Haemostatic effects of recombinant coagulation factor VIIa

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Lay-out: Pre Press, Baarn

Cover illustration by Janine Mariën
Inside illustrations by Geert Donker

ISBN: 90-393-3192-8

Haemostatic effects of recombinant coagulation factor VIIa

Bloedstelping door recombinant stollingsfactor VIIa
(Met een samenvatting in het Nederlands)

PROEFSCHIFT

Ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus,
Prof. Dr. W.H. Gispen,
in gevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op dinsdag 17 december 2002 des ochtends te 10.30 uur.

door

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Geboren op 10 Maart 1976, te Arnhem

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The studies described in this thesis were supported in part by an unrestricted educational grant from Novo Nordisk.
Financial support by Novo Nordisk for the publication of this thesis is gratefully acknowledged.

I've got a pen in my pocket does that make me a writer
Standing on the mountain doesn't make me no higher
Putting on gloves don't make you a fighter
And all the study in the world
Doesn't make it science

Paul Weller

Contents

Chapter 1

General Introduction

1) The haemostatic process

The vascular system is of vital importance for functioning of the human body. Blood components are involved in the transport of oxygen, nutrients, hormones, and other signalling molecules to all organs and tissues, and also in the removal of waste products. Also, blood plays a vital role in the maintenance of body temperature. When the integrity of the vascular tree is disturbed, a complex process referred to as haemostasis is initiated to stop the bleeding and to heal the damaged blood vessel(s).
The haemostatic process is tightly regulated; the haemostatic plug formed closes the rupture in the vessel, but not the vessel itself. A disbalanced haemostatic system can be a consequence of congenital deficiencies or dysfunctionalities of the system, or may be due to acquired defects induced by unrelated diseases or by drugs affecting haemostasis. Disturbance of the haemostatic balance results in either a bleeding or a thrombotic tendency (or a combination of both in rare situations).
Haemostasis can be divided into 5 distinct processes.

1) On rupture of a vessel, vasoconstriction is initiated as a first measure to limit blood loss.
2) Blood platelets adhere to subendothelial components and aggregate to form a platelet plug, which closes the damaged vessel.
3) The formation of a fibrin clot stabilizes the primary haemostatic plug.
4) The generation of plasmin through the fibrinolytic system results in breakdown of the clot.
5) Repair and regeneration of the damaged vessel.

Although the haemostatic process is presented here as a sequence of distinct events, the actual process involves a complex interplay between these haemostatic sub-processes.

Vasoconstriction

The role of vessel wall contraction in haemostasis is poorly understood. Also, the agents involved in vasoconstriction are poorly defined. Molecules derived from endothelium (endothelins)[1], platelets (serotonin and thromboxane A_2)[2,3], or the coagulation system (fibrinopeptide B, bradykinin)[4] may be involved in vasoconstriction mediated by the contraction of smooth muscle. Vasoconstriction might be more significant in arteries than in veins, due to the presence of bigger muscular coats in arteries. Although it is conceivable that vasoconstriction contributes to haemostasis, as the reduction of flow might facilitate other haemostatic processes, the significance of this process remains to be elucidated.

Platelet adhesion and aggregation

Blood platelets circulate in a quiescent state, which is maintained by both active and passive inhibitory elements provided by intact endothelium. Endothelial cells excrete the platelet inhibitors prostaglandin I_2[5], and nitric oxide[6]. In addition, endothelial cells express the transmembrane ecto-ADPase CD39[7], which inhibits platelet function by hydrolyzing ADP from activated platelets, thereby preventing ADP-induced platelet activation (see below). Moreover, platelet adhesion to intact endothelium is prevented by its negative charge, which is provided by heparan sulphate moieties present on the luminal side of the cells.

Rupture of a vessel exposes subendothelial components to the blood. A number of proteins, which are adhesive to platelets, reside in the subendothelium. These include collagen[8], von Willebrand factor (vWF)[9], fibronectin[10], vitronectin[11], laminin[12], and thrombospondin[13]. Platelets adhere to the subendothelium, become activated, and subsequently platelet-platelet interactions are formed, resulting in a platelet aggregate, which serves as a first measure to close the damaged vessel.

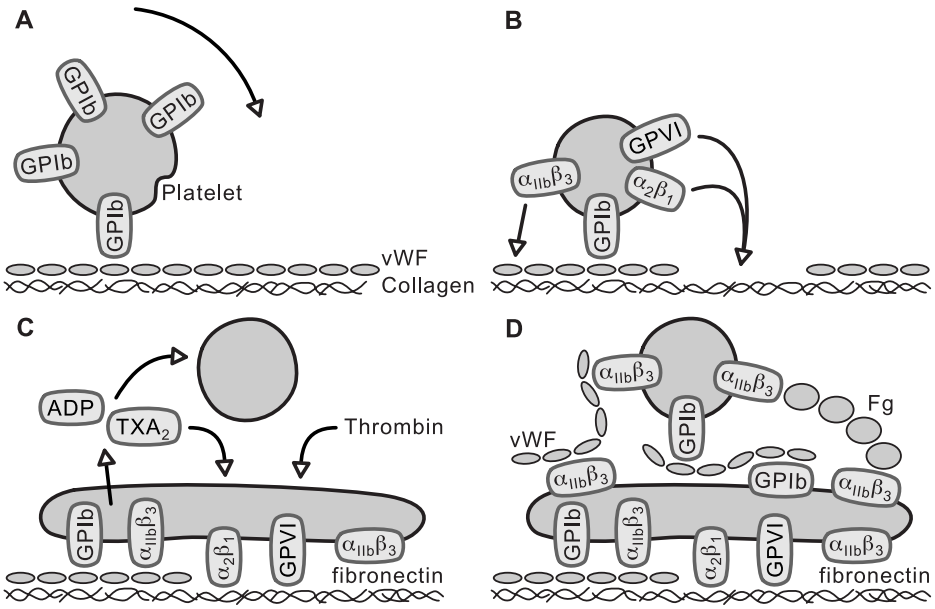

Figure 1. Schematic representation of platelet adhesion and aggregation under flow conditions. A) Rolling of platelets over collagen-bound vWF mediated by GPIb. B) Firm attachment mediated by $\alpha_2\beta_1$ and glycoprotein VI (GP VI) binding to collagen, and by $\alpha_{IIb}\beta_3$ binding to collagen-bound vWF. C) Platelet activation, secretion, and spreading. D) Aggregate formation.

Adhesion of platelets to the vessel wall presumably proceeds in a two-step mechanism (Figure 1). First, platelets are slowed down by transient binding of platelet glycoprotein Ib to von Willebrand factor bound to subendothelial collagen[14]. Subsequently, a stationary contact between platelet and subendothelium is accomplished by binding of one or more platelet integrins and other receptors to their ligands in the subendothelium. Specifically, integrin $\alpha_2\beta_1$ and glycoprotein VI can bind to collagen, $\alpha_{IIb}\beta_3$ can bind to vWF, fibrinogen, vitronectin, and thrombospondin, $\alpha_5\beta_1$ can bind to fibronectin, $\alpha_6\beta_1$ can bind laminin, and $\alpha_v\beta_3$ can bind vitronectin[11].

After adhesion, platelets become activated by a number of agonists including collagen and thrombin. Platelet activation is propagated by excretion of thromboxane A_2, which is synthesized in the platelet on stimulation, and by factors excreted from intracellular granules, such as ADP and serotonin. Platelet activation involves binding of the herefore mentioned agonists to their specific receptors. These receptors are either seven transmembrane G-protein-coupled receptors (GPCR), or receptors coupled to tyrosine kinases. A well-characterised GPCR is the classical thrombin receptor, also referred to as protease activated receptor-1 (PAR-1)[15]. An interesting feature of PAR-1 is that it carries its own ligand. Thrombin cleaves an N-terminal peptide from the receptor, thereby exposing a new N-terminus which binds to another region of the receptor thereby activating it[16]. PAR-1 cleavage by thrombin is strongly enhanced when thrombin is bound to glycoprotein Ib[17].

Platelet activation results in signal transduction pathways, which eventually lead to shape change, aggregation, release of granule contents, and synthesis of propagators of platelet activation.

Platelet activation is accompanied by expression of procoagulant surface, i.e., negatively charged phospholipids. In unactivated platelets, anionic phospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) are restricted to the inner leaflet of the platelet membrane through the cooperative action of an ATP-dependent aminophospholipid translocase and an ATP-dependent floppase. On platelet activation, PS and PE are translocated to the outer leaflet by activation of a calcium-dependent phospholipid scramblase (for a review see[18]). Exposure of procoagulant surface facilitates thrombin generation required for enhancement of platelet activation and the generation of fibrin (see below).

A second mechanism by which platelet activation supports fibrin generation is the excretion of proteins involved in coagulation as well as inhibitors of clot breakdown from alpha granules.

Platelet activation results in activation and clustering of $\alpha_{IIb}\beta_3$, which subsequently can

bind fibrinogen and vWF. Bridging of fibrinogen and vWF between two platelets results in aggregation, which leads to the formation of a stable platelet plug.

Fibrin formation

Blood coagulation, or the process of fibrin formation, consists of a complicated sequence of enzymatic reactions, eventually leading to the generation of thrombin. Thrombin is not only responsible for platelet activation, but also for the cleavage of fibrinogen into fibrin. Fibrin spontaneously polymerizes and forms an insoluble mesh, which stabilizes the platelet plug.

In 1964, two groups independently proposed the cascade model of coagulation[19,20]. In this model, the generation of thrombin involved a sequential series of steps in which activation of one clotting factor led to the activation of another. Through sequential activation of clotting factors an enormous amplification of the initial signal could be accomplished. The first signal was thought to be initiated by either intrinsic activation via coagulation factor XII, or extrinsic activation via the tissue factor-VIIa complex. Although these two activation pathways are nowadays still highly significant in the diagnostic laboratory (i.e., intrinsic and extrinsic activation are used in the APTT and

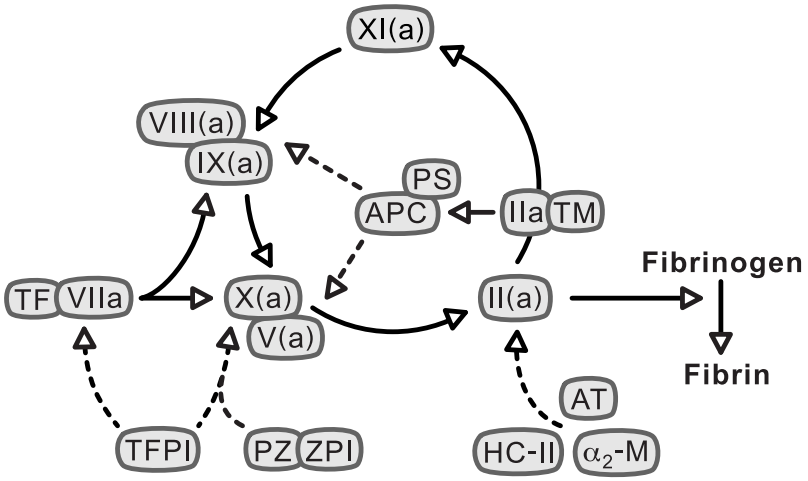

Figure 2. Schematic representation of tissue factor-induced coagulation. Activation steps leading to the generation of thrombin are represented by the uninterrupted lines. The interrupted lines represent inhibitory systems. TF: tissue factor, TFPI: tissue factor pathway inhibitor, PZ: protein Z, ZPI: protein Z-dependent protease inhibitor, APC: activated protein C, PS: protein S, TM: thrombomodulin, AT: antithrombin, HC-II: heparin cofactor II, α_2-M: α_2-macroglobulin.

PT assay, respectively), it is currently believed that the extrinsic pathway is the physiological initiator of haemostasis[21]. The intrinsic pathway seems not to play a role in normal haemostasis, but might become important in pathological situations such as sepsis[22]. A schematic representation of the TF-induced coagulation pathway is presented in figure 2.

Coagulation is initiated when cells expressing the transmembrane receptor tissue factor (TF) are exposed to the blood. Traditionally, it is believed that under normal conditions tissue factor expressing cells are located exclusively extravascular[21], although TF synthesis can be induced in endothelial cells and monocytes by bacterial components or inflammatory cytokines[23,24]. However, recent data suggested that tissue factor expressing cells may circulate in the bloodstream under normal conditions[25]. In an intact vasculature, the tissue factor in blood cells is thought to be 'encrypted' (i.e., inactive), presumably due to a lipid environment not supporting initiation of coagulation[26]. It has also been suggested that encryption of TF is a consequence of dimerisation. Upon activation of TF-bearing cells, TF has been shown to monomerise, and it has been suggested that this is the event that triggers TF decryption[27].
When active (decrypted) tissue factor is in contact with blood, its complex with factor VIIa initiates coagulation. Factor VII circulates in the blood in both zymogen and activated form. Activated factor VIIa does not have any relevant enzymatic activity on its own. Only on binding to tissue factor and in the presence of anionic phospholipids, factor VIIa is able to activate its substrates, factors IX and X, by limited proteolysis. The activation of factor VII proceeds when it is bound to tissue factor. A number of mechanisms by which factor VII is activated have been proposed, including activation by TF-VIIa[28], factor Xa[29], factor IXa[29], thrombin[30], hepsin[31] and a poorly characterised 'factor VII activating protease'[32]. Factor Xa, however is nowadays believed to be the physiological activator of factor VII during injury[33]. Factor IXa is thought to be responsible for the maintenance of low levels of factor VIIa in circulation[34]. The TF-VIIa complex activates factors IX and X. The efficiency of activation depends on the concentration of TF. At low concentrations of TF factor IX is predominantly activated, whereas at high TF factor X is the preferred substrate[35]. Factors IXa and Xa then assemble into the tenase and prothrombinase complex, respectively. The tenase complex, consisting of the enzyme factor IXa, cofactor VIIIa, and zymogen factor X bound to a lipid surface containing anionic phospholipids, results in the activation of factor X by factor IXa. Similarly, the prothrombinase complex (enzyme factor Xa, cofactor Va, and zymogen factor II assembled on a procoagulant surface)

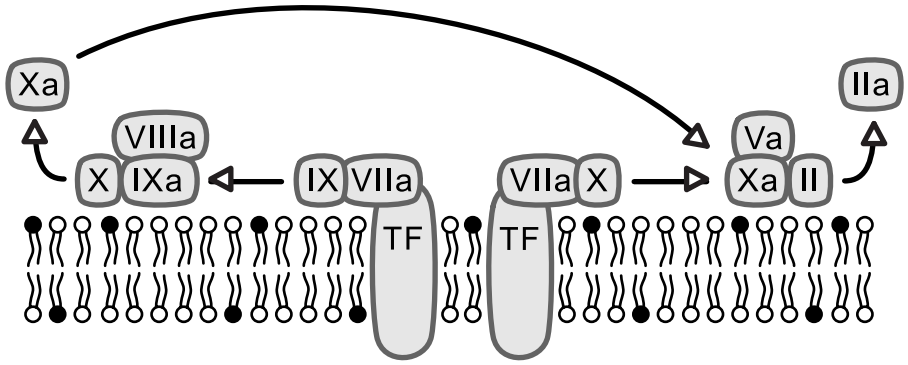

Figure 3. Tissue factor-induced coagulation, and the assembly of tenase and prothrombinase complexes. The TF-VIIa complex can activate factors IX and X. Activated factor IX enters the tenase complex consisting of enzyme factor IXa, activated cofactor VIIIa, substrate factor X, and the catalytic phospholipid surface containing anionic phospholipids. Activated factor X, resulting from tenase activity or from TF-VIIa activity enters the prothrombinase complex consisting of enzyme factor Xa, activated cofactor Va, substrate factor II, and the catalytic phospholipid surface, leading to the generation of thrombin.

results in the generation of thrombin. Figure 3 shows a schematic representation of initiation of coagulation and assembly of tenase and prothrombinase complexes.

The procoagulant surface is required for all steps in thrombin generation so far described. The function of the procoagulant surface is not only to localize thrombin generation at the site of injury, but also to accelerate reaction velocity by producing a profound drop in the Km of the reaction[36,37]. Binding of (pro)enzymes to an anionic lipid surface is facilitated by the presence of γ-carboxylated glutamic acid residues (Gla residues) in the N-terminal part of these proteins. Besides factors VII, IX, X, and II, also the anticoagulant proteins C, S, and Z (see below), and the amplifier of platelet activation GAS6 contain Gla residues. The posttranslational modification of glutamic acid residues to Gla residues is regulated by a vitamin K-dependent carboxylase[38]. Gla residues bind calcium ions, and binding of Gla proteins to a lipid surface is a consequence of calcium bridge formation between protein and lipid surface.
The role of the cofactors Va and VIIIa in the prothrombinase and tenase complex, respectively, is to accelerate velocity of substrate conversion (Vmax), presumably by inducing a conformational change in their respective enzymes[36,37].

The thrombin generated by TF-induced coagulation amplifies its own generation by

activating the procofactors V and VIII, and by activation of factor XI. Factor XIa can activate factor IX, which can subsequently participate in a tenase complex. A relatively small amount of thrombin is required for the cleavage of fibrinogen into fibrin, platelet activation, factor V activation, and factor XIII activation[39]. Activated factor XIII is a transglutaminase which stabilizes polymerized fibrin by crosslinking gamma or alpha chains in adjacent fibrin molecules by introducing ε(γ-glutamyl)lysyl isopeptide bonds[40]. In vitro experiments indicate that thrombin generation continues after clot formation, and that in fact the bulk of thrombin is formed after clot formation[39,41]. These higher concentrations of thrombin are required for the activation of TAFI (see below).

The generation of thrombin is controlled and terminated by a number of inhibitory mechanisms including the presence of blood flow, which removes (activated) clotting factors from the site of injury, and a number of inhibitory protein systems, acting on different steps in the coagulation cascade.

The activity of the TF-VIIa complex is regulated by the tissue factor pathway inhibitor (TFPI)[42]. TFPI is a kunitz type inhibitor and inhibits coagulation in a two step mechanism. First, TFPI binds to activated factor Xa, thereby inhibiting its proteolytic activity. Next, the TFPI:Xa complex binds with high affinity to factor VIIa in the TF-VIIa complex, resulting in a fully inhibited quaternary TF:VIIa:TFPI:Xa complex.

Propagation of coagulation is inhibited by the protein C system, which inactivates cofactors Va and VIIIa[43]. Activation of the protein C system is initiated when thrombin binds to the endothelial cell surface receptor thrombomodulin. Once bound to thrombomodulin, thrombin loses its procoagulant properties, and becomes able to activate protein C and TAFI (see below). Activated protein C, together with its cofactor protein S, inactivates factors Va and VIIIa by limited proteolysis. Localisation of protein C to the endothelial surface is facilitated by the transmembrane receptor EPCR (endothelial protein C receptor). Protein S also displays anticoagulant activity independently of protein C, presumably involving displacement of coagulation factors from the procoagulant surface[44].

Antithrombin regulates thrombin generation by inactivating thrombin, factor Xa, factor IXa, factor XIa, and factor VIIa by forming a stable 1:1 complex, thereby inactivating the enzyme[45]. The inhibitory potential of antithrombin is greatly accelerated by heparin or the heparin-like glycosaminoglycans present on endothelial cells.

Two other plasma proteins, heparin cofactor II and α_2-macroglobulin are also stoichiometric inhibitors of thrombin[46,47].

Finally, a recently characterized inhibitory system of coagulation is the protein Z/protein

Z dependent protease inhibitor (ZPI) system. ZPI inhibits factor Xa, and protein Z serves as a cofactor for this inhibitory reaction[48].

Recently, the above-described model of coagulation has been refined to a cell-based model[49]. In this model, emphasis is placed on the surfaces on which the different coagulation reactions occur. Coagulation initiates on tissue factor-bearing cells, such as fibroblasts or smooth muscle cells. A small amount of thrombin is generated on the (procoagulant) surface of these cells, and the thrombin generated results in platelet activation, and activation of factors Va and VIIIa. For full thrombin generation, the procoagulant surface of the activated platelet is required. Factor IXa formed on the TF-bearing cell diffuses to the platelet surface. Factor IXa, but not factor Xa is assumed to be able to transport from the TF bearing cell to the platelet[50], as factor Xa in solution is rapidly inactivated by antithrombin or TFPI, but factor IXa in solution has a relatively long half life. Factor IXa combines on the platelet surface with factor VIIIa, and starts activating factor X, which in its turn combines with factor Va to generate thrombin.

Fibrinolysis

A formed fibrin clot needs to be removed when the integrity of the vessel is restored. Fibrin degradation proceeds by a process referred to as fibrinolysis (see figure 4 for a schematic overview). Fibrinolysis is initiated by the conversion of the plasma protein plasminogen into plasmin. Plasmin is a rather unspecific serine protease, able to cleave multiple peptide bonds in the fibrin molecule, but it also can proteolyse a number of other proteins, including coagulation factors V[51] and VIII[52], TFPI[53], and others.
The physiological activator of fibrinolysis is thought to be tissue type plasminogen activator (tPA)[54]. tPA is synthesized by endothelial cells, which both constitutively excrete and store this molecule[55]. tPA is found in plasma in small quantities in complex with its physiological inhibitor plasminogen activator inhibitor 1 (PAI-1)[56]. Presumably, the tPA excreted by endothelial cells near the site of the injury is responsible for fibrinolysis in vivo. Alternatively, plasminogen can be activated by urokinase plasminogen activator (uPA). uPA circulates as a single chain proenzyme (scuPA), and is activated by plasmin or by kallikrein generated via the contact activation system of coagulation[57]. uPA can bind to a specific receptor (uPAR), which is present on many cell types[58]. Binding of uPA to uPAR localizes plasminogen activation, but also leads to intracellular signalling which has been shown to play an important role in tissue development[59]. It has been suggested that uPA is primarily

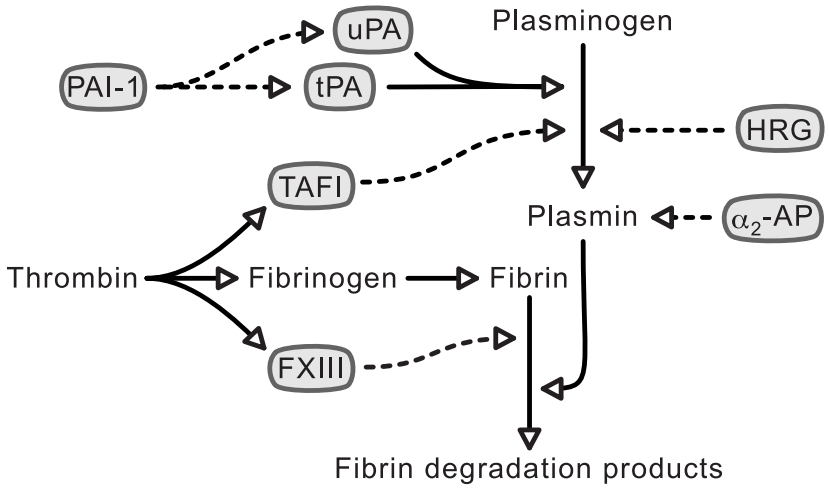

Figure 4. Schematic representation of the fibrinolytic system, and its link with the coagulation system. Activation pathways leading to the generation of plasmin, and subsequent breakdown of the clot are represented by uninterrupted lines. Inhibitory pathways are represented by interrupted lines. PAI-1: plasminogen activator inhibitor-1, tPA: tissue-type plasminogen activator, uPA: urokinase, TAFI: thrombin activatable fibrinolysis inhibitor, HRG: histidine-rich glycoprotein, α_2-AP: α_2-antiplasmine.

responsible for plasminogen activation in tissue, whereas tPA is responsible for clot lysis in the circulation. PAI-1 is most probably also the physiological relevant inhibitor of uPA[60].

tPA and plasminogen can bind to lysine and arginine residues on partially degraded fibrin[61,62]. Binding of tPA and plasminogen to the fibrin clot markedly enhances the plasminogen activating activity of tPA. Moreover, these interactions serve to localize plasmin to the fibrin clot. In solution, plasmin is rapidly inhibited by the plasma protein α_2-antiplasmin. Bound to fibrin, plasmin is relatively resistant to inhibition by α_2-antiplasmin. uPA does not bind specifically to fibrin.

Fibrin clots are made more resistant to fibrinolysis by two mechanisms involving thrombin. Firstly, thrombin activates factor XIII, which stabilizes the fibrin clot by crosslinking gamma or alpha chains in adjacent fibrin molecules by introducing $\varepsilon(\gamma$-glutamyl)lysyl isopeptide bonds[40]. Activated factor XIII also crosslinks α_2-antiplasmin to the fibrin clot, thereby contributing to the inhibition of fibrinolysis. Secondly, thrombin (or thrombomodulin-bound thrombin) can activate thrombin activatable fibrinolysis inhibitor (TAFI)[63]. Activated TAFI cleaves C-terminal lysine and arginine

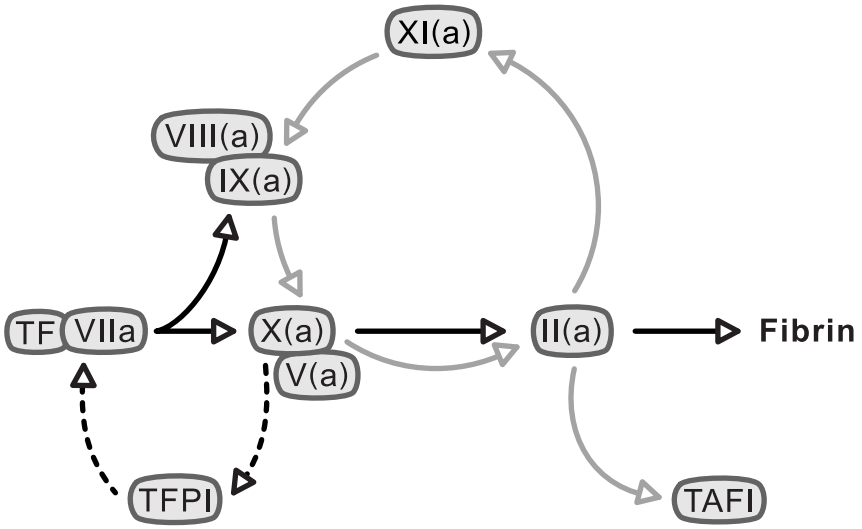

Figure 5. The role of factor XI in the thrombomodulin-independent activation of TAFI. Tissue factor-initiated coagulation results in the generation of a small amount of thrombin required for clot formation (dark arrows). After formation of the clot, thrombin generation continues via a factor XI-dependent pathway (grey arrows). The generation of this secondary thrombin burst results in the activation of TAFI. (Redrawn from Bouma et al.[194])

residues from partially degraded fibrin, thereby preventing tPA and plasminogen binding[64]. Relatively high concentrations of thrombin are required for TAFI activation. It is believed that these high amounts of thrombin are generated after clot formation via thrombin-mediated factor XI activation (see figure 5)[65]. TAFI can also be activated by thrombomodulin-bound thrombin. TAFI activation by thrombomodulin-bound thrombin is approximately 1200 more efficient than TAFI activation by thrombin[66]. It is possible that both activation pathways contribute to TAFI activation in vivo, depending on the amount of thrombomodulin expressed near the site of injury. It has to be noted that thrombomodulin-bound thrombin can also express profibrinolytic properties mediated by protein C activation. Activated protein C stimulates fibrinolysis by inhibiting thrombin generation, and thus TAFI activation. It has been shown that the concentration of thrombomodulin determines its preference for TAFI or protein C activation[67].

