UNIVERSITY RESEARCH DOCTORATE (PhD) DISSERTATION

THE REGULATION OF BLOOD COAGULATION FACTOR XIII BY HUMAN NEUTROPHIL PROTEASES

Zsuzsa Bagoly, MD

Research supervisor: László Muszbek, MD, PhD
PhD program supervisor: László Muszbek, MD, PhD

UNIVERSITY OF DEBRECEN
MEDICAL AND HEALTH SCIENCE CENTER
CLINICAL RESEARCH CENTER
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ABBREVIATIONS

α_1AT: α_1-antitrypsin

α_2PI: α_2-plasmin inhibitor

FV: factor V

FVII: factor VII

FVIII: factor VIII

FIX: factor IX

FX: factor X

FXII: factor XII

Factor XIII abbreviations:

- FXIII: factor XIII
- pFXIII: blood (plasma) coagulation FXIII
- cFXIII: cellular FXIII
- FXIIIa: activated FXIII
- FXIII-A: FXIII A subunit
- FXIII-B: FXIII B subunit
- AP-FXIII: FXIII activation peptide
- FXIII-A‘: truncated FXIII A subunit
- FXIII-A*: truncated active form of FXIII A subunit
- FXIII-A_2‘: truncated FXIII-A dimer
- FXIII-A_2*: truncated active form of the FXIII-A dimer
- G38-FXIII-A_2*: truncated active form of the FXIII-A dimer, truncated at G38
- N40-FXIII-A_2*: truncated active form of the FXIII-A dimer, truncated at N40
- FXIII-A_2°: non-truncated active form of the FXIII-A dimer
- FXIIIa[e]: HNE-activated FXIII
- FXIIIa[t]: thrombin-activated FXIII

fMLP: N-formyl-Met-Leu-Phe

HNE: human neutrophil elastase

MALDI TOF: matrix-assisted laser desorption/ionization time-of-flight

MMPs: matrix metalloproteases

MMP-9: matrix metalloprotease 9

PMNs: polymorphonuclear granulocytes

SDS PAGE: sodium dodecyl sulphate polyacrilamide gel electrophoresis

tPA: tissue plasminogen activator

uPA: urokinase plasminogen activator

INTRODUCTION AND REVIEW OF LITERATURE

Blood coagulation factor XIII (pFXIII) is a protransglutaminase of tetrameric structure containing two types of subunits (A_2B_2) [1,2]. The potentially active A subunit of FXIII (FXIII-A) has a molecular mass of ~83 kDa and its activated form catalyzes an acyl transfer reaction. The B subunit (FXIII-B) has a molecular mass of ~80 kDa and fulfills a carrier/protective function. FXIII-A is primarily synthesized by cells of bone marrow origin, while FXIII-B is synthesized by hepatocytes. In normal conditions, only complexed FXIII-A is present in the plasma, while the B subunits are in excess, about 50% of them circulates in an un-complexed form. Cellular FXIII (cFXIII) is a homodimer of FXIII-A. It is present in platelets, monocytes, in their bone marrow precursor cells and in monocyte-derived macrophages [3-6]. FXIII-A consists of five sequentially folded and well-defined domains: activation peptide (1-37 amino acids), beta sandwich (38-184 amino acids), catalytic core (185-515 amino acids), barrel 1 (516-628 amino acids) and barrel 2 domains (629-730 amino acids) [7]. FXIII-B is a glycoprotein consisting of 10 so-called „sushi-domain" repeats, approximately 60 amino acids each with 2 disulfide bridges. „Sushi-domains" have been found in more than 20 other proteins, and it has been suggested that these domains are involved in binding the molecule to another protein.

pFXIII becomes activated by the concerted action of thrombin and Ca^{2+} in the final phase of the clotting cascade. Thrombin cleaves off the N-terminal activation peptide (AP-FXIII) by hydrolyzing the peptide bond of R37-G38 in FXIII-A. Then, in the presence of Ca^{2+} the B subunits dissociate and the resulting truncated FXIII-A dimer (FXIII-A_2') assumes an enzymatically active configuration (G38-FXIII-A_2*). During this conformational change the active site cysteine, originally buried within the catalytic core domain, becomes unmasked and available for reaction with its substrates. The intracellular activation of cFXIII is a slower process, it does not require proteolytic splitting; the increase of Ca^{2+} concentration is sufficient to transform FXIII-A_2 into an active form (FXIII-A_2°) [1]. In extracellular conditions cFXIII could be activated by thrombin and Ca^{2+} the same way as pFXIII, of course, excluding the dissociation of the B subunits. Interestingly, the release of AP-FXIII from one of the two A subunits is sufficient to induce full transglutaminase activity by

Ca^{2+} [8]. The presence of polymerized fibrin (fibrin I or II, devoid of fibrinopeptide A or fibrinopeptides A and B, respectively) greatly accelerates the thrombin-induced cleavage of FXIII-A, therefore it has an important enhancing effect on FXIII activation [9]. In the case of cFXIII activation, fibrin has no such accelerating effect, indicating that in the presence of fibrin FXIII-B could influence the orientation of the A_2B_2 complex and its interaction with thrombin [9,10].

The rate of thrombin-induced truncation of both pFXIII and cFXIII is also influenced by a common polymorphism in FXIII-A resulting in V/L replacement at position 34 in the activation peptide [11]. This site is located just three amino acids upstream from the thrombin cleavage site, therefore it is not surprising that the polymorphism influences the effect of thrombin on FXIII activation. It was demonstrated with both cFXIII [12] and pFXIII [13,14] that in the case of the L34 variant the truncation of FXIII-A together with the release of AP-FXIII and the consequent activation of FXIII proceeds at an approximately 2.5-folds higher rate than in the case of the V34 variant. Such facilitation of the proteolytic activation of FXIII was found to be independent of the presence or absence of fibrinogen although fibrinogen itself increases the catalytic efficiency [14].

Thrombin is not the only serine protease that could cleave and activate FXIII in the presence of Ca^{2+}. Several other proteases, including batroxobin marajoensis [15], thrombocytin [16], trypsin [17,18] and activated factor X [19] have been reported to be able to activate FXIII. These proteases have substrate specificity similar to that of thrombin, they cleave polypeptides at arginine and lysine residues. Although the cleavage site of these enzymes on FXIII-A has not been identified, based on their substrate specificity and on the Mr of the truncated FXIII-A, it is assumed that the active form of FXIII-A produced by these proteases is also G38-FXIII-A_2*, just like in the case of thrombin. In fact, no other active forms of truncated FXIII-A, other than G38-FXIII-A_2* has ever been identified. It is also not known whether G38 and the subsequent N terminal amino acids of FXIII-A* are essential for the formation of an enzymatically active FXIII-A.

Activated FXIII (FXIIIa), a transglutaminase, catalyzes an acyl transfer reaction [1,2]. The reaction consists of two major steps. In the first step a peptide-bound glutamine substrate forms a binary complex with the enzyme (thioester linkage with the active site cysteine), which is accompanied by ammonia release. In the second step, if a substrate primary amine group is present, the acyl group is

transferred to the acyl acceptor amine and the amine becomes linked to a γ-glutamyl residue via peptide („isopeptide“) bond. In the absence of a substrate amine the end-result of the reaction is the deamidation of the substrate glutamine residue. If an ε-amino group of a peptide bound lysine residue is the primary amine for the reaction, ε(γ-glutamyl)lysyl is formed and the two peptide chains become cross-linked. The latter mechanism is considered to be the physiological function of FXIIIa.

FXIIIa improves the mechanical strength of the fibrin clot and protects it from fibrinolysis by at least two major mechanisms: 1/ FXIIIa cross-links fibrin chains, which renders the clot more resistant to fibrinolysis. 2/ The binding of α_2-plasmin inhibitor (α_2PI) and perhaps other plasma components to fibrin prevents the prompt elimination of fibrin by the powerful fibrinolytic system.

Fibrin cross-linking by FXIIIa results in the formation of γ-chain dimers and α-chain polymers. γ-dimers are formed by a rapid reciprocal intermolecular bond formation between γ406 lysine of one γ-chain and γ398/399 glutamine residue of another aligning γ-chain [20]. α-chain cross-linking is a much slower process, it occurs among multiple glutamine and lysine residues, resulting in α-oligomers and high Mr α-polymers. It is generally accepted that α-chain cross-linking confers the final stability to the fibrin clot allowing strength, rigidity and resistance to fibrinolysis, although γ-chain dimers also contribute to clot stiffness [21,22]. The weaker binding of plasminogen to cross-linked than to non-cross-linked fibrin might also be involved in FXIII-induced resistance to fibrinolysis [23]. In addition, the cross-linking of lysine residues by FXIIIa in the C-terminal domain of fibrin α-chain reduces the number of binding sites [24] which, following proteolysis by plasmin could be used by plasminogen and tPA for binding and promoting plasminogen activation.

It has been demonstrated that extensive cross-linking of fibrin α-chains results in increased fibrinolytic resistance of the fibrin clot [25,26]. If cross-linking by FXIIIa was not regulated, it would result in an over-cross-linked fibrin network, which, together with the extensive cross-linking of other proteins within the thrombus, would lead to its prolonged and undesired persistence. On this basis one would assume the existence of a down-regulating mechanism for the inactivation of FXIIIa within the fibrin clot.

Activated clotting factors may be down-regulated by two major mechanisms. Proteolytically active factors are inhibited by specific serine protease inhibitors, serpins (such as antithrombin III, heparin cofactor II and tissue factor pathway inhibitor), or by less specific protease inhibitors, like α_2-macroglobulin. Due to its unique feature of being a transglutaminase, FXIIIa down-regulation by serpins could not be operative and no other kind of plasma inhibitor of FXIIIa has been discovered, so far. The other way by which activated clotting factors are down-regulated is the inactivation of active factors by proteolytic enzymes. It can be carried out by highly specific proteases, like the cleavage of activated factor V (FVa) and factor VIII (FVIIIa) by activated protein C. The down-regulation could be exerted by less specific proteolytic enzymes, as well. Plasmin, a protease with much broader substrate specificity provides an example; it degrades fibrin, fibrinogen, FVa and FVIIIa. Although all clotting factors have known pathways of inactivation, in the case of FXIIIa, no down-regulating mechanism has been discovered, so far. As, in spite of significant efforts, no plasma protein inhibitor of FXIIIa has been found, one has to consider the proteolytic down-regulatory mechanism. Plasmin could be a candidate for such a role, however, it has been shown by Rider and Mcdonagh that both pFXIII and cFXIII and their activated forms are highly resistant to degradation by plasmin [27]. Besides, the powerful inhibitory effect of fibrin-linked α_2PI also makes it unlikely that, with the exception of the pharmacological condition of thrombolysis, plasmin could play a significant role in the down-regulation of FXIIIa within the fibrin clot.

Recently, much interest has been focused on the role of polymorphonuclear granulocytes (PMNs) in the pathophysiology of thrombus formation, thrombus remodeling and fibrinolysis. Fibrin, present in the thrombus, serves as a potent adhesive substrate for PMN binding [28]. Several other plasma proteins, which become incorporated in the thrombus, including fibronectin, thrombospondin, vitronectin and plasminogen, are also capable of mediating PMN adhesion [29,30]. The mechanism of incorporation of PMNs into the thrombus has been extensively studied most recently. It has been demonstrated under static or flow conditions that both stimulated and un-stimulated PMNs adhere to surface bound fibrinogen and fibrin [31-33]. The binding is supported by cell-surface receptors that belong to the family of integrins, including $\alpha_M\beta_2$ (Mac-1, CD11b/CD18), $\alpha_X\beta_2$ (CD11c/CD18) and

$\alpha_5\beta_1$ [31,34,35]. Although stimulated cells are capable of binding soluble fibrinogen, integrins expressed on resting PMNs do not bind soluble proteins and the prerequisite of binding is the transition of integrins into a high affinity state. Conformational changes in fibrinogen, which occur upon transformation into fibrin or during immobilization might also contribute to the adhesion of un-stimulated cells and their incorporation into fibrin clots [36]. Besides in vitro studies, several in vivo experimental models of thrombosis have shown that PMNs accumulate in the thrombus and they are the first among leukocytes to invade the haemostatic plug [37,38]. PMNs incorporated in the thrombus represent a rich source of proteases and in vivo experiments suggested that PMNs, trapped in the fibrin mesh during clotting, become partially degranulated [39]. It is well documented that proteases stored in PMN granules, such as human neutrophil elastase (HNE), cathepsin G or matrix metalloproteases (MMPs) interact with the haemostatic and fibrinolytic system and possess high fibrinolytic potential after being released [40,41]. PMNs could contribute to endogenous fibrinolysis through a urokinase type plasminogen activator (uPA) dependent pathway as well [42].

HNE and cathepsin G are serine proteases of the chymotrypsin family stored in the primary granules of PMNs. HNE is a major PMN protease with broad substrate specificity that cleaves peptide bonds at short side-chain aliphatic amino acids, preferably at valine [43-45]. Besides being involved in the cleavage of fibrin, interaction of HNE with several clotting and fibrinolytic proteins has been reported. It inactivates factors VII (FVII), VIII (FVIII), IX (FIX), X (FX), XII (FXII), antithrombin III, tissue factor pathway inhibitor, protein C and protein S [46]. HNE can activate FV, which is followed by a subsequent inactivation. It also inactivates thrombin-activated FV [47]. It cleaves plasminogen to form mini-plasminogen, which is more readily activated by plasminogen activators [48,49]. The catalytic activity of HNE in the circulation is regulated primarily by α_1-antitrypsin (α_1AT) and secondarily by α_2-macroglobulin [50,51]. When the down-regulation of HNE activity is impaired, for example, in the case of α_1AT deficiency, severe disease, associated with lung manifestation develops. The decreased anti-protease protection on the airway epithelial surface leads to pulmonary emphysema [52]. In contrast, elevated α_1AT and curtailed HNE function had a negative impact on the fibrinolytic potential in patients with pulmonary thromboembolism [53].

