SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Thrombomodulin-dependent effect of factor V_{Leiden} mutation on factor XIII activation, on the cross-linking of fibrin and alpha-2-plasmin inhibitor and its consequences on fibrinolysis

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INTRODUCTION AND REVIEW OF LITERATURE

Haemostasis has a dual role: on one hand it keeps the blood in liquid form inside of the blood vessel, on the other hand in case of blood vessel injury, it stops the bleeding as soon as possible in order to minimize the blood loss. Normal hemostasis requires fine control by natural procoagulant and anticoagulant systems.

Acquired or inherited factors may influence the procoagulant or anticoagulant mechanism resulting in hemostatic disturbances. Hereditary hemorrhagic diseases with clinical symptoms have been known for centuries, but the majority of hereditary factors associated with thrombosis have been described only in the past half century. Inherited thrombophilia includes activated protein C resistance caused by Leiden mutation of factor V (FV_{Leiden}), the presence of prothrombin G20210A allele, genetic deficiencies of protein C, protein S and antithrombin. In Caucasian population the most common cause of familial thrombophilia is activated protein C (APC) resistance caused by FV_{Leiden} mutation. It is associated with a 5-8-fold increased risk of venous thromboembolism in heterozygotes and with a 50-80-fold increased risk in homozygotes. This point mutation predicts replacement of arginine by a glutamine at position 506 of the FV molecule (p.Arg506Gln), eliminating one of three cleavage sites of APC in activated FV (FVa) heavy chain, and this way impairs the down-regulation of the procoagulant activity of FVa. The mutation also compromises FV cofactor activity, which accelerates APC-catalyzed inactivation of activated factor VIII (FVIIIa). Due to these mechanisms, FV_{Leiden} impairs the down-regulation of thrombin generation by the APC pathway.

An important thrombin substrate is blood coagulation factor XIII (FXIII), which was first described by Lóránd and Laki in 1948 as a „fibrin stabilizing factor”. FXIII is a protransglutaminase, which can be found in two forms in the human body. The plasmatic form of FXIII has a tetrameric structure (A_2B_2), and it consists of two catalytic A subunits

(FXIII-A) and two carrier/inhibitory B subunits (FXIII-B). The other form is cellular FXIII, which is a homodimer of FXIII-A (FXIII-A_2) and it is present in platelets, monocytes and macrophages in cytoplasmic localization. FXIII-A consists of four structural domains (a β-sandwich domain, a catalytic core domain and two β-barrel domains) plus an activation peptide (AP-FXIII) of 37 amino acids at the N-terminus. The catalytic core domain contains the catalytic triad (the active-site Cys314, His373 and Asp396), which in non-activated FXIII is buried and inaccessible for the substrates.

Several polymorphisms with amino acid exchange have been described in the FXIII-A gene. Among them the Val34Leu polymorphism is the best characterized because of its suspected thrombo-protective effect. It is located in the activation peptide only 3 amino acid upstream from the thrombin cleavage site, and it influences the activation of FXIII by thrombin. In the case of Leu34 variant the release of activation peptide is 2.5-fold faster than in the case of Val34 variant. Due to the earlier activation of FXIII Leu34 variant the cross-linking of fibrin $\alpha-$ and $\gamma-$chains and the cross-linking of α_2-PI to fibrin becomes accelerated. Previously, it has been also demonstrated that the structure of fibrin clots is influenced by FXIII-A Val34Leu polymorphism, and this effect is modulated by the concentration of fibrinogen. At high fibrinogen concentrations, plasma samples from homozygotes for the Leu34 allele form clots having looser structure, thicker fibers and increased permeability.

FXIII-B is a glycoprotein; it contains 8.5% carbohydrate. It is a typical mosaic protein consisting of ten so-called sushi domains; each of them is hold together by a pair of disulfide bond. The two subunits form complex in the plasma, in healthy individuals its plasma concentration is 14–28 mg/L. Plasma FXIII circulates in association with fibrinogen (Kd is ~10^{-8} mol/L). It is transformed into an active transglutaminase by the concerted action of thrombin and Ca^{2+}. First thrombin cleaves off an activation peptide of 37 amino acids from the potentially active FXIII-A. Then, in the presence of Ca^{2+}, FXIII-B dissociates and FXIII-

A assumes an enzymatically active configuration (FXIIIa). The presence of fibrin accelerates the activation process 80-100-fold, and in the plasma FXIII is activated on the surface of newly formed fibrin. The formed FXIIIa remains associated with the fibrin clot. In the absence of FXIII-B the cellular form of FXIII does not need proteolytic cleavage, the rise of intracellular Ca^{2+} concentration is sufficient to bring about the active configuration.