Platelet activation also contributes to down-regulation of fibrinolysis by excretion of PAI-1[68] and α_2-antiplasmin from its α-granules[69]. Moreover, platelets contain the a-subunit of factor XIII[70], and platelets are able to bind (and thereby localize) factor

XIII through $\alpha_{IIb}\beta_3$, thereby contributing to the stability of the haemostatic plug and its resistance to fibrinolysis[71].

Repair

Ultimately, the haemostatic plug needs to be replaced by a novel layer of endothelial cells and underlying extracellular matrix. The fibrin clot not only serves to close a damaged vessel wall, but also acts as a scaffold for invading inflammatory, endothelial, and other tissue cells during tissue repair[72]. The formation of a new endothelium proceeds through a process referred to as angiogenesis. A highly similar process referred to as vasculogenesis, is essential during fetal development. Furthermore, angiogenesis plays an important role in pathological conditions such as cancer (the formation of new bloodvessels is essential to supply a tumor with oxygen and nutrients)[73].
Angiogenesis is facilitated by the (cross linked) fibrin clot, which in conjunction with fibronectin forms a provisional matrix for the influx of endothelial and other tissue cells, and inflammatory cells, which are also involved in angiogenesis. These cells use integrin receptors to interact with this matrix. Endothelial cells detach from their established matrix, devide, and subsequently migrate over the fibrin-fibronectin matrix towards the site of injury[74]. The signal for angiogenesis comes from angiogenic growth factors (such as VEGF-A, bFGF, aFGF, or HGF), often in conjunction with cytokines (such as TNF-α). The migration of cells is also facilitated by enzymes of the matrix metalloprotease (MMP) family, and by uPA-mediated plasmin generation, which are able to proteolyse different matrix components, thus making a path for the migrating cells[75,76]. Once the new cells have found their way, the provisional matrix needs to be removed by the fibrinolytic system. A proper balance between the formation of the provisional matrix and its removal is essential for the wound healing process. When the formation of the matrix is disturbed, wound healing is delayed, and defective matrix breakdown may lead to fibrosis.

2) Congenital bleeding disorders

This section will describe congenital defects in the haemostatic system in humans leading to a bleeding diathesis. The classic modalities available to treat these patients during bleeding episodes, or prophylactically during surgery will be discussed.

Platelets

Numerous congenital defects in platelet function, which result in a bleeding diathesis, have been described. Platelet abnormalities found include deficiencies in adhesive receptors, deficiencies in signal transduction pathways or signalling receptors, deficiencies in intracellular granules, and deficiencies in the development of platelet procoagulant potential. Furthermore, a defect in vWF, resulting in defective platelet-vessel wall interaction also results in a bleeding tendency.

A congenital deficiency of the glycoprotein Ib/V/IX complex leads to a severe bleeding disorder referred to as Bernard-Soulier syndrome (BSS)[77]. Its prevalence is extremely rare; a prevalence of less than 1 in 1.000.000 has been estimated from literature. BSS is usually inherited as an autosomal recessive trait. Next to the defective primary platelet-vessel wall interaction, patients with BSS suffer from thrombocytopenia, and their platelets show morphological abnormalities. Specifically, extremely large platelets with enhanced expression of PS on the outer leaflet are found in BSS patients.
A congentital qualitative or quantitative deficiency of the integrin $\alpha_{IIb}\beta_3$ is known as Glanzmann's thrombasthenia (GT)[78]. GT is inherited in a recessive manner. Patients with GT have a severe bleeding tendency as a consequence of defective platelet-vessel wall interaction, and more importantly, the complete absence of platelet-platelet interaction.
Bleeding problems in both BSS and GT consist of epistaxis, petechiae, ecchymoses, menorrhagia, gingival bleeding, gastrointestinal bleeding, and bleeding associated with trauma or surgery.

A deficiency in the collagen receptor $\alpha_2\beta_1$ has been described in only two (female) patients[79,80]. This deficiency is associated with a mild bleeding tendency and partial resolution of the bleeding disorder was seen in both patients. Also, a deficiency in the other known collagen receptor, glycoprotein VI, is associated with a mild bleeding tendency[81].

Defects in platelet membrane receptors involved in signalling pathways have been described. These deficiencies, however, are extremely rare, and usually lead to a mild haemorrhagic disorder. Two groups have reported patients with a hereditary defect in one of the platelet ADP receptors $P2Y_{12}$, resulting in a moderate bleeding tendency[82]. Two Japanese families with a defective thromboxane A2 receptor have been described[83].

Likewise, defects in platelet signal transduction mechanisms are known, but they are also extremely rare. These deficiencies include defects in arachidonic acid metabolism and thromboxane A2 production, defects in Gαq, defects in calcium response, and reduced protein phosphorylation[84].

Patients with various abnormalities in platelet granules have been described[85]. A deficiency of dense granules is referred to as δ-storage pool deficiency (SPD). δ-SPD is a heterogenous, mostly mild bleeding disorder, which can occur both as a primary, autosomal dominant inherited disorder, or as a component of a multisystem disorder such as the Hermansky-Pudlak syndrome, the Chediak Higashi syndrome, and the Wiskott-Aldrich syndrome. As dense granules contain ATP and ADP, a defect in these granules leads to defective propagation of platelet activation.
A deficiency in platelet α-granules (which contain proteins involved in coagulation and fibrinolysis) is referred to as gray platelet syndrome. This mild bleeding disorder is usually accompanied by moderate thrombocytopenia, and enlarged platelets. It is usually inherited in an autosomal recessive manner. A variant α-granule disorder is called Quebec platelet disorder. This bleeding disorder is characterized by excessive proteolysis of α-granule proteins, presumably due to the presence of large amounts of uPA in the α-granules[86].
A combined α,δ-SPD is a rare disorder characterized by moderate to severe defects in both δ and α-granules.

An extremely rare defect in the platelet's potential to develop procoagulant activity is referred to as Scott syndrome[87]. Scott syndrome is a relatively severe bleeding tendency, which is inherited in an autosomal recessive manner. Platelets from patients with Scott syndrome lack the ability to translocate phosphatidylserine from the inner to the outer leaflet, presumably due to a defect in the activation of the phospholipid scamblase. Defective PS expression on activation is also observed in erythrocytes and lymphocytes from these patients. The defective PS expression is accompanied by a reduced ability to shed microvesicles on activation.

The most common inherited platelet-related bleeding disorder is von Willebrand's disease (vWD)[88]. Patients with a qualitative or quantitative defect in vWF have a bleeding diathesis due to defective platelet-vessel wall interaction, and to defective platelet-platelet interaction. The prevalence of vWD is estimated at 1% of the general population. The prevalence of clinically relevant vWD is presumably around 10-fold

lower (i.e., 1:1000). Seventy percent of the clinically relevant vWD is caused by a type 1 vWD, in which decreased levels of vWF (20-50% of normal) are present in plasma. Type 1 vWD is inherited in an autosomal dominant manner, and is associated with a mild bleeding tendency. In type 3 vWD, a complete deficiency of vWF (<1% of normal) is present, accompanied by severely reduced levels of coagulation factor VIII (3-10% of normal). Type 3 vWD is characterized by severe bleeding and appears to be inherited as an autosomal recessive trait. Several subtypes of type 2 vWD are distinguished, all of them are characterized by an abnormal structure or function (i.e., a discordance between antigen and activity). Type 2A is the most common type 2 vWD. It is characterized by an absence of larger (more reactive) multimers. In type 2B vWD, a mutation causes vWF to bind spontaneously to GPIb. The platelet-vWF complexes are rapidly cleared from the circulation. As a consequence, a loss of larger multimers and thrombocytopenia occurs. In type 2N vWD, factor VIII binding to vWF is defective, with a haemophilia A-like phenotype (see below) as a result. Finally, type 2M vWD refers to a defective interaction with GPIb, with normal or nearly normal multimeric pattern.

Standard treatment for all platelet-related bleeding disorders (except for vWD) in case of uncontrolled haemostasis or prophylactically during surgery consists of platelet transfusion. However, platelet transfusion in these patients may lead to alloimmunisation. Antibodies to both HLA determinants and to the deficient protein (e.g., GP Ib in case of BSS) may be formed. The development of these antibodies makes further platelet transfusion ineffective.
1-desamino-8-d-arginine vasopressin (DDAVP) is frequently administered to patients with SPD, BSS, and GT, although efficacy in BSS and GT has not been thoroughly established yet. Antifibrinolytic agents may be useful in oral cavity bleedings. In women with menorrhagia, the use of oral contraceptives may be beneficial.
Treatment of bleeding complications in vWD depends on the type of the disease. Patients with type 1 vWD may be given DDAVP, which increases vWF levels in plasma by inducing vWF release from endothelial cells. Many type 2 and nearly all type 3 vWD patients do not respond to DDAVP. In these patients infusion of vWF-concentrates is indicated. As DDAVP also releases tPA from endothelial cells, adjunctive therapy with antifibrinolytics may be beneficial. Menorrhagia may be treated with oral contraceptives or estrogens, as these compounds tend to increase plasma vWF levels.

Coagulation

Inherited deficiencies in all procoagulant proteins associated with a bleeding diathesis have been described in humans, with the exception of tissue factor. Experiments with TF knockout mice suggest that TF is not only involved in initiating coagulation, but also in the formation of the vascular system[89]. Therefore, it has been suggested that TF deficiency in humans is not compatible with life.
The most common deficiencies in procoagulant proteins are factor VIII and factor IX deficiency (haemophilia A and B, respectively)[90]. Haemophilia A is an X-linked disorder with an estimated prevalence of 1:10.000 males. Its severity depends on the residual FVIII activity. Severe haemophilia (FVIII levels <1%) is a serious bleeding disorder, characterized by spontaneous bleeding primarily in joints and muscles from early infancy. In moderate haemophilia (FVIII levels 1-5% of normal) spontaneous bleeding is uncommon, but patients do bleed after surgery or following trauma. In mild haemophilia (FVIII levels 6-30% of normal) spontaneous bleeding is rare, but bleeding after trauma or surgery does occur.
Haemophilia B is clinically indistinguishable from haemophilia A. This X-linked disorder occurs in 1 out of every 25.000-30.000 male births.
Factor XI deficiency (haemophilia C) has a much milder phenotype than haemophilia A or B[91]. It is inherited in an autosomal recessive manner. Factor XI deficiency is relatively rare, except in Ashkenazi Jews, in which the prevalence of homozygous factor XI deficient individuals is estimated at 0.1-0.3%. Factor XI deficient patients particularly bleed after surgery that involves tissues with high fibrinolytic activity such as the oral cavity, the urinary tract, tonsils and nose. This phenotype might be explained by the role of factor XI in the activation of TAFI[65]. In contrast to haemophilia A and B, the severity of the bleeding diathesis in factor XI deficient patients does not correlate with the residual factor XI activity levels.

The therapy of choice for patients with haemophilia A and B consists of factor replacement therapy. Both prophylactic administration of factor concentrates, as well as an on-demand approach are currently used. With the introduction of recombinant factor VIII and IX, viral safety is warranted. In mild haemophilia, factor VIII levels can be temporarily increased by administration of DDAVP. Antifibrinolytic agents are frequently used as adjunctive therapy, particularly in case of mucous membrane bleeding and dental procedures. Factor replacement therapy may be complicated by the development of inhibitory antibodies against the infused protein. Inhibitory

antibodies develop in 25-30% of patients with haemophilia A[92] and in 1-3% of patients with haemophilia B[93] at some point during their life. Approaches for eradication of inhibitors include immunoabsorption[94], and immune tolerance therapy (infusion of high doses of factor VIII concentrates)[95]. Treatment of bleedings in a patient with inhibitors consists of the infusion of porcine factor VIII[96] (which also carries a risk for inhibitor development), and (activated) prothrombin concentrates[97].
In patients with factor XI deficiency, plasma transfusion is given when major surgical procedures are performed. Factor XI concentrates are available, but administration of these products is associated with development of laboratory signs of DIC[98]. Antifibrinolytic agents may be administered as well.

Relatively rare are deficiencies in coagulation factors VII, X, II, and V[99]. Factor VII deficiency is an autosomal recessive disorder with unknown prevalence. Factor VII deficient patients with low levels of factor VII frequently present with a severe haemorrhagic disorder. Patients with a factor VII level of 5% or more, usually have a mild bleeding tendency. Factor X deficiency, also inherited in an autosomal recessive trait, results in a moderate to severe bleeding tendency, primarily from soft tissues and mucous membranes. Menorrhagia may occur in women with factor X deficiency. Factor II deficiency is a rare disorder, with about 50 patients described worldwide. Factor II deficiency is characterized by mild to moderate mucocutaneous and soft-tissue bleeding. Factor V deficiency (initially called parahaemophilia) is also a rare autosomal recessive disorder, with a prevalence of 1:10.000.000. Factor V deficiency manifests itself as a moderate bleeding tendency, the bleeding complications particularly include ecchymoses, epistaxis, and gingival bleeding.
Bleedings in factor VII, X, II and V deficiency can be controlled by plasma infusion. In factor VII, X, and II deficiency prothrombin concentrates may also be given.

A hereditary deficiency of fibrinogen occurs as an autosomal recessive disorder[100]. Afibrinogenemia is a rare disorder, with a prevalence of 0.5 in a million. Bleeding problems in afibrinogenemia include umbilical cord bleeding, gum bleeding, epistaxis, gastrointestinal bleeding, menorrhagia, and intracranial bleeding. The bleeding diathesis varies from minimal to severe, and especially intracranial haemorrhage may be fatal. Paradoxically, thrombotic complications have also been reported in young patients with afibrinogenemia[101].
Replacement therapy with fibrinogen concentrates or plasma may be required, but has been reported to be associated with thromboembolic complications.

A deficiency of factor XIII occurs with a prevalence of 0.5 in a million[102]. This disease, which is inherited in an autosomal recessive manner, results in a moderate to severe bleeding disorder. Manifestations of factor XIII deficiency include umbilical cord bleeding, ecchymosis, hematomas, prolongued bleeding following trauma (which may occur 12 to 36 hours after the event), and intracranial bleeding. Also, a higher rate of spontaneous abortion, and problems with wound healing may occur.
Replacement therapy with factor XIII concentrates is convenient, because of the long half-life of factor XIII, and the small quantities of factor XIII required for effective haemostasis.

Fibrinolysis

Hereditary deficiencies of the fibrinolytic inhibitors α_2-antiplasmin and PAI-1 have been described in only a small number of patients[103,104]. Both deficiencies result in a life long bleeding disorder, with manifests itself as delayed bleeding after surgery or trauma, or spontaneous bleeding such as ecchymosis and epistaxis. Bleedings in these patients are mainly controlled by antifibrinolytic agents. A hereditary deficiency of the recently discovered fibrinolytic inhibitor TAFI has not yet been reported. A complete lack of phenotype in the TAFI knockout mouse may suggest that an isolated TAFI deficiency in humans is not associated with a serious bleeding tendency[105].
Isolated reports as well as a family with a bleeding tendency due to excessive plasma tPA activity have been reported[106,107]. In these cases, treatment with antifibrinolytic drugs proved effective.

3) Acquired or drug-induced bleeding disorders

Defects in platelet number or function, coagulation, fibrinolysis, or a combination may be a consequence of disease states of which the underlying cause is not primarily related to haemostasis. This chapter will discuss some common disease states, which are frequently associated with a bleeding tendency.
A common complication of treatment with drugs used in treatment or prevention of thromboembolic events is the occurrence of bleeding complications. The bleeding complications associated with antiplatelet, anticoagulant, and profibrinolytic drugs will be briefly described.

Liver disease

Both chronic and acute hepatic failure induce severe changes in the haemostatic system. Platelets, coagulation, and fibrinolysis all become affected. In chapter 3 of this thesis, an extensive overview of the haemostatic abnormalities found in liver disease will be given.

Renal failure

Renal disease is associated with a bleeding tendency, which is ascribed to a combination of abnormalities in platelet function, abnormal platelet-vessel wall interaction, anemia, and abnormalities in the endothelium[108-111]. After the introduction of dialysis to treat patients with renal failure, the extent and severity of bleeding complications in these patients decreased tremendously. However, excessive bleeding after trauma, during surgery, or following smaller invasive procedures such as renal biopsy is still frequently seen. Also gastrointestinal bleeding is common in these patients. Next to bleeding complications, patients with chronic renal failure also experience thrombotic complications, such as vascular access thrombosis, cardiovascular disease, and renal vein thrombosis[111]. The thrombotic tendency in uremic patients presumably is caused by a combination of general risk factors such as hyperlipidemia, hypertension, and glucose intolerance, and the presence of a hypercoaguable state caused by decreased levels of AT, protein C, and protein S, and the presence of hypofibrinolysis.

Platelet abnormalities found in patients with uremia include reduced in vitro aggregation and secretion in response to a variety of agonists, decreased development of platelet procoagulant activity on stimulation, and impaired clot retraction. Toxins that accumulate in plasma from patients with renal failure are implicated in the development of these platelet defects. Guanidinosuccinic acid, a product of an alternative pathway of ammonia detoxification has been suggested to play a role in the in vitro platelet defects. Moreover, it has been demonstrated in animal and in human studies that this compound may be involved in elevated production of nitric oxide by endothelial cells, thereby contributing to an in vivo platelet defect.

Abnormal platelet-vessel wall interaction in uremia has been observed using in vitro perfusion systems, possibly also related to the presence of uremic toxins in plasma. Part of this defect is caused by the low haematocrit in uremic blood. Also, part of the adhesion defect is compensated for by the presence of elevated concentrations of vWF in uremic plasma[112].

Endothelial abnormalities that might contribute to the bleeding tendency in uremia

include enhanced production of prostacyclin (presumably also triggered by uremic toxins), and nitric oxide.

Management of bleeding in uremia consists of the correction of the anemia, either by the administration of erythropoietin, or by red cell transfusion. Furthermore, DDAVP and vWF concentrate appear to be effective in improving haemostasis even though vWF levels are normal in uremia.

Acquired thrombocytopenia

Thrombocytopenia is defined as a peripheral platelet count of less than 150.000/µl. Three distinct mechanisms can cause acquired thrombocytopenia: decreased platelet production, decreased platelet survival, and increased pooling of platelets in the spleen. Furthermore, hereditary forms of thrombocytopenia have been described (either as isolated disorder, or associated with other defects), but these forms will not be discussed here.

Thrombocytopenia as a consequence of decreased platelet production is usually caused by damage to megakaryocytes in the bone marrow by e.g., malignancies, chemicals, drugs, or viral infections.

Thrombocytopenia caused by decreased platelet survival is due to either increased platelet destruction, or increased platelet consumption. Increased platelet destruction can be drug induced, and usually immune mechanisms are involved. A classical example is heparin-induced thrombocytopenia[113]. In this syndrome, antibodies against the heparin-platelet factor 4 complex mediate platelet activation and subsequent aggregation via binding to platelet Fc receptors. Increased platelet consumption may be a consequence of disseminated intravasular coagulation (see below) or by thrombotic thrombocytopenic purpura (TTP) or the hemolytic uremic syndome (HUS). TTP and HUS are poorly characterized systemic disorders of the microcirculation, in which an unusally high amount of high molecular weight multimers of vWF is responsible for spontaneous platelet aggregation in the arterial microvasulature. Autoantibodies against or mutations in the vWF-cleaving protease (ADAMTS-13) are presumably responsible for accumulation of high molecular weight multimers[114,115].

Thrombocytopenia as a consequence of increased platelet pooling in the spleen is the consequence of enlargement of the spleen most often occuring as a complication of liver disease (see chapter 3).

As acquired thrombocytopenia usually is associated with an underlying disease, treatment of the primary condition is the treatment of choice. Platelet transfusions may be required in case of bleeding complications, but may be ineffective if antibodies against platelets are present. For TTP and HUS, plasma exchange results in normalisation of vWF protease levels, and consequently in normalisation of vWF multimeric pattern.

Disseminated intravascular coagulation

Severe infection, sepsis, trauma, malignancies, and various other disease states may be accompanied by disseminated intravascular coagulation (DIC)[116]. In DIC, the underlying condition results in systemic activation of coagulation, with important roles for tissue factor and inflammatory cytokines[117,118]. Clotting activation results in micothrombosis in a diversity of organs, leading to multiple organ failure. As a consequence of systemic activation of coagulation, consumption of platelets and clotting factors leads to a hypocoagulant state, with bleeding complications as a consequence.

Treatment of coagulation abnormalities in DIC is difficult, as both a procoagulant and hypocoagulant state are present. Inhibition of the procoagulant state in DIC may be accomplished by anticoagulants such as heparin, antithrombin concentrates, protein C concentrates, TFPI concentrates, and active site inactivated factor VIIa[119]. Bleeding episodes may be controlled by infusion of platelet concentrates and plasma.

Antiplatelet drugs

Treatment of thrombotic manifestations, particularly in atherosclerothic cardiovascular disease, may be assisted by use of drugs inhibiting platelet function. The oldest anti platelet drug is aspirin (acetylsalicylic acid), which inhibits platelet function by blocking the enzyme cyclooxygenase involved in the conversion of arachidonic acid to thromboxane A_2. Novel platelet inhibitors include inhibitors of $\alpha_{IIb}\beta_3$, such as abciximab (Fab fragments of a humanized antibody), Eptifibatide (a cyclic peptide containing a KGD sequence), and tirofiban (a peptidomimetic molecule mimicking an RGD sequence). Finally, inhibitors of $P2Y_{12}$, such as ticlopidine and clopidogrel, are currently in use in different clinical settings[120].
Aspirin and $P2Y_{12}$ inhibitors are used in secondary prevention of thrombosis in patients

with established vascular disease. Aspirin also has been shown to be effective in primary prevention of myocardial infarction. Inhibitors of $\alpha_{IIb}\beta_3$ are only used in acute situations (e.g., myocardial infarction) or during interventional procedures (e.g., stenting, percutaneous coronary revascularisation).
An important complication of the use of antiplatelet drugs is an increased bleeding risk, especially after surgery or trauma[121].
Aspirin use is associated with spontaneous gastrointestinal bleeding, presumably as a consequence of both inhibition of platelet function and ulceration and mucosal damage by the drug. The effect of aspirin in the surgical setting is less clear. The bleeding risk may be reversed simply by withdrawing the drug, but as aspirin irreversably inhibits platelet function, it takes several days for the aspirin effect to be completely reversed.
Spontaneous bleeding associated with the use of $\alpha_{IIb}\beta_3$-inhibitors is uncommon, unless the patient is also receiving heparin (see below)[122,123]. Also, surgical bleeding has been reported to be elevated with the use of $\alpha_{IIb}\beta_3$ drugs. An important complication associated with the prolonged use of $\alpha_{IIb}\beta_3$-inhibitors (especially abciximab) is the development of thrombocytopenia, presumably initiated by immune mechanisms.
Bleeding complications in patients receiving anti-$\alpha_{IIb}\beta_3$ drugs may be controlled simply by withdrawing infusion of the drug. Due to the relatively short half-life of these compounds, the induced platelet defect is rapidly reversed. When urgent bleeding problems occur, platelet transfusion has been shown to reverse abciximab's inhibitory effect. However, in case of eptifibatide and tirofiban, which are dosed so that the peak concentration is very high relative to the amount of $\alpha_{IIb}\beta_3$ molecules present in circulation, transfused platelets are most likely inhibited rapidly after infusion by free circulating drug[124].
Spontanous bleeding associated with the use of ADP receptor blockers has been reported to be less frequent compared to patients treated with aspirin. Surgical bleeding has been shown to be increased. Specific therapy to reverse bleeding has not been established in humans, but animal experiments suggest that the use of aprotinin might be beneficial.

Drugs targeting the coagulation system

Inhibition of thrombin generation is considered an important aspect of antithrombotic treatment. Traditionally, heparin or low molecular weight heparins have been used to downregulate the coagulation system. These agents function by enhancing the activity of antithrombin towards both factor Xa and thrombin. If prolongued anticoagulation

is required, treatment with heparin is replaced by administration of oral anticoagulants (OAC's) such as warfarin[125]. OAC's interfere with γ-carboxylation of vitamin K dependent coagulation factors (FVII, IX, X, II, protein C, S, and Z), thereby reducing plasma levels of functional vitamin K-dependent proteins.
Recently, a synthetic pentasaccharide (Org31540/SR90107A, fondaparinux sodium, Arixtra, PENTA), which selectively inhibits factor Xa in an antithrombin dependent manner, has been introduced as an alternative to traditional low molecular weight heparins (LMWH's)[126]. Alternative strategies aiming at down-regulation of coagulation include direct inhibition of thrombin, by low molecular weight inhibitors such as D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone (PPACK)[127] or hirudin[128], direct inhibition of factor X[129], or inhibition of the tissue factor pathway by active site inactivated factor VIIa (ASIS, FFRck-VIIa)[130] or recombinant full length tissue factor pathway inhibitor (rFL-TFPI)[131].
The major complication of anticoagulant therapy using any of the mentioned anticoagulant drugs is bleeding. In case of heparin use, bleeding may be treated with protamine, which instantly neutralizes the anticoagulant function of heparin. LMWH is not effectively neutralized by protamine. Reversal of oral anticoagulants may be accomplished by administration of vitamin K. However, it takes time for the body to synthesize new, functional coagulation factors. Administration of prothrombin complex concentrates can be used if immediate reversal of OAC's is required[132]. However, the use of PCC's is associated with a thrombotic risk[133]. For the new generation anticoagulant drugs no consensus over reversal strategies exist. Most probably, the use of PCC's is also in these settings the treatment of choice.

Thrombolytic drugs

Treatment of arterial thrombosis, particularly myocardial infarction, but also of venous thrombosis may be facilitated by the use of thrombolytic therapy[134]. Thrombolytic drugs activate plasminogen into plasmin, thereby facilitating breakdown of a thrombus. Recombinant tPA and uPA, and variants such as reteplase are human-derived thrombolytic agents. Also, bacterial products such as streptokinase and staphylokinase are currently in clinical use. A major complication of the use of thrombolytic agents is the development of systemic haemorrhagic effects. Intracranial haemorrhage is an important side effect of thrombolytic therapy, affecting 5 to 10 of 1000 patients treated[135]. Bleeding episodes induced by thrombolytic drugs may be treated with plasma infusion. Withdrawal of the infusion of the thrombolytic agent may be

sufficient to neutralize the fibrinolytic state due to the short half-life of these drugs, and immediate reversal of the effect of the fibrinolytic agent may be accomplished by antifibrinolytic drugs such as tranexamic acid or ε-aminocaproic acid.