Cleavage of several haemostatic factors, including fibrinogen, FV, FVII, FVIII, FX, protein C and protein S by cathepsin G has also been reported. The cleavage of activated FVII, protein C and protein S results in the inactivation of the cleaved protein, while in the case of FV, FX and FVIII activation of the clotting factors was reported [54,55]. Just like HNE, cathepsin G is also inactivated by α_1AT [50,51].

MMPs constitute a tightly regulated family of zinc dependent endopeptidases that function in diverse proteolytic processes [56]. The members of the MMP family are divided into five classes based on their structure and substrate specificities. These are collagenases, gelatinases, stromelysins, membrane type MMPs and others. Of the members of the gelatinase family, matrix metalloprotease 9 (MMP-9) is present in PMNs and its role in arterial remodeling has been confirmed in MMP-9 deficient mice [57]. PMN-derived MMP-9 was also associated with aortic wall degeneration and aneurysm formation [39].

The effect of PMN proteases on FXIII within the fibrin clot has not yet been investigated. A few early studies have shown that HNE and cathepsin G proteolytically degrades, but does not activate FXIII [17,58,59]. Another study, using qualitative activity-staining technique suggested that in non-physiological conditions, i.e. in the presence of EDTA, a potentially active form of pFXIII is formed transiently [60]. Identification of cleavage sites was not attempted in these studies. It is not known, whether proteases released from PMNs in fibrin clots could cleave FXIII, and whether it has an effect on FXIII activity. It is also a question whether PMN proteases could exert any effect on FXIII in the more complex environment of plasma clot, where α_1AT exerts a potent inhibitory effect. It has not been investigated whether PMN proteases have any influence on the fibrin cross-linking process and therefore the final structure and the ageing of the clot.

THE AIM OF THE STUDY

To evaluate the possible role of PMN proteases in the regulation of FXIII activity.

1. To study how the proteolytic cleavage by HNE affects pFXIII and cFXIII.
2. To find out whether cleavage of FXIII by HNE, the substrate specificity of which is substantially different from that of thrombin, could result in an active truncated FXIII different from G38-FXIII-A* and to provide new pieces of information on the structural requirement of transglutaminase activity of FXIIIa.
3. To study the effect of PMN proteases on purified FXIIIa: to find out whether proteolytic degradation of FXIII occurs and study its relation with FXIII activity.
4. To demonstrate the release and the proteolytic activity of PMN proteases in fibrin clots.
5. To investigate the effect of PMN proteases on activated FXIII within the fibrin clot. To determine the relative importance of certain individual PMN proteases when interacting with FXIIIa.
6. To test the effect of PMN proteases on FXIIIa in fibrin clots with/without the addition of α_1AT and in plasma clots.
7. To investigate whether PMN proteases influence the extent of cross-linking in fibrin clots and whether it has any physiological implications.

MATERIALS AND METHODS

Materials

Fibrinogen from human plasma (plasminogen free, containing FXIII), human thrombin (3093 NIH units/mg), α_1AT, cathepsin G substrate (N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide), N-formyl-Met-Leu-Phe (fMLP) and recombinant hirudin (11500 units/mg) were from Sigma-Aldrich (St. Louis, MO). The FXIII content of the fibrinogen preparation was 6.2 μg/mg fibrinogen as determined by a sandwich ELISA method, specific for the tetrameric pFXIII [61]. Fibrinogen depleted of plasminogen, fibronectin and FXIII was the product of American Diagnostica (Stamford, CT). Highly purified HNE (20-22 units/mg), HNE substrate (Methoxysuccinyl-Ala-Ala-Pro-Val-p-Nitroanilide), fluorogenic matrix metalloproteinase substrate III (Dabcyl-Gaba-PQGL-E(Edans)-AK-NH_2; MMP substrate III), HNE inhibitor IV (ONO 5046), cathepsin G inhibitor I, MMP-9 inhibitor and rabbit anti-human FXIII-A were purchased from Calbiochem (La Jolla, CA). The proteolytic activity of HNE was fully inhibited by its specific inhibitor ONO 5046 demonstrating that no other proteolytic enzyme contaminated the preparation. Batroxobin moojeni, isolated from the venom of Bothrops moojeni was purchased from Pentapharm (Basel, Switzerland). Dextran 500, Ficoll-Paque^{TM} Plus and enhanced chemiluminescence reagent (ECL Plus+) were obtained from Amersham Biosciences (Uppsala, Sweden). Complete protease inhibitor cocktail was the product of Roche Diagnostics (Penzberg, Germany). Rabbit anti-human FXIII-B polyclonal antibody was from Diagnostica Stago (Assiéres, France). Vectastain ABC reagent was the product of Vector (Burlingame, CA). Highly purified human pFXIII was prepared from the pooled plasma of healthy volunteers according to Lorand et al. [62]. cFXIII was prepared from human placenta [63]. For certain experiments pFXIII was also prepared from the plasma of individuals with wild type FXIII-A (V34) and from individuals homozygous for the L34 allele (L34). The FXIII-A V34L genotype was determined by real-time polymerase chain reaction method developed in our laboratory [64].

Preparation of PMN leukocytes and their supernatant

Blood was collected from healthy donors who had not received any medication for at least 2 weeks. Drawing blood for the experiments was approved by the Ethical Review Board of the University of Debrecen, Medical and Health Science Center and blood donors signed informed consent. Nine volumes of blood were anticoagulated with 1 volume of 3.8 % trisodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation at 120 g for 25 min. PMN cells were isolated from the remaining blood by dextran sedimentation, followed by gradient centrifugation on Ficoll-Paque and hypotonic lysis of erythrocytes. To eliminate adhering platelets PMNs were washed 3-times in HEPES Tyrode buffer (140 mM NaCl, 10 mM $NaHCO_3$, 2.5 mM KCl, 0.5 mM NaH_2PO_4, 5.6 mM dextrose, 10 mM HEPES, pH 7.4) containing 5 mM EDTA, and finally resuspended in HEPES Tyrode buffer supplemented with 0.1 mM $MgCl_2$. Cell suspensions contained an average of 95% PMN granulocytes. To obtain supernatant of activated cells, $5x10^6$/ml or $20x10^6$/ml PMNs were incubated with 2 μM fMLP and 2 mM $CaCl_2$ for 2 min at 37 °C. The cells were then pelleted (1000 g, 1 min) and the supernatant was removed for further use. To obtain platelet-poor plasma (PPP) PRP was centrifuged at 1,300 g for 15 min at room temperature. In order to remove residual platelets the centrifugation was repeated and the second supernatant was allowed to filter through a membrane filter of 0.2 μm pore size by gravity.

SDS PAGE and Western blotting

Aliquots of denatured plasma/serum, fibrin or FXIII samples were analyzed by SDS PAGE. Extent of fibrin cross-linking or fibrin degradation was followed on 10 % SDS gel, stained with Coomassie brillant blue. When the proteolytic degradation of purified FXIII by the supernatant of activated PMNs were studied, the gels were silver stained. After SDS PAGE part of the samples were subjected to Western blotting. Rabbit polyclonal antibodies against FXIII-A or FXIII-B were used as primary antibodies. The immuno-reaction was developed by biotinylated anti-rabbit goat antibody and avidin-biotinylated peroxidase complex (components of the Vectastain ABC kit) and visualized by enhanced chemiluminescence reagent according to the manufacturer's instructions. In certain experiments results were quantified by GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA).

Measurement of HNE, cathepsin G and MMP-9 activity

HNE and cathepsin G activity in the supernatant of fMLP activated PMN cells was measured in the presence of 0.66 mM HNE or cathepsin G substrate at 405 nm according to the manufacturer's instructions. For the measurement of MMP-9 activity the fluorescence resonance energy transfer (FRET) peptide substrate (MMP III substrate) was used. This substrate has an EDANS/Dabcyl fluorophore/quencher combination. After the cleavage of G-L bond by MMP-9 the quenching effect becomes abrogated and the increase in fluorescence (λ_{ex}=340 nm, λ_{em} = 485 nm) reflects the MMP-9 activity. To detect the release of proteolytic enzymes from PMNs within fibrin clots, 2.1 mg/ml fibrinogen containing 13 μg/ml plasma FXIII and $20x10^6$/ml PMN granulocytes in HEPES Tyrode was supplemented with one of the following protease substrates: 0.66 mM HNE substrate, 0.66 mM cathepsin G substrate or 5 μM MMP substrate III. Fibrinogen was clotted by 1.2 U/ml thrombin and 2 mM $CaCl_2$. Clots were incubated for 30 min at 37 °C, then were centrifuged and the amount of p-nitroanilide liberated from the chromogenic HNE or cathepsin G substrate was measured in the clot liquor at 405 nm. MMP-9 activity was determined by measuring the fluorescence intensity in the clot supernatant. To test the extent of inhibition by specific protease inhibitors in the supernatant of activated PMNs or within the clot the above experiments with HNE, cathepsin G and MMP-9 substrates were also carried out in the presence of the respective protease inhibitor (10 μM HNE inhibitor IV, 10 μM cathepsin G inhibitor I or 0.1 μM MMP-9 inhibitor I). All inhibitors were applied in concentrations more than one magnitude higher than their IC 50 for the respective human PMN enzymes (IC50 for ONO 5046: 44 nM, for cathepsin G inhibitor I: 53 nM, for MMP-9 inhibitor I: 5 nM) and no further inhibition was achieved by raising the inhibitor concentration.

Measurement of transglutaminase activity

FXIII activity was measured according to a spectrophotometric transglutaminase assay [65] using REA-chrom FXIII assay kit (Reanal-ker, Budapest, Hungary). In experiments where the activation of FXIII by HNE was investigated, thrombin and GPRP were omitted from the reagent mixture. In the assay the ammonia released by FXIIIa from a glutamine containing dodecapeptide that corresponds to the N-terminal sequence of α_2PI was continuously monitored by the NADPH-dependent glutamate

dehydrogenase reaction. After a 5 min lag time the decrease of NADPH absorbance at 340 nm became a linear function of transglutaminase activity. The results were expressed as U/l and as percentage of maximal transglutaminase activity obtained following thrombin activation. In the latter case FXIII was activated by 10 U/ml thrombin and after 5 min thrombin was blocked by 15 U/ml hirudin. To compensate for the innate activity of zymogen FXIII [66], samples with non-activated zymogen were run in parallel and the innate activity was deducted from the results. As it has been reported, the innate activity measured with low-molecular-weight amine substrate was minimal [66], in our case, it corresponded to 2-3% of HNE-induced maximal FXIII activity.

The effect of HNE on FXIII

25 μg/ml pFXIII or cFXIII was incubated with various concentrations of HNE in 50 mM HEPES, 100 mM NaCl buffer (pH 7.4) containing 2.5 mM $CaCl_2$ at 37 °C for 20 min. In certain experiments 2.5 mg/ml fibrinogen and/or 1.5 mg/ml α_1AT was also included into the incubation mixture, in this case the effect of 5 μg/ml HNE was tested. In kinetic experiments various concentrations of pFXIII and cFXIII, up-to 400 μg/ml, and 2.5 μg/ml HNE were used. After 20 min 10 μM ONO 5046 was added to the incubation mixture to block HNE, and aliquots were removed for FXIII activity measurements and for Western blotting.

Evaluation of the fibrin cross-linking capacity of HNE-activated FXIII using batroxobin moojeni

Fibrin cross-linking was tested in fibrin prepared from 2.5 mg/ml FXIII-depleted fibrinogen solution (in 50 mM HEPES, 100 mM NaCl, pH 7.4) supplemented with 25 μg/ml non-activated, HNE-activated or thrombin-activated FXIII. Fibrin prepared from non-supplemented fibrinogen served as control. HNE-activated FXIII was prepared by incubating 100 μg/ml pFXIII with 10 μg/ml HNE in 50 mM HEPES, 100 mM NaCl buffer (pH 7.4) containing 2.5 mM $CaCl_2$ at 37 °C. After 20 min the reaction was stopped by 10 μM ONO 5046. In the case of thrombin-activated FXIII, 10 U/ml thrombin was applied instead of HNE, and after 5 min incubation thrombin was blocked by 15 U/ml hirudin. Fibrinogen solutions were clotted by either 1.2 U/ml thrombin or by batroxobin moojeni of equivalent clotting activity (6.7 BU/ml). Clots

were incubated for 30 min at 37 °C, and the reactions were stopped by adding 7 volumes of SDS PAGE sample buffer containing 8 M urea. The samples were analyzed by SDS PAGE in reducing condition. To find out if batroxobin moojeni exerted any proteolytic effect on FXIII-A, fibrinogen samples supplemented with non-activated FXIII clotted by batroxobin moojeni were also analyzed by Western blotting for FXIII-A.

Identification of HNE cleavage sites in FXIII-A by MALDI TOF mass spectrometry and N-terminal sequencing

100 µg/ml pFXIII or cFXIII was incubated with 10 µg/ml HNE and 2.5 mM $CaCl_2$ at 37 °C and after various intervals the reaction was terminated by ONO 5046. Samples were boiled for 5 min, heat-precipitated proteins were removed by centrifugation and the supernatants were filtered through disposable centrifuge filter device of 0.2 µm pore size (Whatman, Maidstone, UK). Peptides that remained in the filtrate were purified and desalted using reversed-phase C18 matrix in pipette tips (ZipTip™, Millipore, Billerica, MA) according to the manufacturer's recommendations. After washing, the bound peptides were eluted with 1:4 dilution of saturated α-cyano-4-hydroxycinnamic acid (in 50 % acetonitrile, 0.1 % trifluoroacetic acid). The eluted samples were directly spotted on stainless steel MALDI sample plate and allowed to air-dry. MALDI-TOF mass spectrometry was done on a Voyager DE STR (Applied Biosystems, Foster City, CA) operated in positive linear and reflectron mode.