Like all transglutaminases, FXIIIa catalyzes an acyl-transfer reaction, in which the γ-carboxamide group of a peptide-bound glutamine residue is the acyl donor and a primary amine is the acyl acceptor. If the substrate primary amine is an ε-amino group of a peptide-bound lysine residue the end-result is the cross-linking of two peptide chains by ε(γ-glutamyl)lysyl cross-links.

The primary physiological substrates of FXIIIa are fibrin and α_2-plasmin inhibitor (α_2-PI). In addition, there is a number of other protein substrates of FXIIIa, such as blood FV, plasminogen activator inhibitor-2, plasminogen, lipoprotein(a), thrombin activatable fibrinolysis inhibitor (TAFI), adhesive proteins (von Willebrand factor, fibronectin, vitronectin, thrombospondin), cytoskeletal proteins (actin, myosin). However, cross-linking of most of these proteins has no physiological relevance.

In addition to hemostasis, FXIII is also important for wound healing and plays an essential role in maintaining pregnancy. The main hemostatic function of FXIII prevails in the formation of final clot structure and regulation of fibrinolysis. Cross-linking of fibrin chains and α_2-PI to fibrin by FXIII improves the mechanical stability of fibrin clot, increases its resistance to shear stress and protects it from prompt elimination by fibrinolytic degradation. Fibrin cross-linking by FXIIIa results in the formation of γ-chain dimers and high molecular weight α-chain polymers. γ-chain dimmerization is a rapid process, while the multiple cross-linking of α-chains occures much slower and results in α-chain oligomers and high Mr α-

chain polymers. α-chain cross-linking confers stability to the fibrin clot and enhances its strength, rigidity and resistance, γ-dimers formation contributes to clot stiffness.

In theory, FXIIIa may influence fibrinolysis by three mechanisms: 1/ Fibrin α-chain cross-linking into a high Mr α-polymers may have a direct effect on the susceptibility of fibrin clot to fibrinolysis. 2/ Cross-linking of fibrin fibers by FXIIIa decreases the binding of plasminogen to fibrin, and consequently, decreases plasminogen activation by tissue plasminogen activator (tPA). 3/ The binding of α_2-PI to fibrin prevents its prompt proteolytic degradation by the fibrinolytic enzyme, plasmin.

α_2-PI, the main physiological inhibitor of plasmin, is an excellent substrate of FXIIIa, it becomes rapidly cross-linked to fibrin α-chains soon after fibrin polymerization. Then the α_2-PI-fibrin α-chain heterodimer becomes incorporated into α-chain polymers. The protein is a serpin, synthesized and secreted by hepatocytes. It consists of 491 amino acids with a methionine at the N-terminus (Met1-α_2-PI). In the plasma a protease, antiplasmin cleaving enzyme cleaves off a 12 amino acids oligopeptide from the N-terminus and Met1-α_2-PI becomes transformed into Asn1-α_2-PI. Both Met1-α_2-PI and Asn1-α_2-PI exist in the human plasma, the ratio of the two isoforms is 3:7. Asn1-α_2-PI isoform is an excellent substrate of FXIIIa, while the Met1-α_2-PI isoform is a poor substrate. During the formation of the fibrin clot, the Asn1-α_2-PI Gln2 site becomes cross-linked to Lys303 of α-chain of fibrin(ogen). This Lys residue is not involved in the formation of α-chain polymers. The incorporation of α_2-PI into fibrin α-chain precedes fibrin α-chain cross-linking. Cross-linked α_2-PI retains its full inhibitory activity toward plasmin, and plays a key role in the protection of the fibrin clot from elimination by fibrinolysis. The cross-linking of lysine residues by FXIIIa, especially in the C-terminal domain of fibrin α-chain, reduces the binding sites for plasminogen and also for tPA and this mechanism might also play a role in FXIIIa-induced resistance to fibrinolysis.

