement of FXIII levels and the onset of donor cornea neovascularization, which was detected 3-12-month later, seems to exclude a direct relationship. However, the higher basal FXIII levels and the ability to respond to stimuli with increased elevation of FXIII level might well represent a risk of neovascularization following PKP.

SUMMARY

Highly sensitive chemiluminscent ELISAs were developed to be able to measure FXIII subunits and FXIII-A_2B_2 complex at low concentration in a few microliter volume with good precision. These methods are suitable for the measurement of FXIII subunits and their complex in tears and, very likely, also in other body fluids. The methods provide promising new tools for monitoring the changes of FXIII and FXIII subunit concentrations in pathological conditions. The concentrations of FXIII species were measured in non-stimulated and stimulated tears of 60 healthy volunteers. The results demonstrate that in non-stimulated tears of healthy individuals FXIII-A and FXIII-B are present in a low, but consistent concentration. At such low concentration only a minor portion of the two subunits forms complex. FXIII levels decreased following the stimulation of tear production, but values normalized to total protein did not change. FXIII levels in tears are greatly increased following corneal transplantation. The elevation is more pronounced in patients who later developed neovascularization of donor cornea. It is suggested that FXIII in normal tears could be involved in the repair of micro-injuries and higher FXIII concentrations observed after PKP could promote the healing of surgical wounds. However, above a certain concentration the effect of FXIII might be harmful by impairing fibrin elimination and promoting angiogenesis leading to neovascularization.

Candidate: Zsuzsanna Zita Orosz

Neptun ID: XSIIKF

Doctoral School: Kálmán Laki Doctoral School

<u>**List of publications related to the dissertation**</u>

1. **Orosz, Z.Z.**, Katona, É., Facskó, A., Módis jr., L., Muszbek, L., Berta, A.: Factor XIII
   subunits in human tears: Their highly elevated levels following penetrating keratoplasty.
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