For N-terminal sequencing experiments 100 µg/ml cFXIII was incubated with 10 µg/ml HNE and 2.5 mM $CaCl_2$ for 10 minutes. Samples were subjected to SDS PAGE analysis in 7.5 % gels and proteins were blotted onto PVDF membrane. The membranes were stained with Coomassie brillant blue, the band corresponding to HNE-cleaved FXIII-A was excised and the truncated protein was sequenced using an Applied Biosystems protein sequencer (model Procise 494) employing Edman degradation program [67].

Molecular modeling of cFXIII structure around the primary HNE cleavage site

The cFXIII geometry was obtained from the protein crystallographic database (www.rcsb.org, accession code: 1F13) [68]. The few missing residues at the cleavage site and the N- and C-terminal parts were completed by means of loop searching and

protein-building tools implemented in the SYBYL modeling package (SYBYL 7.0, Tripos Inc., St. Louis, MO). The figure was generated using "new cartoon" representation and POV3 (Persistence Of Vision) rendering options in the 1.8.6 (April, 2007) version of the VMD (Visual Molecular Dynamics) [69] software suite.

The effect of activated PMN supernatant on FXIIIa

100 μg/ml pFXIII was activated by 40 U/ml thrombin and 10 mM $CaCl_2$ for 5 min at 37 °C in 500 μl final volume. Then, 2 ml supernatant of $5x10^6$/ml fMLP-activated PMNs was added and the mixture was incubated at 37 °C. At various intervals aliquots were removed for FXIII activity measurements and for SDS PAGE.

The effect of proteases released by PMNs on thrombin activated FXIII within fibrin clots

2.1 mg/ml fibrinogen containing 13 μg/ml pFXIII in HEPES Tyrode was supplemented with PMN suspensions ($5x10^6$/ml or $20x10^6$/ml). Fibrinogen was clotted with 1.2 U/ml thrombin and 2 mM $CaCl_2$. Clots were incubated at 37 °C for various times, dissolved in sample buffer and subjected to Western blotting for both FXIII subunits. The above experiments were also carried out in the presence of protease inhibitors. To block HNE, cathepsin G or MMP-9, 10 μM ONO 5046, 10 μM cathepsin G inhibitor I or 0.1 μM MMP-9 inhibitor I were included in the incubation mixture, respectively. In addition, inhibition experiments were also performed in the presence of 1.5 g/l α_1AT.

Activation of FXIII in plasma and the recovery of fibrin

100 μl aliquots of normal platelet-free plasma were mixed with 20 μl thrombin-$CaCl_2$ to give final concentrations of 0.1-0.3 U/ml thrombin and 18 mM $CaCl_2$. After incubations for various intervals at 37 °C, the reaction was stopped by an equal volume of inhibitor cocktail to block thrombin and FXIIIa and to prevent fibrinolysis. The samples were centrifuged, the supernatants were removed and added to 9 volumes of SDS PAGE sample buffer. The remaining fibrin clot, if present, was thoroughly washed with physiological NaCl solution and then dissolved in sample buffer. Samples were analyzed by SDS PAGE and Western blotting for FXIII-A. Similar experiments were also carried out in plasma supplemented with PMNs. In this

case platelet-free plasma was incubated with or without PMNs ($5x10^6$/ml or $20x10^6$/ml) at 37 °C and samples were clotted with 1.2 U/ml thrombin and 18 mM $CaCl_2$. After certain intervals the reaction was stopped by adding protease inhibitor cocktail and fibrin was recovered after extensive washing.

RESULTS

HNE-induced activation of FXIII

In these series of experiments we tested the effect of purified HNE on purified pFXIII and cFXIII. HNE activated pFXIII (Fig. 1) and cFXIII (the latter is not shown) in a concentration dependent manner; the Km values were 2.73 μM and 3.10 μM, respectively. The maximum transglutaminase activity, obtained by 5 μg/ml HNE, was approximately 50% of that achieved by full thrombin activation. At higher concentrations of HNE the transglutaminase activity started to decline, i.e., further proteolytic cleavage of FXIII by HNE resulted in the inactivation of FXIIIa.

The time course of 25 μg/ml pFXIII and cFXIII activation by 5 μg/ml HNE was followed by Western blotting (Fig. 2A) and by activity measurements (Fig. 2B).
In the absence of HNE only non-activated FXIII-A was present even after 3-hour incubation. A considerable amount of truncated FXIII-A appeared after 5 min incubation with HNE. At the same time, significant transglutaminase activity was measured demonstrating that, due to the concerted action of proteolytic cleavage by HNE and Ca^{2+}, part of FXIII molecules became activated, i.e. transformed into FXIII-A*. The Mr of HNE-truncated FXIII-A (~78 kD) roughly corresponded to the Mr of thrombin-activated FXIII-A. The amount of FXIII-A* was maximal between 10-20 min, then it gradually decreased due to further proteolysis. After 3 hours only a faint band corresponding to FXIII-A* was detected. The transglutaminase activity was maximal after 10 min incubation, reaching 52.5% and 67.4% of thrombin-activated pFXIII and cFXIII, respectively. The truncation of FXIII-A and the appearance of transglutaminase activity did not run completely parallel. This is very likely due to the full enzyme activity of the dimer consisting of a cleaved and an un-cleaved FXIII-A [8]. After reaching the maximum, the activity gradually declined and reached zero after 3 hours. In the case of cFXIII (in the absence of B subunit), the activation

seemed to occur slightly faster than in the case of pFXIII, however, the difference was not significant.

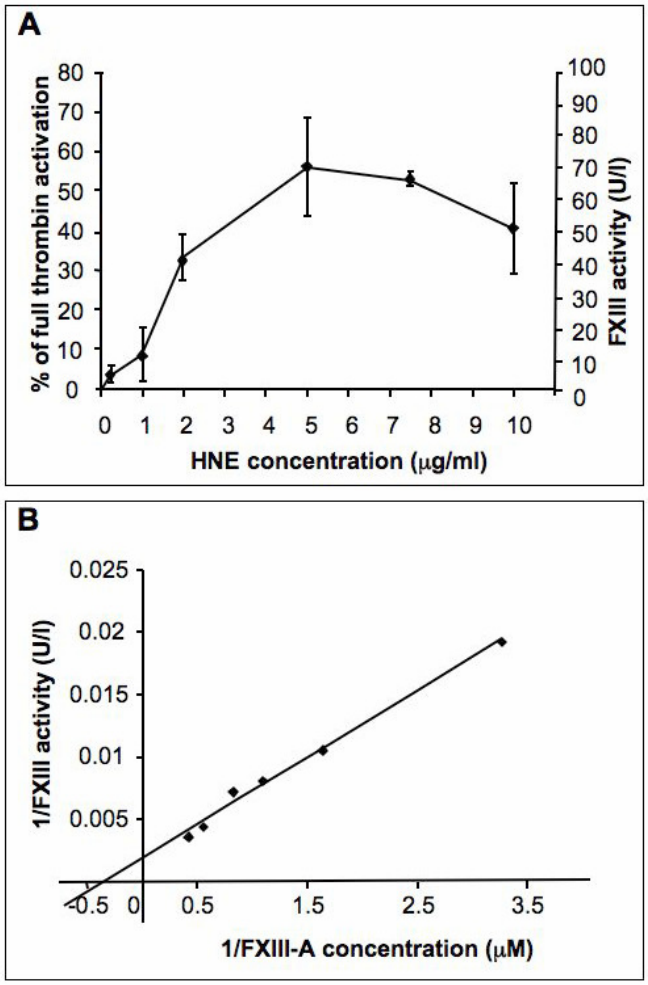


Figure 1. Activation of pFXIII by HNE. Panel A: dependence of FXIII activation on HNE concentration. Results represent means of three parallel experiments and standard deviations. Panel B: Lineweaver-Burk plot of the substrate dependence of plasma FXIII activation by HNE. Km values of 2.64 μM and 2.81 μM were calculated from two parallel experiments.

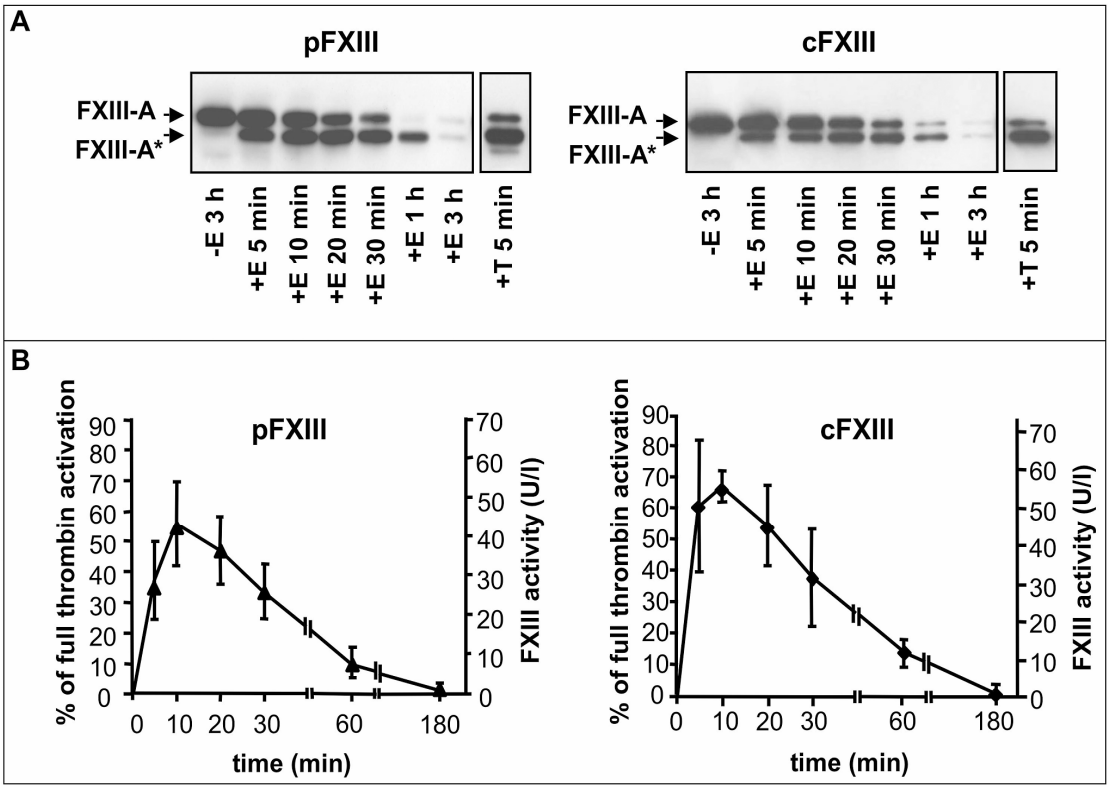


Figure 2. Time-course of the proteolytic cleavage and activation of pFXIII and cFXIII by HNE. pFXIII and cFXIII was incubated with or without HNE (+E or –E) as described in the text. Incubation of pFXIII and cFXIII with 10 U/ml thrombin (+T) for 5 min was used to obtain full activation. Panel A: Western blotting for FXIII-A. Panel B: measurement of transglutaminase activity. Activity measurements represent means of six parallel experiments and standard deviations.

It was interesting to see if FXIII activation by HNE could take place in a more complex environment, e.g. when fibrinogen is present, and also in the presence of the main physiological inhibitor of HNE, α_1AT. Maximal activation of pFXIII by HNE in the absence and in the presence of physiological fibrinogen concentration resulted in 62.5±5.5 U/l and 67.3±4.6 U/l (n=4 in both cases) transglutaminase activity, respectively (not shown). The presence of plasma concentration of α_1AT completely blocked HNE-induced FXIII activation.

Fibrin cross-linking by HNE-activated pFXIII

The next question was, if HNE-activated FXIII is able to cross-link fibrin, i.e. if it is able to exert the main physiological function of FXIIIa. In these experiments thrombin could not be used to form fibrin, since its presence would result in FXIII activation. Batroxobin moojeni is a thrombin-like proteolytic enzyme, which cleaves off fibrinopeptide A, but not B from fibrinogen and the resulting fibrin monomer then spontaneously polymerizes. It has been claimed not to cleave and activate FXIII [70,71]. Fibrinogen samples, supplemented with either non-activated FXIII, thrombin-activated FXIII (FXIIIa[t]) or HNE-activated FXIII (FXIIIa[e]) were prepared as described in the section "Materials and Methods". As shown on Figure 3, batroxobin moojeni induced fibrin formation without cleaving the Bβ-chain. Neither thrombin nor batroxobin moojeni induced cross-linking of fibrin that was formed from FXIII-depleted fibrinogen in the presence of Ca^{2+}. When non-activated FXIII was added to the incubation mixture, and fibrin formation was induced by batroxobin moojeni, only a slight fraction of γ-chains was dimerized, which could theoretically be due to the innate activity of FXIII [66] or to minor contamination of the batroxobin moojeni preparation. Western blotting analysis demonstrated no proteolysis of zymogen FXIII-A by batroxobin moojeni. This finding suggests that the observed γ-chain dimerization was due to the innate activity of zymogen FXIII. In the presence of FXIIIa[e], practically all γ-chains appeared as γ-dimer, and α-chains became transformed into high Mr oligomers/polymers. The effect of FXIIIa[e] was comparable to that of FXIIIa[t], with the exception that in the latter case the cross-linking of α-chains was more advanced.