Human tPA is a single-chain glycoprotein with Mr ~70 kDa and it is secreted by endothelial cells into the blood, where 80% of tPA is connected to plasminogen activator type 1 (PAI-1) and the remaining 20% circulates free. tPA catalyzes the conversion of plasminogen to plasmin. tPA contains "kringle-domain", which has a key role in the interaction with its cofactor, fibrin. It is a poor enzyme in the absence of fibrin, but the presence of fibrin strikingly enhances the activation of plasminogen, higher efficiency of plasminogen activation increases by up to three orders of magnitude in the presence of fibrin. Like other activators of plasminogen, tPA cleaves Arg561-Val560 bond of plasminogen, resulting in active plasmin. Plasmin contains five „kringle-domains". The active centrum containing the catalytic triad (Ser741, His603, Asp646) is located in the C-terminal region of the molecule. Plasmin cleaves fibrin along the lysine carboxyl-groups, perpendicular to the fibrin strands. As the process progresses in time, more and more C-terminal lysine chains become free and available for further linking of plasminogen and tPA, which promotes plasminogen activation. Formation of tPA-plasminogen-fibrin complex represents a positive feedback for plasmin formation and leads to increased fibrinolysis.

Inhibitors, such as α_2-PI, PAI-1 and TAFI, are also involved in the regulation of fibrinolysis. Newly formed free plasmin is rapidly inhibited by α_2-PI (half-life about 0.1 s), while plasmin bound to the surface of fibrin becomes inactivated by α_2-PI only slowly (half-life of about 10-100 sec). PAI-1 produced by hepatocytes, endothelial cells and megakaryocytes also belongs to the family of serpins; large amount of it is found in platelets. Its most important role is the inhibition of fibrinolysis during the course of platelets-plug formation.

TAFI is one of the most recently discovered fibrinolysis inhibitors, which represents a link between coagulation and fibrinolysis. TAFI is a Mr ~60 kDa single chain protein, also known as procarboxypeptidase U, procarboxypeptidase B or procarboxypeptidase R. It is activated by thrombin, a process that is more than 1,000-fold enhanced in the presence of

thrombomodulin (TM). TAFI could also be activated by trypsin or plasmin. The active enzyme is relatively unstable under physiological conditions its half-life is approximately 10 min. Activated TAFI cleaves off C-terminal lysines and arginine residues from fibrin fragments; these residues in partially digested fibrin are essential for the binding of plasminogen and/or t-PA to fibrin.

The downstream consequences of FV_{Leiden}, which might be connected to increased thrombin generation, have been revealed only partially. It has been shown that APC also exerts a profibrinolytic effect and this mechanism, at least in part, operates through the down-regulation of TAFI. FV_{Leiden} impairs the profibrinolytic response to APC. In subjects with FV_{Leiden}, the impaired fibrinolysis is associated with the lack of APC effect on thrombin generation and, consequently, on TAFI activation. The increased activation of TAFI in the presence of FV_{Leiden} depends on TM concentration. These mechanisms might contribute to the increased risk of venous thromboembolism associated with FV_{Leiden} mutation.

THE AIM OF THE STUDY

Since the effect of FV_{Leiden} mutation on the formation of fibrin cross-links has not been studied and its effect on fibrinolysis has only been partially explored, our aim was to examine:

1. The effect of FV_{Leiden} on the activation of FXIII in the presence and absence of recombinant human TM (rhTM).
2. How the effect of FV_{Leiden} mutation on FXIII activation influences the cross-linking of fibrin chains.
3. How FV_{Leiden} influences the cross-linking of α_2-PI to fibrin.
4. What is the relative contribution of TAFI and FXIII to the impaired tPA induced fibrinolysis observed in the plasma of FV_{Leiden} mutants in the presence rhTM.

MATERIAL AND METHODS

Subjects

Fifteen apparently healthy individuals of known FV genotypes (5 wild type, 5 FV_{Leiden} heterozygotes and 5 FV_{Leiden} homozygotes) with no history of thrombosis or bleeding disorders were recruited for the study (3 men and 12 women; age 17-67 years). None of them were on anticoagulant therapy, and they did not take any medication for at least 2 weeks prior to blood sampling. All individuals had normal coagulation screening tests; their fibrinogen, factor XIII activity and antigen values were in the reference interval. As the rate of FXIII activation is modified by FXIII-A Val34Leu polymorphism, only subjects being wild type for this polymorphism were selected for the study. The study protocol was approved by the Ethics Committee of the University of Debrecen.

Preparation of plasma samples

18 mL blood was collected by venipuncture into 2 mL 0.105 M trisodium citrate. In order to suppress contact activation of coagulation, 50 μg/mL corn trypsin inhibitor (Gentaur, Brussels, Belgium) was added immediately to the blood sample. Citrated blood was centrifuged at 1,400 g for 20 min, then the upper two-third of platelet poor plasma was transferred to another centrifuge tube and centrifuged again at 1,400 g for 20 min. The upper two-third of the second supernatant was considered as platelet-depleted plasma (PDP).