4) Recombinant factor VIIa (rFVIIa) – a universal haemostatic agent?

The development of recombinant factor VIIa to treat inhibitor-complicated haemophilia

In the 1970's, factor VIII and IX concentrates became available to provide replacement therapy for patients with haemophilia. This was a major step forward in the treatment of these patients, reducing morbidity and mortality. An important complication of replacement therapy, however, is the development of inhibitory antibodies against factor VIII or IX, as a result of which continuation of replacement therapy will be ineffective[92,93].

Initially, PCC's and activated PCC's were used to bypass the lack of intrinsic tenase activity in inhibitor patients. However, these agents have a rather low efficacy rate (50-60%)[136,137] and are associated with thromboembolic complications[133].

It was hypothesized that factor VIIa might be a useful alternative to treat inhibitor-complicated haemophilia, as factor VIIa is virtually proteolytic inactive by itself, and would thus not induce systemic coagulation, whereas coagulation would be enhanced at sites of vascular injury where tissue factor is expressed. In 1983, the first publication appeared in which factor VIIa purified from human plasma was succesfully used to treat two patients with haemophilia A and high titre inhibitors[138]. The use of plasma-derived factor VIIa for treating a large population of haemophilia patients would, however, not be a realistic option, due to the low plasma concentration of factor VII and the low yield of purification.

In 1986, factor VII was cloned[139], and cDNA was transfected into baby hamster kidney cells[140]. The factor VII produced by this expression system is currently purified using four chromatograpic steps (Q-sepharose, Immunoaffinity, and two anion-exchange steps)[140]. Also a virus inactivation step using triton X-100 is included. During purification, activation of factor VII, i.e., hydrolysis of a single peptide bond (Arg152-Ile153) in the single chain protein to yield two-chain VIIa, is accomplished by an incompletely understood mechanism[141]. Most probably, trace amounts of VIIa are generated by cellular proteases during fermentation. These trace amounts of VIIa could initiate VII autoactivation[142]. The obtained product is a highly purified protein preparation with an extremely high degree of virus safety[143].

The amino acid sequence of rFVIIa was found to be identical to that of plasma derived VIIa[140]. The posttranslational modifications of rFVIIa and plasma derived VIIa (such as glycosylation and γ-carboxylation) were highly comparable, and the enzymatic properties of the two products were indistinguishable[144].
In 1988, the first publication of the successful use of rFVIIa in a patient with inhibitor-complicated haemophilia A undergoing surgery appeared[145]. In 1989, a compassionate use program was initiated (patients with life or limb threatening bleedings in whom all conventional therapy had failed and patients undergoing elective surgery were included) and at the same time regular clinical studies were initiated. All studies showed that rFVIIa was a safe and highly effective treatment both during bleeding episodes, and prophylactically during surgery[146-150]. rFVIIa (NovoSeven®) was approved in Europe in 1996, in the United States in 1999, and in Japan in 2000.
The advised dosing schedule currently consists of bolus injections of 90-120 μg/kg bodyweight every two hours. However, recent reports suggest higher dosages (up to 300 μg/kg) to be more effective[151]. Alternatively, continuous infusion may be administered after a single bolus dose, but the efficacy of that approach seems less effective, at least in some cases[152]. Adjunctive therapy with antifibrinolytic agents such as tranexamic acid is frequently used. Recovery and clearance rate of rFVIIa may vary substantially between patients. Especially in paediatric patients, clearance of rFVIIa seems significantly higher than in controls[153]. Few options for laboratory monitoring of rFVIIa treatment are currently available. A standard one-stage clotting assay, or an assay employing a TF mutant incapable of supporting FVII autoactivation can be used to determine rFVIIa activity in plasma[154]. Treatment should be aimed at achieving peak levels of 60-90 U/ml (corresponding to 1.2-1.8 μg/ml or 24-36 nM)[155].
Since approval, around 200.000 standard doses of rFVIIa have been administered, and the number of reported serious side effects is extremely low. Only two haemophilia patients with thromboembolic complications and three with acute myocardial infarction have been reported in association with the use of rFVIIa. In all of them, predisposing factors such as previous cardiovascular disease and advanced age were present[155].

Novel indications for rFVIIa

After the encouraging results of the use of rFVIIa in patients with haemophilia A and B, case reports and small studies were published describing succesful use of rFVIIa in a diversity of congenital and aqcuired bleeding disorders. Moreover, rFVIIa has been

used in patients without any coagulopathy either to arrest bleeding, or to reduce blood loss during surgery. It has been suggested that rFVIIa might become a universal haemostatic agent. This section will discuss the current experience with rFVIIa in clinical settings other than haemophilia.

Deficiencies of other coagulation factors

A number of patients with factor VII deficiency have been succesfully treated with rFVIIa[156]. Only one patient, who was treated with an accidental overdose of 800 μg/kg developed antibodies against VIIa[157]. Also, patients with factor XI deficiency[158,159], and a single patient with amyloid-induced factor X deficiency[160] have been reported to benefit from rFVIIa infusion.

Platelet-related bleeding disorders

Patients with various platelet-related bleeding disorders have been treated with rFVIIa. Several patients with severe thrombocytopenia have been succesfully treated with rFVIIa[161]. Moreover, dozens of patients with Glanzmann's thrombasthenia have been succesfully treated with rFVIIa during bleeding episodes and surgery[162]. It must be noted, however, that these patients often receive adjunctive therapy such as antifibrinolytic drugs and local measures such as topical thrombin and fibrin glue. Treatment of a single patient with Bernard Soulier syndrome with rFVIIa for recurring nosebleeds has been reported[163]. Finally, several case reports of patients with von Willebrand's disease treated with rFVIIa have been published[164,165].

Hepatic failure and liver transplantation

The use of rFVIIa in patients with liver disease and during liver transplantation has gained a lot of interest. Patients with liver failure may suffer from esophageal bleedings and may be at increased risk of bleeding from small invasive procedures such as liver biopsy. A blind randomised study dealing with safety and efficacy of the use of rFVIIa during liver biopsy[166], and several case reports describing efficacy of rFVIIa in patients with liver failure during bleeding episodes have been published[167,168].

Liver transplantation is despite major improvements in surgical techniques still associated with substantial blood loss. A pilot study has shown that a single bolus dose of rFVIIa significantly reduced transfusion requirements during liver transplantation[169]. A drawback of this study, however, was the comparison with historic controls. More extensive double blind, randomised clinical trials are currently ongoing.

Renal failure

Two case reports describing the succesful use of rFVIIa to arrest bleeding in patients with uremia have been published[170,171].

Antiplatelet and anticoagulant drugs

rFVIIa has been implicated in treatment of bleedings induced by antithrombotic drugs. Also, reversal of the anticoagulated state of these patients may be required for urgent surgery. The actual experience with rFVIIa in these patients is, however, scarce. A single case in which a patient who had received an antiplatelet drug (tirofiban) was treated with rFVIIa has recently been reported[18]. rFVIIa has been administered to healthy volunteers who were anticoagulated with acenocoumarol and reversal of laboratory values such as the international normalized ratio (INR) was observed[172]. A single case report of succesful use of a bleeding episode in a patient receiving oral anticoagulation has been reported[173]. rFVIIa has been implicated in reversal of anticoagulation by pentasaccharide, and reversal of laboratory values have been shown in a phase I trial (Dr. M. Levi, personal communication).

Trauma, uncontrollable bleeding, and surgery in absence of preexisting coagulopathy

The possible use of rFVIIa in patients with uncontrollable bleeding due to severe trauma has gained interest since the first report in 1999 on a patient with extensive bleeding due to an intra-abdominal gun-shot wound who stopped bleeding on rFVIIa infusion after all other possible treatment options were unsuccesful[174]. After this first case report seven other critically ill, multitransfused trauma patients (gunshot wounds, stab wounds, and trauma induced by car accidents) were succesfully treated with rFVIIa[175].

A limited number of case reports described the use of rFVIIa in uncontrollable post-surgical bleeding, such as gastrointestinal bleeding, in patients without preexisting coagulopathy[176-178]. One centre reported the use of rFVIIa in 5 patients undergoing heart valve replacement therapy[179]. rFVIIa was administered during or after surgery while the patients experienced excessive and uncontrollable bleeding. In all cases rFVIIa substantially reduced subsequent blood loss, and haemostasis was achieved in all cases.

Finally, preliminary results from one randomised, double blind, placebo controlled, dose escalating, single centre study has been reported in which rFVIIa was used during prostatectomy[180,181]. This study showed a significant reduction in blood loss and transfusion requirements.

5) Mechanism of action of recombinant factor VIIa

The molecular mechanisms responsible for the haemostatic efficacy of rFVIIa in its various clinical applications are poorly understood. It is also still unclear why relatively high plasma concentrations of rFVIIa are required to induce effective haemostasis in patients with haemophilia, and whether these concentrations are also required to induce haemostasis in different patient populations. In this section the current hypotheses on the mechanism of action of rFVIIa will be discussed.

Tissue factor-dependent enhancement of coagulation

Initial concepts on the mechanism of action of rFVIIa in haemophilia were based on tissue factor-dependent enhancement of thrombin generation. The extremely low incidence of thromboembolic complications on rFVIIa administration was explained by the requirement for tissue factor for VIIa to be enzymatically active. It was subsequently shown that rFVIIa infusion in non-bleeding chimpansees resulted in significant increases in plasma levels of prothrombin fragment 1+2, and the activation peptides of factor IX and X[182]. The effects for rFVIIa infusion could be abolished by preinfusing an inhibitory antibody against tissue factor. These results pointed to a TF-dependent mechanism, although it was still not clear why relatively high rFVIIa concentrations are required for haemostatic efficacy. Moreover, experiments in actively bleeding animals or in haemophilia A or B animals adressing the question of TF-dependency have to our knowledge not been performed.
In vitro experiments provided an explanation for the requirement of relatively high rFVIIa concentrations. Van 't Veer et al. showed that endogenous zymogen factor VII inhibits tissue factor-induced coagulation at low concentrations of TF[183]. In the absence of factor VIII, the presence of zymogen factor VII significantly inhibited thrombin generation, and addition of rFVIIa in pharmacologically relevant concentrations restored thrombin generation to levels found in the presence of factor VIII. A subsequent publication from the same group, however, showed that rFVIIa is not able to restore thrombin generation in a haemophilia model but only restores clotting time in a whole blood model[184]. In the same paper it was concluded that the efficacy of rFVIIa in haemophilia blood is dependent of TF as in the absence of TF the concentration of rFVIIa to reach similar thrombin generation than in its presence was around 10^4 times higher.

Tissue factor-independent enhancement of coagulation

In the early phase of development of rFVIIa it was observed that rFVIIa not only has an effect on the prothrombin time, but that the activated partial thromboplastin time (APTT) was also shortened by rFVIIa. This phenomenon could be explained by a tissue factor-independent activation of factor X by rFVIIa[185]. Indeed, also in purified systems it had already been shown that FVIIa was able to activate factor X in the presence of calcium and phospholipids, albeit at a much slower rate than in the presence of TF[186]. It was hypothesized that a TF-independent mechanism of action of rFVIIa could be physiologically relevant, as a TF-dependent mechanism could not explain relatively high plasma concentrations of rFVIIa required for haemostatic efficacy. As the Kd for VIIa binding to TF is around 0.5 nM[187], and the concentrations of rFVIIa required for effective haemostasis are at least an order of magnitude higher, it seemed unlikely that TF-rFVIIa interaction plays an important role in inducing haemostasis.

The TF-independent mechanism of rFVIIa was further formulated using a cell-based in vitro model. It was shown that rFVIIa could activate FX on the surface of freshly isolated monocytes independently of tissue factor[188]. An endothelial cell line was not able to support TF-independent FX activation and this finding provided an explanation why rFVIIa treatment is not complicated by systemic induction of coagulation. Thus, rFVIIa is able to activate FX independently of TF, provided that a suitable cellular surface is provided. Whether the surface requirements only depend on phospholipid composition, of if cellular receptors for rFVIIa are also involved remains unclear.

When it was shown that rFVIIa is also able to bind directly to the surface of activated platelets (with a Kd of around 90 nM) and activate FX independently of TF[189], a refined model for the TF-independent mechanism of action of rFVIIa was formulated. In the cell-based model of coagulation as proposed by Hoffman, Monroe, and Roberts[49], coagulation is initiated on a tissue factor-bearing cell (e.g., a fibroblast or smooth muscle cell). Binding of FVIIa to TF results in the generation of a small amount of FXa and subsequently a small amount of thrombin is generated. This small amount of thrombin activates platelets, and cofactors Va and VIIIa, but is not sufficient to generate fibrin. The FXa generated on the TF bearing cell is rapidly inhibited by TFPI or antithrombin if it leaves the surface. On the other hand, the factor IXa generated by the TF bearing cell is relatively protected from inhibitors when it leaves the surface. This factor IXa can bind to the activated platelets, and activate factor X, which

subsequently combines with factor Va, leading to large-scale thrombin generation. According to this model, haemophilia is a defect in platelet-mediated thrombin generation. The efficacy of rFVIIa can according to the cell-based model be explained by TF-independent activation of FX on the platelet surface facilitated by direct binding of rFVIIa to activated platelets. The same concept is postulated for the efficacy of rFVIIa in the various other indications[190]. Theoretically, for all indications, TF-independent enhancement of thrombin generation on activated platelets by rFVIIa could compensate for the haemostatic defect.

Enhancement of clot stability

Novel hypotheses on the mechanism of action of rFVIIa are not focussed on enhancement of thrombin generation, but on the consequences of the enhancement of thrombin generation. As obviously a damaged vessel wall is not closed by thrombin, but by a haemostatic plug consisting of platelets and fibrin, it makes sense to investigate the composition, structure, and stability of haemostatic plugs formed in absence or presence of rFVIIa.

It has been demonstrated that the rate of thrombin generation determines fibrin structure and stability[191]. In the presence of little thrombin the fibrin consists of thick fibres which are easily dissolved by the fibrinolytic system, whereas thin, stable fibres are formed in the presence of a higher concentration of thrombin[192]. Recently, He et al. demonstrated that rFVIIa could normalize fibrin permeability in haemophilia plasma, directly indicating that rFVIIa affects fibrin structure in this system[193]. The decrease in fibrin porosity on rFVIIa addition might indicate enhancement of clot stability.

The enhancement of thrombin generation might enhance the stability of the haemostatic plug by enhancing platelet activation and aggregation, factor XIII activation and TAFI activation. Enhancement of platelet activation and aggregation might improve the mechanical stability of the clot. Also, enhanced platelet activation might lead to an increase in exposure of procoagulant surface, which might lead to a further enhancement of thrombin generation. Enhanced fibrin crosslinking and enhanced TAFI activity could result in a further protection of the haemostatic plug against breakdown by the fibrinolytic system. Furthermore, enhanced platelet activation might lead to enhanced secretion of PAI-1 and α_2-antiplasmin from platelet α-granules, which also contributes to down-regulation of fibrinolysis.

Whether the above mentioned processes are indeed involved in rFVIIa-mediated enhancement of clot stability in vivo remains to be investigated.

6. Aim of this thesis

In this thesis, novel hypotheses on the mechanism of action of rFVIIa in different haemostatic disorders will be described. In the major part of this thesis, the focus will be on the involvement of TAFI-mediated down-regulation of fibrinolysis in in vitro systems representing various haemostatic disorders, and the effect of rFVIIa. In chapter 2, the effect of rFVIIa on coagulation and fibrinolysis in plasma from patients with severe haemophilia A has been investigated. In an appendix to chapter 2, similar experiments were performed using mutants of rFVIIa with enhanced intrinsic (i.e., TF-independent) activity. In chapters 3,4, and 5 the haemostatic disorders induced by liver failure, the involvement of TAFI in the coagulopathy of liver failure, and the effects of rFVIIa on TAFI-mediated down-regulation of fibrinolysis in patients with cirrhosis and during liver transplantation are described. Chapter 6 deals with the effects of antithrombotic drugs targeting different steps in the coagulation cascade on TAFI-mediated down-regulation of fibrinolysis, and in chapter 7 the reversal of anticoagulant and profibrinolytic effects of the novel heparin-derivative fondaparinux by rFVIIa is described. In chapter 8, the focus is on the effects of rFVIIa on platelet adhesion under flow conditions. More specifically, a model for the mechanism of action of rFVIIa in patients with Glanzmann's thrombasthenia, and patients treated with anti-$\alpha_{IIb}\beta_3$ drugs is proposed based on results of in vitro perfusion studies. In chapter 9, the results presented in the previous chapters are discussed in a broader context. The advances in the understanding of the mechanism of action of rFVIIa in its different applications are discussed.

Chapter 2

Inhibition of fibrinolysis by recombinant factor VIIa in plasma from patients with severe haemophilia A

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Blood 2002; 99; 175-179.

Abstract

Recombinant factor VIIa (rFVIIa) is a novel prohaemostatic drug for patients with haemophilia who have developed inhibitory antibodies. The postulation has been made that haemophilia is not only a disorder of coagulation, but that hyperfibrinolysis due to a defective activation of thrombin activatable fibrinolysis inhibitor (TAFI) might also play a role. In this in vitro study, the potential of rFVIIa to downregulate fibrinolysis via activation of TAFI was investigated. rFVIIa was able to prolong clot lysis time in plasmas from 17 patients with severe haemophilia A. The prolongation of clot lysis time by rFVIIa was completely abolished by addition of an inhibitor of activated TAFI. The concentration of rFVIIa required for half maximal prolongation of clot lysis time ($C_{lys½}$-VIIa) varied widely between patients (median 73.0 U/ml; range 10.8-250). The concentration of rFVIIa required for half maximal reduction of clotting time ($C_{clot½}$-VIIa) was approximately 10-fold lower than the $C_{lys½}$-VIIa value (median 8.4 U/ml; range 1.7-22.5). Inhibition of TFPI with a polyclonal antibody significantly decreased $C_{lys½}$-VIIa values (median 2.6 U/ml; range 0-86.9), whereas $C_{clot½}$-VIIa values did not change (median 7.2 U/ml; range 2.2-22.5). On addition of 100 ng/ml recombinant full length TFPI, a nonsignificant increase of $C_{lys½}$-VIIa values was observed (median 119.2 U/ml; range 12.3-375.0), whereas $C_{clot½}$-VIIa values did not change (median 8.8 U/ml; range 2.6-34.6). In conclusion, this study shows that rFVIIa both accelerates clot formation and inhibits fibrinolysis by activation of TAFI in factor VIII-deficient plasma. However, a large variability in antifibrinolytic potential of rFVIIa exists between patients.

Introduction

Treatment of patients suffering from haemophilia is often complicated by the development of inhibitory antibodies. In approximately 25-30% of patients with haemophilia A[92] and in approximately 1-3% of the patients with haemophilia B[93], inhibitors develop during their lifetime. A novel way to treat haemophilia patients with inhibitors is the administration of recombinant factor VIIa (rFVIIa, NovoSeven®)[195]. rFVIIa has been shown to be a safe and effective prohaemostatic drug during bleeding episodes or surgery[148,196]. Advantages over traditional treatment (i.e., factor concentrates) are the lack of antigenicity and viral safety of rFVIIa[143,197].

rFVIIa exerts its prohaemostatic effect via enhancement of the extrinsic coagulation pathway in a tissue factor-dependent manner[182]. On binding of factor VIIa to tissue

factor, factor VIIa is able to activate both factors IX and X, thereby leading to a primary generation of thrombin. At high concentrations of tissue factor, factor X is the preferred substrate for the tissue factor-VIIa complex, whereas at low tissue factor concentrations factor IX is preferably activated. (for a review see [21]). The ability of high-dose rFVIIa to overcome the inhibitory effect of plasma factor VII on coagulation might play a role in the enhancement of thrombin generation by rFVIIa[183]. After formation of the fibrin clot, thrombin generation proceeds via the intrinsic pathway through activation of factor XI by thrombin[198]. The secondary burst of thrombin, which is formed via this factor XI feedback loop, results in the activation of TAFI (thrombin activatable fibrinolysis inhibitor)[65]. Activated TAFI down-regulates fibrinolysis by cleaving C-terminal lysine and arginine residues from partially degraded fibrin, thereby attenuating t-PA mediated fibrinolysis[199]. Activation of TAFI is stimulated approximately 1200-fold on thrombin binding to the cell surface receptor thrombomodulin[66].

The discovery of TAFI as an important link between coagulation and fibrinolysis stimulated the development of new concepts on the regulation of the haemostatic system. A clotting factor deficiency such as haemophilia A might not only result in defective clot formation, but also in accelerated clot degradation due to diminished TAFI activation. Indeed, it has been shown that factor VIII-deficient plasma shows premature lysis of tissue factor-induced clots due to a lack of TAFI activation, which could be restored by addition of factor VIII[200,201]. Clinical evidence that supports the hypothesis of haemophilia A being a defect in both coagulation and fibrinolysis has been obtained from skin biopsy studies in patients with severe haemophilia A in which abnormalities in a skin wound could not be observed until 2 hours after the wound was made[202]. Although these abnormalities were initially explained by the decreased fibrin forming capacity of these patients, an increased fibrinolytic potential could also explain these observations. Another indication of disturbed down-regulation of fibrinolysis in haemophilia is the efficacy of antifibrinolytic drugs like tranexamic acid and ε-aminocaproic acid in controlling bleedings in regions of the body with high fibrinolytic activity, such as the oral cavity[203,204].

Alternative mechanisms for the therapeutic effects of rFVIIa have been proposed. A potential mechanism, hypothesized in the literature[205], involves enhancement of activation of TAFI. Another possible alternative mechanism involves thrombin formation on activated platelets or monocytes independently of tissue factor[188,189].

In this study, the antifibrinolytic potential of rFVIIa in plasma from different patients with severe haemophilia A was explored. Also, the involvement of tissue factor pathway inhibitor on rFVIIa mediated clot protection was investigated.

Materials and Methods

Patients

Plasma samples from 17 patients with severe haemophilia A were used. Of these 17, eight patients had an inhibitor titre above 1 BU/ml. Patients had not received treatment with factor VIII containing products for at least 72 hours before blood sampling. Pooled normal plasma was obtained by combining plasma from 40 healthy volunteers. Blood samples were obtained by venipuncture from the antecubital vein into 3.2% sodium citrate (9:1, v/v). To obtain platelet poor plasma, the samples were centrifuged twice at 2000g for 15 minutes. Plasma samples were stored at –70°C until use.

Materials

Recombinant human tissue factor (Innovin) was from Dade Behring GmbH (Marburg, Germany). Carboxypeptidase inhibitor from potato (CPI) was purchased from Calbiochem (La Jolla, CA). Tissue type plasminogen activator (t-PA) was from Chromogenix (Mölndal, Sweden). Recombinant factor VIIa, recombinant full length tissue factor pathway inhibitor (rFL-TFPI), a polyclonal inhibitory antibody against tissue factor, and a polyclonal inhibitory antibody against human factor VIII were generous gifts from Drs U. Hedner and M. Kjalke (tissue factor/factor VII research, Novo Nordisk, Denmark). A polyclonal inhibitory antibody against TFPI (13 mg/ml) was a generous gift from Dr Walter Kisiel (University of New Mexico, Albuquerque, NM, USA) and was used in a dilution of 1:100. Purified human factor VIII (Monoclate-P) was from Armour Pharmaceuticals (Collegeville, PA, USA).

TAFI antigen levels were determined by a sandwich-type ELISA, using a monoclonal capturing antibody and a polyclonal detection antibody as described[206]. TAFI levels were expressed as percentage of pooled normal plasma. TFPI activity levels were determined according to Sandset[207]. Factor II, VII, and X activity levels were determined by a one stage clotting assay using factor II and factor X deficient plasma from Boeringer Mannheim GmbH (Mannheim, Germany), and factor VII deficient plasma from Helena Laboratories (Beaument, TX). Thromborel S from Dade Behring (Mannheim, Germany) was used as thromboplastin reagent. Fibrinogen levels were determined according to Clauss[208], using thrombin obtained from Sigma (St. Louis, MO). Levels of plasminogen were determined using the Coamatic plasminogen kit from Chromogenix (Mölndal, Sweden). Soluble thrombomodulin levels were determined using the asserachrom Thrombomodulin ELISA kit from Diagnostica Stago (Asnières, France).

Phospholipid vesicles consisting of 40% L-α-dioleoylphosphatidylcholine, 20% L-α-dioleoylphosphatidylserine and 40% L-α-dioleoylphosphatidylethanolamine (all from Sigma, St. Louis, MO) were prepared according to Brunner[209] with minor modifications as described by van Wijnen[210]. Total phospholipid content of the vesicles was determined by phosphate analysis according to Rouser[211].

Clot lysis assay

Lysis of a tissue factor induced clot by exogenous t-PA was studied by monitoring changes in turbidity during clot formation and subsequent lysis essentially as described previously[212]. A mixture of tissue factor (diluted Innovin, final dilution 10^5 times), $CaCl_2$ (final concentration 17 mM), t-PA (final concentration 30 U/ml; 56 ng/ml) and phospholipid vesicles (final concentration 10 μM) was added to 75 μl of citrated plasma. The volume was adjusted to 150 μl with Hepes buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 3 mM $CaCl_2$, 0.1% BSA, pH 7.4), resulting in a final plasma concentration of 50%. After mixing thoroughly, 100 μl of this mixture was transferred to a microtiter plate and turbidity at 405 nm was measured in time at 37ºC in a Spectramax 340 kinetic microplate reader (Molecular Devices Corporation, Menlo Park, CA, USA). Clot lysis time was defined as the time from the midpoint of the clear-to-maximum-turbid transition, which is defined as clotting time, to the midpoint of the maximum-turbid-to-clear transition. To block possible residual factor VIII activity in haemophilic plasma which did not contain an inhibitor, 10 BU/ml of a polyclonal antibody against human factor VIII was added and preincubated at room temperature for 45 minutes. To assess the contribution of TAFI activation to clot lysis time, experiments were performed in which CPI (25 μg/ml), a specific inhibitor of activated TAFI[199], was added to the plasma. The effect of rFVIIa on fibrinolysis in haemophilic plasma was determined by performing clot lysis assays with factor VIII deficient plasma to which different concentrations of rFVIIa was added.

Statistical analysis

Statistical analysis was performed using the GraphPad InStat (San Diego, USA) software package. Statistical significance of the increase in mean clot lysis time on addition of rFVIIa was determined by a repeated measures ANOVA followed by Dunnett's post test. Statistical differences between clotting times in the presence or absence or an inhibitory antibody against tissue factor was determined by standard t-test. Correlations were calculated using the Pearson correlation coefficient. Statistical significance between differences in $C_{lys½}$-VIIa or $C_{clot½}$-VIIa on manipulation of TFPI was calculated by a repeated measures ANOVA followed by the Tukey post test. P values <0.05 were considered statistically significant.