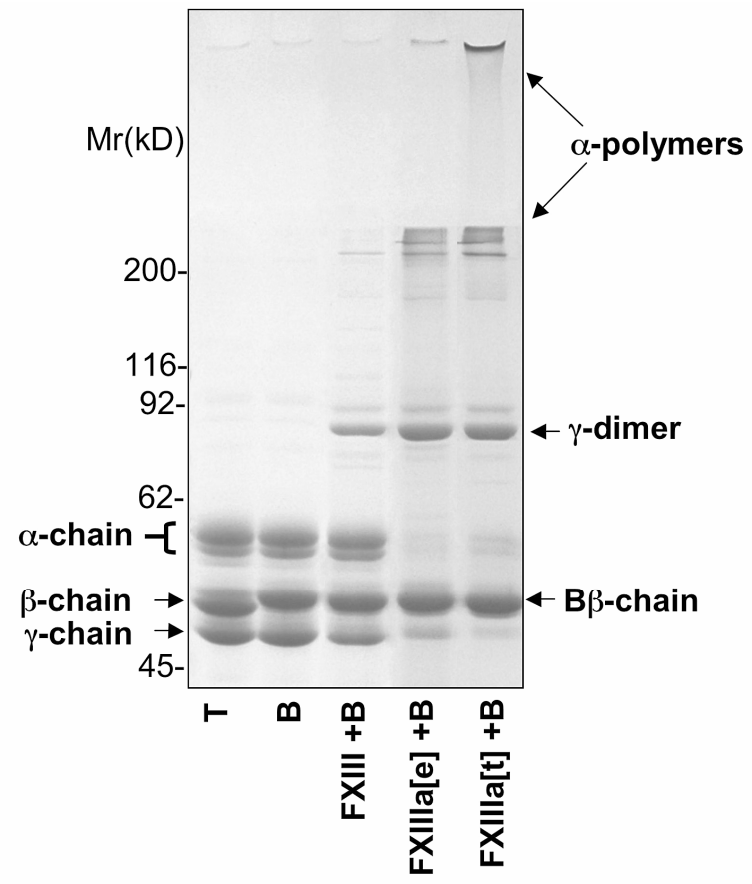


Figure 3. Fibrin cross-linking by HNE-activated pFXIII. FXIII-free fibrinogen was clotted by thrombin (T) or batroxobin moojeni (B) in the presence of Ca^{2+}. To selected fibrinogen samples non-activated (FXIII), HNE-activated FXIII (FXIIIa[e]) or thrombin-activated FXIII (FXIIIa[t]) was added. The clots were dissolved and analyzed by SDS PAGE (10% gel). Fibrinogen/fibrin chains and their cross-linked forms are indicated on the Figure.

HNE cleavage sites in FXIII-A

Although the Mr of the HNE-truncated FXIII-A roughly corresponded to the Mr of the thrombin-activated FXIII-A on the Western blot, the cleavage site of HNE on FXIII-A was surmised to be different from the known thrombin cleavage site, due to the different substrate specificity of HNE. To determine the primary (and possible secondary) cleavage site(s) of HNE in FXIII, two series of experiments were performed. The peptides cleaved from cFXIII by HNE were investigated with MALDI-TOF, and the amino acid sequence of the truncated form of FXIII-A was

analyzed by the N-terminal sequencing of the HNE-activated FXIIIa. It was revealed that a peptide corresponding to the N-terminal 39 amino acids of FXIII-A was the primary split product (Fig. 4A). Peptide 1-39 was measured as a double peak with a mass difference of 14 D, due to the presence of V/L 34 variants in the cFXIII preparation. The initially released peptide 1-39 became further cleaved by HNE at position T6-A7 and subsequently at position V34-V35 (see 20 min sample). The facts that peptide 7-34 appeared only as a single peak and the relative proportion of L34 variant of peptide 7-39 increased by time suggest that L34 peptide variant was not cleaved at position L34-V35. Although in the V34 variant one of the secondary cleavage sites was the peptide bond at V34-V35, this cleavage did not occur in the intact protein and was strictly limited to the N-terminal peptide that had been released. Investigations using such a technique were also carried out on pFXIII and the fragmentation pattern of FXIII-A was identical to that observed with cFXIII (not demonstrated).

The V39-N40 primary cleavage site was confirmed by the N-terminal sequencing of HNE-activated FXIII (Fig. 4B). The truncated FXIII-A that was excised from the blot had asparagine as the single N-terminal and the subsequent amino acid sequence was identical with that of the expected fragment. This finding strongly suggests that FXIII-A_2 truncated at V39-N40 by HNE is the dimer of FXIII-A derivative that is transformed by Ca^{2+} into active transglutaminase (N40-FXIII-A_2*). N40-FXIII-A_2* is a novel, active form of FXIII, two amino acids shorter that the known thrombin-cleaved active transglutaminase. Primary and secondary HNE cleavage sites are summarized on Fig. 4C.

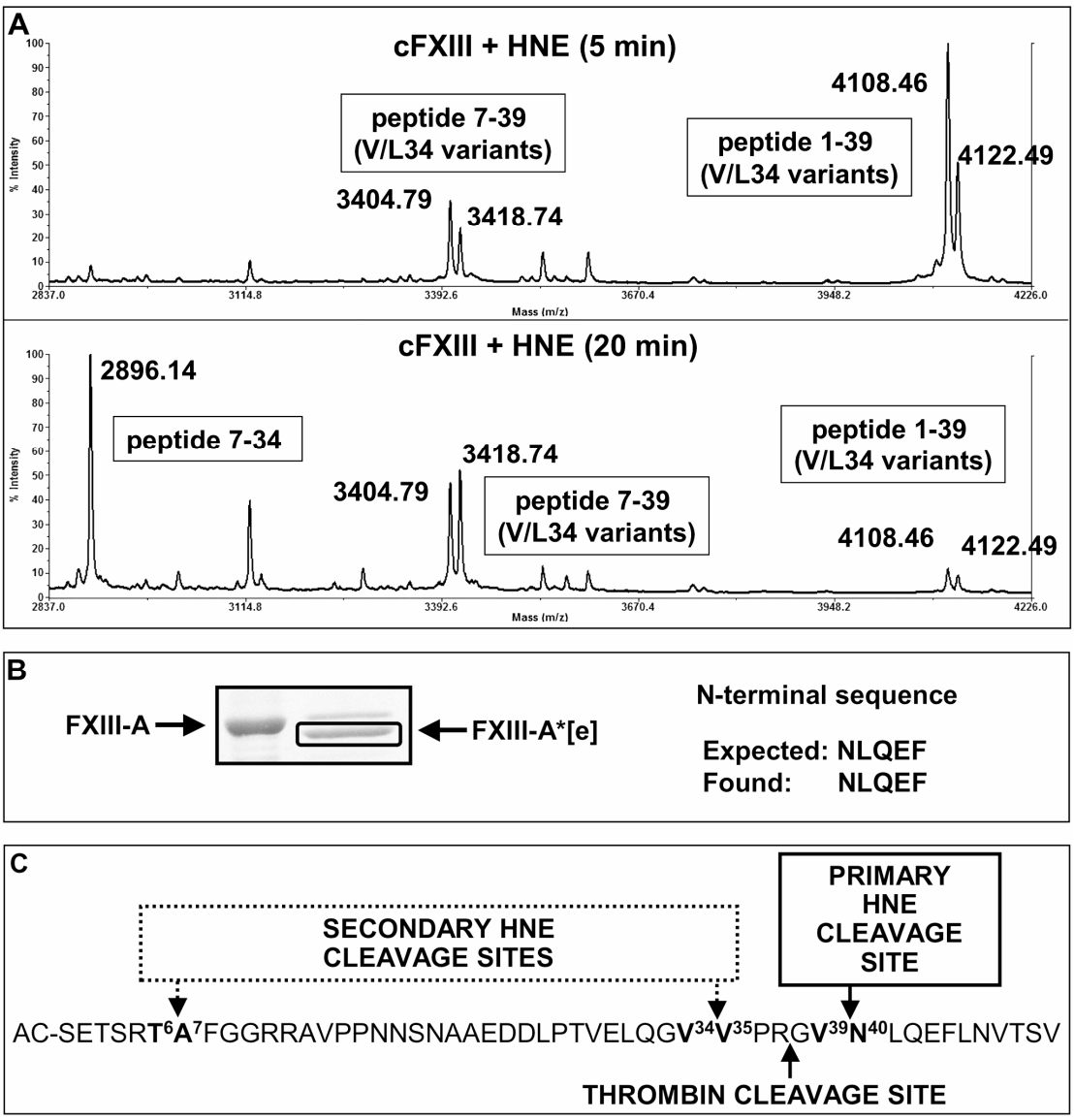


Figure 4. Analysis of FXIII-A split products formed during HNE-induced fragmentation of cFXIII. Panel A: identification of the oligopeptides released from FXIII-A by HNE using MALDI TOF analysis. Peptides released within 5 and 20 min incubation are shown. Panel B: N-terminal sequencing of HNE-truncated FXIII-A (FXIII-A*[e]). cFXIII before and after digestion with HNE is shown on the left side and the band cut out for N-terminal sequencing is depicted in a rectangle. Expected and determined N-terminal sequence of HNE-truncated cFXIII is shown on the right side. Panel C: primary and secondary HNE cleavage-sites in the sequence of FXIII-A.

The effect of FXIII-A V34L polymorphism on the activation of FXIII by HNE

As shown above, no primary cleavage occurred at V34 residue, which is a site of a major polymorphism in FXIII-A. Since V34L polymorphism significantly influences the rate of FXIII-A cleavage by thrombin, and it is located just five amino acids upstream from the primary HNE cleavage site, it was interesting to see if the polymorphism influences the rate of FXIII activation by HNE. Western blotting experiments (Fig. 5A) and FXIII activity measurements (Fig. 5B) showed that neither the cleavage of FXIII-A nor the activation of pFXIII by HNE was influenced by FXIII-A V34L polymorphism.

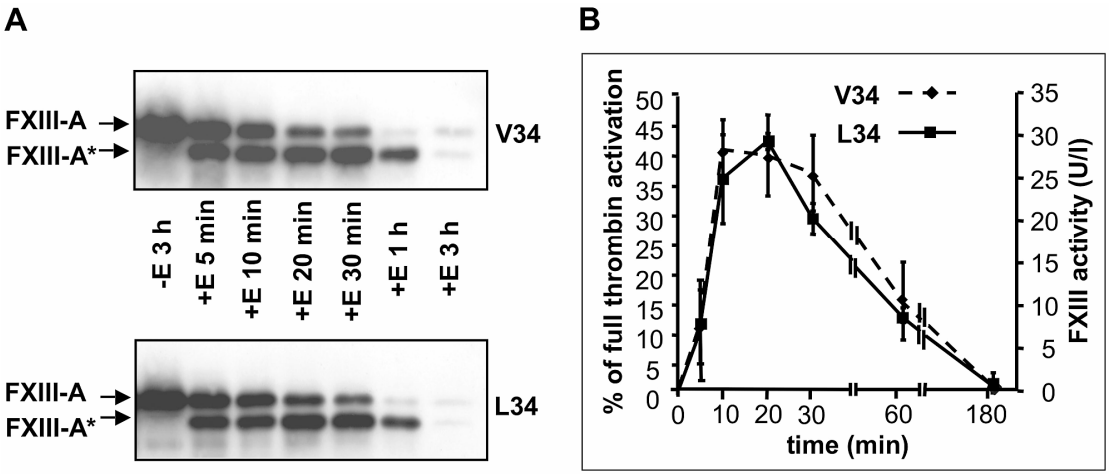


Figure 5. The proteolytic cleavage and activation of pFXIII of different FXIII-A V34L genotypes by HNE. 25 μg/ml FXIII prepared from the plasma of individuals with FXIII-A V34 and FXIII-A L34 genotypes was incubated with 5 μg/ml HNE (E) for various intervals. Then, HNE activity was blocked and aliquots were removed for Western blotting (Panel A) and for the measurement of the transglutaminase activity (Panel B). The results of activity measurements are expressed as U/l or as percentage of maximal thrombin-induced FXIII activity. Data points represent means of 4 parallel experiments and the standard deviations. Broken line with diamonds: V34 FXIII, solid line with squares: L34 FXIII. FXIII-A: intact A subunit of FXIII, FXIII-A*: truncated active form of FXIII-A.

Molecular modeling of cFXIII structure around the primary HNE cleavage site

Besides V39, the primary cleavage site of HNE on FXIII-A, there are four other valine residues in the vicinity of this site in the molecule. The question why HNE prefers V39, as primary cleavage site, to other nearby valine residues, V34, V35, V47 or V50, was addressed by molecular modeling. As it is shown on Figure 6, the V39 residue is located in the middle of a flexible loop, it is freely accessible to the active center of HNE, and the neighboring residues can easily adopt the conformation required for enzyme-substrate interaction. In contrast, V47 and V50 residues are located on a β-sheet at the beginning of β-sandwich domain, the side chains of these residues point to the inner region of the domain what makes them unavailable for HNE. The V34 and V35 residues are close to the N-terminal end of the Q32-L45 loops and partially hidden by the core and the 1st β-barrel domains of FXIII-A dimer. Compared to the V39 residue, they are located in a less flexible region and their accessibility for interaction with HNE is reduced.