Preparation of crude factor V from pooled plasma

Partial purification of FV from the pooled plasma of wild type or FV_{Leiden} homozygous individuals was carried out essentially as described by Dahlback. 35 ml blood from each of three individuals of the same genotype was collected in acid-citrate-dextrose Vacutainer tubes

(ACD-B, Becton Dickinson, Franklin Lakes, NJ). PDP was prepared as described above. PDP samples of the same genotypes were pooled and one tablet of protease inhibitor cocktail (Complete, Mini, EDTA-free, Roche Diagnostics, Indianapolis, IN) was added to each 10 ml of pooled plasma. Barium citrate adsorption was carried out by the drop-wise addition of 0.8 ml 1 M $BaCl_2$/10 ml plasma. After the mixture had been stirred for 1 h, the barium citrate was removed by centrifugation at 6,000 g for 10 min. Next, PEG-6,000 fractionation was performed. First, solid PEG-6,000 (Sigma-Aldrich, St. Louis, MO) was added to the supernatant to obtain a final concentration of 80 g/l. After stirring for 1 h, the precipitate was removed by centrifugation at 6,000 g for 10 min. Solid PEG-6,000 was added to the supernatant, again, to bring the PEG concentration to 120 g/l. The solution was stirred for 1 h, then, the precipitate was collected by centrifugation (6,000 g, 10 min). The FV containing plasma precipitate was washed twice with 120 g/l PEG-6,000 in dist. water. The whole preparation procedure was carried out at 4 °C. The precipitate containing wild type FV or FV_{Leiden} was dissolved in one-tenth of the original plasma volume of immunodepleted FV deficient plasma (Technoclone, Vienna, Austria). The FV activity of the mixture was determined by one-stage clotting assay, and it was set to 90% by further dilution in FV deficient plasma in both cases.

Factor XIII activation in plasma samples

As the activation of FXIII takes place exclusively on the surface of fibrin and after its formation FXIIIa remains associated with fibrin, the activation of FXIII was measured in recovered fibrin clots. Fibrin clots were generated by the activation of tissue factor (TF) pathway in the presence and absence of rhTM (American Diagnostica, Stamford, CT). An rhTM concentration that inhibits thrombin generation in the plasma of FV wild type individuals by approximately 50% was selected as described by Dielis et al. Using the selected rhTM concentration (final concentration 1.5 nM) the thrombin generation was

determined in the plasma of FV wild type individuals and 45.4% mean inhibition (range 40-61%) by sTM was obtained. Trombin generation was measured by Thrombin S-2238 chromogenic substrate (Chromogenix, Lexington, MA).

Aliquots of 150 µl PDP were incubated with 40 µl activator cocktail at 37 ^{o}C for various intervals and the reaction was stopped by an equal volume of inhibitor cocktail. To produce an activator cocktail Technothrombin® TGA RC Low (Technoclone GmbH, Vienna, Austria) was dissolved in 62.5 mM $CaCl_2$, with or without 7.5 nM rhTM. The dissolved Technothrombin® TGA RC Low contained ~5 pM recombinant human TF, low concentration of phospholipid micelles in Tris-Hepes-NaCl buffer. The inhibitor cocktail contained 20 mM benzamidine, 0.1 mM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), 2 mM iodoacetamide, 50 mM ε-aminocaproic acid, 100 mM NaCl, 50 mM EDTA in 50 mM HEPES buffer, pH 7.5. The reaction was stopped at various intervals after the initiation of coagulation by the activator cocktail. Polymerized fibrin, if present, was fully recovered by centrifugation of the formed clots; the clots were then exhaustively washed with physiological NaCl and dissolved in SDS-PAGE sample buffer.

SDS-PAGE and Western blotting technique

The dissolved fibrin samples were subjected to SDS-PAGE in 7.5% gel. The extent of FXIII activation was monitored by Western blotting using affinity purified sheep anti-human FXIII-A antibody as primary antibody (Affinity Biologicals, Ancaster, Canada). The immunoreaction was developed by biotinylated rabbit anti-sheep IgG and avidin-biotinylated peroxidase complex (components of Vectastain ABC kit, Vector, Burlingame, CA, USA) and it was visualized by enhanced chemiluminescence detection (ECL Plus+, Amersham, Little Chalfont, UK) according to the manufacturer's instructions. The determination of fibrin bound activated FXIII-A was used as parameter to study the kinetics of FXIII activation. The

amount of truncated, proteolytically activated FXIII-A (FXIII-A') was determined in each sample by quantitative densitometry using GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA) and expressed as percentage of the maximal amount of FXIII-A' formed in the clot. To compare the effect of rhTM on the rate of FXIII activation in different FV genotypes quantitatively, the time required for half maximal activation of FXIII was calculated ($T_{1/2}$ values).