Results

rFVIIa mediates down-regulation of fibrinolysis and acceleration of clot formation in factor VIII-deficient plasma

To investigate whether rFVIIa restores down-regulation of fibrinolysis in factor VIII-

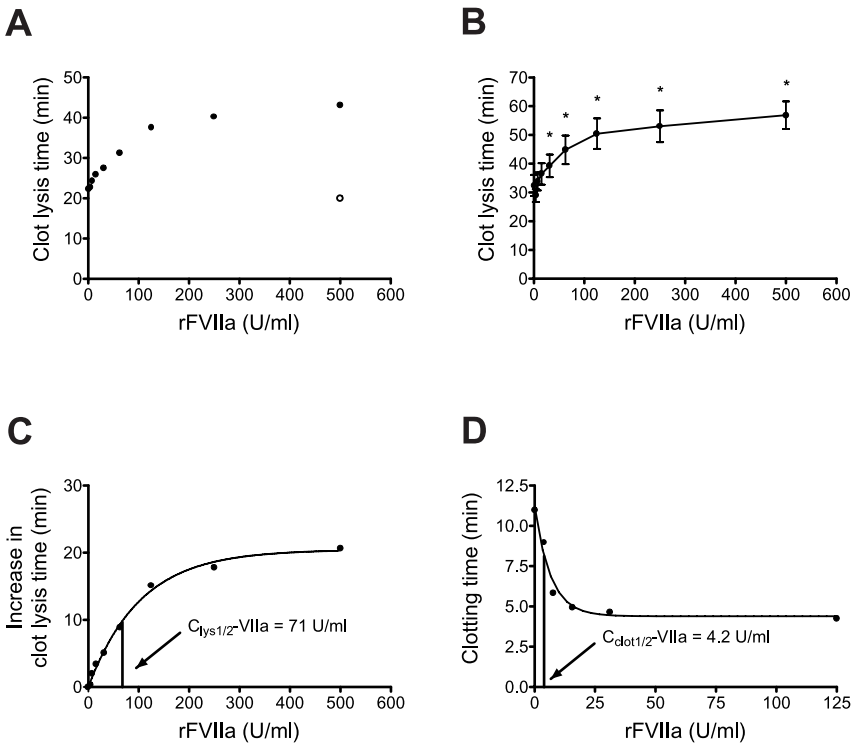

Figure 1. Effect of rFVIIa on clot formation and clot lysis time in plasma from a patient with severe haemophilia A. Panel A shows the increase in clot lysis time upon addition of increasing concentrations of rFVIIa (closed symbols). The increase in clot lysis time upon addition of 500 U/ml of rFVIIa could be completely abolised by addition of CPI (open symbol). Panel B shows mean clot lysis times of 17 patients with severe haemophilia A upon addition of increasing concentrations of rFVIIa. At concentrations of rFVIIa of 31.3 U/ml and higher, the increase in clot lysis time was statistically significant. Error bars indicate standard error of mean. * $p<0.01$ vs clot lysis time in the absence of rFVIIa. Panel C shows the data from panel A fitted by an exponential function. From this curve the concentration of rFVIIa required for half maximal prolongation of clot lysis time ($C_{lys½}$-VIIa) was calculated. Panel D shows the decrease in clotting time upon addition of rFVIIa. These data were fitted by an exponential function and from this curve the concentration of rFVIIa required for half maximal reduction of clotting time ($C_{clot½}$-VIIa) was calculated.

deficient plasma, clot lysis assays were performed using plasma from 17 patients with severe haemophilia A in the presence of different concentrations of rFVIIa. A polyclonal inhibitory antibody to human factor VIII was added to the plasma samples in which no inhibitor was present, because a previous study showed that as little as 0.01% of factor VIII present in pooled normal plasma is sufficient to completely restore down-regulation of fibrinolysis in factor VIII-deficient plasma[201]. A typical example of the effect of rFVIIa on clot lysis time in haemophilic plasma is shown in figure 1A. rFVIIa was able to prolong clot lysis time in all patient samples. The rFVIIa-mediated prolongation of clot lysis time could be completely abolished by the addition of a specific inhibitor of activated TAFI (CPI). Figure 1B shows mean clot lysis times for the 17 patient samples at increasing concentrations of rFVIIa. At rFVIIa concentrations of 31.3 U/ml and higher the increase in clot lysis time was statistically significant ($p<0.01$). From the rFVIIa titration curves the concentrations of rFVIIa required for half maximal prolongation of clot lysis time ($C_{lys½}$-VIIa) were determined as shown in figure 1C.
A wide variation of $C_{lys½}$-VIIa values was observed as shown in figure 2A (median 73.0 U/ml; range 10.7-250.0). $C_{lys½}$-VIIa values were similar in plasma samples from patients without (median 73.0 U/ml; range 17.4-185.9) and with (median 81.8 U/ml; range 10.7-250.0) inhibitory antibodies. To examine whether the antifibrinolytic potential of rFVIIa remained constant over time in a single patient, $C_{lys½}$-VIIa values of two unrelated patients were determined in 4 plasma samples collected at

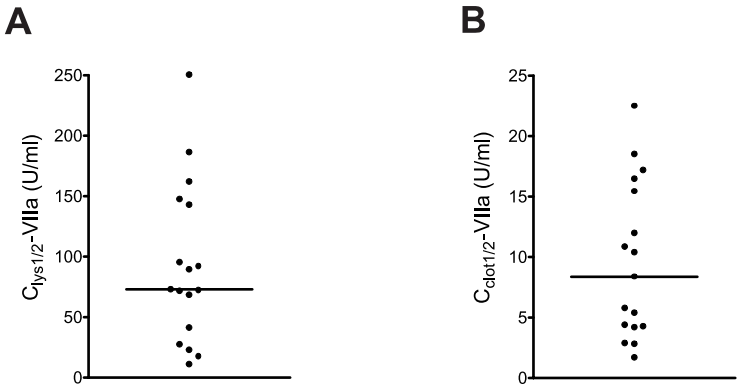

Figure 2. Variability in antifibrinolytic and procoagulant effect of rFVIIa in 17 plasma samples from patients with severe haemophilia A. Panel A: $C_{lys½}$-VIIa values were determined in plasma samples from 17 patients with severe haemophilia A following the example in figure 1B. Panel B: $C_{clot½}$-VIIa values were determined in plasma samples from 17 patients with severe haemophilia A following the example in figure 1C. The horizontal line indicates medians.

intervals of approximately 3 months. These experiments showed that $C_{lys½}$-VIIa values remained constant in a period of 1 year (patient 1: range 238-267 U/ml, patient 2: range 91-150 U/ml).
To investigate the effect of tissue factor concentration on $C_{lys½}$-VIIa values, clot lysis assays were performed using plasma of a single patient in which coagulation was initiated by increasing amounts of tissue factor. $C_{lys½}$-VIIa was 85 U/ml at the tissue factor dilution used throughout this study. When the tissue factor concentration was doubled or quintupled, $C_{lys½}$-VIIa values decreased to 26 and 11 U/ml, respectively.

Correlation of $C_{lys½}$-VIIa values with levels of several plasma proteins
To study possible parameters that influence $C_{lys½}$-VIIa values, levels of several plasma proteins were measured and the correlation with $C_{lys½}$-VIIa values was determined. $C_{lys½}$-VIIa values were not correlated with TFPI activity (r=0.190, p=0.468), TAFI antigen (r=0.089, p=0.729), soluble thrombomodulin antigen (r=0.032, p=0.895), fibrinogen (r=0.054, p=0.861), factor II activity (r=0.071, p=0.785), factor VII activity (r=0.286, p=0.265), factor X activity (r=0.302, p=0.238), and plasminogen activity (r=0.032, p=0.902) levels.
To exclude the possibility that differences in trace amounts of factor VIII activity left in the plasma samples account for the large variation in $C_{lys½}$-VIIa values, an experiment was performed using a plasma sample from a patient with severe haemophilia A due to an intron 22 gene inversion, who did not receive any treatment for over a year. The plasma in this patient was considered to completely lack factor VIII activity. $C_{lys½}$-VIIa values were determined in this plasma sample in the presence of the polyclonal inhibitory antibody in the absence or presence of 1 mU/ml factor VIII, which represents 0.1% of the factor VIII found in pooled normal plasma. $C_{lys½}$-VIIa did not change upon addition of factor VIII, indicating sufficient inhibition of 0.1% factor VIII by the antibody (data not shown).

Improvement of clot formation requires around 10-fold less rFVIIa than improvement of fibrinolysis
In all patients, a significant reduction in clotting time on addition of rFVIIa was observed. A typical example of the effect of rFVIIa on clotting time is shown in figure 1D. $C_{clot½}$-VIIa values were defined as the concentration of rFVIIa required to half-maximally reduce clotting time. $C_{clot½}$-VIIa values were approximately 10-fold lower than $C_{lys½}$-VIIa values as shown in figure 2B (median 8.4 U/ml; range 1.7-22.5). $C_{clot½}$-

VIIa values were not correlated with $C_{lys½}$-VIIa values (r=0.187, p=0.470). $C_{clot½}$-VIIa for pooled normal plasma was 9.9 U/ml.

$C_{clot½}$-VIIa values correlate with endogenous factor VII levels

$C_{clot½}$-VIIa values were positively correlated with endogenous factor VII levels as shown in figure 3 (r=0.653, p<0.005). $C_{clot½}$-VIIa values were not correlated with plasma levels of factor X (r=0.311, p=0.223), factor II (r=0.164, p=0.530), and fibrinogen (r=0.206, p=0.499).

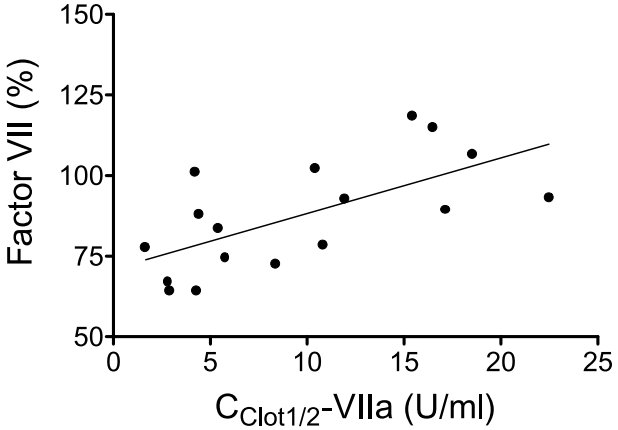

Figure 3. Correlation between $C_{clot½}$-VIIa levels and endogenous factor VII levels.

Tissue factor dependency of clot formation in haemophilic plasma

To investigate whether clot formation in factor VIII-deficient plasma was completely dependent on tissue factor, clot lysis assays were performed using plasma samples from 4 patients (all with inhibitory antibodies against factor VIII) in the presence of increasing concentrations of rFVIIa and in the presence or absence of an inhibitory antibody against tissue factor (500 μg/ml). As shown in figure 4, clot formation was significantly inhibited (clotting time >30 min) on inhibition of tissue factor. When rFVIIa was added to the plasma, clotting did occur during the time span of our experiment when tissue factor was inhibited. Inhibition of tissue factor attenuated clot formation at all rFVIIa concentrations tested. However, at 125 and 250 U/ml rFVIIa the increase in clotting time upon inhibition of tissue factor was no longer statistically significant.

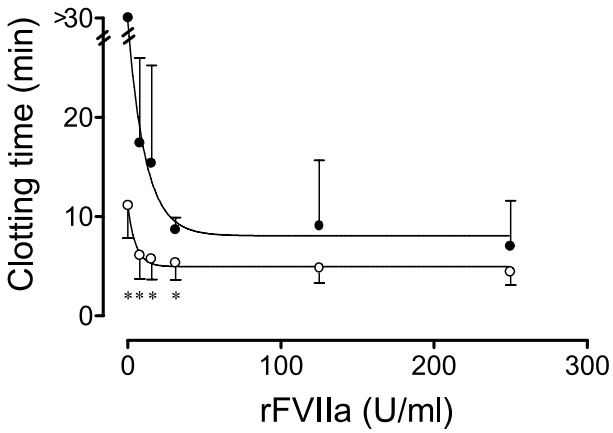

Figure 4. Effect of the inhibition of tissue factor on the clotting time in plasma from four patients with severe haemophilia A at different rFVIIa concentrations. Clotting times were determined using the same conditions as used for the clot lysis assay in the absence (open circles) or presence (closed circles) of a polyclonal inhibitory antibody against tissue factor. Error bars indicate standard deviation. * indicates $p<0.05$.

Effects of modulation of TFPI on $C_{lys½}$-VIIa and $C_{clot½}$-VIIa values

The effect of TFPI on $C_{lys½}$-VIIa and $C_{clot½}$-VIIa values was investigated in all patient samples. As shown in figure 5A, addition of rFL-TFPI resulted in an increase in $C_{lys½}$-VIIa values (median 119.2 U/ml; range 12.3-375 U/ml), but this did not reach statistical significance. Inhibition of TFPI by an inhibitory antibody resulted in a significant decrease of $C_{lys½}$-VIIa values (median 2.6 U/ml; range 0-86.9, $p<0.05$).

As shown in figure 5B, addition of rFL-TFPI did not change $C_{clot½}$-VIIa values (median 7.2 U/ml; range 2.2-20.5). Also addition of a blocking antibody against TFPI did not change $C_{clot½}$-VIIa values (median 8.8 U/ml; range 2.6-34.6). However, clotting times in the absence of rFVIIa were decreased by addition of a blocking antibody against TFPI, whereas clotting times in the absence of rFVIIa increased by addition of rFL-TFPI (data not shown).

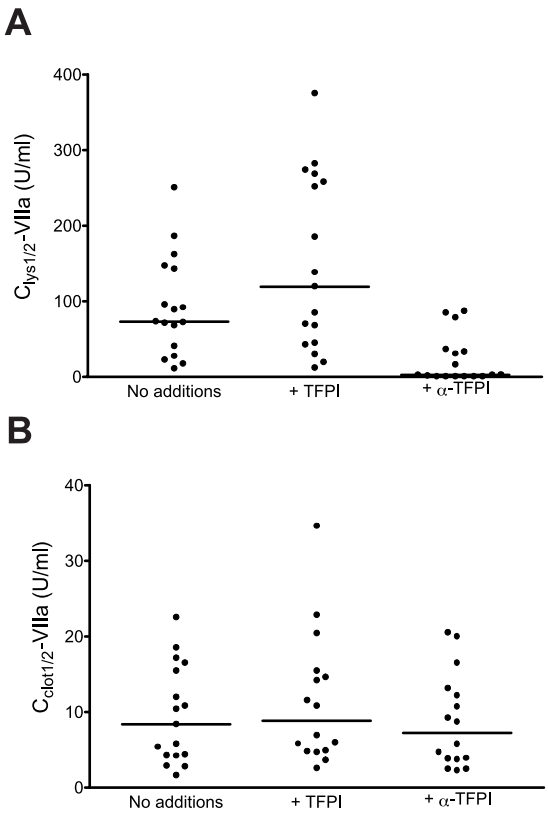

Figure 5. Effect of TFPI on antifibrinolytic and procoagulant potential of rFVIIa. Panel A shows $C_{lys½}$-VIIa values of the 17 plasma samples as shown in figure 2A (No additions), and $C_{lys½}$-VIIa values of the same samples in the presence of 100 ng/ml rFL-TFPI (+ TFPI), or an inhibitory antibody against TFPI (+ α-TFPI). Panel B shows $C_{clot½}$-VIIa values of the 17 plasma samples as shown in figure 2B (No additions), and $C_{clot½}$-VIIa values of the same samples in the presence of 100 ng/ml rFL-TFPI (+ TFPI), or an inhibitory antibody against TFPI (+ α-TFPI).

Discussion

This in vitro study shows that rFVIIa both accelerates clot formation and inhibits fibrinolysis by activation of TAFI in factor VIII-deficient plasma. In agreement with previous studies we have shown a lack of TAFI activation in plasma from patients with severe haemophilia A at low tissue factor concentrations, implicating that haemophilia is a disorder of both clot formation and down-regulation of clot

breakdown. In a previous study we have shown that as little as 0.01% of the amount of factor VIII present in pooled normal plasma completely restores TAFI-mediated down-regulation of fibrinolysis in factor VIII-deficient plasma[201]. Here, we showed that high rFVIIa levels are required for inhibition of fibrinolysis. Values required for half maximal effect range from therapeutical (10.7 U/ml) to supratherapeutical (250 U/ml) levels.

We have not been able to determine specific factors, which determine $C_{lys½}$-VIIa values. We speculate that the antifibrinolytic potential of rFVIIa is determined by a combination of both thrombin-generating capacity and plasma fibrinolytic potential.

We have shown that the amount of rFVIIa required to restore fibrinolysis is dependent on the concentration of tissue factor. The efficacy of a certain dose of rFVIIa in a patient is probably highly dependent on the site of injury and the extent of vascular damage. It is likely that also in vivo rFVIIa is more effective in downregulation of the fibrinolytic system at wounds where large amounts of tissue factor are exposed. Another important factor in the efficacy of rFVIIa is the amount of thrombomodulin present. Thrombomodulin is capable of enhancing TAFI activation by approximately 3 orders of magnitude[66]. However, a high concentration of thrombomodulin down-regulates TAFI activation by protein C activation[67]. Thus, it is likely that both tissue factor and thrombomodulin present at the site of injury determine the antifibrinolytic potential of rFVIIa in a patient with haemophilia A.

The regulatory effect of TFPI in rFVIIa-mediated inhibition of fibrinolysis becomes evident on evaluation of $C_{lys½}$-VIIa values, which are determined in the presence of a blocking antibody against TFPI. In the absence of the inhibitory activity of TFPI, little rFVIIa is needed to restore fibrinolysis, and in some patients fibrinolysis was already maximally inhibited in the absence of rFVIIa. The addition of rFL-TFPI did not lead to a significant inhibition of the antifibrinolytic effect of rFVIIa, probably because TFPI concentrations present in plasma were nearly saturating at the low tissue factor concentrations used in our assay.

The effect of rFVIIa on clot formation was more pronounced than the effect on TAFI activation; the levels of rFVIIa required to half maximally reduce clot formation were approximately 10-fold lower than those required to half maximally inhibit fibrinolysis. Moreover $C_{lys½}$-VIIa values were not correlated with $C_{clot½}$-VIIa values. This indicates that primary and secondary thrombin formation in factor VIII-deficient plasma are differently regulated processes. This phenomenon is supported by the observation that modulation of TFPI activity clearly effects $C_{lys½}$-VIIa values, whereas $C_{clot½}$-VIIa values are unchanged. The inhibitory activity of TFPI on clot formation is overruled by rFVIIa. This might be due to kinetic limitations combined with the rate-limiting concentration of

factor Xa. The thrombin generation that occurs after formation of the fibrin clot is efficiently inhibited by TFPI even in the presence of rFVIIa, as inhibition of TFPI markedly reduces $C_{lys½}$-VIIa values.

The hypothesis that primary and secondary thrombin formation in our system are differently regulated processes is further supported by the observation that endogenous factor VII levels positively correlate with $C_{clot½}$-VIIa but not with $C_{lys½}$-VIIa values. The correlation of endogenous factor VII levels with $C_{clot½}$-VIIa corresponds to the inhibitory activity of zymogen factor VII on tissue factor-induced coagulation as described by van 't Veer et al.[183] Apparently this inhibitory activity is not important for secondary thrombin generation.

In conclusion, the efficacy of rFVIIa infusion in patients with haemophilia who developed inhibitory antibodies may be explained by a combination of the following factors: 1) enhancement of clot formation via the tissue factor pathway, possibly by overruling the inhibitory activity of zymogen factor VII, and 2) down-regulation of fibrinolysis by TAFI activation, which contributes to the stability of the clot. However, until now we have no evidence that the antifibrinolytic effect of rFVIIa also occurs in the in vivo situation. More sophisticated techniques for TAFI activation in vivo will be required to adress this question. Whether the in vitro variations in antifibrinolytic potential of rFVIIa may have clinical relevance (e.g., in predicting clinical efficacy of rFVIIa in patients with severe haemophilia) will be investigated in future studies.

Acknowledgements

The authors like to thank Drs. U. Hedner and M. Kjalke for their generous gift of rFVIIa, rFL-TFPI and the antibodies against factor VIII and tissue factor, and Dr. W. Kisiel for his generous gift of the antibody against TFPI.

Appendix to chapter 2

Enhanced procoagulant and antifibrinolytic potential of superactive variants of recombinant factor VIIa in plasma from patients with severe haemophilia A

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Recombinant factor VIIa (rFVIIa, NovoSeven®, Novo Nordisk, Bagsværd, Denmark) has recently become available for the treatment of patients with inhibitor-complicated haemophilia[195]. rFVIIa has been shown to be safe and effective in the treatment of bleeding episodes, and prophylactically during surgery[148,196].

The mechanism of action of rFVIIa in patients with haemophilia is still a matter of debate. It has been proposed that tissue factor (TF)-dependent enhancement of coagulation is responsible for the therapeutic effect of rFVIIa in haemophilia[182]. This was supported by the in vitro observation that endogenous zymogen factor VII is a physiological inhibitor of coagulation, and that rFVIIa overcomes the inhibitory effect of zymogen VII by competing for TF binding[183]. Alternatively, it has been proposed that the therapeutic efficacy of rFVIIa could be explained by a mechanism involving tissue factor-independent thrombin generation triggered by rFVIIa directly bound to activated platelets or monocytes[188,189].

If this latter mechanism of action would have physiological relevance, genetically modified analogues of rFVIIa with enhanced intrinsic (i.e., TF independent) activity, might be beneficial in treating haemophilia patients. Recently, several mutant rFVIIa molecules with substantially enhanced intrinsic activity have been described[213,214]. In the presence of TF, these mutants displayed similar proteolytic activity compared to wild type rFVIIa.

It has been hypothesized that haemophilia is not only a disorder of coagulation, but that accelerated fibrinolysis due to a lack of activation of thrombin activatable fibrinolysis inhibitor (TAFI) also contributes to the bleeding diathesis of these patients[200,201]. In a previous publication, we have shown that rFVIIa does not only have procoagulant but also antifibrinolytic properties in plasma from patients with severe haemophilia A[215]. These antifibrinolytic properties were attributable to enhancement of activation of thrombin activatable fibrinolysis inhibitor (TAFI) as a consequence of enhanced thrombin generation. However, in our assay system, the procoagulant potential of rFVIIa was much more pronounced than its antifibrinolytic activity.

We have compared the procoagulant and antifibrinolytic potential of two analogues of rFVIIa with substantially increased TF-independent activity with those of wild-type rFVIIa in plasma from patients with severe haemophilia A using a plasma-based clot lysis assay as described previously[215]. We investigated M298Q-rFVIIa, which had a 7-fold increased intrinsic proteolytic activity compared to wild type, and K337A/V158D/E296V/M298Q-rFVIIa (K337A-rFVIIa$_{IIa}$), which had a 56-fold increased proteolytic activity compared to wild type[213]. In short, in a 96-well microtitre plate, plasma was allowed to clot by the addition of tissue factor (Innovin, Dade Behring GmbH,

Marburg, Germany, 1:100.000 times diluted), calcium chloride (17 mM), and phospholipid vesicles (10 μM), fibrinolysis was induced by addition of tPA (Chromogenix, Mölndal, Sweden, 30 U/ml). Coagulation and clot lysis were monitored by continuous turbidity measurements at 405 nm, and clotting times and clot lysis times were determined as described[215]. Titration curves of wild type and mutant rFVIIa were made in plasma from 6 different patients with severe haemophilia A, who had an inhibitor titre >1 BU/ml at the time of sampling. From these titration curves, rFVIIa concentrations required for half maximal reduction of coagulation time ($C_{clot½}$-VIIa) and rFVIIa concentrations required for half maximal increase in clot lysis time ($C_{lys½}$-VIIa) were calculated.

As shown in figure 1, the rFVIIa analogues had a significantly larger procoagulant potential compared to wild-type rFVIIa ($C_{clot½}$-VIIa: median [range], wt-rFVIIa: 363 [130-604] ng/ml, M298Q-rFVIIa: 70 [34-190] ng/ml, K337A-rFVIIa$_{IIa}$: 17 [10-31] ng/ml, $p<0.01$ wt vs mutants, paired ANOVA with Dunnett's post test). Also, the amount of rFVIIa analogues required for downregulation of fibrinolysis was substantially decreased compared to wild type ($C_{lys½}$-VIIa: median [range], wt-rFVIIa: 5288 [1000-10120] ng/ml, M298Q-rFVIIa: 641 [220-1367] ng/ml, K337A-rFVIIa$_{IIa}$: 101 [49-379] ng/ml, $p<0.01$ wt vs mutants, paired ANOVA with Dunnett's post test).

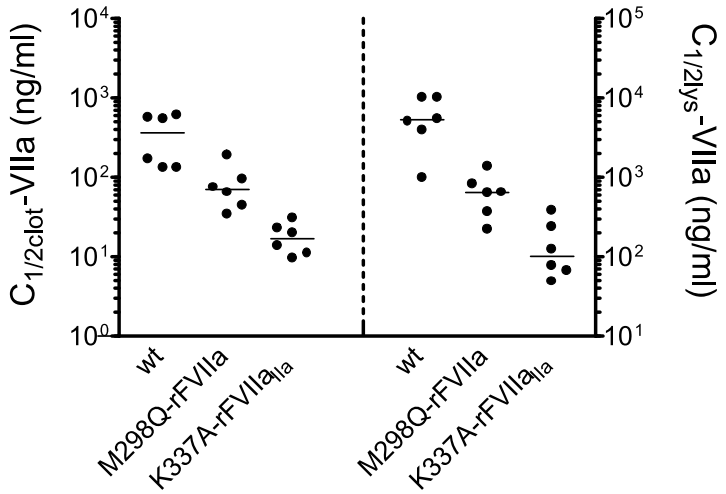

Figure 1. Enhanced procoagulant and antifibrinolytic potential of rFVIIa analogues compared to wild-type in factor VIII deficient plasma. In plasma from 6 patients with severe haemophilia A, the rFVIIa concentrations required for half maximal reduction of clotting time ($C_{clot½}$-VIIa) and the rFVIIa concentrations required for half maximal prolongation of clot lysis time ($C_{lys½}$-VIIa) were determined. Horizontal bars indicate medians.

Next, the TF dependency of clot formation was investigated in plasma from a single patient with severe haemophilia A (George King Bio-Medical, Inc., Overland Park, KS). Titration curves of wild type and mutant rFVIIa were made in absence or presence of an inhibitory antibody against TF as described previously (figure 2)[215]. In the absence of TF activity, no clot formation was observed during the time-course of the experiment. Clotting did occur in the absence of TF, when as little as 20 ng/ml wild type or mutant rFVIIa was added to the plasma. At lower concentrations of rFVIIa, clotting was significantly slower when TF activity was blocked. However, at increasing concentrations of rFVIIa clotting times were no longer significantly affected by inhibition of TF. On inhibition of TF, the rFVIIa analogues displayed a similar increase in procoagulant potential compared to the situation in which TF activity was present.