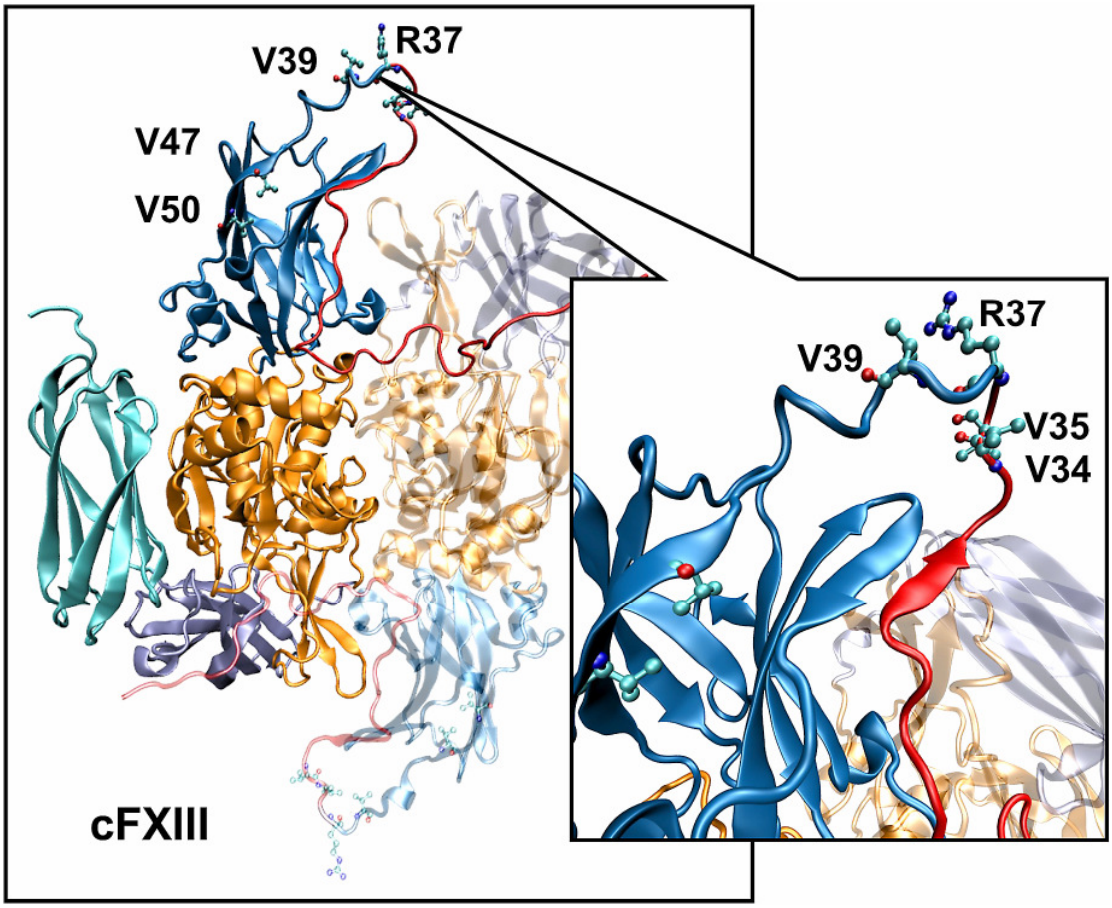
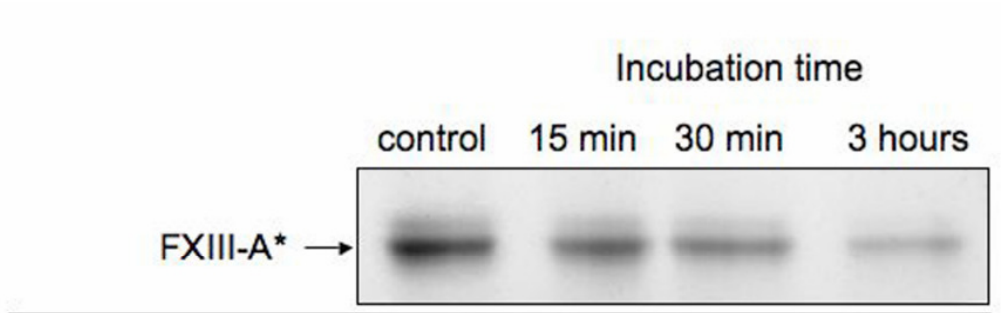


Figure 6. Molecular graphic representation of HNE cleavage site and its surroundings in cFXIII (FXIII-A_2 homodimer). The β-sandwich, core, the β-barrel1 and β-barrel2 domains were colored blue, orange, violet and cyan, respectively. The activation peptide is depicted in red. To distinguish between the two subunits, one of the A subunits is shown in lighter colors. The insert is a magnification of the area around the HNE cleavage site. For the V34, V35, R37, V39, V47 and V50 residues CPK representation was used.

Proteolytic degradation of FXIIIa by the supernatant of activated PMNs

In the previous experiments it was shown that prolonged action of HNE degrades and inactivates FXIIIa. Besides HNE, PMNs contain an armory of proteolytic enzymes that are released upon activation. In the followings it was investigated if PMN proteases could be responsible for the down-regulation of FXIIIa within the clot. As a first step we intended to study the effect proteases released from stimulated PMNs on FXIIIa. In these experiments the supernatant of fMLP-activated PMNs were

incubated with thrombin-activated pFXIII. As demonstrated on Figure 7, PMN proteases degraded the thrombin-activated form of FXIII (FXIII-A*) and the proteolytic degradation was time dependent as followed by SDS PAGE and quantitative densitometry. FXIII activity decreased in parallel with the diminution of FXIII-A* band suggesting that the proteolytic degradation fragments of FXIII-A* do not possess FXIII activity. In non-reducing conditions, FXIII-A* and FXIII-B are well separated, only FXIII-A* is shown on the figure. FXIII-B was also degraded by PMN proteases, of the original amount of undigested FXIII-B, 73.6% and 50.3% could be detected after 30-min and 3-h incubation, respectively (not shown).



Intact FXIII-A* (%)	100	60.1	57.0	26.0
FXIII activity (%)	100	60.8	57.6	27.1

Figure 7. Proteolytic degradation of FXIIIa by the supernatant of fMLP-activated PMNs. The picture shows the results of SDS PAGE analysis in non-reducing conditions, where the gels were silver stained and the relative amount of intact FXIII-A* was determined by quantitative densitometry. The results are expressed as percentage of control in which HEPES Tyrode buffer was replaced for PMN supernatant and incubated for 3h. In controls the amount of FXIII-A* did not change during the 3 h of incubation (not demonstrated). The figure depicts three separate experiments with similar results.

Release of proteolytic enzymes from PMNs in the fibrin clot

The next step was to show that PMN cells incorporated into the fibrin clot become activated and release proteolytic enzymes. In order to prove this, two series of experiments were conducted. First, it was shown by SDS PAGE that in the presence of PMNs powerful lysis of fibrin clot occurs (Fig 8A). We used plasminogen free fibrinogen and no proteolytic degradation of fibrin was observed in the absence of PMN cells, thus the results indicate that the proteolytic degradation of fibrin was carried out by proteases released from PMNs. In the second series of experiments HNE, cathepsin G and MMP-9 activities were measured in the fibrin clot as described in "Materials and Methods". Considerable HNE, cathepsin G and MMP-9 activity could be measured in the clot in the presence (Fig. 8B), but not in the absence of PMNs. It is to be noted that the MMP III substrate used in the MMP-9 assay is also effectively cleaved by MMP-2, however this MMP is not present in PMNs [72] and therefore does not contribute to the measured enzyme activity. HNE and MMP-9 activities were almost completely abrogated by their specific inhibitors ONO 5046 and MMP-9 inhibitor I, respectively (Fig. 8B). In contrast, high concentration of cathepsin G inhibitor I only partially inhibited the enzyme activity within the clot, although it caused complete inhibition of cathepsin G in the supernatant of fMLP activated PMN cells (data not shown). The incomplete inhibition might be due to the inaccessibility of the surface bound enzyme in the clot for inhibitor molecules [73].

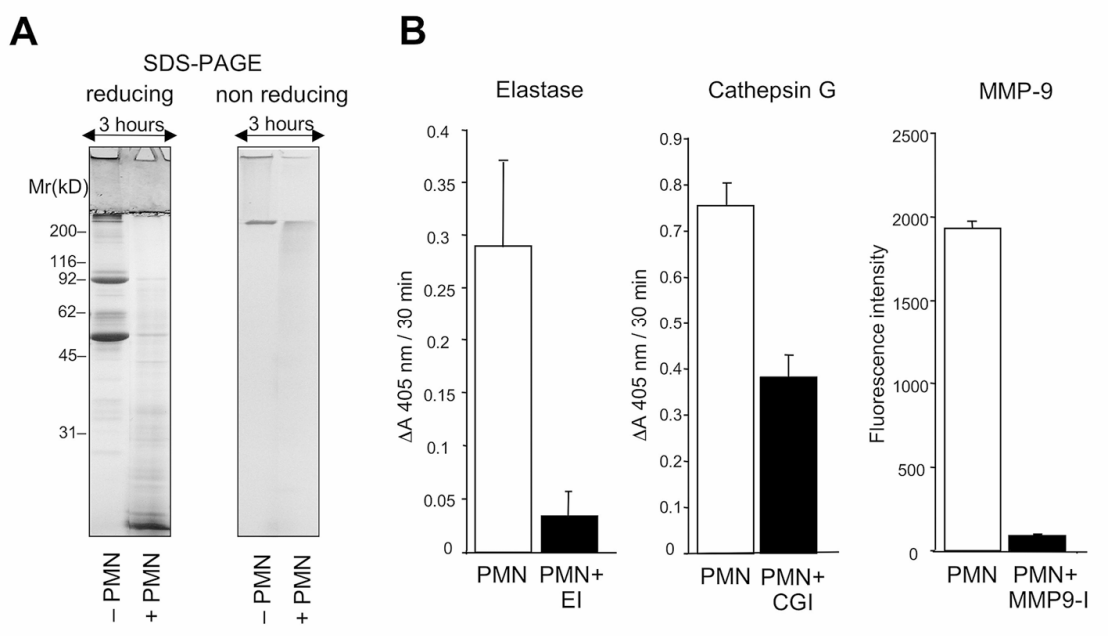


Figure 8. The release of proteolytic enzymes from PMNs incorporated into fibrin clot. Panel A: fibrinolysis induced by PMN proteases released from intact cells in the fibrin clot. Fibrinogen solutions containing FXIII in the absence (-PMN) or in the presence (+PMN) of intact PMNs ($20x10^6$/mL) were clotted by thrombin and Ca^{2+} and incubated for 3 hours. Proteolytic degradation of fibrin was followed by SDS PAGE in reducing and non-reducing conditions. Panel B: demonstration of the presence of enzymatically active specific proteases in the fibrin clot containing $20x10^6$/ml PMN granulocytes (open bars) using chromogenic (HNE and cathepsin G) and fluorogenic (MMP-9) substrates and their inhibition by specific inhibitors (solid bars), ONO 5046 (EI), cathepsin G inhibitor I (CGI), MMP-9 inhibitor I (MMP9-I). No enzyme activity was measured in the absence of PMNs (not shown). The results represent the means±SD of three separate experiments.

Proteolytic degradation of FXIII subunits in fibrin clots supplemented with PMNs

To test the effect of proteases released from PMNs on thrombin activated FXIII within fibrin clots, pFXIII containing fibrinogen solution was supplemented with PMN suspensions and clotted with thrombin and $CaCl_2$. Clots were analyzed by Western blotting. As it is demonstrated on Figure 9, initially only non-activated FXIII-A was present. It became gradually activated during the course of incubation and at 30 min after the initiation of clotting almost all FXIII-A was in the truncated active form. If PMNs were also incorporated in the clot a time-, and cell count-dependent proteolytic degradation of FXIII-A occurred. In the presence of $5x10^6$/ml PMNs, gradual proteolysis of FXIII-A/A* occurred and after 3 hours only a faint band represented FXIII-A*. $20x10^6$/ml PMNs digested activated FXIII-A considerably within 15 min and full proteolytic degradation was observed in 3 hours. It was interesting that no fragment of intermediate Mr was detectable and only a small amount of low Mr split products could be demonstrated on the immunoblot after three hours (Fig. 10). This finding suggests that a group of proteolytic enzymes of different substrate specificity that are released from PMNs quickly degrade FXIII-A* to low Mr peptides. FXIII-B, which during FXIII activation dissociates from FXIII-A' and from fibrin, was also degraded in the presence of PMNs.

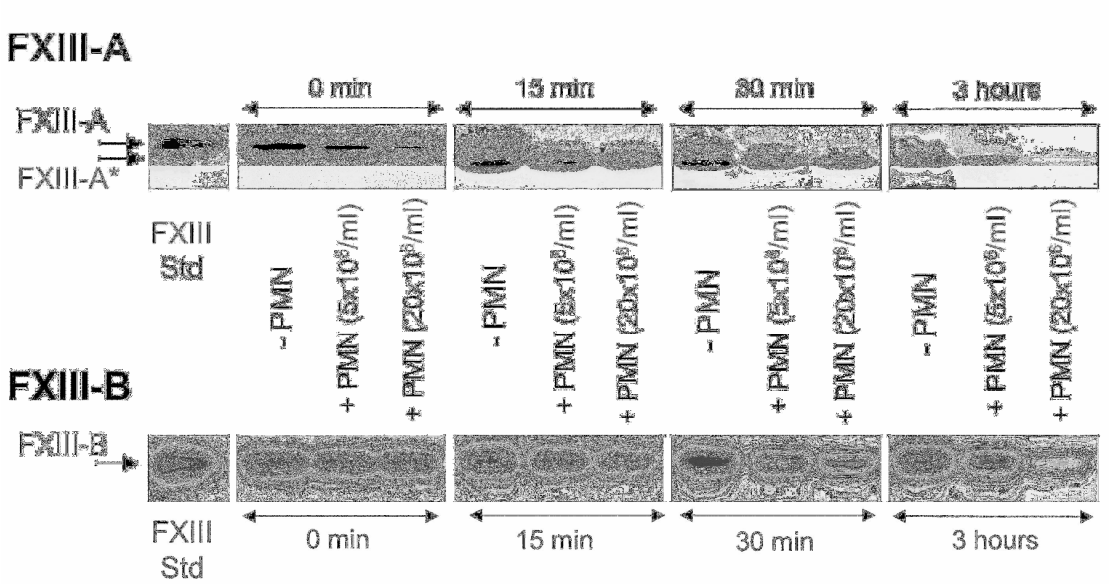


Figure 9. Proteolytic degradation of FXIII-A and FXIII-B by PMNs incorporated in the fibrin clot. Western blotting analysis of fibrinogen solutions containing FXIII in the absence (lanes -PMN) or in the presence (lanes +PMN) of intact PMNs ($5x10^6$/ml or $20x10^6$/ml), clotted by thrombin and Ca^{2+} and incubated for various time. FXIII-A*: FXIII-A activated by thrombin and Ca^{2+}.