The cross-linking of α_2-PI to fibrin was monitored by Western blotting using affinity purified goat anti-human α_2-PI as primary antibody (Affinity Biologicals, Ancaster, Canada). The immunoreaction was developed by biotinylated rabbit anti-goat IgG and avidin-biotinylated peroxidase complex (components of Vectastain ABC kit, Vector, Burlingame, CA). The immune reaction was visualized by enhanced chemiluminescence detection (ECL Plus+, Amersham, Little Chalfont, UK). The extent of α_2-PI-fibrin α-chain heterodimer formation and the relative amount of total cross-linked α_2-PI were determined by quantitative densitometry and expressed as percentage of maximal amount of the respective cross-linked products. $T_{1/2}$ values, the intervals required for 50% of α_2-PI cross-linking, were calculated.

Clot lysis assay

Tissue plasminogen activator (tPA) induced clot lysis was investigated in a turbidimetric assay system. Fibrinolysis was induced by mixing 10 μL tPA (American Diagnostica, Stamford, CT; final concentration: 5 nM), to the plasma immediately before the addition of activator cocktail with or without rhTM. The process of clotting and fibrinolysis was monitored for two hours in a temperature-controlled microplate reader (405 nm, 37 °C). 50% clot lysis times were determined as the time elapsed between the maximum turbidity and the midpoint of the maximum turbid-to-clear transition. To inhibit TAFIa, certain experiments were carried out in the presence 25 μg/mL carboxypeptidase inhibitor (CPI) from potato

tubers (Sigma, St. Louis, MO). When indicated, FXIIIa activity was blocked by the addition of 2 mM iodoacetamide to the reaction mixture.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (La Jolla, CA). Kruskal-Wallis one-way analysis of variance was used to determine differences among groups. The extent of significance was determined by Mann-Whitney U test; $p<0.05$ was considered as significant.

RESULTS

1. The effect of rhTM dependent FV_{Leiden} mutation on FXIII activation

1.1. The effect of rhTM on FXIII activation in plasma from individuals with different FV genotype

The kinetics of FXIII activation was followed by quantitative densitometry of Western blots and the results were expressed as percentage of maximal FXIII activation. To be able to compare the effect of rhTM on FXIII activation in the plasma samples of individuals with different FV genotypes quantitatively, the mean time required for the activation of half of the FXIII molecules present in the plasma ($T_{1/2}$ FXIII activation) was determined and the difference of $T_{1/2}$ values measured in the presence and absence of rhTM, ($T_{1/2}$ rhTM+)-($T_{1/2}$ rhTM-), was calculated.

In the absence of rhTM no relevant differences were seen in the rate or in the pattern of FXIII activation in the plasma of individuals with different FV genotype and the $T_{1/2}$ of FXIII activation did not differ significantly among genotypes. In the plasma of wild type individuals, the activation of FXIII occurred considerably later in the presence of rhTM than

in its absence. The effect of rhTM was considerably less in the case of heterozygous and homozygous individuals. $T_{1/2}$ values for FXIII activation measured in the presence of rhTM were more prolonged in the plasma samples of wild type individuals than in the plasma samples of FV_{Leiden} heterozygotes or homozygotes, but, due to considerable individual variation and the low sample number, the differences among genotypes were not statistically significant. However, when homozygotes and heterozygotes were grouped, the difference between FV_{Leiden} carriers and wild type individuals became significant. As individual variations in FXIII activation may partially obscure the genotype-dependent effect of rhTM, the delay in $T_{1/2}$ activation of FXIII, caused by rhTM was calculated for each individual and the effect of rhTM was expressed as the difference between $T_{1/2}$ values obtained in its presence ($T_{1/2}$rhTM+) and absence ($T_{1/2}$rhTM-) of rhTM. The mean difference between $T_{1/2}$rhTM+ and $T_{1/2}$rhTM- values was significantly more pronounced in the case of FV wild type individuals than in the cases of FV_{Leiden} heterozygotes and homozygotes (175 sec, 39 sec and 25 sec, respectively). Although the difference between heterozygotes and homozygotes was noticeable, it was not statistically significant.