With these experiments we show that the superactive rFVIIa variants do not only display a substantially enhanced procoagulant potential compared to wild type, but that the antifibrinolytic potential of the mutants is also significantly enhanced. This confirms our earlier observations that rFVIIa-mediated thrombin generation in our assay system is at least partially tissue factor-independent[215]. However, when lower concentrations of either wild type or mutant rFVIIa is used, the presence of TF is required for expression of full procoagulant potential.

In conclusion, should a TF-independent mechanism be involved in the efficacy of rFVIIa in patients with haemophilia, the herein described superactive VIIa analogues might offer improved clinical benefit as both procoagulant and antifibrinolytic potential are significantly enhanced compared to wild type.

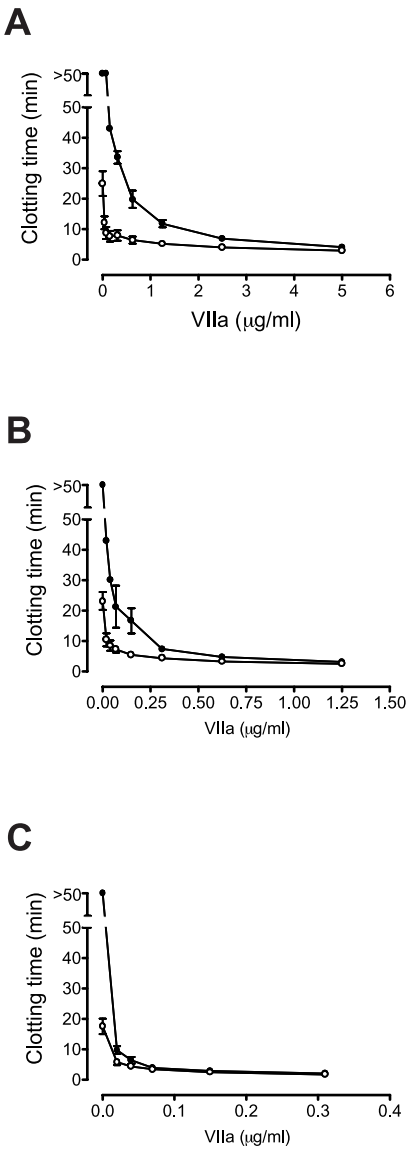

Figure 2. Procoagulant effect of wt-rFVIIa (panel A), M298Q-rFVIIa (panel B), and K337A-rFVIIa$_{IIa}$ (panel C) in presence (closed symbols) or absence (open symbols) of an inhibitory antibody against tissue factor. Wild type or mutant VIIa was added in different concentrations to plasma from a single patient with severe haemophilia A, and clotting times were determined. TF activity was inhibited by a polyclonal antibody (500 μg/ml, 45 minutes preincubation at room temperature). Shown are mean values of 3 independent experiments. Error bars indicate standard error of mean.

Chapter 3

Haemostatic abnormalities in patients with liver disease

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J. Hepatol. 2002; 37(2): 280-287.

1) Introduction

Haemostasis consists of a complicated sequence of events designed to prevent excessive blood loss in case of vessel wall injury. The haemostatic system is in a delicate balance between pro- and antihaemostatic processes. Alterations in the system may lead to either a bleeding diathesis or thrombotic disorder.
Liver failure is accompanied by multiple changes in the haemostatic system. As many proteins involved in haemostasis are synthesized by the liver, reduced plasma levels are found when the synthesis function of the liver becomes compromised. The diseased liver also has a reduced capacity to clear activated haemostatic proteins or protein-inhibitor complexes from the circulation. Finally, the number and function of platelets can be affected in patients with liver disease. In this review the haemostatic changes occurring in patients with chronic or acute hepatic failure will be discussed. Furthermore, the occurrence and treatment of bleeding complications in these patients will be reviewed.

2) Normal haemostasis – current insights

Primary haemostasis

Blood platelets are of crucial importance in preventing blood loss upon vessel wall damage. Platelets are produced from megakaryocytes in the bone marrow. Platelet production is regulated by thrombopoietin, a hormone synthesized by the liver. When subendothelial components are exposed to the bloodstream, platelets rapidly adhere and aggregate to form a primary haemostatic plug. The formation of a platelet plug starts when platelets from the circulation are slowed down by transient binding of platelet glycoprotein Ib to von Willebrand factor (vWF) bound to subendothelial collagen (figure 1A). Subsequently, a stationary contact between platelet and subendothelium is accomplished by binding of one or more platelet receptors to their ligands in the subendothelium (figure 1B). After adhesion, platelets become activated by a number of agonists including collagen and thrombin. Platelet activation is propagated by excretion of thromboxane A_2, which is synthesized and excreted by the platelet upon stimulation, and by ADP excreted from platelet α-granules (figure 1C). Platelet activation results in activation and clustering of the integrin $\alpha_{IIb}\beta_3$, which subsequently can bind fibrinogen and vWF. Bridging of fibrinogen and vWF between two platelets results in aggregation, which leads to the formation of a stable platelet plug (figure 1D).

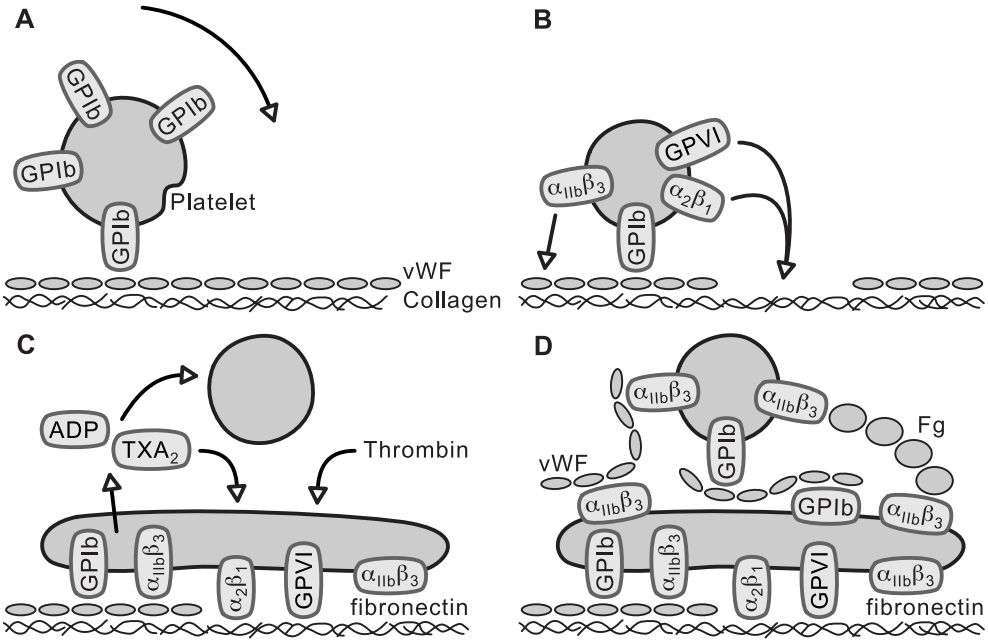

Figure 1. Schematic representation of platelet adhesion to subendothelium under flow conditions (see text for details). A) Rolling of platelets over collagen-bound vWF mediated by GPIb. B) Firm attachment mediated by $\alpha_2\beta_1$ and glycoprotein VI (GP VI) binding to collagen, and by $\alpha_{IIb}\beta_3$ binding to collagen-bound vWF. C) Platelet activation, secretion, and spreading. D) Aggregate formation.

Secondary Haemostasis

Blood coagulation, or the process of fibrin formation, consists of a sequence of enzymatic reactions, eventually leading to the generation of thrombin. Thrombin is not only involved in platelet activation, but can also cleave fibrinogen into fibrin. Fibrin spontaneously polymerizes and forms an insoluble mesh, which stabilizes the platelet plug.

Currently, it is believed that fibrin formation is initiated by the exposure of tissue factor to the bloodstream. Factor VII binds to tissue factor and is rapidly activated by plasma proteases. The physiological relevant activator of factor VII presumably is coagulation factor Xa. The tissue factor-VIIa complex activates factors IX and X, which then assemble with their cofactors VIIIa and Va onto an anionic phospholipid surface. The resulting tenase and prothrombinase complexes activate factor X and II (prothrombin), respectively. Prothrombin amplifies its own generation by activating

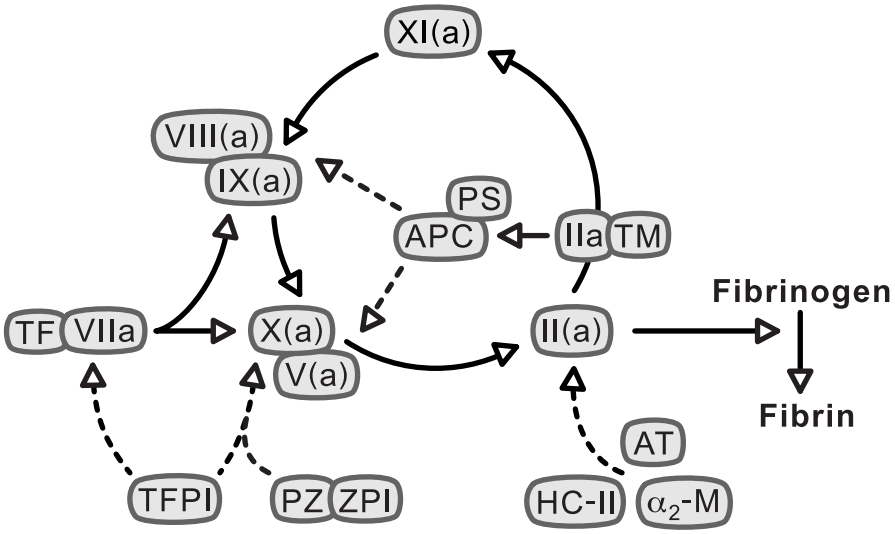

Figure 2. Schematic overview of tissue factor induced coagulation. (See text for details). Uninterrupted lines indicate activation, interrupted lines indicate inhibition.

cofactors V and VIII, and also by activating factor XI, which once activated can also activate factor IX (figure 2).

The regulation of thrombin formation proceeds through a number of plasma inhibitors of coagulation. The tissue factor pathway is regulated by the kunitz type inhibitor TFPI (tissue factor pathway inhibitor). TFPI binds activated factor X, and the TFPI-Xa complex subsequently binds to the TF-VIIa complex resulting in a fully inhibited quaternary TFPI-Xa-TF-VIIa complex. Antithrombin (AT) is a serine protease inhibitor present in plasma, which inactivates thrombin and factors IXa, Xa, and XIa. Its inhibitory action is potentiated by heparin or glycosaminoglycans present on the vessel wall. Other plasma thrombin inhibitors are heparin cofactor II (HCII) and α_2-macroglobulin (α_2-M). The inhibitory activity of HCII is also enhanced by heparin. HCII exclusively inhibits thrombin. α_2-M is a more general serine protease inhibitor, also able (among other proteins) to inhibit factor Xa and plasmin. Activated protein C, which is activated by thrombomodulin-bound thrombin, together with its cofactor protein S inhibits thrombin generation by inactivating coagulation cofactors Va and VIIIa. Protein C circulates in plasma in zymogen form, and is activated by thrombomodulin-bound thrombin. Finally, the protein Z-dependent protease inhibitor (ZPI) inactivates factor Xa with protein Z as mandatory cofactor.

Fibrinolysis

The dissolution of a fibrin clot is facilitated by plasmin, an enzyme formed from the plasma protein plasminogen. The physiological activator of plasminogen is presumably tissue type plasminogen activator (tPA). tPA circulates in plasma in low levels in complex with its plasma inhibitor plasminogen activator inhibitor-1 (PAI-1). tPA is synthesized and both constitutively excreted and stored by endothelial cells, and the tPA released locally from endothelial cells is thought to be responsible for plasmin activation in vivo. Both tPA and plasminogen need to bind to fibrin, in order to accelerate plasminogen activation.
Fibrinolysis is regulated by a number of plasma inhibitors. Firstly, plasminogen activation is regulated by PAI-1, a stoichiometric inhibitor of tPA, which is synthesized and constitutively extreted by endothelial cells. PAI-1 is also present in substantial amounts in platelet α-granules. Histidine rich glycoprotein (HRG) has also been implicated in regulation of plasmin activation, by its ability to bind to lysine residues on plasminogen, thereby preventing binding to fibrin and hence delaying its activation. The physiological relevance of HRG is unclear at present. The proteolytic activity of plasmin is regulated by the circulating inhibitor α_2-antiplasmin.
Fibrin clots are made more resistant to fibrinolysis by two mechanisms involving thrombin. Firstly, thrombin activates factor XIII, which stabilizes the fibrin clot by crosslinking gamma or alpha chains in adjacent fibrin molecules by introducing ε(γ-glutamyl)lysyl isopeptide bonds. Activated factor XIII also crosslinks α_2-antiplasmin to the fibrin clot, thereby contributing to the inhibition of fibrinolysis. Secondly, thrombin (or thrombomodulin bound thrombin) can activate thrombin activatable fibrinolysis inhibitor (TAFI). Activated TAFI cleaves C-terminal lysine and arginine residues from partially degraded fibrin, thereby preventing tPA and plasminogen binding (figure 3).

3) Defects in primary haemostasis in liver disease

Thrombocytopenia and platelet function defects

Mild to moderate thrombocytopenia (i.e., platelet counts between 50.000 and 150.000 per μl) can be seen in both chronic and acute liver failure[216,217]. In patients with cirrhosis, the major cause for thrombocytopenia is thought to be increased platelet sequestration in the spleen due to congestive splenomegaly[218]. Decreased production of thrombopoietin (tpo)

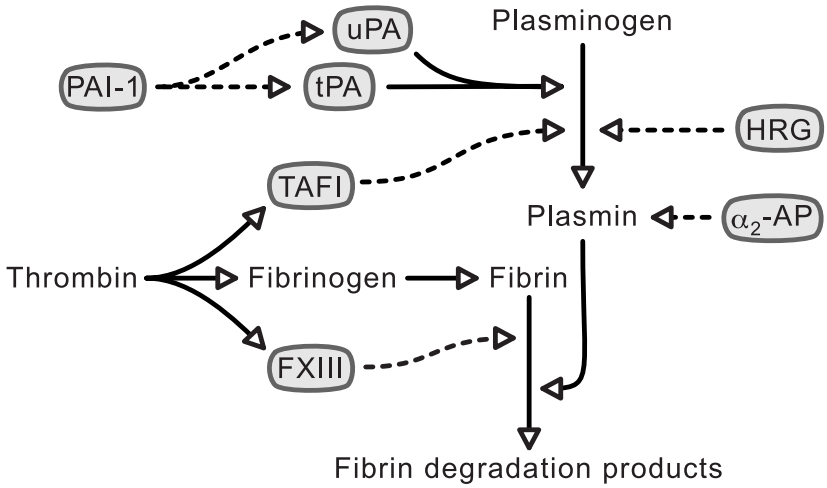

Figure 3. Schematic representation of the fibrinolytic system and its link with the coagulation system (see text for details). Uninterrupted lines indicate activation, interrupted lines indicate inhibition

by the diseased liver has been suggested to contribute to the low platelet count in patients with chronic or acute liver failure. However, both decreased[219,220], increased[221] and normal[222,223] plasma tpo levels in patients with chronic liver failure have been reported. A recent publication showed that the return to normal platelet count after liver transplantation was accompanied by an increase in plasma tpo levels, and that the peripheral platelet count increased irrespective of spleen size, indeed suggesting that decreased platelet production as a consequence of low levels of tpo might be an important cause of the low platelet count in end stage liver disease[224]. A reduced platelet half life has been shown in some patients[225], and autoimmune mechanisms may be involved[226]. In patients with alcohol-induced cirrhosis, the reduced platelet count may also be a consequence of folic acid deficiency[227] or decreased platelet production due to direct toxic effects of ethanol on megakaryocytopoiesis[228]. Another suggested cause of thrombocytopenia in liver disease is the presence of chronic (low-grade) disseminated intravascular coagulation (DIC)[229]. However, the presence of DIC in patients with hepatic failure is still a matter of controversy[230].

Platelet function defects are often encountered in patients with chronic or acute liver disease. In vitro platelet aggregation in response to ADP, arachidonic acid, collagen, and thrombin has been shown to be defective[231,232]. Also, platelet-vessel wall interaction studied under flow conditions has been shown to be impaired[233]. Impaired

aggregation might be caused by defective platelet signal transduction mechanisms[234], an acquired storage pool deficiency[235], and decreased levels of arachidonic acid (required for thromboxane A_2 production) in the platelet membrane[236]. Furthermore, increased production of prostacyclin and nitric oxide (both powerful platelet inhibitors) by endothelial cells may contribute to impaired platelet function in vivo[237,238]. Finally, platelet-vessel wall interaction may be defective in patients with liver disease due to proteolysis of platelet receptors by plasmin[239,240], or due to the presence of a reduced haematocrit[241].

Von Willebrand factor

Plasma levels of vWF are substantially increased in patients with liver failure, presumably due to endothelial dysfunction, which may be triggered by endotoxemia[242]. Although reduced levels of the vWF cleaving protease, which cleaves high molecular weight multimers of vWF in less reactive smaller multimers, have been found in cirrhotic patients[243], the multimeric structure of vWF is found to be either normal[242], or defective in high molecular weight multimers[244]. Other proteases (such as plasmin and elastase, see section on fibrinolysis) have been implicated in increased proteolytic degradation of vWF[244]. Studies on vWF function in patients with cirrhosis are conflicting. Both normal[242] and reduced[231] platelet agglutination to ristocetin have been found, and in one study hyperagglutination of platelets towards botrocetin was observed[245].

4) Defects in secondary haemostasis in liver disease

Defects in procoagulant pathways

As the liver is assumed to be the site of synthesis for all proteins involved in thrombin generation, decreased plasma concentrations for all proteins except for factor VIII are observed in patients with hepatic failure. Although the liver is probably the principle site of synthesis for factor VIII, as haemophilia can be resolved by liver transplantation[246], other tissues have also been shown to produce factor VIII mRNA, and protein synthesis in these tissues may be stimulated by liver disease[247]. Another possibility is that, as factor VIII is not only produced by hepatocytes, but also by sinusoidal endothelial cells, the latter cell type sustains its capacity for factor VIII

synthesis even when liver function becomes impaired[247]. Alternatively, reduced clearance might lead to elevated factor VIII levels.

Besides low levels of clotting factors due to impaired synthesis capacity, also dysfunctional proteins are found in patients with liver failure. Factors II, VII, IX, and X as well as the anticoagulant factors protein C, protein S, and protein Z (see below) contain γ-carboxyglutamic acid (gla) residues which facilitate binding to anionic phospholipids. Gla residues are introduced into the protein by a vitamin K dependent carboxylase present in the hepatocyte[248], which catalyses the attachment of a second carboxyl group to specific N-terminal glutamic acid residues. It has been shown that in liver disease part of the circulating prothrombin[249] and protein C[250] lack their gla residues, and this is presumably also true for the other gla containing proteins. Abnormal γ-carboxylation might be the consequence of an intrinsic enzymatic defect, or to a vitamin K deficiency.

Factor V, a vitamin K independent coagulation factor is also reduced in both chronic and acute hepatic failure. Factor V levels in acute liver failure has been shown to correlate with survival[251].

Fibrinogen is believed to be synthesized exclusively in the liver. Fibrinogen levels are within the normal range in patients with stable chronic liver disease, but decreased levels are found in patients with advanced cirrhosis or acute hepatic failure[252]. Accelerated clearance and consumption due to intravascular coagulation may also contribute to the hypofibrinogenemia seen in these patients. Both in chronic and acute liver failure, abnormal fibrinogen molecules are commonly found[253]. These dysfibrinogens are characterised by an excessive content of sialic acid residues, which lead to impaired fibrin polymerisation[254]. The exact cause of fibrinogen hyperglycosilation is not clear, but it might be a consequence of elevated tissue levels of glycosyltransferases[255].

Defects in anticoagulant pathways

Plasma levels of TFPI in patients with liver disease are found to be normal[256], although also significantly decreased levels have been reported[257]. As endothelial cells (and not hepatocytes) are considered the primary site of synthesis of TFPI[258], normal levels would be expected.

AT levels are decreased in liver failure[252], as are HCII and α_2-M levels[259], thus resulting in reduced thrombin inhibition.

Decreased levels of both protein C and S are found in patients with liver disease as a

consequence of reduced synthesis. Moreover, protein C and S molecules lacking γ-carboxylation may be produced.
Reduced levels of protein Z have been reported in patients with liver failure[260], but to our knowledge ZPI levels have not been measured in this patient population.
Low levels of anticoagulant proteins might not only compensate for the defective thrombin generating capacity due to low levels of procoagulant proteins in patients with liver failure, reduced thrombin inhibition might also facilitate thrombosis. The clinical consequence of the concomitant reduction in both pro- and anticoagulant proteins is unknown. Although patients with hepatic failure in general are thought to have a bleeding tendency, there are also thrombotic complications, such as portal vein thrombosis known to occur in this patient population[261]. Although these thrombotic complications are rare, they are considered serious events.

5) Disorders of the fibrinolytic system

All proteins involved in fibrinolysis, except for tPA and PAI-1 are synthesized in the liver, and indeed reduced plasma levels of plasminogen[225], α_2-antiplasmin[212], HRG[262], factor XIII[263] and TAFI[212] have been found in patients with liver disease. Plasma tPA[264] levels are often elevated in patients with liver failure, which may be a result of either enhanced secretion from endothelial cells or diminished clearance by the diseased liver. PAI-1 levels are slightly elevated in plasma from patients with liver disease and do not seem to balance the elevated plasma tPA levels[265,266], except in acute hepatic failure, in which plasma PAI-1 levels are dramatically increased[267].
The net effect of the changes in the fibrinolytic system in patients with chronic liver failure is thought to be a hyperfibrinolytic state (reviewed in [268]). In contrast, in patients with acute hepatic failure, a shift towards hypofibrinolysis is observed, presumably as a consequence of substantially elevated plasma PAI-1 levels[212,267]. Already in 1914 it was reported that clotted whole blood of patients with liver disease showed premature fibrinolysis in vitro[269]. Hyperfibrinolysis was subsequently shown to be present in at least part of the patients with liver failure by many studies, in which different clot lysis assays were used. These assays include thromboelastography using whole blood[230], diluted whole blood clot lysis assays[265,270], and assays in which the euglobulin fraction of plasma is used (euglobulin clot lysis time and fibrin plate method)[271]. However, these commonly used in vitro fibrinolysis assays have major drawbacks, and caution should be taken in interpreting shortened clot lysis times. For example, classical thromboelastography assays whole, nonanticoagulated blood, and caution must be

taken in interpreting this assay as clot formation is initiated by (non physiological) contact activation. More recent variations on this technique make use of tissue factor-induced coagulation of citrated whole blood, but to our knowledge tissue factor-induced thromboelastrography has not yet been used for determination of fibrinolytic potential in patients with liver failure. The dilute whole blood clot lysis time is performed in the absence of calcium, and a clot is formed by addition of thrombin. Clot lysis time is therefore independent of coagulation and, as a consequence, independent of TAFI. Finally, accelerated clot lysis times in clot lysis assays using the euglobulin fraction from plasma (such as the euglobulin clot lysis time and fibrin plate method) must be interpreted with caution. As the euglobulin fraction does not contain inhibitors of fibrinolysis, enhanced clot lysis times presumably only reflect elevated tPA levels.

Recently, our group has used a plasma-based clot lysis assay in which coagulation was initiated by tissue factor, and exogenously added tPA was used to induce clot lysis to investigate fibrinolytic potential in patients with liver disease. In a large group of patients with cirrhosis of different severity and etiology, we could not demonstrate accelerated fibrinolysis compared to lysis in plasma clots from healthy volunteers[212]. However, the absence of platelets in our assay makes it difficult to refute a hyperfibrinolytic state in vivo based on our experiments, as the reduced platelet count in patients with cirrhosis might affect fibrinolysis by a reduced availability of platelet derived PAI-1[272].

A different line of evidence supporting the concept of a hyperfibrinolytic state in patients with liver failure is the appearance of elevated levels of indicators of fibrinolysis such as plasmin-α_2-antiplasmin complexes[273], fibrin(ogen) degradation products[274], and D-Dimers[275] in plasma from patients with cirrhosis. However, accumulation of these molecules may also be a reflection of a reduced capacity of the diseased liver to clear these substances.

The presence of hyperfibrinolysis in cirrhosis has been suggested to be coupled to activation of the coagulation system, presumably triggered by endotoxemia[276,277]. The presence of low-grade disseminated intravascular coagulation (DIC) in liver disease has been proposed to be responsible for ongoing fibrinolysis, but the presence of DIC in liver disease is highly controversial[230]. Although DIC-like laboratory features (elevated markers of coagulation and fibrinolysis, such as thrombin-antithrombin complexes, plasmin-α_2-antiplasmin complexes and D-Dimers[273]) are observed, this might be due to a reduced clearance capacity of the diseased liver. Furthermore, autopsy studies have shown little evidence for fibrin deposition in organs, which is the hallmark of 'classical' DIC[278].

The in vivo significance of hyperfibrinolysis in cirrhosis have been shown by Francis and Feinstein[270], and by Violi and coworkers[279], who reported an increased occurrence of bleeding in patients in whom hyperfibrinolysis was detected in vitro. However, it could be that the presence of in vitro hyperfibrinolysis is merely a reflection of a greater severity of the disease. Another argument against the presence of hyperfibrinolysis in liver disease, is that these patients do not show a typical hyperfibrinolytic bleeding tendency, i.e., delayed bleeding after trauma or surgery, as observed in α_2-antiplasmin[280] or PAI-1[281] deficiency. Bleeding problems in patients with cirrhosis, e.g., after liver biopsy, is usually immediate[282].

6) Bleeding complications associated with defective haemostasis in liver disease

Table 1 summarizes the pro and anti haemostatic changes observed in patients with liver disease. The net outcome of these alterations in the haemostatic system is a bleeding diathesis, although thrombosis of the portal vein is also frequently seen in patients with cirrhosis[261]. However, the development of thrombosis might also be a consequence of local disturbances, such as a reduced blood flow in the portal vein.
As liver disease has multiple effects on the haemostatic system, it is difficult to determine which factors contribute most to the bleeding diathesis. Bleeding problems in patients

Haemostatic changes impairing haemostasis	Haemostatic changes promoting haemostasis
• Low platelet count • Impaired platelet function and platelet vessel wall interaction • Enhanced platelet inhibition by nitric oxide and prostacyclin • Low levels of coagulation factors II, V, VII, IX, X, XI • Quantitative and qualitative abnormalities in fibrinogen • Low levels of α_2-antiplasmin, TAFI, HRG • Elevated levels of plasma tPA, which are not balanced by PAI-1 levels	• Elevated levels of factor VIII and vWF • Decreased levels of protein C, protein S, protein Z, antithrombin, α_2-macroglobulin, and heparin cofactor II • Low levels of plasminogen

Table 1.
Changes in the haemostatic system promoting bleeding (left) and counteracting bleeding (right)

with liver failure may be a consequence of the disbalanced haemostatic system. However, also bleeding due to mechanical reasons, such as bleeding from esophageal varices, occurs frequently in patients with cirrhosis[283]. The contribution of the haemostatic defects to variceal bleeding is unclear, but it has been suggested that laboratory signs of hyperfibrinolysis are associated with an increased risk to variceal bleeding[284].