The relative importance of certain PMN proteases in the proteolytic degradation FXIII-A

Among the protease inhibitors investigated, ONO 5046 exerted the most effective protection against the proteolytic degradation of both FXIII-A and FXIII-B (Fig. 10). Only this inhibitor was able to prevent the complete proteolytic degradation of FXIII subunits within 3 hours of incubation. However, even in this case a significant portion of the FXIII subunits became degraded. Some protection provided by the cathepsin G inhibitor could be demonstrated at 30 min. The diminution of the band representing intact FXIII-A* was not influenced by the MMP-9 inhibitor, however its presence (and to a lesser extent the presence of cathepsin G inhibitor) resulted in the accumulation of intermediate split products of 63 and 61 kD, not seen in samples with no inhibitor or with HNE inhibitor. This result suggests that HNE is involved in the primary proteolytic cleavage of FXIII-A, while the main task of MMP-9 (and to a lesser extent that of cathepsin G) is further proteolysis of primary split products.

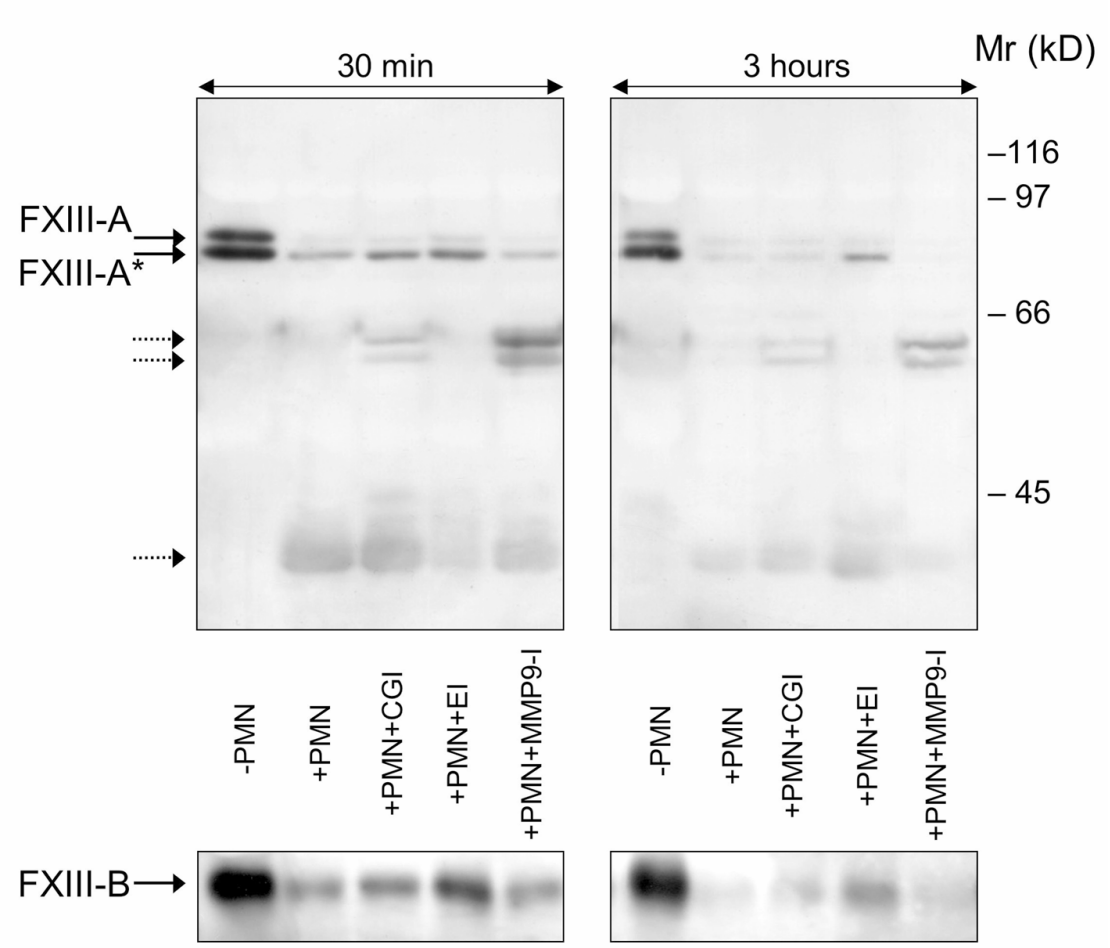


Figure 10. The protective effect of HNE, cathepsin G and MMP-9 inhibitors against the proteolytic degradation of FXIII-A (upper panel) and FXIII-B (lower panel) by PMN granulocytes. Fibrin containing FXIII was incubated for 30 min or 3 hours in the absence (-PMN) and in the presence (+PMN) of $20x10^6$/ml PMNs. Samples containing inhibitors of cathepsin G, HNE or MMP-9 are labeled as CGI, EI and MMP9-I, respectively. Arrows with dotted line indicate lower Mr split products.

Activation of FXIII in plasma and its association with fibrin

To study the effect of PMNs on FXIIIa in plasma clots, first we had to prove that activated FXIII remains attached to fibrin. This was an important question because in order to evaluate the degradation of FXIII in clots made from whole plasma, clots had to be extensively washed to remove plasma proteins that were not bound to fibrin (especially albumin) and would cause interference with SDS PAGE and Western blotting. The onset and the rate of thrombin induced FXIII activation in combination with fibrin formation was investigated using low thrombin concentrations. Figure 11. illustrates the relative proportion of activated/non-activated FXIII in fibrin clots and in plasma/serum samples together with the time course of fibrin formation. In accordance with earlier reports [74,75], polymerization of α-chains occurred immediately after the removal of fibrinopeptide A from fibrinogen Aα-chain, while at this stage most parts of the Bβ-chain had not been yet cleaved. FXIII bound instantly to the newly formed fibrin polymer, even in its native non-truncated form.

The cleavage and activation of FXIII took place on the surface of the newly formed fibrin clot within a short lag phase. It is to be emphasized that truncation of FXIII-A never occurred in the soluble phase of the investigated plasma samples (n=23) and the truncated form of FXIII never appeared in the serum. This result proves that the physiological activation of FXIII takes place exclusively on the surface of fibrin and after its formation FXIIIa remains associated with its substrate.

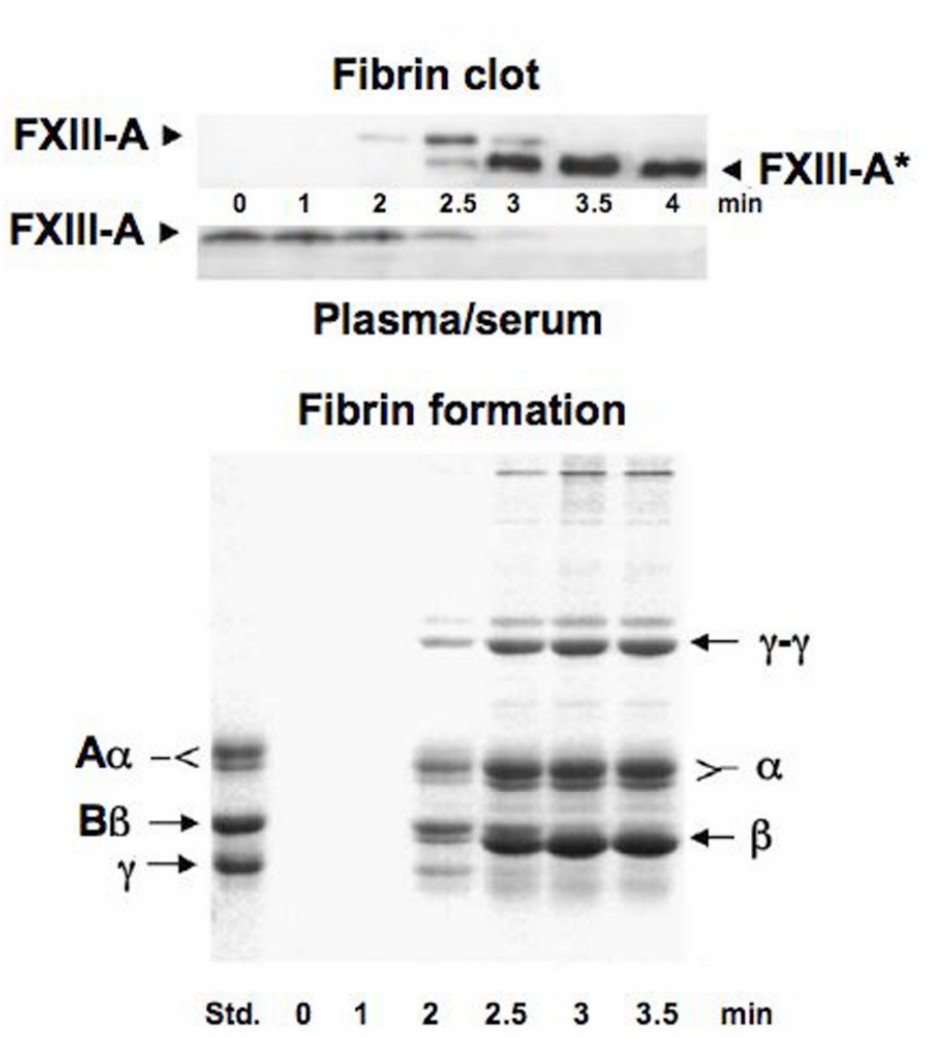


Figure 11. Fibrin formation and the proteolytic activation of FXIII by thrombin in whole plasma. Upper panel: intact FXIII-A and its truncated form (FXIII-A') on the Western blot of a representative plasma/serum and fibrin sample obtained at different times after the addition of thrombin. Lower panel: fibrin formation at different times following the addition of thrombin to a representative plasma sample. The picture represents SDS PAGE analysis of the fibrin clot in 10% gel stained with Coomassie brilliant blue. Aα, Bβ: fibrinogen chains in the fibrinogen standard (Std.). α, β: fibrin chains, γ: fibrinogen/fibrin chain, γ-γ dimer: dimer of γ-chain cross-linked by FXIIIa.

Proteolytic degradation of FXIII by PMNs in plasma clot

It was a question whether the FXIIIa down-regulating mechanism exerted by PMN proteases in clots made from purified fibrinogen could also operate in the more complex environment of clots made from whole plasma. Despite the presence of a considerable amount of natural serine protease inhibitor(s) in the plasma, a well detectable proteolytic degradation of FXIII-A* occurred in the plasma clot if $5x10^6$/ml PMNs were present (Fig. 12). A more intense proteolysis was exerted by $20x10^6$/ml PMNs although its extent was somewhat smaller than that observed in clots made of purified fibrinogen (Fig. 9).

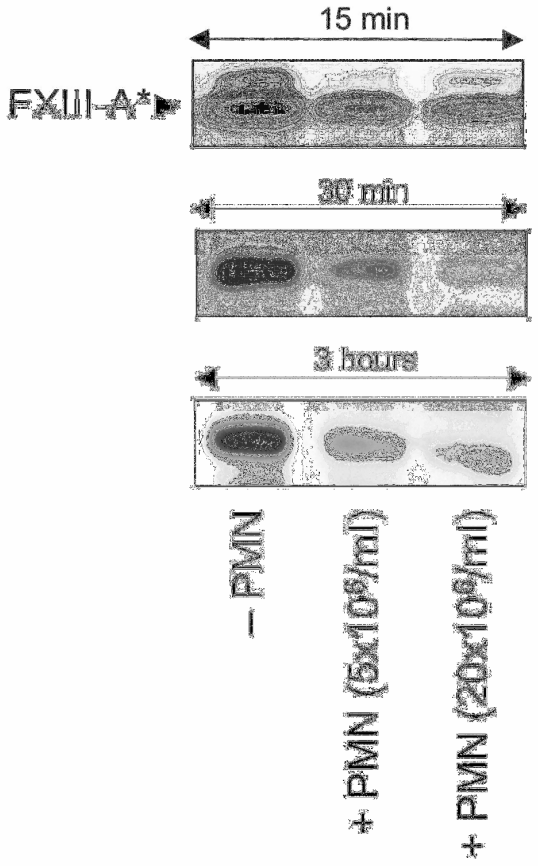


Figure 12. Proteolytic degradation of FXIII-A induced by PMN granulocytes incorporated into the plasma clot. Clots made from platelet-free plasma were incubated for various intervals without (-PMN) and with (+PMN) of $5x10^6$/ml or $20x10^6$/ml PMNs. The proteolytic degradation was demonstrated by Western blotting using a monospecific polyclonal antibody against FXIII-A.

The effect of α_1AT on the proteolytic degradation of FXIII subunits by PMNs

As α_1AT is the main serine protease inhibitor responsible for the inhibition of PMN proteases (HNE, and to a lesser extent cathepsin G and proteinase 3) in plasma [50,51], it was interesting to see how the proteolysis of FXIII subunits was influenced by α_1AT in fibrin clots. To address this question proteolytic degradation experiments were carried out on fibrin clots made from FXIII-containing fibrinogen solution supplemented with PMN cells and α_1AT. Physiological concentration of α_1AT inhibited the proteolytic degradation of FXIII subunits by PMN proteases, however the inhibition was only partial (Fig. 13). Even in the presence of α_1AT significant proteolysis of FXIII-A occurred within 30 min and only a fraction of non-digested protein remained in the fibrin clot after three hours. The protection provided by α_1AT against the proteolytic degradation of FXIII-B was more effective than in the case of FXIII-A.