1.2. rhTM effect of FXIII activation in FV deficient plasma supplemented with isolated wild type FV or FV_{Leiden}

In order to eliminate individual variations that influence clot formation we also followed a different approach. FV deficient plasma was supplemented with FV isolated from pooled plasma of individuals either wild type or homozygous for FV_{Leiden}. These supplemented plasma samples were used to study how FV of different genotypes influences the effect of rhTM on FXIII activation in the very same plasmatic environment. In the absence of rhTM, in the plasma with FV_{Leiden} FXIII activation occurred somewhat earlier than in the plasma with wild type FV, which might be related to some differences in the composition of the two partially purified FV preparations. In FV deficient plasma

supplemented with wild type FV rhTM significantly delayed FXIII activation, the difference between $T_{1/2}$ values was 185 sec. In the plasma supplemented with FV_{Leiden} the effect of rhTM was more moderate (the difference between $T_{1/2}$ values was only 65 sec).

2. rhTM dependent effect of FV_{Leiden} mutation on the formation of fibrin crosslinks catalyzed by FXIIIa

In the followings we tested how rhTM influences the cross-linking of fibrin chains in plasma samples from individuals of different FV genotypes. rhTM considerably delayed the dimerization of fibrin γ-chains in plasma samples containing wild type FV, but was much less effective in the plasma of FV_{Leiden} homozygotes. The effect of rhTM on the formation of fibrin α-chain polymers seemed to follow a similar pattern, however the slow formation of fully polymerized α-chain polymers was out of the timeframe of the experiments and quantitative measurement could not be performed. Similarly to the evaluation of FXIII activation, the time intervals required for half maximal γ-chain dimerization were used for quantitative comparisons. In the plasma samples of wild type individuals there was a considerable difference between $T_{1/2}$ obtained in the presence and absence of rhTM, while in FV_{Leiden} carriers only slight differences could be observed. In the plasma samples from FV_{Leiden} homozygotes rhTM delayed γ-chain dimerization to a lesser extent than in the plasma of heterozygotes, but the difference was not statistically significant.

3. rhTM dependent effect of FV_{Leiden} mutation on the FXIIIa-induced formation of crosslinks between α_2-PI and fibrin

Knowing that FXIII is a key regulator of fibrinolysis, in the followings we tested the effect of rhTM on fibrinolysis in plasma samples of individuals with different FV genotypes.

3.1 The effect of rhTM on the formation of α_2-PI-fibrin crosslinks in plasma samples form individuals with different FV genotype

In the plasma samples from FV wild type individuals rhTM delayed the formation of α_2-PI-fibrin α-chain heterodimer, it starts and becomes intensive significantly later than in the absence of rhTM. There is also a significant difference in the appearance of high Mr α_2-PI containing polymers. In contrast, the difference between samples with and without rhTM disappears in patients homozygous for FV_{Leiden} mutation and even the presence of one mutant allele significantly decreases the difference. For quantitative comparison, we calculated the mean time required for the crosslinking of 50% of α_2-PI to fibrin α-chain in the presence ($T_{1/2}$rhTM+) and absence ($T_{1/2}$rhTM-) of rhTM. The average differences in the $T_{1/2}$ values ($T_{1/2}$rhTM+)-($T_{1/2}$rhTM-) between wild type individuals and heterozygotes or homozygotes are statistically significant. The two FV_{Leiden} carrier groups did not differ significantly. Similar FV_{Leiden} mutation-dependence was obtained when the formation of total cross-linked α_2-PI (α_2-PI-fibrin α-chain heterodimers plus oligomers) was measured in the presence and absence of rhTM.

3.2. The effect of rhTM on α_2-PI-fibrin cross-linking in FV deficient plasma supplemented with wild type FV or FV_{Leiden}

In order to eliminate individual variations that influence clot formation, a different approach was also followed. FV was isolated from pooled plasma of individuals either wild type or homozygous for FV_{Leiden} and these isolated crude FV preparations were used to supplement FV deficient plasma. The supplemented plasma samples, which differed only in the genotype of FV, were used to study how FV_{Leiden} mutation influences the effect of rhTM on α_2-PI cross-linking. In FV deficient plasma supplemented with wild type FV rhTM significantly delayed α_2-PI cross-linking. In the plasma supplemented with FV_{Leiden} the effect

of rhTM was strongly diminished.