The relative contribution of the changes in platelet number and function, coagulation, and fibrinolysis to the bleeding tendency in these patients is unclear. The mild to moderate thrombocytopenia is of little clinical relevance, but the low platelet count is often accompanied by poorly defined platelet function defects, and this combination might contribute to some extent to the bleeding diathesis in liver failure. The substantially reduced levels of procoagulant proteins in these patients is probably a major contributor to the bleeding tendency, even though the defects in procoagulant potential is partly compensated for by decreased levels of anticoagulant proteins. The contribution of alterations in the fibrinolytic system to the bleeding problems in liver disease is uncertain. Although associations between in vitro hyperfibrinolysis and bleeding in patients with cirrhosis have been demonstrated, this association might not be causal, as described in the previous section.

The bleeding diathesis in patients with acute hepatic failure or advanced cirrhosis manifests itself by epistaxis, gingival bleeding, ecchymoses, and gastrointestinal bleeding. Patients undergoing small invasive procedures, such as liver biopsy have a small but significant bleeding risk[282], and it is recommended that if a serious coagulopathy is detected, it is corrected by transfusion of platelets or plasma before the procedure. A larger bleeding risk is encountered in patients undergoing major abdominal surgery, including liver transplantation. During liver transplantation major changes in the already disturbed haemostatic system occur, and despite major improvements in the surgical techniques and available pro-haemostatic agents, liver transplantation may be accompanied by substantial intraoperative bleeding[285].

7) Treatment of bleeding complications in liver disease

Patients in whom platelet count is above 50.000/μl are usually not at risk for acute bleeding or bleeding during small invasive procedures[286]. Whenever platelet counts are below 50.000/μl, platelet transfusion is indicated during bleeding episodes or prior to invasive procedures (e.g., liver biopsy)[287]. The increase in platelet count upon platelet transfusion may be markedly less in a patient with liver failure compared to patients with intact liver function as a consequence of sequestration of the transfused platelets

in the enlarged spleen, and platelet count should always be measured before a procedure is initiated. Splenectomy has been shown to improve platelet count, but this procedure is contraindicated in patients with portal hypertension and hypersplenism, due to high surgery-related morbidity and mortality[288]. The prolonged bleeding time in patients with liver failure may be corrected by infusion of 1-deamino-8-D-arginine vasopressin (DDAVP), but the exact mechanism as well as the clinical relevance of this finding is unclear[289]. DDAVP has failed to prove efficacy in acute variceal bleeding in cirrhotic patients[290], and the clinical usefulness of DDAVP in other procedures has not been demonstrated yet.

The deficiencies in coagulation factors may be corrected by administration of fresh frozen plasma (FFP) or prothrombin complex concentrates (PCC). Disadvantages of FFP are the large volumes required for correction of a severe coagulopathy and the brief duration of action because of the short biological half life of some clotting factors, in particular factor VII. Fluid overload may be a complication upon FFP administration. Disadvantages of PCC's, which contain only the vitamin K dependent coagulation factors, are the fact that the coagulopathy is only partially corrected and the risk of thromboembolic complications, including DIC[291]. In patients with acute liver failure and haemostatic complications, plasmapheresis has been used as adjunctive therapy to correct haemostasis and to prevent fluid overload, but this procedure is not routinely used[292].

A novel approach to treat the coagulopathy in patients with liver disease is the administration of recombinant factor VIIa (rFVIIa). rFVIIa has been shown to correct a prolonged prothrombin time in patients with stable cirrhosis[293], or patients who are actively bleeding from esophageal varices[294]. It must be stressed, however, that prolongation of the prothrombin time by rFVIIa does not necessarily reflect haemostatic efficacy. Anecdotal reports do support the haemostatic potential of rFVIIa in patients with liver disease[295,296]. Moreover, rFVIIa has been shown to significantly reduce transfusion requirements during liver transplantation[297]. A limitation of this study, however, was the comparison of the rFVIIa treated patients with a historic control group.

Antifibrinolytic agents such as ε-aminocaproic acid (eACA), tranexamic acid, and aprotinin may be administered if a hyperfibrinolytic state is shown. However, since a hyperfibrinolytic state in patients with chronic liver failure is still somewhat controversial, and because of the risk for thromboembolic complications, the general therapeutic value of antifibrinolytics in these patients remains uncertain. On the other hand, both aprotinin[298] and tranexamic acid[299] have been shown effective in reducing

transfusion requirements during liver transplantation. However, whether the efficacy of these agents is exclusive due to their antifibrinolytic capacity, or that other mechanisms (such as modulation of vascular tone) are involved is unclear[300].

Chapter 4

Thrombin activatable fibrinolysis inhibitor deficiency in cirrhosis is not associated with increased plasma fibrinolysis

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Gastroenterology. 2001; 121: 131-139.

Abstract

Background & aims

The bleeding tendency of patients suffering from cirrhosis is in part ascribed to accelerated fibrinolysis. In this study, the role of the recently discovered inhibitor of fibrinolysis, thrombin activatable fibrinolysis inhibitor (TAFI) in cirrhosis was examined.

Methods

In 64 patients with cirrhosis of varying severity, TAFI antigen levels were measured by ELISA, and compared with TAFI levels in control subjects. Furthermore, a plasma-based fibrinolysis assay was performed in the presence and absence of a specific inhibitor of activated TAFI.

Results

TAFI levels were decreased in cirrhosis. Mean TAFI levels were 66% in Child's A, 55% in Child's B, 47% in Child's C cirrhosis, and 26% in acute liver failure. Decreased TAFI antigen levels were highly correlated with antithrombin and α_2-antiplasmin activity levels. Clot lysis times and clot lysis ratio (defined as ratio between clot lysis time in the absence and presence of a specific inhibitor of activated TAFI) of cirrhotics were not significantly different from healthy controls.

Conclusions

Despite decreased levels of TAFI and other components of the fibrinolytic system, no evidence of increased plasma fibrinolytic potential in cirrhosis is observed using the plasma-based assay of this study. The reduction of antifibrinolytic factors in cirrhosis is compensated by the concomitant reduction in profibrinolytics.

Introduction

Bleeding problems are frequently encountered in cirrhotic patients and can be ascribed to a defective haemostasis, or to complications of portal hypertension such as esophageal varices[301]. Spontaneous bleeding associated with haemostatic abnormalities is not common, but patients may bleed severely during invasive procedures like liver biopsy, dental extraction or venipuncture. An even greater challenge to haemostasis is encountered during liver transplantation, which can be accompanied by severe blood loss[302].

Frequently encountered haemostatic abnormalities present in cirrhosis include thrombocytopenia and platelet function defects, deficiencies in clotting factors, deficiencies of inhibitors of coagulation, dysfibrinogenemia, and abnormalities in the fibrinolytic system[216,303,304]. Moreover, clearance of activated coagulation factors and enzyme-inhibitor complexes may be reduced. An imbalance of the fibrinolytic system has been observed using in vitro clot lysis assays, which show accelerated fibrinolysis in part of the patient population[230,270,271]. In vivo indications for hyperfibrinolysis includes increased plasmin-α_2-antiplasmin (PAP) complexes, increased D-Dimer levels and fibrin(ogen) degradation products in plasma from cirrhotics (for a review see Leebeek[268]). However, increased levels of PAP, D-Dimer, and fibrin(ogen) degradation products may also be a consequence of impaired clearance. The nature of enhanced fibrinolysis in cirrhosis is not completely clear. An imbalance between t-PA and PAI-1 activity in favor of t-PA, accompanied by reduced levels of α_2-antiplasmin leading to decreased binding of α_2-antiplasmin to fibrin might explain the apparent hyperfibrinolytic state of these patients[266]. Hyperfibrinolysis in cirrhosis might be a primary phenomenon, but could be secondary to disseminated intravascular coagulation (DIC), which might be triggered by endotoxemia[277]. However, controversy on the presence of (low-grade) DIC in cirrhosis exists[230].

In this study, the role of the recently discovered inhibitor of fibrinolysis, thrombin activatable fibrinolysis inhibitor (TAFI)[305], which is also known as plasma procarboxypeptidase B[306], procarboxypeptidase U[307] or procarboxypeptidase R,[308] was examined. Activated TAFI inhibits fibrinolysis by cleaving C-terminal arginine and lysine residues from partially degraded fibrin, thereby inhibiting t-PA-mediated plasminogen activation[64]. TAFI is synthesized as zymogen in the liver[306] and can be activated by relatively high levels of thrombin[305]. TAFI can also be activated by the thrombin-thrombomodulin complex. This reaction is approximately 1200 times more efficient than the activation of TAFI by thrombin alone[66]. Both in vitro and in vivo experiments indicate that the thrombin required for TAFI activation is formed via thrombin-mediated activation of factor XI[65,309]. This factor XI feedback loop generates additional thrombin after formation of the fibrin clot resulting in down-regulation of fibrinolysis by activation of TAFI. Tissue factor-induced clots formed from plasma with deficiencies in factor VIII, IX, X or XI, have been shown to lyse prematurely because of a lack of TAFI activation, indicating the necessity for an intact coagulation pathway for down-regulation of fibrinolysis[200]. The clinical importance of TAFI is not established so far. However, it has been shown that increased levels of TAFI are a mild risk factor for venous thrombosis[310]. Also, inhibition of TAFI has been shown

to improve thrombolytic therapy in animal thrombosis models, indicating in vivo importance of down-regulation of the fibrinolytic system by TAFI[311,312].

Because TAFI is synthesized in the liver, we hypothesized that TAFI levels are decreased in patients with cirrhosis. Premature fibrinolysis in cirrhosis might be explained in part by a defective down-regulation of fibrinolysis by activated TAFI as a consequence of both reduced levels of TAFI and reduced thrombin generation caused by deficiencies in the thrombin-generating system. In this study, we examined TAFI antigen levels of patients with cirrhosis of varying severity. Moreover, plasma clot lysis assays were performed in the presence of exogenous t-PA to examine the contribution of TAFI to the inhibition of fibrinolysis in cirrhosis.

Materials and methods

Patients

Sixty-four patients in stable condition with biopsy-proven cirrhosis of various etiology – alcohol abuse (18), viral hepatites (26), autoimmune hepatites (2), primary biliary cirrhosis (4), cryptogenic cirrhosis (10), and others (4) – were included in this study. The patients were classified according to Pugh's modification of the Child's classification[313]. Nineteen patients with Child's A cirrhosis, 20 patients with Child's B cirrhosis, and 25 patients with Child's C cirrhosis were studied. In addition, 4 patients with acute liver failure were included in this study. Twenty healthy volunteers from our laboratory served as a control group. Pooled normal plasma was obtained by combining plasma from 40 healthy volunteers. Plasma from a patient with a congenital homozygous functional α_2-antiplasmin deficiency (α_2-antiplasmin Enschede) was also used in the study[280]. This patient has normal antigen levels of α_2-antiplasmin, but α_2-antiplasmin activity is < 4%.

Blood samples were obtained by venipuncture from the antecubital vein into 3.2% sodium citrate (9:1, v/v). To obtain platelet poor plasma, the samples were centrifuged twice at 2000g for 15 minutes. Plasma samples were stored at –70°C until use.

Materials

Fresh frozen plasma was obtained from the local blood bank. Tissue type plasminogen activator (t-PA) was from Chromogenix (Mölndal, Sweden). Recombinant human tissue factor (Innovin) was from Dade Behring GmbH (Marburg, Germany), and carboxypeptidase inhibitor from potato (CPI) was purchased from Calbiochem (La Jolla, CA). Recombinant active plasminogen activator inhibitor 1 (PAI-1) was a

generous gift from Dr. Thomas M. Reilly (DuPont Pharmaceuticals Company, Wilmington, DE). Antithrombin deficient plasma, and purified plasminogen were obtained from American Diagnostica Inc. (Greenwich, CT).
Phospholipid vesicles consisting of 40% L-α-dioleoylphosphatidylcholine, 20% L-α-dioleoylphosphatidylserine and 40% L-α-dioleoylphosphatidylethanolamine (all from Sigma, St. Louis, MO) were prepared according to Brunner[209] with minor modifications as described by van Wijnen[210]. Total phospholipid content of the vesicles was determined by phosphate analysis according to Rouser[211].
TAFI was purified from fresh frozen plasma by immunoaffinity chromatography followed by further purification on protein G-Sepharose and Q-Sepharose as described[314]. TAFI antigen levels were determined by a sandwich-type ELISA, using a monoclonal capturing antibody and a polyclonal detection antibody as described[206]. TAFI levels were expressed as percentage of pooled normal plasma. TAFI deficient plasma was prepared by passing plasma over a Sepharose column to which monoclonal antibody NIK 9H10 was coupled. Undiluted flow-through fractions were shown to be deficient in TAFI by ELISA.
Plasminogen deficient plasma was prepared by passing plasma over a lysine-Sepharose column. Since this procedure also resulted in a partial depletion of TAFI from the plasma, purified TAFI was added to the plasma to reach a final concentration of 100%. The concentration of α_2-antiplasmin in the plasminogen-depleted plasma was 104%.
Antithrombin was purified from human plasma using DEAE-Sepharose chromatography followed by heparin-Sepharose chromatography according to de Swart[315]. Levels of α_2-antiplasmin were determined using the Coamatic plasmin inhibitor kit from Chromogenix (Mölndal, Sweden). Antithrombin levels were measured with the Coamatic thrombin inhibitor kit from Chromogenix (Mölndal, Sweden). PAI-1 antigen levels were determined using the PAI-1 ELISA kit from Technoclone GmbH (Vienna, Austria). D-Dimer levels were determined using the Asserachrom D-dimer ELISA kit from Boehringer Mannheim GmbH (Mannheim, Germany).

Clot lysis assay

Lysis of a tissue factor-induced clot by exogenous t-PA was studied by monitoring changes in turbidity during clot formation and subsequent lysis essentially as described previously[198]. A mixture of tissue factor (diluted Innovin, final dilution 10^5 times), $CaCl_2$ (final concentration 17 mM), t-PA (final concentration 30 U/ml; 56 ng/ml) and

phospholipid vesicles (final concentration 10 μM) was added to 75 μl of citrated plasma. The volume was adjusted to 150 μl with Hepes buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 3 mM $CaCl_2$, 0.1% BSA, pH 7.4), resulting in a final plasma concentration of 50%. After thorough mixing, 100 μl of this mixture was transferred to a microtiter plate and turbidity at 405 nm was measured in time at 37ºC in a Spectramax 340 kinetic microplate reader (Molecular Devices Corporation, Menlo Park, CA, USA). Clot lysis time was defined as the time from the midpoint of the clear to maximum turbid transition, which characterizes clot formation, to the midpoint of the maximum turbid to clear transition, which represents clot lysis. To assess the contribution of TAFI activation to clot lysis time, experiments were performed in which CPI (25 μg/ml), a specific inhibitor of activated TAFI[199], was added to the plasma. The contribution of TAFI to the clot lysis assay was quantified by calculating the clot lysis ratio, which is defined as the ratio between clot lysis time in absence and presence of CPI. To assess the influence of PAI-1 on clot lysis times, clot lysis experiments were performed with pooled normal plasma to which different amounts of active PAI-1 were added. To examine the influence of α_2-antiplasmin on clot lysis times, clot lysis experiments were performed with plasma from a patient with severe α_2-antiplasmin deficiency, which was mixed with different amounts of pooled normal plasma. Clot lysis times with varying plasminogen concentrations were determined by mixing plasminogen-deficient plasma with different amounts of purified plasminogen. The contribution of antithrombin to clot lysis times was assayed using antithrombin-deficient plasma to which different amounts of purified antithrombin was added. The effect of a 50% reduction of both TAFI and antithrombin on clot lysis time was assayed by mixing equal amounts of TAFI and antithrombin-deficient plasma.

Statistical analysis.

Statistical analysis was performed using the GraphPad InStat (San Diego, USA) software package. Differences in TAFI antigen levels were assayed by standard one-way analysis of variance (ANOVA) using the Tukey-Kramer test. As clot lysis times and clot lysis ratio showed Gaussian distribution with significant differences between standard deviations between groups, statistical analysis on clot lysis times and clot lysis ratios was performed using Kruskal-Wallis ANOVA test, with Dunn's post test. P values <0.05 were considered statistically significant. Correlations between TAFI antigen levels and antithrombin or α_2-antiplasmin levels were determined by Pearson's correlation coefficient.

Results

TAFI antigen levels are decreased in cirrhosis

TAFI antigen levels were determined in plasma samples from 64 patients with cirrhosis of varying severity. Also, TAFI antigen levels in 4 patients with acute liver failure were determined. TAFI levels were significantly reduced in plasma from patients with Child's A ($66 \pm 13\%$; mean $\pm$ sd), Child's B ($55 \pm 22\%$), as well as Child's C ($47 \pm 18\%$) cirrhosis compared with TAFI levels in the control group, in which mean TAFI level was $103 \pm 20\%$ ($p<0.001$, for mild, moderate, and severe cirrhosis compared with control). As shown in figure 1, TAFI levels decreased significantly with increasing severity of the disease (mild vs. severe cirrhosis $p<0.05$). In 4 patients studied who suffered from acute liver failure, mean TAFI levels were $26 \pm 11\%$ ($p<0.001$ compared to control).

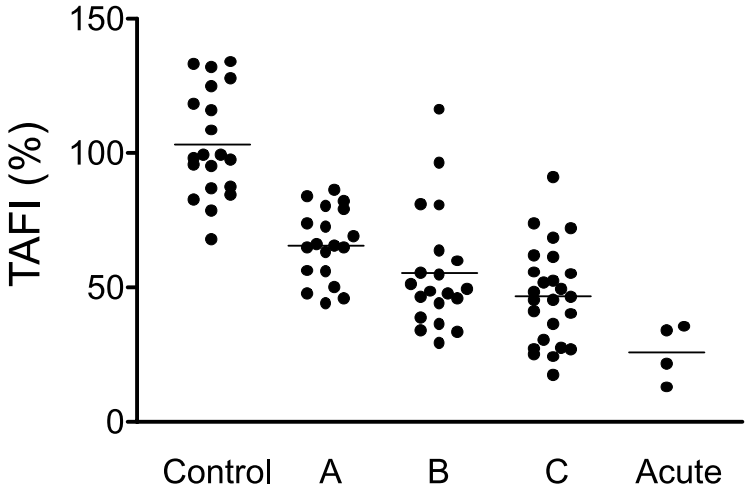

Figure 1. TAFI antigen levels of patients with Child's A (A), Child's B (B) or Child's C (C) cirrhosis and of patients suffering from acute liver failure (Acute) compared with TAFI antigen levels in healthy controls (Control). TAFI levels were determined by ELISA and are expressed as a percentage of pooled normal plasma. The horizontal line represents the mean of each group.

Correlation of TAFI antigen levels with antithrombin and α_2-antiplasmin activity levels

TAFI antigen levels correlated with antithrombin ($r=0.701$, $p<0.0001$) and α_2-antiplasmin ($r=0.730$, $p<0.0001$) activity levels as shown in figure 2.

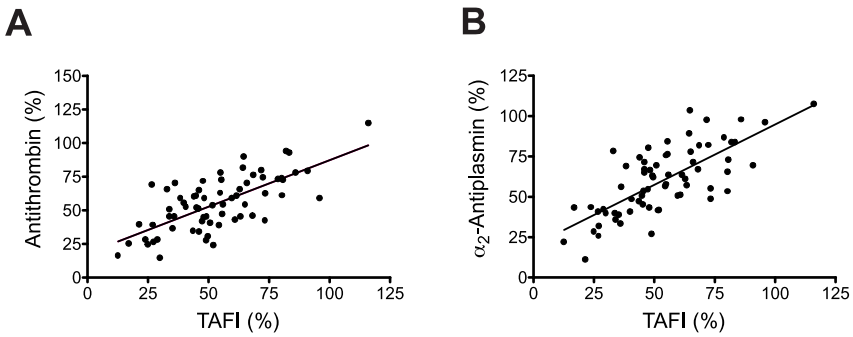

Figure 2. Correlation of TAFI antigen levels with antithrombin (A) and α_2-antiplasmin (B) activity levels.

Clot lysis assay

Clot lysis times were determined by measuring the turbidity profile in time of a tissue factor-induced clot, which was lysed by exogenous t-PA. Clot lysis times were determined for all patients and controls both in the presence and absence of the TAFI inhibitor CPI. Figure 3 shows typical examples of clot lysis curves in plasma of healthy subjects (panel A) and in plasma of cirrhotic patients (panel B). Despite decreased TAFI levels and decreased thrombin formation, down-regulation of fibrinolysis by TAFI is seen in cirrhotic plasma because clot lysis times can be significantly reduced by the

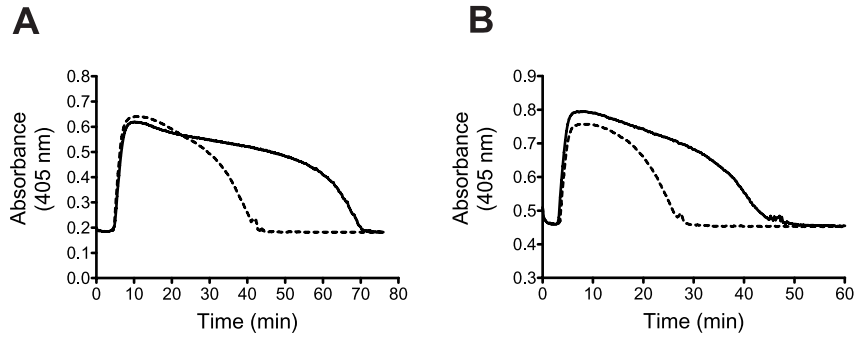

Figure 3. Typical example of a clot lysis curve in normal (panel A) and cirrhotic (panel B) plasma in the absence (uninterrupted line) or presence (interrupted line) of CPI. Coagulation was initiated by tissue factor (10^5 diluted Innovin), phospholipid vesicles (10 μM), and calcium chloride (17 mM). Fibrinolysis was initiated by the addition of tPA (30 U/ml). Fibrin formation and subsequent lysis were measured in time as the change in turbidity at 405 nm.

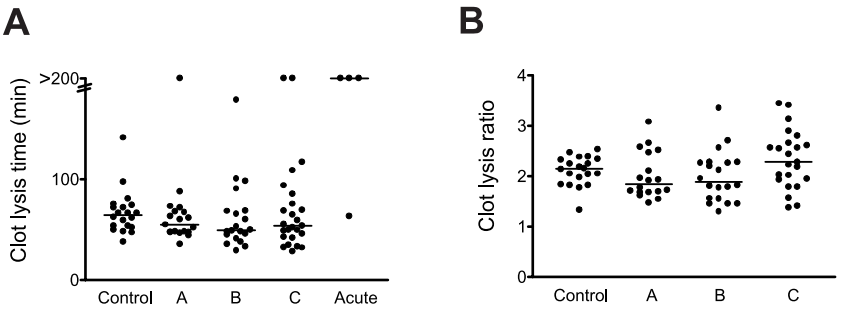

Figure 4. A. Clot lysis times of patients with Child's A (A), Child's B (B) or Child's C (C) cirrhosis and in patients suffering from acute liver failure (Acute) compared with clot lysis times in healthy controls (Control). B. Clot lysis ratio of patient samples as shown in panel A. Clot lysis ratio is defined as clot lysis time divided by clot lysis time in the presence of CPI. Because 3 of 4 plasma samples from patients with acute liver failure did not lyse at all during the time course of the experiment both in presence and absence of CPI, no clot lysis ratios are given for this group. Horizontal lines represent medians of each group.

addition of CPI. As shown in figure 4A, clot lysis times do not differ between cirrhotic patients and controls. A few patients showed almost no lysis during the time span of our experiment. These plasma samples contain high PAI-1 levels (data not shown), which can cause complete inhibition of clot lysis in our assay (see below). When clot lysis results are expressed as clot lysis ratio, i.e., the ratio of clot lysis time in absence and presence of CPI, a specific inhibitor of activated TAFI, no differences between the patient groups and the control group are seen (figure 4B). Because all clot lysis ratios are significantly higher than 1, TAFI activation occurs in all plasma samples obtained from cirrhotic patients. In 3 of 4 plasma samples from patients with acute liver failure, no lysis could be detected during the time course of our experiment, both in presence and absence of CPI. This can most likely be attributed to very high PAI-1 levels in these plasma samples (data not shown).

Elevated D-Dimer levels in cirrhosis

To investigate if the patient population used in this study was comparable to patient populations used in previous studies in which patients with cirrhosis are found to be hyperfibrinolytic, D-Dimer levels were measured in patients and controls. As shown in figure 5, D-Dimer levels increase with increasing severity of the disease. A hyperfibrinolytic state (i.e., D-Dimer levels above 500 ng/ml) was detected in 47% of the patient with Child's A, 75% of the patients with Child's B, and 100% of the patients with Child's C cirrhosis.

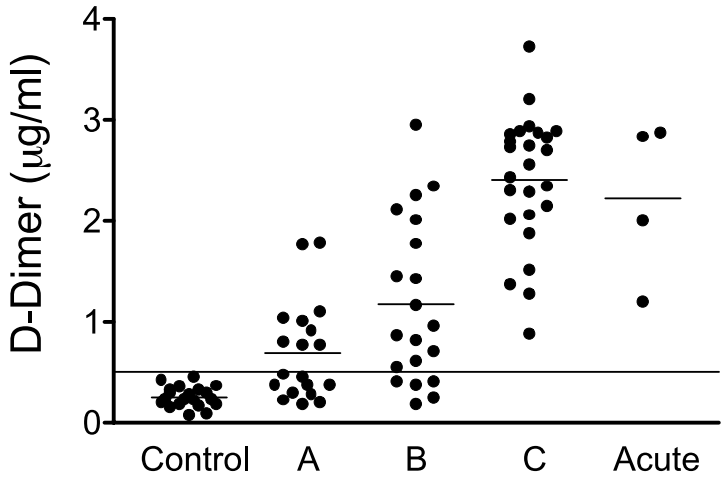

Figure 5. D-Dimer levels in patients with Child's A (A), Child's B (B) or Child's C cirrhosis and of patients suffering from acute liver failure (Acute) compared to D-Dimer levels in healthy controls. D-Dimer levels >500 ng/ml were considered to represent hyperfibrinolysis. The horizontal line represents the mean of each group.

PAI-1, α_2-antiplasmin, plasminogen and antithrombin levels influence both clot lysis time and clot lysis ratio

As TAFI levels decrease with increasing severity of the disease, clot lysis times and ratios were also expected to decrease. However, as clot lysis times and ratios in different stages of cirrhosis did not differ from those in healthy controls, we examined the effect of PAI-1, α_2-antiplasmin, plasminogen and antithrombin on clot lysis time and ratio.