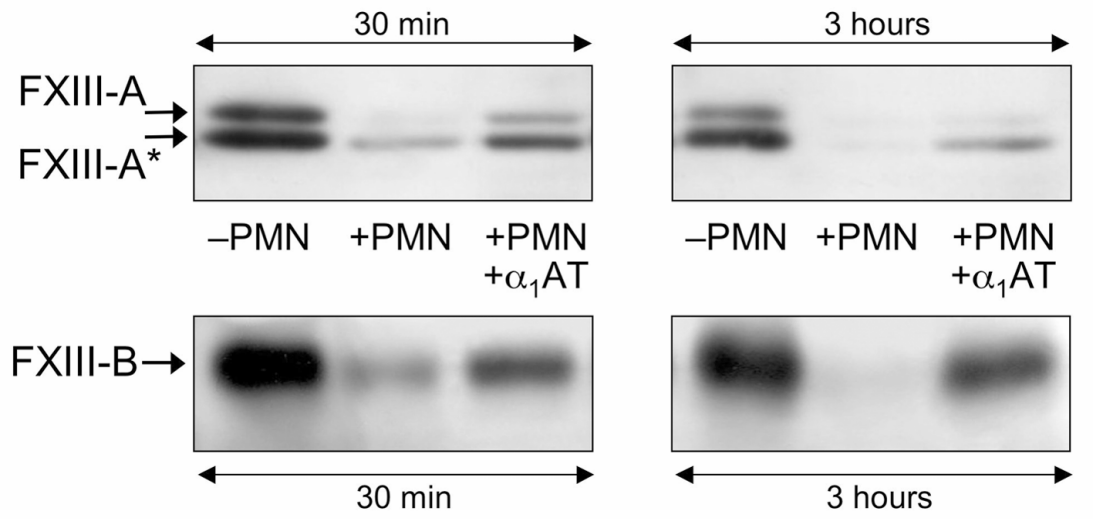


Figure 13. The effect of α_1AT on the proteolytic degradation of FXIII subunits by PMNs in fibrin clot. Fibrinogen solutions containing FXIII were clotted by thrombin and Ca^{2+} in the absence (-PMN) and in the presence (+PMN) of $20x10^6$/ml PMN granulocytes. In some samples α_1AT (+α_1AT) was also included. Samples were subjected to SDS PAGE in reducing conditions and Western blotted for FXIII-A (upper panel) or FXIII-B (lower panel).

The influence of PMN proteases on the fibrin cross-linking process

To explore if the proteolytic degradation of FXIII by PMN proteases influences the cross-linking process we analyzed the time course of fibrin cross-linking and the proteolysis of FXIII-A at physiological PMN count ($5x10^6$/ml PMN) and also in the presence of physiological concentration of α_1AT. Full cross-linking of fibrin γ-chain needs only a few minutes and only γ-chain dimers were seen after half an hour (Fig. 14A). In contrast to $20x10^6$/ml PMNs (see Fig 8A), incubation with $5x10^6$/ml PMNs even for three hours only slightly diminished the amount of γ-chain dimers. The cross-linking of fibrin α-chain is a much slower process, high Mr cross-linked products appeared on the upper part of the separating gel and on the top of the concentrating gel, but three hours was not enough for the complete cross-linking of α-chains (Figs 14A-C). PMN proteases preferentially digested the α-chain, but α_1AT exerted a significant, although not complete, protective effect that allowed the accumulation of cross-linked products in the upper part of separating and also some highly cross-linked polymers on the top of the concentrating gel. Fibrin cross-linking and digestion of fibrin occurs at the same time, however in the presence of α_1AT the total amount of cross-linked proteins did not diminish even after three hours and the amount of highly cross-linked α-polymers increased, although at a much slower rate than in the absence of PMNs. In the presence of α_1AT the digestion of FXIII-A by PMN proteases slowed down, especially in the initial phase (Fig. 14D). However, after 1 hour and 3 hours the amount of intact FXIII remained only 52% and 21%, respectively. Comparison of the proteolytic degradation of FXIII-A and the amount of cross-linked fibrin demonstrates that in the fibrin clot with α_1AT FXIII-A was degraded significantly faster than cross-linked fibrin. In this period newly formed cross-linked products kept balance with their proteolysis and the amount of highly cross-linked species still increased.

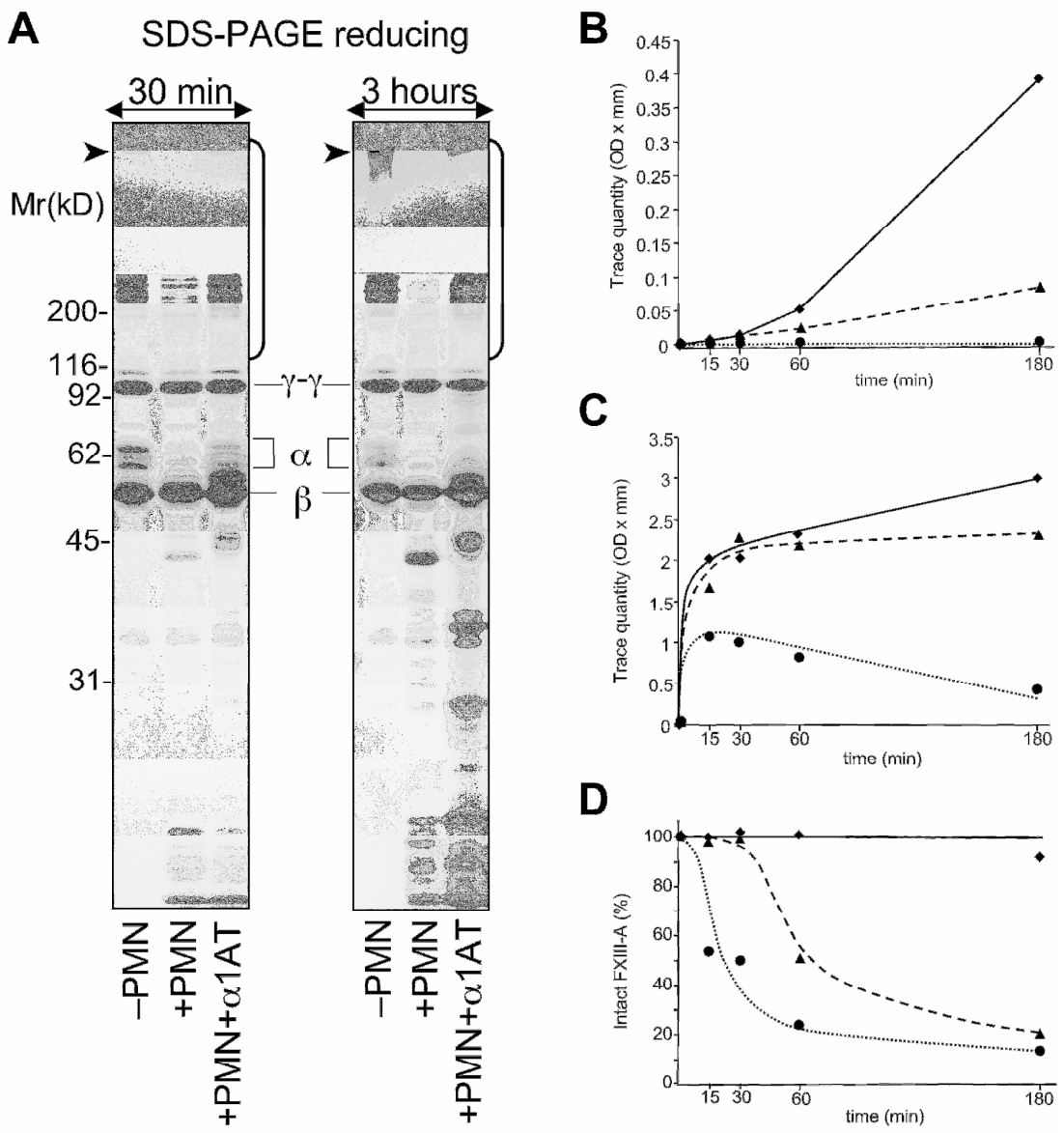


Figure 14. Comparison of the time course of fibrin cross-linking and the proteolytic degradation of FXIII-A. Fibrinogen solutions containing FXIII were clotted by thrombin and Ca^{2+} in the absence (-PMN) and in the presence (+PMN) of $5x10^6$/ml PMN granulocytes and incubated for various time intervals. In some samples containing PMNs, α_1AT (+α_1AT) was also included. Samples were subjected to SDS PAGE in reducing conditions and stained with Coomassie blue or Western blotted for FXIII-A. The accumulation of the total amount of cross-linked proteins above fibrin γ-chain dimers (indicated by brackets) and the highly cross-linked fibrin α-chain polymers

remaining on the top of the concentrating gel (indicated by arrowheads) was evaluated by quantitative densitometry. The amount of cross-linked proteins was expressed as trace quantity (OD x mm), while the amount of non-digested FXIII-A/A* at different intervals was expressed as percentage of the amount of FXIII-A in zero-time sample. Panel A: SDS PAGE of fibrin samples after 30 min and 3 hour incubation. The positions of fibrin α-chain, β-chain and γ-chain dimers are indicated. Panel B: the amount of highly cross-linked fibrin α-chain polymers on the top of concentrating gel. Panel C: the total amount of cross-linked protein products with Mr higher than that of fibrin γ-chain dimer. Panel D: the amount of non-digested FXIII-A/A*. In panels B-D solid lines with diamonds represent samples without PMNs and α_1AT, dotted lines with circles represent samples with PMNs but without α_1AT, and broken lines with triangles represent samples with PMNs and α_1AT.

DISCUSSION

Proteolysis plays a central role in the regulation of blood coagulation, the activation and inactivation of many factors and cofactors is effectuated by proteolytic cleavages in the blood coagulation pathway. Few early studies described the effect of PMN proteases on FXIII. The inactivation of a FXIII preparation by purified HNE and its partial inactivation by cathepsin G have been reported [58] and the proteolytic degradation of FXIII by these enzymes has also been demonstrated [59]. An early preliminary study by Henriksson et al. suggested that in non-physiological conditions, i.e. in the presence of EDTA, HNE could transiently activate FXIII [60]. Our results unequivocally demonstrated that in the presence of Ca^{2+} both pFXIII and cFXIII are activated by HNE, and the peptide bond, the cleavage of which is responsible for activation was found to be different from the thrombin cleavage site.

Cleaving off AP-FXIII from the A subunit is an essential step in the activation of pFXIII which makes the Ca^{2+}-induced dissociation of FXIII-B from the heterotetramer and the quick transformation of the remaining FXIII-A_2' into an active enzyme, FXIII-A_2*, possible [76,77]. In the case of cFXIII, truncation of FXIII-A by thrombin highly accelerates the Ca^{2+}-induced activation process. So far, only G38-

FXIII-A_2* has been demonstrated to be an active truncated FXIII species. Attempts in a number of laboratories, including ours, to express active forms of FXIII-A in transfected cells and to investigate different truncated forms of FXIII-A failed to succeed. Here we took a different approach and tried to obtain truncated FXIII-A, different from G38-FXIII-A_2*, by proteolytic cleavage using HNE. Here we show that the primary cleavage of FXIII-A by HNE, the substrate specificity of which is different from that of thrombin, occurs at V39-N40 and results in a novel active truncated form, N40-FXIII-A_2*. The preferred P1 residue at the cleavage-site of HNE is valine. In addition to V39, there are four more valine residues (V34, V35, V47 and V50) in the neighborhood of thrombin cleavage-site. Molecular modeling revealed that the position of these valine residues is less favorable for the interaction with HNE than the position of V39 residue.

The transglutaminase activity of N40-FXIII-A_2* indicates that G38 and V39 amino acid residues are not essential for the assumption of enzymatically active configuration. As the action of HNE is less restricted than that of thrombin and activation as well as degradation occur in parallel, it is not possible to establish the specific activity of HNE-activated FXIII-A_2*. The measured maximal activity values in the ammonia release assay, which were over 50% of the activity of FXIIIa formed by maximal thrombin activation of both pFXIII and cFXIII, suggest that the specific activity of FXIIIa[e] is comparable to that of FXIIIa[t]. FXIIIa[e] also cross-linked fibrin chains effectively, i.e. it was able to exert the main function of FXIIIa.

X-ray diffraction studies on crystallized FXIII-A_2 suggested that after thrombin activation AP-FXIII remains with the truncated protein, but the cleavage itself makes the active site (and the catalytic triad) accessible for interaction with the substrate glutamine residue [78]. In contrast, a most recent study indicates that in solution AP-FXIII dissociates from FXIII-A_2* opening up a passage through which high Mr substrates can enter and their substrate glutamine residue can interact with the catalytic triad [79]. HNE cleaves off 39 amino acids from the N-terminus of FXIII-A and only after this cleavage takes further fragmentation of the peptide place. If the activation peptide cleaved off by HNE dissociated from the active truncated form, originally hidden cleavage sites could become accessible for HNE and/or the released peptide in solution could assume a flexible configuration making enzyme-substrate interaction more effective. Alternatively, if the activation peptide remained

with the parent molecule, AP-FXIII could move to a somewhat different location on the FXIII-A surface rendering new sites more available for HNE-cleavage.