4. FV_{Leiden} dependent effect of rhTM on tPA induced fibrinolysis

As the cross-linking of α_2-PI to fibrin has a major impact on fibrinolysis, in the followings we investigated the effect of rhTM on tPA induced clot lysis. In the absence of rhTM there was no difference between the two genotypes (wild type and FV_{Leiden} homozygote). rhTM significantly prolonged the clot lysis time in both genotypes, i.e., the fibrinolysis was significantly decreased if rhTM was present. However, in the case of FV_{Leiden} homozygotes rhTM was significantly more effective than in the case of wild type individuals. In the former case 50% lysis occurred 40 minutes later than in the plasma of FV wild type individuals. A major effect of TM on fibrinolysis is exerted through affecting the activation of TAFI. To prove that its effect on α_2-PI cross-linking also plays a role in the regulation of fibrinolysis, experiments were also carried out in the presence of CPI, a TAFIa inhibitor and also in condition when both TAFIa and FXIIIa were inhibited. In sharp contrast with the results obtained without TAFIa inhibitor, when TAFIa was inhibited rhTM resulted in accelerated fibrinolysis in FV wild type plasma and rhTM did not accelerated fibrinolysis in plasma samples from individuals with FV_{Leiden} mutation. It seemed very likely that when TAFIa was blocked, the acceleration of fibrinolysis by rhTM in wild type plasma samples was due to the delay in the cross-linking of α_2-PI, which did not occur in plasma from FV_{Leiden} mutants. To prove the role of FXIII, i.e., α_2-PI cross-linking, in the effect of rhTM in TAFIa blocked plasma samples, the clot lysis assay was also carried out in the presence of FXIIIa inhibitor. In this case clot lysis was equally and highly accelerated in all plasma samples and the difference between wild type plasma samples with and without rhTM disappeared.

DISCUSSION

TM could influence FXIII activation by direct and an indirect mechanisms. In the absence of fibrinogen high concentration of thrombin is required for the activation of purified plasma FXIII and complex formation with TM impairs the FXIII activating capability of thrombin. If fibrinogen is also present a different picture emerges. The activation of plasma FXIII is enhanced by polymerizing fibrin approximately 100-fold. There is a clear correlation between fibrin formation and FXIII activation and in plasma the onset of fibrin formation is followed by the onset of FXIII activation. It has also been shown that the activation of FXIII in plasma occurs only on the surface of polymerized fibrin. The binding to fibrin makes the orientation of both FXIII and thrombin favorable for the proteolysis of FXIII-A at Arg37-Gly38 and accelerates the thrombin-induced cleavage of FXIII-A. Consequently, much lower concentration of thrombin is sufficient to activate FXIII in plasmatic conditions. Here TM exerts its effect primarily through different mechanisms. It competes with fibrin for the binding of exosite 1 in thrombin. As the binding of fibrin to certain amino acid side-chains present in exosite 1 is essential to its cofactor activity on FXIII-A truncation by thrombin, TM inhibits the acceleration of FXIII activation on the surface of fibrin. An additional indirect mechanism, by which TM influences FXIII activation, operates through enhancing the thrombin-induced activation of protein C. APC proteolytically cleaves FVa and FVIIIa and down-regulates the promotion of thrombin generation by these activated clotting factors. Then, impaired thrombin generation might lead to decreased/delayed activation of FXIII.

In our experiments the presence of rhTM caused a significant delay of FXIII activation and fibrin cross-linking only in the plasma of individuals wild type for FV. This finding suggests that in whole plasma it is not the direct inhibitory effect of TM on thrombin, but the suppression of thrombin generation and fibrin formation that play the primary role in delaying

FXIII activation by TM, i.e., in the plasma from wild type individuals the formation of both the activator protease and the enhancer fibrin becomes impaired. In the case of FV_{Leiden} the down-regulation of thrombin generation and fibrin formation through the APC pathway is lost and rhTM delayed FXIII activation and fibrin cross-linking only to a minimal extent.

Based on our experiments although we found difference between FV_{Leiden} hetero-and homozygotes, but this was not statistically significant. This is partly explained by the small number of subjects. FV_{Leiden} homozygotes, wild type for FXIII Val34Leu polymorphism and not on anticoagulant treatment, were very hard to recruit and five was the maximal number which we were able to select for the study from over three thousands genotyped individuals. On the other hand, in the experimental set-up the detection of FV_{Leiden} mutation's effect was the prime consideration, not the examination of differences between hetero- and homozygous state. By varying of the experimental conditions we could have found conditions under which the difference between heterozygotes and homozygotes were more pronounced, but in this case, due to the small number of subjects, we might have lost the statistical significance of the difference between heterozygotes and wild type individuals.