To determine the effect of PAI-1 on clot lysis time, increasing concentrations of active PAI-1 were added to pooled normal plasma, and clot lysis times were determined in presence and absence of CPI. As shown in figure 6A, PAI-1 dose dependently prolongs clot lysis time. PAI-1 levels of 500 ng/ml or higher completely inhibit clot lysis during the time span of the experiment (4 hours). On inhibition of activated TAFI, a decrease in fibrinolysis time is seen at PAI-1 concentrations up to 500 ng/ml. Clot lysis ratio increases at PAI-1 levels up to 500 ng/ml. At higher PAI-1 levels no lysis is seen even in the presence of CPI.

To assay the effect of α_2-antiplasmin on clot lysis times, plasma from a patient with a severe α_2-antiplasmin deficiency (TAFI antigen level of 82%) was mixed with pooled normal plasma, and the clot lysis assay was performed in the presence and absence of CPI. As shown in figure 6B, the clot lysis time increases with increasing amounts of

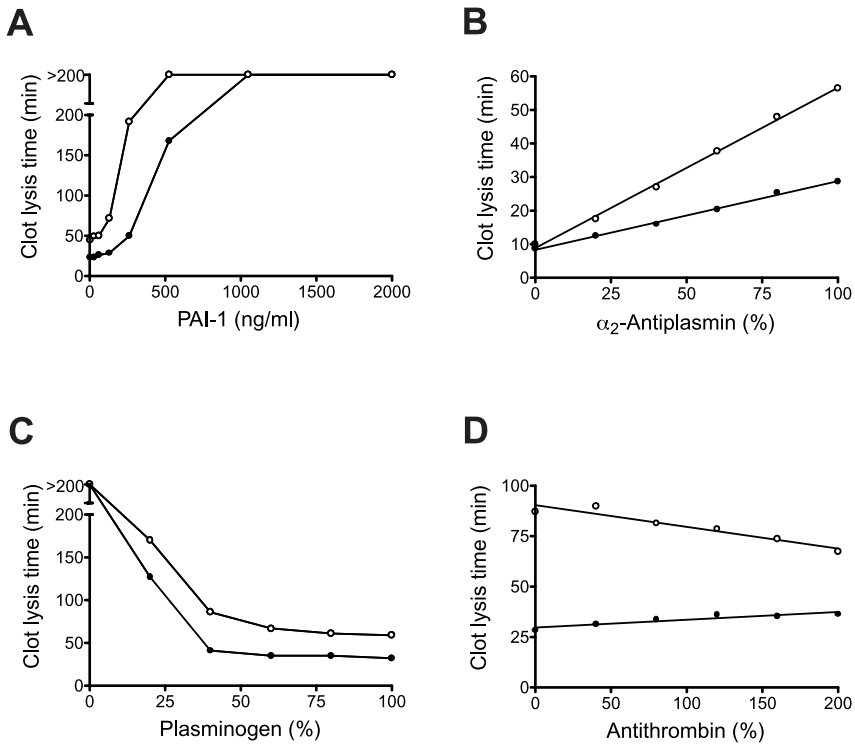

Figure 6. A. The effect of PAI-1 on clot lysis time in the absence (open circles) or presence (closed circles) of CPI. Different amounts of active PAI-1 were added to pooled normal plasma and the clot lysis times were determined. Lysis times >200 min mean no detectable lysis in the time span of the experiment. B. The effect of α_2-antiplasmin on clot lysis time in the absence (open circles) or presence (closed circles) of CPI. Plasma from a patient with a dysfunctional α_2-antiplasmin molecule was mixed with pooled normal plasma and clot lysis times were determined. C. The effect of plasminogen on clot lysis time in absence (open circles) or presence (closed circles) of CPI. Purified plasminogen was added to plasminogen depleted plasma, and clot lysis times were determined. Lysis time >200 min means no detectable lysis in the time span of the experiment. D. Antithrombin moderately accelerates clot lysis in normal plasma. Clot lysis assays were performed in antithrombin depleted plasma, which was reconstituted with different amounts of purified antithrombin. Clot lysis times were determined in the absence (open circles) and presence (closed circles) of CPI.

functional α_2-antiplasmin. Moreover, clot lysis ratio increases with increasing concentrations of functional α_2-antiplasmin.

The effect of plasminogen on clot lysis time was determined by addition of purified plasminogen to plasminogen deficient plasma. As shown in figure 6C, when plasminogen concentration is reduced to 60% of normal, no effect on clot lysis time is observed.

However, when plasminogen concentration is reduced to 40% or less, a dramatic increase in clot lysis time is seen, and on complete depletion of plasminogen no lysis is seen at all. The influence of thrombin inhibition by antithrombin on clot lysis time was assayed in antithrombin-deficient plasma (TAFI antigen level of 83%) to which different amounts of purified antithrombin were added. As shown in figure 6D, increasing amounts of antithrombin moderately decrease clot lysis time, as well as clot lysis ratio.

A reduction of both TAFI and antithrombin does not affect clot lysis time

Because TAFI levels are decreased in cirrhosis, a decrease in clot lysis times in cirrhotics compared to controls would have been expected. However, clot lysis time is influenced by both the level of antithrombin and the levels of fibrinolytic proteins. It was hypothesized that clot lysis times of cirrhotics are not decreased compared to controls because of compensatory mechanisms for the decrease in antifibrinolytics. For example, decreased TAFI levels might be counterbalanced by decreased levels of antithrombin. To assay the effect of a 50% reduction of both TAFI and antithrombin in normal plasma, mixing experiments were performed with TAFI-deficient, antithrombin-deficient, and pooled normal plasma. As shown in figure 7, the decrease in clot lysis time due to a decrease in TAFI levels to 50% can be balanced by a simultaneous decrease of antithrombin levels to 50%.

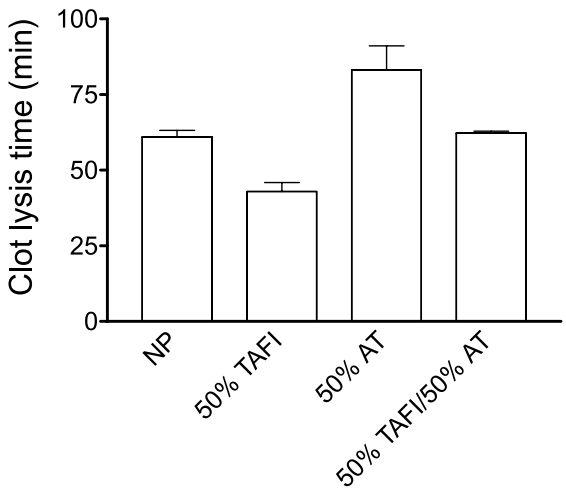

Figure 7. A simultaneous reduction of TAFI and antithrombin to 50% of pooled normal plasma levels does not affect clot lysis time, whereas reduction of either TAFI or antithrombin to 50% results in a reduction or an increase in clot lysis time, respectively. Values shown represent means $\pm$ standard deviation (n=3).

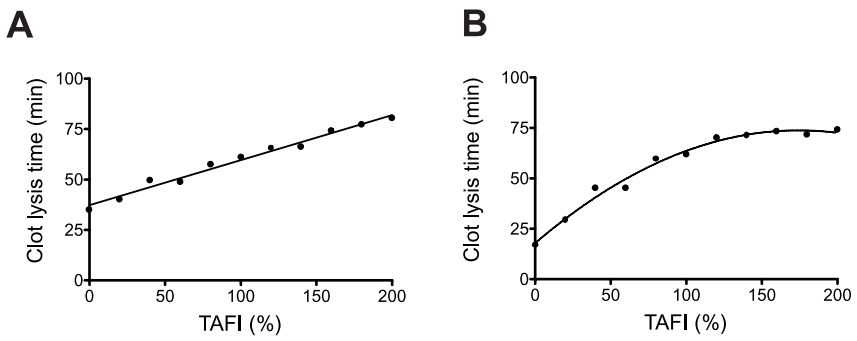

Figure 8. Effect of TAFI concentration on clot lysis time in normal (panel A) or cirrhotic (panel B) plasma. Different concentrations of purified TAFI were added to TAFI depleted plasma from a healthy volunteer or TAFI depleted plasma from a patient suffering from Child C cirrhosis after which clot lysis times were determined. TAFI concentrations are expressed as percentage of pooled normal plasma.

Down-regulation of fibrinolysis in cirrhotic plasma is TAFI mediated

To show that activation of TAFI indeed results in a down-regulation of fibrinolysis in cirrhotic plasma, different concentrations of TAFI were added to TAFI-depleted plasma from a single cirrhotic patient who suffered from Child's C cirrhosis. As shown in figure 8, increasing amounts of TAFI prolong clot lysis times in both normal (panel A) and cirrhotic (panel B) plasma. However, in plasma from the patient with cirrhosis no clear prolongation of clot lysis time is seen at TAFI concentrations above 100% of pooled normal plasma, whereas in normal plasma prolongation of clot lysis time is seen with TAFI concentrations up to 200% of pooled normal plasma.

Discussion

This study shows reduced plasma levels of TAFI antigen in a large group of patients suffering from cirrhosis, which is related to the severity of the disease. We hypothesized that reduced TAFI levels might contribute to the bleeding diathesis seen in cirrhotic patients, which is partly ascribed to accelerated fibrinolysis[268,270]. However, despite reduced TAFI levels and a reduced ability to generate thrombin, as characterized by a prolonged prothrombin and activated partial thromboplastin time, we still observed down-regulation of fibrinolysis by activated TAFI in our fibrinolysis assay because clot lysis times could be significantly reduced by addition of a specific inhibitor of activated TAFI. Apparently, sufficient 'free' thrombin is generated to activate TAFI,

most probably caused by reduced antithrombin levels in these patients. This hypothesis is supported by the observation that depletion of antithrombin from normal plasma moderately prolongs clot lysis time. In summary, we find no indication of accelerated plasma fibrinolysis in patients suffering from cirrhosis because clot lysis times do not differ from healthy controls.

Although the concept of a hyperfibrinolytic state in cirrhosis is widely accepted, the evidence from the literature is not convincing. In vitro indications for hyperfibrinolysis in cirrhosis are based on decreased clot lysis times in fibrinolysis assays, such as dilute whole blood clot lysis time[265], euglobulin clot lysis time[271] and thromboelastography[230]. It has been proposed that increased t-PA activity relative to PAI-1 activity, accompanied by decreased α_2-antiplasmin levels cause accelerated fibrinolysis in cirrhosis[266]. Commonly used fibrinolysis assays have major drawbacks, and caution should be taken in interpreting shortened clot lysis times. For example, the dilute whole blood clot lysis time is performed in the absence of calcium, and a clot is formed by addition of thrombin. Clot lysis time is therefore independent of coagulation and, as a consequence, independent of TAFI. Because the euglobulin fraction used in fibrinolysis assays, such as euglobulin clot lysis time and fibrin plate method, do not contain inhibitors of fibrinolysis, these tests only assay increased plasma t-PA levels. Although thromboelastography assays whole, nonanticoagulated blood, caution must be taken in this assay as the clot formed is initiated by (nonphysiological) contact activation.

The appearance of indicators of fibrinolysis in plasma like D-Dimers[275] fibrin(ogen) degradation products[274], and PAP complexes[273] has been used as in vivo evidence for a hyperfibrinolytic state in patients with cirrhosis. This study confirms the presence of elevated levels of D-Dimers in plasma of patients with cirrhosis. The appearance of D-Dimers in our patient population is consistent with the concept of accelerated fibrinolysis preceded by clotting activation[276]. The appearance of elevated plasma levels of prothrombin fragment 1+2[277] and thrombin-antithrombin complexes[273] has been used as evidence for in vivo clotting activation in cirrhosis. However, accumulation of indicators of both clotting activation and fibrinolysis could also be the consequence of a reduced clearance of these molecules by the diseased liver.

Clinically, the bleeding manifestations in cirrhotic patients do not resemble bleeding problems seen in a clear hyperfibrinolytic state like α_2-antiplasmin[280] or PAI-1[281] deficiency, in which, among other bleeding manifestations, delayed bleeding after trauma or surgery is observed. In cirrhotic patients, bleeding complications after invasive procedures like biopsy are immediate, whereas delayed bleeding is uncommon. Using a plasma-based clot lysis assay, in which coagulation is initiated by the

physiological initiator of coagulation (i.e., tissue factor), we have found no evidence of accelerated plasma fibrinolysis in cirrhosis. Because our assay uses exogenous t-PA to initiate fibrinolysis, slightly enhanced endogenous t-PA levels are not detected. Because vessel wall injury immediately leads to massive release of t-PA from the endothelium, this assay set up more accurately reflects the physiological condition than do fibrinolysis assays in which endogenous t-PA is responsible for clot lysis.

The lack of hyperfibrinolysis in cirrhotic plasma is most likely explained by a balance of profibrinolytic and antifibrinolytic factors. Defective inhibition of fibrinolysis by reduced levels of TAFI and α_2-antiplasmin is balanced by a defective fibrinolytic activation caused by reduced plasminogen levels and increased PAI-1 levels. Moreover, we have shown that antithrombin also functions as profibrinolytic agent because of its ability to inhibit thrombin activity and thus TAFI activation and that decreased antithrombin levels are able to counterbalance decreased TAFI levels with respect to clot lysis time. This balance is also observed on evaluation of clot lysis ratio of cirrhotic plasma. Because PAI-1, α_2-antiplasmin, plasminogen and antithrombin levels all have an effect on clot lysis ratio, no decrease in clot lysis ratio is seen with increasing severity of the liver damage. This decrease would be expected if clot lysis ratio was solely dependent on TAFI levels.

Whether the absence of plasma hyperfibrinolysis can be translated to the in vivo situation remains uncertain. Platelets have also been shown to contribute to inhibition of fibrinolysis by PAI-1 release and by PAI-1-independent mechanisms[68,272]. Because patients with cirrhosis also suffer from thrombocytopenia and platelet function defects, it is possible that impaired platelet-mediated inhibition of fibrinolysis contributes to the development of a hyperfibrinolytic state in vivo.

In conclusion, in this study we do not observe plasma hyperfibrinolysis in patients with cirrhosis. Our data implicate that despite decreased levels of TAFI, accompanied by deficiencies in the thrombin-generating system, down-regulation of fibrinolysis by TAFI can take place in patients with cirrhosis. Abnormalities in coagulation, platelet number, and platelet function may be more important in the bleeding diathesis of cirrhotics, although the contribution of platelet defects to a hyperfibrinolytic state cannot be ruled out.

Acknowledgements

The authors like to thank Dr. Thomas M. Reilly for his generous gift of human recombinant active PAI-1.

Chapter 5

Recombinant factor VIIa improves clot formation but not fibrinolytic potential in patients with cirrhosis and during liver transplantation

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Hepatology 2002; 35: 616-621.

Abstract

Cirrhosis is associated with a bleeding tendency, which is particularly pronounced during orthotopic liver transplantation (OLT). A novel approach to treat the bleeding diathesis of patients with cirrhosis is administration of recombinant factor VIIa (rFVIIa). This study examined whether the efficacy of rFVIIa in cirrhosis might be explained in part by enhanced down-regulation of fibrinolysis by thrombin activatable fibrinolysis inhibitor (TAFI). Addition of therapeutic or supratherapeutic doses of rFVIIa to plasma of 12 patients with stable cirrhosis did not result in a prolongation of clot lysis time, though clotting times were significantly reduced. Also, clot lysis assays of plasma samples taken during and after OLT, which was performed with or without a single bolus dose of rFVIIa, did not show any effect of rFVIIa on plasma fibrinolytic potential. In conclusion, this study shows no evidence for an antifibrinolytic effect of rFVIIa in cirrhotic patients or in patients undergoing OLT.

Introduction

Cirrhosis results in severe changes in the haemostatic system. The haemostatic defects in cirrhosis include thrombocytopenia and platelet function defects, deficiencies of clotting factors and inhibitors, dysfibrinogenemia, and deficiencies of fibrinolytic proteins[216,266,303]. Bleeding complications in cirrhosis may be a consequence of a defective haemostatic system, but patients may also bleed due to complications of portal hypertension such as esophageal varices[301]. The bleeding diathesis of cirrhotic patients becomes strikingly apparent during orthotopic liver transplantation (OLT), which is frequently accompanied by excessive blood loss[302].

Management of bleeding in patients with cirrhosis includes the administration of vitamin K, fresh frozen plasma, fibrinogen concentrate and platelet concentrates. Whenever a hyperfibrinolytic state is shown, treatment with antifibrinolytic drugs such as ε-aminocaproic acid, tranexamic acid or aprotinin is an option. Both tranexamic acid and aprotinin have been shown to reduce blood loss during liver transplantation[298,299].

A novel approach to treat the haemostatic defects in cirrhosis is administration of recombinant factor VIIa (rFVIIa; NovoSeven®). rFVIIa was shown to normalise a prolonged PT in stable cirrhotic patients[293], and in patients with alcoholic cirrhosis who are bleeding from esophageal varices[294]. Also, rFVIIa was shown to be effective in inducing haemostasis during small invasive procedures[295]. A recent report showed that rFVIIa reduced the need for transfusion of red blood cells and plasma during OLT[169].

rFVIIa was originally developed for the treatment of haemophiliacs with inhibitors during bleeding episodes or surgery, in whom it was shown to be safe and effective[145,146,195]. rFVIIa exerts its pro-haemostatic effect via enhancement of the extrinsic coagulation pathway in a tissue factor-dependent manner[182]. The ability of high-dose rFVIIa to overcome the inhibitory effect of plasma factor VII on coagulation might play a role in the enhancement of thrombin generation by rFVIIa[183]. Alternative mechanisms for the therapeutic effects have been proposed. A possible alternative mechanism involves thrombin formation on activated platelets or monocytes independently of tissue factor[188,189]. Another mechanism for the efficacy of rFVIIa might be down-regulation of the fibrinolytic system via (enhanced) activation of thrombin activatable fibrinolysis inhibitor (TAFI). We have previously shown that rFVIIa serves as an antifibrinolytic agent in factor VIII deficient plasma by enhancing secondary thrombin generation required for TAFI activation[215]. Whether this mechanism also applies to cirrhotic plasma remains speculative[304].

TAFI is activated by high concentrations of thrombin[305] or by the thrombin-thrombomodulin complex[66]. Activated TAFI down-regulates fibrinolysis by cleaving C-terminal lysine and arginine residues from partially degraded fibrin[64]. These lysine and arginine residues are essential cofactors in tissue-type plasminogen activator (t-PA) mediated fibrinolysis. We previously observed significantly reduced levels of TAFI antigen in patients with cirrhosis[212]. However, despite reduced levels of TAFI antigen accompanied by a reduced ability to generate thrombin, optimal TAFI activation could be demonstrated in a plasma-based fibrinolysis assay. Even more, plasma clot lysis times were not significantly shorter in cirrhotic plasma compared to plasma of control subjects. In this report, we investigated a possible (further) TAFI-mediated down-regulation of fibrinolysis by rFVIIa in non-bleeding cirrhotic patients and in patients undergoing OLT.

Materials and methods

Patients

Twelve patients in stable condition with biopsy-proven cirrhosis of different etiologies - viral hepatitis (6), alcohol abuse (4), auto-immune hepatitis (1), and cryptogenic cirrhosis (1)- were included in this study. The patients were classified according to Pugh's modification of the Child classification[313]. Four patients with Child A, 4 patients with Child B, and 4 patients with Child C cirrhosis were studied. Twelve healthy volunteers from our laboratory served as a control group. Pooled normal plasma was obtained by combining plasma from 40 healthy laboratory volunteers.

Twelve patients with Child B and C cirrhosis who underwent OLT were also included in this study. Six of them underwent a transplantation after receiving a single bolus dose of rFVIIa (80 μg/kg; 4000 U/kg), whereas 6 others served as a control group. The clinical evaluation of these patients has been described by Hendriks et al.[169]. Patients and controls were matched according to expected blood loss and transfusion requirements. Baseline characteristics of patients and controls were similar. Transfusion of red blood cells, platelets, plasma and fibrinogen concentrate was performed according to predefined criteria, and treatment with rFVIIa significantly reduced transfusion requirements as described previously[169]. No antifibrinolytic drugs such as aprotinin were administered. Plasma samples from 6 time points during and after surgery were studied; 30 minutes after induction of anaesthesia (A), 30 minutes after the start of the anhepatic phase (B), 30 minutes after reperfusion (C), at the end of surgery (D), 24 hours after the end of surgery (E) and at day 5 after surgery (F). rFVIIa was given 10 minutes before to start of the surgery (i.e., between time-points A and B). In the rFVIIa-treated group time-point B corresponds to 4.5 hours (median; range 4-5.75), time point C to 7 hours (range 5.5-8.5), and time point D to 9 hours (range 7.75-11.75) after rFVIIa infusion. The duration of the different phases in the control group was similar.
Blood was collected in 3.8% sodium citrate (9:1, v/v) as the anticoagulant. To obtain platelet poor plasma, samples were centrifuged twice at 2000 g for 10 minutes. Plasma samples were stored at -80°C until use.

Materials
Tissue type plasminogen activator (t-PA) was from Chromogenix (Mölndal, Sweden). Recombinant human tissue factor (Innovin) and the prothrombin fragment 1+2 kit (Enzygnost) were from Dade Behring GmbH (Marburg, Germany). Carboxypeptidase inhibitor from potato (CPI) was purchased from Calbiochem (La Jolla, CA, USA). Trypsin inhibitor from corn (CTI; >30.000 trypsin inhibitory units/ml) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Recombinant factor VIIa was a generous gift of Novo Nordisk (Bagsvaerd, Denmark). Recombinant hirudin was a generous gift from R. Wallis (Ciba Geigy, Horsham, UK).
Phospholipid vesicles consisting of 40% L-α-dioleoylphosphatidylcholine, 20% L-α-dioleoylphosphatidylserine and 40% L-α-dioleoylphosphatidylethanolamine (all from Sigma, St. Louis, MO, USA) were prepared according to Brunner[209] with minor modifications as described by van Wijnen[210]. Total phospholipid content of the vesicles was determined by phosphate analysis according to Rouser[211].

Plasminogen activator inhibitor-1 (PAI-1) antigen levels were determined using the PAI-1 ELISA kit from Technoclone GmbH (Wien, Austria). TAFI antigen levels were determined by a sandwich type ELISA, using a monoclonal capturing antibody and a polyclonal detection antibody as described[206]. TAFI levels were expressed as percentage of pooled normal plasma. Antithrombin levels were measured using the Coamatic thrombin inhibitor kit from Chromogenix (Mölndal, Sweden).

Clot lysis assay

Lysis of a tissue factor-induced clot by exogenous t-PA was studied by monitoring changes in turbidity during clot formation and subsequent lysis essentially as described[198]. Fifty microliters of plasma was pipetted into Immulon-2 flatbottom microtiter plates (Dynatech Laboratories Inc, Chantilly, VA). These plates do not support contact activation[316]. To further suppress contact activation CTI, a potent inhibitor of coagulation factor XIIa[317] was added to the plasma throughout this study. Fifty microliters of a mixture containing tissue factor (diluted Innovin, final dilution 10^5 times), $CaCl_2$ (final concentration 17 mM), t-PA (final concentration 30 U/ml; 56 ng/ml), phospholipid vesicles (final concentration 10 μM), and CTI (final dilution 1:25) diluted in Hepes buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 3 mM $CaCl_2$, 0.1% BSA, pH 7.4) was added. After thorough mixing, turbidity at 405 nm was measured in time at 37°C in a Spectramax 340 kinetic microplate reader (Molecular Devices Corporation, Menlo Park, CA, USA). Coagulation time was defined as the time to reach the midpoint of clear to maximal turbid transition. Clot lysis time was defined as the time from the midpoint of the clear to maximum turbid transition, which characterises clot formation, to the midpoint of the maximum turbid to clear transition, which represents clot lysis. To assess the contribution of activated TAFI to clot lysis time, experiments were performed in which CPI (25 μg/ml), a specific inhibitor of activated TAFI[199], was added to the plasma. To assay the influence of rFVIIa on clot lysis in cirrhotic plasma, 60 U/ml of rFVIIa, which represents the double amount of peak values of rFVIIa observed in cirrhotic patients who received a bolus dose of 80 μg/kg (4000 U/kg) rFVIIa[293], was added to the plasma.

Prothrombin fragment 1+2 measurement during clot lysis assay

Prothrombin activation during the clot lysis assay was monitored by performing a clot lysis assay not in 96-well microtitre plates, but in eppendorf tubes. Coagulation was induced in cirrhotic plasma or pooled normal plasma in the presence or absence of rFVIIa (60 U/ml). At different time intervals, thrombin formation was terminated by

the addition of 100 µl of HEPES buffer containing 20 mM EDTA and 50 U/ml hirudin. After thorough mixing for 1 minute, the clot was spun down (2 minutes, 15.000 g). Prothrombin activation was quantified by assaying the concentration of prothrombin fragment 1+2 in the supernatant by ELISA.

Statistical analysis

Statistical analysis was performed using the GraphPad InStat (San Diego, USA) software package. The statistical significance of differences in coagulation or clot lysis time upon addition of rFVIIa was evaluated using a paired Student's t-test. A *P* value of <0.05 was considered statistically significant.

Results

Effect of rFVIIa on coagulation during the clot lysis assay in cirrhotic and control plasma. In 12 patients with cirrhosis of varying severity and in 12 controls, a clot lysis assay was performed in presence and absence of rFVIIa (60 U/ml). As shown in figure 1, coagulation times in cirrhotics, which were derived from the turbidity profiles of the clot lysis assay, were similar to those in healthy controls. Upon addition of rFVIIa, a significant decrease in clotting time was seen in both cirrhotic and control plasma ($p<0.0001$ for controls; $p<0.0001$ for cirrhotics, paired t-test).

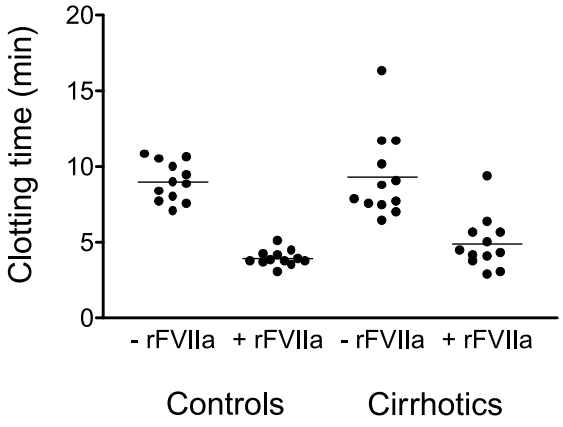

Figure 1. Coagulation times in cirrhotic and control plasma derived from clot lysis turbidity profiles in absence or presence of recombinant factor VIIa (rFVIIa; 60 U/ml). Coagulation was initiated by addition of calcium (17 mM), phospholipids (10 µM) and tissue factor (Innovin; 10^5 times diluted). Corn trypsin inhibitor was added to the reaction mixture to suppress contact activation. Horizontal bars represent means.