As FXIII-A V34L polymorphism significantly influences the activation of pFXIII and cFXIII by thrombin, it was interesting to see if the polymorphism has any effect on the activation of FXIII by HNE. Although the V34L polymorphism in FXIII-A resides just five amino acids upstream from the HNE cleavage site, the absence of significant difference in the rate of HNE-induced activation of V34 and L34 FXIII variants suggests that the polymorphism is practically without influence on the binding of HNE to FXIII-A and on its proteolytic effect. It was also shown that once the N-terminal peptide is cleaved by HNE, it becomes available for further cleavage at V34, while no cleavage occurred at this position in the L34 variant. Evidently, this difference did not influence the rate of activation of FXIII by HNE. It is interesting that, as opposed to V34 no secondary cleavage occurred in its immediate vicinity at V35. This finding suggests that the sequence of amino acid residues surrounding V35 does not seem to fulfill the requirement of HNE substrate specificity.

Although the primary aim of our experiments with HNE was to find a novel truncated active form of FXIII-A and thereby to provide pieces of information regarding the structural requirement of the biochemical function of FXIIIa, the results raise the question whether activation of FXIII by HNE could have any physiopathological relevance. The concentrations of FXIII and HNE used in most experiments were physiological. PMNs have been estimated to contain 0.44-2.5 pg of HNE/cell [50,80,81], i.e. 5 x10^6/ml PMNs (normal cell count in the blood) should contain around 5 μg/ml HNE, a significant amount of which is released upon activation [82]. The reference interval of FXIII in plasma is 14-28 μg/ml [61]. Thus, the concentration of FXIII and HNE used in most of the experiments, 25 μg/ml and 5 μg/ml, respectively, could suggest relevance in physiopathological conditions. In spite of this possibility, we would like to highlight that it is unlikely that HNE significantly contributes to the activation of FXIII within the clot. During blood coagulation thrombin is formed rapidly in an amount that is sufficient to activate pFXIII without the contribution of HNE. The release of HNE is a later event that occurs only after fibrin had been formed. In the extravascular compartment the situation could be different, especially in patients with α_1AT deficiency. The bronchoalveolar lining

fluid contains cFXIII [83], which could theoretically be activated by HNE and could then participate in the cross-linking of substrate adhesive proteins in the bronchoalveolar compartment. Of course, this theory should be tested experimentally.

Although the activation of FXIII by HNE during blood coagulation does not seem likely to occur, the down-regulation of FXIIIa by HNE together with other PMN proteases could represent an important negative feed-back mechanism. This hypothesis was supported by our experiments showing that proteases present in the supernatant of activated PMNs degraded FXIII-A* with the parallel loss of transglutaminase activity.

It has been previously demonstrated that non-stimulated PMNs become incorporated into plasma clots or thrombi and release proteolytic enzymes [33,39] that could interact with several components of the haemostatic and fibrinolytic system [40-42,46]. The release of PMN proteases in fibrin clots was confirmed in our study and their fibrinolytic effect was also demonstrated with plasminogen-free fibrinogen. Released PMN proteases bind to the cell membrane and possibly also to fibrin and they can be only partially recovered in the clot liquor [73,84,85]. To be able to estimate the total activity of bound and unbound fractions of these enzymes and to assess their inhibition by specific inhibitors in the fibrin clot we developed a new approach. Specific substrates were included in the fibrin clot and the absorbance or fluorescence intensity of the leaving groups accumulated within a fixed period of time was measured. Using this system the release or membrane exposure of HNE, cathepsin G and MMP-9 by PMNs in the clot was clearly verified and it was also shown that within the clot HNE and MMP-9 were fully, while cathepsin G was only partially accessible to the respective low Mr inhibitors.

PMNs incorporated into fibrin clots effectively degraded FXIII-A and FXIII-B. As yet, the role of free or released FXIII-B is not known, thus the importance of the latter finding remains unclear. The relative importance of certain individual PMN proteases in the degradation of FXIIIa was studied by testing the protective effect of specific inhibitors. Among the inhibitors tested, HNE inhibitor exerted the most effective, but still partial protection. The importance of cathepsin G is more difficult to estimate because it was only partially inhibited by its specific inhibitor in the fibrin clot. The MMP-9 inhibitor hardly influenced the proteolytic degradation of intact FXIII-A*, however, in its presence intermediate Mr proteolytic fragments accumulated. These results suggest that HNE plays a dominant role in the primary

proteolytic cleavage of FXIII-A*, while MMP-9, and perhaps to a lesser extent cathepsin G, are involved in the further proteolysis of the primary split products.

Despite the presence of α_1AT, the down-regulation of FXIIIa by PMN proteases was also operative in plasma clots. To confirm this finding, we supplemented fibrin clots, made from purified fibrinogen with physiological concentration of α_1AT, and also in this case the protease inhibitor only decreased the rate of the proteolytic degradation of FXIIIa. It is to be emphasized that, when fibrin or fibrinogen were not present, α_1AT completely abrogated the proteolytic effect of purified HNE or the supernatant of activated PMNs. This is in accordance with previous findings describing that α_1AT prevented the effect of PMN proteases on platelets when PMN supernatants or purified cathepsin G was used but it was much less effective when intact PMNs were present in the mixed cell suspensions [86]. The limited effectiveness of α_1AT against proteases released from PMN cells within fibrin clots might be due to several reasons: a) to the inaccessibility of the cell-surface bound proteases to α_1AT [73], b) to the non-proteolytic oxidative inactivation of α_1AT by PMN myeloperoxidase [87] c) to its proteolytic inactivation by MMP-9 [88]. Another reason for the limited effectiveness of α_1AT could be accounted to the fact that fibrin reduces the rate at which α_1AT inhibits HNE, thus, proteolysis by surface-bound HNE remains efficient even in the presence of the inhibitor [48]. The results of these series of experiments suggest that FXIIIa down-regulation by PMN proteases could have pathophysiological relevance in the thrombus. The concentrations of PMNs that were used in the experiments most likely represent the conditions that occur in physiological/pathological environments. We supplemented the plasma or fibrinogen solutions with $5x10^6$/ml and $20x10^6$/ml PMNs, which represent normal or moderately elevated PMN counts even if PMNs were only passively trapped into the clot/thrombus. However, it has been shown in a series of excellent studies that in the circulation thrombi become enriched in PMN leukocytes; in addition to passive trapping, PMNs actively accumulate in thrombi by direct binding to fibrin and by P-selectin-dependent binding to platelets [28,89,90]. Thus, in vivo the density of PMNs in thrombi could even be higher than in the clots formed under the experimental conditions used in the study.

The action of PMN proteases in the clot is a complex process that includes the proteolysis of both the cross-linking enzyme, FXIIIa and its cross-linked substrate.

The time course of FXIIIa degradation by PMN proteases makes it unlikely that the proteolytic degradation of FXIII by PMN proteases would interfere with initial cross-linking events. The dimerization of γ-chains and the cross-linking of α_2PI to fibrin α-chain goes to completion within a few minutes. The degradation of FXIIIa by proteases released during PMN activation is a much slower process, which is further slowed down by α_1AT and is unlikely to influence the initial cross-linking. However, the proteolytic inactivation of FXIIIa could be involved in the limitation of the much slower cross-linking process of fibrin α-chains, providing a down-regulatory mechanism of fibrin cross-linking. It is presumed that this down-regulating mechanism allows fibrin and α_2PI-fibrin cross-linking required for the production of fibrin clot resistant to prompt fibrinolysis by plasmin to occur, but prevents the formation of highly cross-linked clot that would be difficult to eliminate during the healing process.

In summary, a complex regulatory role exerted by PMN proteases in the activation/inactivation of FXIII was described, providing new pieces of information on the biochemical function of FXIII and on the down-regulation of the fibrin cross-linking process in the thrombus. Experiments with HNE showed that cleavage of inactive FXIII results in a novel active truncated form of FXIII (N40-FXIII-A*), two amino acids shorter than the thrombin cleaved form, but still possessing significant transglutaminase activity. For the first time a mechanism, which down-regulates FXIIIa in the clot was described. Proteases released by PMNs effectively degraded FXIIIa in fibrin as well as in plasma clots. This down-regulating mechanism could prevent the formation of over-cross-linked plasma clots and thus facilitate the elimination of fibrin when it is no longer needed.

SUMMARY

Blood coagulation factor XIII (FXIII) is a protransglutaminase of tetrameric structure (A_2B_2). The first step in the activation of pFXIII is the cleavage of R37-G38 bond in the A subunit (FXIII-A) by thrombin, which makes the subsequent formation of an active transglutaminase possible. No active form of FXIII-A, other than G38-FXIII-A* has been identified. The main task of activated FXIII (FXIIIa) in hemostasis is the cross-linking of fibrin chains, which, together with the cross-linking α_2 plasmin inhibitor to fibrin renders the clot resistant to fibrinolysis. Although all activated clotting factors have known pathways of inactivation, in the case of FXIII, no such mechanism has been reported. As the hemostatic plug contains polymorphonuclear granulocytes (PMNs) rich in proteolytic enzymes, we tested if these proteases are released in fibrin clots and if they become involved in the regulation of FXIII activity.

Purified human neutrophil elastase (HNE) induced a limited cleavage of the inactive FXIII, resulting in the rapid activation of FXIII, followed by a much slower inactivation. HNE-activated FXIII cross-linked fibrin γ- and α-chains in the clot formed by batroxobin moojeni. MALDI-TOF analysis and N-terminal sequencing identified V39-N40 as the primary cleavage site and N40-FXIII-A* as a novel active form of FXIII.

The supernatant of stimulated PMNs proteolytically degraded FXIIIa, resulting in the parallel loss of transglutaminase activity. It was demonstrated that in the fibrin clot HNE, cathepsin G and matrix metalloprotease-9 (MMP-9) were released from PMNs, they exerted a fibrinolytic effect and degraded both FXIII subunits. It was shown that HNE is involved in the down-regulation of FXIIIa within the fibrin clot, while the task of MMP-9 and to a lesser extent that of cathepsin G is the further degradation of the split products. The proteolytic degradation of FXIII by PMNs was also significant when clots were made from whole plasma or from fibrinogen supplemented with α_1-antitrypsin (α_1AT). In the presence of α_1AT the degradation of FXIIIa by PMN proteases occurred significantly faster than that of cross-linked fibrin. These results suggest that proteases released from PMNs could effectively be involved in the inactivation of FXIIIa within the fibrin clot. For the first time, a mechanism, which down-regulates FXIIIa in the clot, was described. This mechanism could prevent the formation of over-cross-linked fibrin clot difficult to eliminate when it is no longer needed.

ÖSSZEFOGLALÁS

A véralvadás XIII-as faktora (FXIII) egy tetramer struktúrájú zymogen (A_2B_2). A FXIII aktivációja során első lépésként a trombin hidrolizálja az R37-G38 peptidkötést az A alegységben (FXIII-A), mely lehetővé teszi az aktív transzglutamináz kialakulását. Az így létrejött G38-FXIII-A* az egyetlen eddig ismert aktív FXIII-A forma. Az aktivált FXIII (FXIIIa) fő funkciója a fibrin láncok keresztkötése és az α_2 plazmin inhibitor fibrin láncokhoz való kötése, mely stabilizálja a fibrint és védi a fibrinolízissel szemben. Bár az összes aktivált alvadási faktornak van ismert inaktivációs szabályozó mechanizmusa, a FXIII esetében erről eddig nem született közlés. Mivel a trombusban proteolitikus enzimekben gazdag polymorphonucleáris (PMN) granulocyták halmozódnak fel, megvizsgáltuk, hogy a PMN sejtekből felszabaduló proteázoknak van e szerepük a FXIII aktivitás szabályozásában.

Tisztított humán neutrophil elasztáz (HNE) az inaktív FXIII-at részlegesen hasította, a FXIII gyors aktivációját majd lassabb inaktivációját eredményezve. A HNE-aktivált FXIII a fibrin γ-, és α-láncait keresztkötötte a batroxobin moojenivel megalvasztott fibrin alvadékban. MALDI TOF analízis és N-terminális szekvenálás segítségével azonosítottuk az elsődleges hasítási helyet (V39-N40) és egy új, aktív FXIII formát írtunk le (N40-FXIII-A*).

Stimulált PMN sejtek felülúszójának hatására a FXIIIa proteolitikusan degradálódott, a transzglutamináz aktivitás parallel csökkenésével. Kimutattuk, hogy a fibrin alvadékban HNE, katepszin G és mátrix metalloproteáz 9 (MMP-9) szabadul fel a PMN sejtekből, melyek a fibrint bontották és degradálták mindkét FXIII alegységet. Kimutattuk, hogy a FXIIIa down-regulációjában a HNE részt vesz, míg az MMP-9 és a katepszin G elsődleges szerepe a hasítási termékek további lebontása. A FXIII PMN proteázok által bekövetkező degradációja teljes plazmából illetve α_1-antitripszinnel kiegészített fibrinogénből készült alvadékok esetén is jelentős volt. Az α_1-antitripszint is tartalmazó fibrin alvadékban a FXIIIa degradációja szignifikánsan gyorsabban ment végbe, mint a keresztkötött fibrin degradációja.

Eredményeink azt bizonyítják, hogy a PMN sejtekből felszabaduló proteázok szerepet játszhatnak a FXIIIa inaktiválásában a fibrin alvadékban. A FXIII PMN proteázok által bekövetkező degradációja a FXIII down-regulációjának egyetlen eddig ismert, új mechanizmusa, mely meggátolhatja a túlzottan keresztkötött, nehezen eliminálható fibrin alvadék kialakulását.

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XXIst Congress of the International Society on Thrombosis and Haemostasis, Geneva, Switzerland, 06-12 July, 2007

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KEYWORDS

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TÁRGYSZAVAK

Faktor XIII

Faktor XIII-A V34L polimorfizmus

Fibrinogén

Fibrin

Polymorphonucleáris granulocyták

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APPENDIX

Original publications related to the dissertation