The effect of TM on FXIII activation and fibrin cross-linking might be important in the regulation of fibrinolysis and the impairment of this regulatory mechanism in FV_{Leiden} carriers might contribute to the increased risk of venous thromboembolism conferred by the mutation. Indeed, one of the prothrombotic effects of FV_{Leiden} is the inhibition of fibrinolysis, which has been demonstrated both in vitro and in vivo in transgenic mice. Two key down-regulators of fibrinolysis are TAFI and FXIII. TAFI, is activated by thrombin, a process that is more than 1,000-fold enhanced in the presence of TM. Activated TAFI cleaves off C-terminal lysines from partially digested fibrin, which are required for efficient plasminogen activation. TM regulates TAFI by an intricate mechanism. It increases the rate of TAFI activation by thrombin, but at the same time, through the activation of PC, it decreases the generation of thrombin required for TAFI activation. These effects of TM are concentration

dependent, at low TM concentrations the activation of TAFI is predominantly stimulated, whereas at high concentrations, the decreased thrombin generation, due to the effect of APC on FV, results in decreased TAFI activation. Accordingly, at low TM concentrations its antifibrinolytic/prothrombotic effect, while at high TM concentrations its profibrinolytic/antithrombotic effect prevails. In the case of FV_{Leiden} this antithrombotic effect is abrogated, i.e., TM at high concentration does not influence the activation of TAFI. Similarly, FV_{Leiden} also abrogates the profibrinolytic/antithrombotic effect of TM exerted through FXIII activation and fibrin cross-linking. The diminished delaying effect of TM on FXIII activation in FV_{Leiden} carriers could represent a novel downstream molecular mechanism that, in addition to the effect on TAFI activation, contributes to the increased risk of thrombosis in these individuals.

SUMMARY

FV_{Leiden} mutation is common among Caucasians and it is associated with a 5-8-fold increased risk of venous thromboembolism in heterozygotes and with a 50-80-fold risk among homozygotes. FV_{Leiden} is associated with an impaired down-regulation of the procoagulant activity exerted by activated FV and with the loss of FV anticoagulant function, hereby it compromises the down-regulation of thrombin generation. The downstream consequences of FV_{Leiden}, which might be connected to increased thrombin generation, have been revealed only partially. The effect of FV_{Leiden} on FXIII activation, on the cross-linking of fibrin chains and α_2-PI to fibrin have not been investigated.

In the plasma samples of fifteen healthy individuals with known FV_{Leiden} genotype the physiological, extrinsic pathway of coagulation was initiated by recombinant human tissue factor, phospholipids and Ca^{2+} in the presence and absence of rhTM. Clots were recovered after various time intervals, the extent of FXIII activation, fibrin cross-linking and α_2-PI-fibrin cross-linking were analyzed by SDS-PAGE and Western blotting, then quantified by densitometry. The experiments were also carried out using FV deficient plasma supplemented with purified wild type FV or $FV_{Leiden.}$ The effect of rhTM on tPA induced clot lysis was measured by turbidimetric method.

rhTM significantly delayed the activation of FXIII, fibrin cross-linking and the formation of α_2-PI-fibrin α-chain heterodimers/oligomers in the plasma from FV wild type individuals. This effect of rhTM was impaired in the presence of FV_{Leiden}. rhTM delayed tPA-induced clot lysis and this effect of rhTM was more pronounced in plasma containing FV_{Leiden}. When TAFIa was inhibited by potato carboxypeptidase inhibitor, rhTM accelerated clot lysis in the presence of wild type FV, which is explained by the delayed α_2-PI-fibrin cross-linking. This effect of rhTM did not prevail in the presence of FV_{Leiden}.

FV_{Leiden} abrogates the delaying effect of rhTM on FXIII activation, on fibrin chain cross-linking and on α_2-PI-fibrin cross-linking, which contribute to the impaired fibrinolysis. Our results reveal a novel mechanism that contributes to the increased thrombosis risk in FV_{Leiden} carriers.

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2. **Koncz, Z.**, Bagoly, Z., Haramura, G., Mezei, Z.A., Muszbek, L.: Thrombomodulin-dependent effect of factor V Leiden mutation on the cross-linking of alfa2-plasmin inhibitor to fibrin and its consequences on fibrinolysis.
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3. **Koncz, Z.**, Bagoly, Z., Haramura, G., Mezei, Z.A., Muszbek, L.: Thrombomodulin-dependent effect of factor VLeiden mutation on factor XIII activation.
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