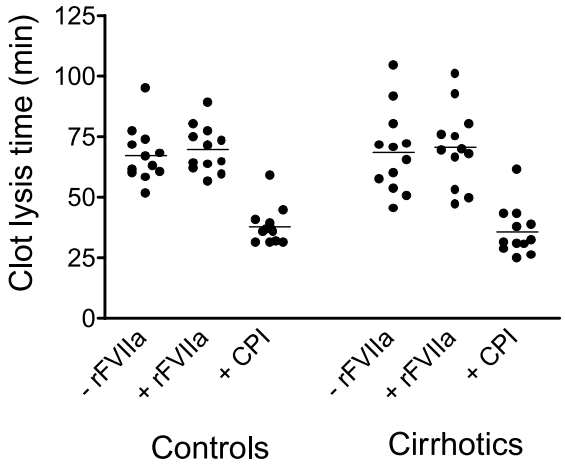

Figure 2. Clot lysis times of cirrhotic and control plasma. Coagulation was initiated by addition of calcium (17 mM), phospholipids (10 μM) and tissue factor (Innovin; 10^5 times diluted), fibrinolysis was induced by the addition of tissue-type plasminogen activator (t-PA; 30 U/ml). Clot lysis times were determined in plasma spiked with buffer, recombinant factor VIIa (rFVIIa; 60 U/ml), or carboxypeptidase inhibitor (CPI; 25 μg/ml). Corn trypsin inhibitor (CTI) was added to the reaction mixture to suppress contact activation. Horizontal bars represent means.

Effect of rFVIIa on clot lysis time in cirrhotic and control plasma.

As shown in figure 2, addition of 60 U/ml rFVIIa had no effects on clot lysis time in both cirrhotic and control plasma. Addition of supratherapeutic doses of rFVIIa (up to 400 U/ml) to cirrhotic plasma also did not result in a prolongation of clot lysis time (data not shown). Also, clot lysis times did not differ between cirrhotics and controls, despite a pronounced difference in TAFI antigen levels (controls $102 \pm 22\%$ (mean $\pm$ SD); cirrhotic patients $62 \pm 23\%$). In both groups a down-regulation of fibrinolysis by activated TAFI was present, since clot lysis times could be significantly reduced by an inhibitor of activated TAFI (CPI).

Prothrombin fragment 1+2 formation during the clot lysis assay

Prothrombin activation during the clot lysis assay was quantified by measurement of prothrombin fragment 1+2 (F_{1+2}) at different time points after initiation of the clot lysis assay. As shown in figure 3, only a very small amount of prothrombin was activated when the plasma started to clot (which is at approximately 5 minutes) for both cirrhotic and pooled normal plasma. The bulk of the thrombin was formed after clot formation. On addition of rFVIIa to both cirrhotic and pooled normal plasma,

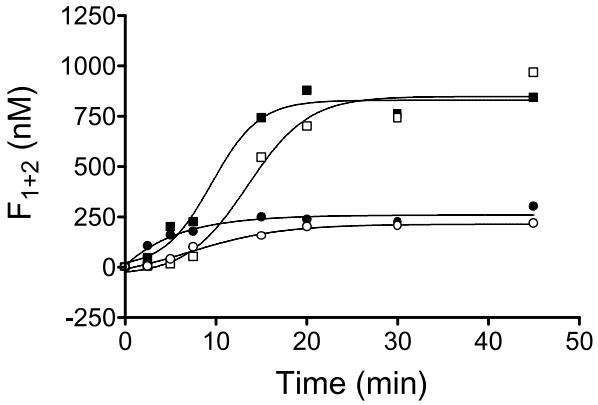

Figure 3. Prothrombin fragment 1+2 formation during clot lysis assay in pooled normal plasma (squares) or cirrhotic plasma (circles) in absence (open symbols) and presence of recombinant factor VIIa (rFVIIa; 60 U/ml; closed symbols).

prothrombin activation increased in the first 15 minutes, but was not increased at later time-points, when all available prothrombin had been converted. The amount of F_{1+2} formed in cirrhotic plasma was much smaller than that in pooled normal plasma, due to the low prothrombin level in the cirrhotic plasma.

TAFI antigen levels during and after OLT

Figure 4A shows TAFI antigen levels at 6 selected time-points during and after OLT of six patients who did not receive rFVIIa. TAFI levels decreased during the anhepatic and post-reperfusion phases, and rapidly normalized after surgery. Figure 4B shows TAFI antigen levels in 6 patients undergoing OLT after a single bolus dose of rFVIIa. Although basal levels of TAFI were lower in this group, a similar pattern of the course of TAFI antigen levels during and after the transplantation was observed in both groups. The course of TAFI antigen levels was comparable to levels of other proteins involved in coagulation and fibrinolysis such as antithrombin (figures 4C and D) plasminogen and α_2-antiplasmin (data not shown), and represent the improvement of the synthesis function of the liver.

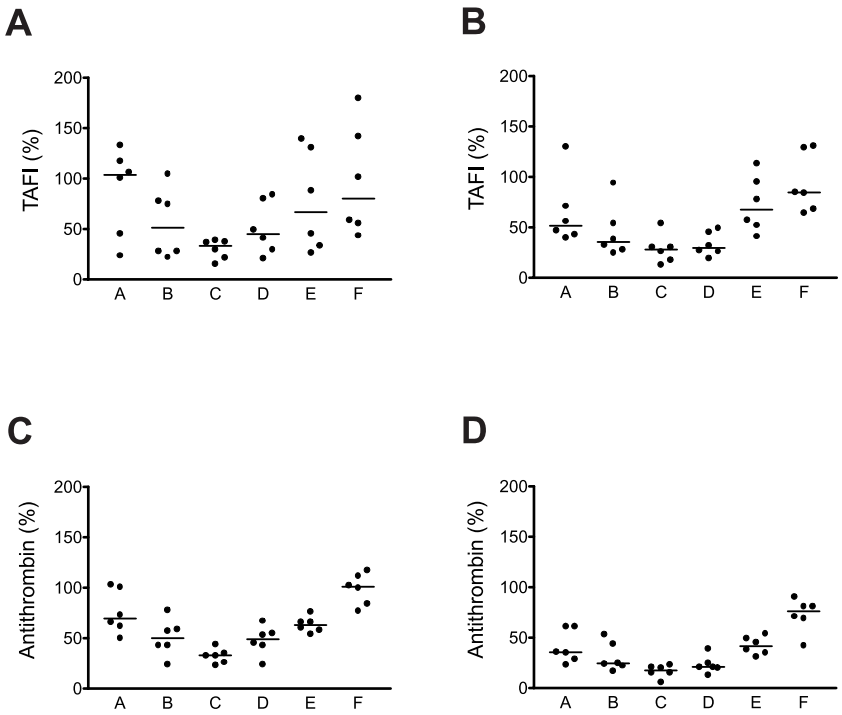

Figure 4. TAFI antigen and antithrombin activity levels during and after OLT performed without (panels A and C) or with (panels B and D) a single bolus dose of recombinant factor VIIa (rFVIIa; 80 μg/kg; 4000 U/kg). TAFI and antithrombin levels were measured in samples taken 30 minutes after the induction of anesthesia (A), 30 minutes after the start of the anhepatic phase (B), 30 minutes after reperfusion (C), at the end of surgery (D), at day 1 (E), and day 5 after surgery (F). rFVIIa was injected 10 minutes before the start of the surgery (between time points A and B). In the rFVIIa-treated group time point B corresponds to 4.5 hours (median; range 4-5.75), time point C to 7 hours (range 5.5-8.5), and time point D to 9 hours (range 7.75-11.75) after rFVIIa infusion. The duration of the different phases in the control group was similar. The horizontal bars represent medians.

Clot lysis times during and after OLT

Clot lysis times were determined in plasma samples taken at 6 time-points during and after OLT in the presence or absence of a specific inhibitor of activated TAFI. Figure 5A shows clot lysis times of patients who did not receive rFVIIa. Clot lysis times decreased during the anhepatic and post-reperfusion phase, indicating the development of a hyperfibrinolytic state. At the end of surgery, clot lysis times increased, and most samples showed no lysis at all during the time course of our experiment. The inhibition of clot lysis during this phase was associated by a transient

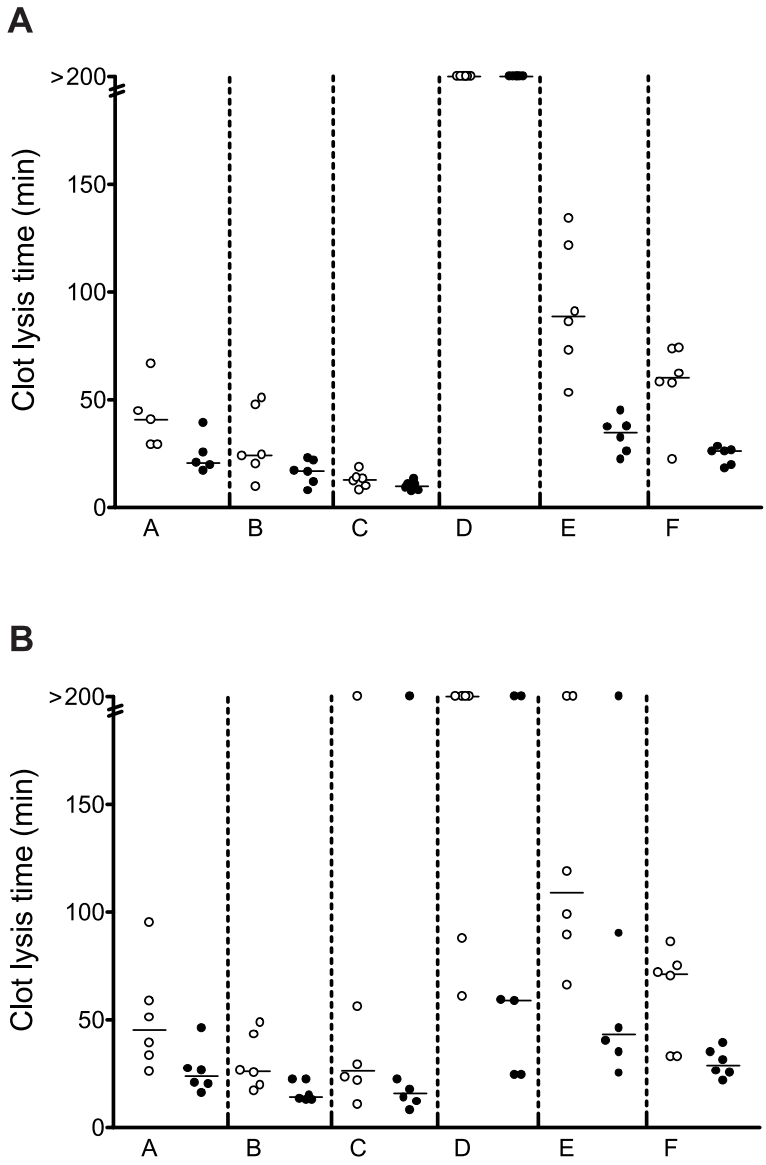

Figure 5. Clot lysis times during and after OLT performed without (panel A) or with (panel B) a single bolus dose of recombinant factor VIIa (rFVIIa; 80 μg/kg; 4000 U/kg) in the absence (open symbols) or presence (closed symbols) of the specific TAFIa inhibitor CPI. Clot lysis times were determined in samples taken 30 minutes after the induction of anesthesia (A), 30 minutes after the start of the anhepatic phase (B), 30 minutes after reperfusion (C), at the end of surgery (D), at day 1 (E), and day 5 after surgery (F). The horizontal bars represent medians.

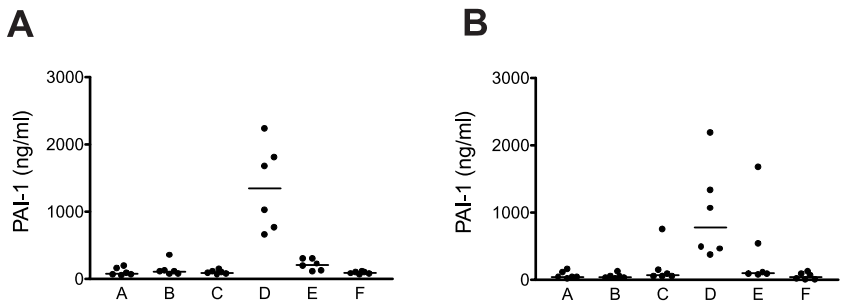

Figure 6. Plasminogen activator inhibitor type 1 (PAI-1) antigen levels during and after OLT performed without (panel A) or with (panel B) a single bolus dose of recombinant factor VIIa (rFVIIa; 80 μg/kg; 4000 U/kg). PAI-1 levels were determined in samples taken 30 minutes after the induction of anesthesia (A), 30 minutes after the start of the anhepatic phase (B), 30 minutes after reperfusion (C), at the end of surgery (D), at day 1 (E), and day 5 after surgery (F). The horizontal bars represent medians.

increase in plasminogen activator inhibitor-1 (PAI-1) levels as shown in figure 6. In a previous report we have shown that very high PAI-1 levels can completely inhibit clot lysis[212]. At day 5 after surgery clot lysis times were still above basal levels. As shown in figure 5B, the rFVIIa treated patients showed the same pattern of clot lysis time during and after OLT as the control group, except for clot lysis times during the postreperfusion phase, which were longer in the rFVIIa treated group (not significant). In all samples, except for those who did not show any lysis during our experiment, clot lysis times could be significantly shortened by addition of a specific inhibitor of activated TAFI (CPI).

Discussion

The mechanism behind the efficacy of rFVIIa in patients suffering from cirrhosis[295] as well as patients undergoing OLT[169] is still unclear. We have explored the potential of rFVIIa to serve as an antifibrinolytic agent in plasma from non-bleeding cirrhotic patients. Also, the fibrinolytic potential of plasma samples taken during and after OLT, which was performed with or without the use of rFVIIa was assayed. This study shows, by using a plasma-based clot lysis assay, no evidence for enhanced activation of TAFI by therapeutic or even supratherapeutic doses of rFVIIa in these patient groups. On the other hand, rFVIIa does result in enhancement of the rate of clot formation, as reflected by a shortening of the clotting times.

Two separate stages of thrombin formation during tissue factor-induced coagulation can

be distinguished. The primary formation of thrombin results in cleavage of fibrinogen, and activation of factor XIII, leading to the assembly of a stable fibrin clot. After the fibrin clot is formed, thrombin formation continues via thrombin-mediated activation of factor XI and subsequent thrombin generation via the intrinsic pathway[198]. This secondary burst of thrombin results in the activation of TAFI[65].

In both control and cirrhotic plasma, primary thrombin formation was accelerated by the addition of rFVIIa. Acceleration of primary thrombin formation was observed both as a shortening of coagulation time as well as an increase in prothrombin fragment 1+2 release in the first 10 minutes of incubation. Basal coagulation times in our experimental set-up were similar in patient and control plasma, though prothrombin times of the patient plasma were significantly prolongued. The assay performed in this study is a very dilute prothrombin time assay. On dilution of the tissue factor source, clotting time is no longer dependent only on levels of the procoagulant proteins, but also on the levels of anticoagulants like antithrombin. It is likely that in our assay the decreases in procoagulant factors in cirrhotic plasma are compensated by the decreases in anticoagulant factors, resulting in clotting times comparable to control levels.

Our results show that secondary thrombin formation was already optimal for TAFI activation in both control and cirrhotic plasma because addition of rFVIIa did not result in an increase in clot lysis time. All available prothrombin is already converted after 30 minutes of incubation in both control and cirrhotic plasma in the absence or rFVIIa.

Although we have questioned the presence of a hyperfibrinolytic state in non-bleeding cirrhotic patients[212], we did observe development of a hyperfibrinolytic state in patients undergoing OLT, consistent with previous studies[285,318]. In this study we showed accelerated fibrinolysis during the anhepatic and postreperfusion phase of OLT. During these critical phases of OLT, TAFI levels, which were already decreased at baseline, decreased even more. However, TAFI was still contributing to down-regulation of fibrinolysis during these phases, as clot lysis times significantly decreased on addition of a specific TAFI inhibitor (CPI). At the end of surgery, a transient strong increase in plasma PAI-1 levels was seen, which leads to a complete inhibition of clot lysis. Clot lysis times in OLT patients who received a single bolus dose of rFVIIa were similar to those in controls, except in the postreperfusion phase in which a trend towards higher clot lysis times in the rFVIIa treated group was seen. Because rFVIIa levels during this phase had almost returned to baseline[169], this apparent difference is unlikely to be a consequence of a direct rFVIIa effect.

In this study we did not find any evidence for a rFVIIa-mediated downregulation of fibrinolysis in cirrhosis, both in stable patients as well as in patients undergoing OLT. The beneficial effect of rFVIIa during OLT can probably be mainly ascribed to its potential to enhance fibrin formation. The reduced thrombin inhibition due to low levels of antithrombin in cirrhotic patients facilitates enhancement of fibrin formation by rFVIIa. As treatment with rFVIIa does not alter the development of a hyperfibrinolytic state during anhepatic and post-reperfusion phase, a combination of rFVIIa and antifibrinolytics during OLT might be an option. However, it should be considered that this combination might increase the risk of thrombosis, particularly of the donor liver vessels. The absence of hyperfibrinolysis in cirrhotics limits the rational use of antifibrinolytics in stable cirrhosis.

Acknowledgements

The authors thank Dr R. Wallis for his generous gift of recombinant hirudin.

Chapter 6

Enhancement of fibrinolytic potential in vitro by anticoagulant drugs targeting factor Xa, but not by those inhibiting thrombin or tissue factor

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Abstract

Tissue factor-induced coagulation leads to the generation of a small amount of thrombin via tenase and prothrombinase activity, resulting in the formation of a fibrin clot. After the fibrin clot has formed, thrombin generation continues via a factor XI-dependent pathway. The resulting secondary burst of thrombin activates TAFI (thrombin activatable fibrinolysis inhibitor), leading to downregulation of fibrinolysis.

In this study the effect of anticoagulant drugs targeting different steps in the coagulation cascade on clot formation and subsequent breakdown was investigated using a plasma-based clot lysis assay. All drugs tested significantly delayed clot formation. However, only those drugs targeting factor Xa (tissue factor pathway inhibitor, the pentasaccharide compound fondaparinux sodium, and low molecular weight heparin) accelerated fibrinolysis. Anticoagulant drugs targeting tissue factor (active site inactivated factor VIIa) or thrombin (hirudin and the low molecular weight compound PPACK) did not affect clot lysis time. In accordance with these findings, it was shown that total thrombin generation, as quantified by the endogenous thrombin potential, was only affected by anticoagulant drugs targeting factor Xa when all drugs were used in a concentration with an equal potential to inhibit clot formation.

Induction of hyperfibrinolysis by anticoagulant drugs directed against factor Xa might be beneficial as increased clot breakdown might facilitate thrombolysis or prevent reocclusion. On the other hand, the induction of hyperfibrinolysis by these compounds might increase the risk of bleeding complications.

Introduction

Numerous approaches for the treatment or prevention of venous and arterial thromboembolism have become available in the last decades. Among those are inhibitors of platelet function[319], inhibitors of coagulation[320], and a diversity of thrombolytic agents[321]. Inhibition of thrombin generation is considered an important aspect of antithrombotic treatment. Traditionally, heparin or low molecular weight heparins have been used to downregulate the coagulation system. These agents function by enhancing the activity of antithrombin towards both factor Xa and thrombin.

Recently, a synthetic pentasaccharide (Org31540/SR90107A, fondaparinux sodium, Arixtra, PENTA), which selectively inhibits factor Xa in an antithrombin dependent manner has been introduced as an alternative to traditional low molecular weight heparins (LMWH's)[126]. Alternative strategies aiming at downregulation of

coagulation include direct inhibition of thrombin, by low molecular weight inhibitors such as D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone (PPACK)[127] or hirudin[128], or inhibition of the tissue factor pathway by active site inactivated factor VIIa (ASIS, FFRck-VIIa)[130] or recombinant full length tissue factor pathway inhibitor (rFL-TFPI)[131].

The application of the different novel anticoagulants in the clinic has not been fully established yet. An important complication of anticoagulant treatment is the development of bleeding complications[322]. Phase II and III studies have indicated differences between anticoagulant drugs with respect towards in vivo anticoagulant potency and risk to benefit ratio[323-325]. Anticoagulant-related bleeding can occur spontaneously, at sites of previous vascular damage (e.g., after surgery). Alternatively, it may be associated with concomitant drug use or comorbid disease such as cancer. Insight into the mechanism by which the different anticoagulants affect the coagulation system may assist treatment in case of bleeding complications.

A thus far underestimated consequence of anticoagulant treatment is its effect on the stability of the fibrin clot, and its resistance to breakdown by the fibrinolytic system. A recently discovered important determinant in clot resistance to fibrinolysis in in vitro sytems is the activation of thrombin activatable fibrinolysis inhibitor (TAFI)[63]. TAFI is activated by high concentrations of thrombin, which is generated after clot formation via thrombin-mediated activation of factor XI[65]. Alternatively, TAFI may be activated by the thrombin-thrombomodulin complex. The latter reaction is approximately 1200 times more efficient than the activation of TAFI by thrombin alone[66].

In vivo relevance of TAFI has been demonstrated in animal thrombosis models, in which inhibition of TAFI by carboxypeptidase inhibitor from potatoe resulted in facilitation of thrombolysis[311,326]. However, TAFI deficiency in mice failed to show an altered phenotype compared to control mice when challenged in different thrombosis models[105].

The effects of the different anticoagulants on the haemostatic system are usually investigated in laboratory assays, which have clot formation as the primary endpoint. However, at the time of clot formation, very little thrombin has been generated[41]. In this study, the effects of different anticoagulants, inhibiting tissue factor (VIIai), the TF-VIIa complex and Xa (TFPI), factor Xa (PENTA), factor Xa and thrombin (LMWH), or thrombin (PPACK and Hirudin) on secondary thrombin generation (i.e., the thrombin generated after clot formation) and TAFI activation have been investigated in an in vitro clot lysis assay. The outcome of these clot lysis experiments were confirmed by investigating total thrombin generation as measured by the

endogenous thrombin potential[327] in the presence of the mentioned anticoagulants.

Materials and methods.

Plasma samples

Pooled normal plasma was obtained by combining plasma from 10 healthy volunteers. Blood samples were obtained by venipuncture from the antecubital vein into 3.2% sodium citrate (9:1, v/v). To obtain platelet poor plasma, the samples were centrifuged twice at 2000g for 15 minutes. Plasma samples were stored at –70°C until use.

Materials

Tissue type plasminogen activator (t-PA) was from Chromogenix (Mölndal, Sweden). Recombinant human tissue factor (Innovin) was from Dade Behring GmbH (Marburg, Germany). Trypsin inhibitor from corn (CTI; >30.000 trypsin inhibitory units/ml) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). The fluorogenic substrate Z-GGR-AMC was from Bachem (Bubendorf, Switzerland).
Phospholipid vesicles consisting of 40% L-α-dioleoylphosphatidylcholine, 20% L-α-dioleoylphosphatidylserine and 40% L-α-dioleoylphosphatidylethanolamine (all from Sigma, St. Louis, MO, USA) were prepared according to Brunner[209] with minor modifications as described by van Wijnen[210]. Total phospholipid content of the vesicles was determined by phosphate analysis according to Rouser[211].

Anticoagulant drugs

Active site inactivated factor VIIa and recombinant full length TFPI (rFL-TFPI) were generous gifts from Drs. M. Kjalke and U. Hedner, TF/VIIa research, Novo Nordisk (Måløv, Denmark). Org31540/SR90107A was from NV Organon (Oss, The Netherlands). LMWH (Fragmin) was from KabiVitrum AB (Stockholm, Sweden). PPACK was obtained from Bachem (Bubendorf, Switzerland). Recombinant desulphato hirudin was a generous gift from Dr. R. Wallis (Ciba Geigy, Horsham, UK).

Clot lysis assay

Lysis of a tissue factor-induced clot by exogenous t-PA was studied by monitoring changes in turbidity during clot formation and subsequent lysis essentially as described, except that a different tissue factor concentration was used[198]. Fifty microliters of plasma was pipetted into Immulon-2 flatbottom microtiter plates (Dynatech Laboratories Inc, Chantilly, VA). These plates do not support contact

activation[316]. To further suppress contact activation CTI, a potent inhibitor of coagulation factor XIIa[317] was added to the plasma throughout this study. Fifty microliters of a mixture containing tissue factor (diluted Innovin, final dilution 1000 times), $CaCl_2$ (final concentration 17 mM), t-PA (final concentration 30 U/ml; 56 ng/ml), phospholipid vesicles (final concentration 10 μM), and CTI (final dilution 1:25) diluted in Hepes buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 3 mM $CaCl_2$, 0.1% BSA, pH 7.4) was added. After thorough mixing, turbidity at 405 nm was measured in time at 37°C in a Spectramax 340 kinetic microplate reader (Molecular Devices Corporation, Menlo Park, CA, USA). Coagulation time was defined as the time to reach the midpoint of clear to maximal turbid transition. Clot lysis time was defined as the time from the midpoint of the clear to maximum turbid transition, which characterises clot formation, to the midpoint of the maximum turbid to clear transition, which represents clot lysis.

Endogenous thrombin potential

The endogenous thrombin potential was determined essentially as described by Hemker et al[327]. In a 96-wells microtitre plate, 80 μl plasma was pipetted. A mixture of tissue factor (1/1000 dilution of innovin), phospholipids vesicles (final concentration 10 μM), CTI (final dilution 1:25), and one of the anticoagulant drugs was added in a total volume of 20 μl. The reaction was started by adding 20 μl of a solution containing 100 mM $CaCl_2$, and 5 mM of the fluorogenic substrate Z-GGR-AMC. Fluorescence was monitored in time in a microtiter plate fluorometer Fluoroskan Ascent, type 374 (Labsystems, Helsinki, Finland) with the excitation filter at 390 nm and the emission filter at 460 nm. The endogenous thrombin potential was determined as described[328].

Statistical analysis

Statistical analyses were performed using the GraphPad InStat (San Diego, USA) software package. Statistical significance of differences in clotting and clot lysis times upon addition of increasing concentrations of anticoagulants was determined by standard ANOVA with Dunnett's post-test. $P<0.05$ was considered statistically significant.

Results

Effect of different anticoagulant drugs on clot formation and clot lysis

Clot lysis assays were performed with increasing concentrations of active site inactivated factor VIIa. Clotting times and clot lysis times were derived from the obtained turbidity profiles. Figure 1A shows a dose dependent increase in clotting time upon addition of VIIai, which was statistically significant at VIIai concentrations of 5 ng/ml and higher ($p<0.01$ vs no VIIai). Clot lysis times showed a small decrease at higher concentrations of VIIai, but this was not statistically significant ($p>0.05$ for 5000 ng/ml VIIai vs no VIIai).

Figure 1B shows clotting times and clot lysis times obtained in the presence of increasing concentrations of recombinant full length tissue factor pathway inhibitor. Clotting times dose dependently increased, and a statistically significant increase in clotting time was observed at rFL-TFPI concentrations of 1 and 2 μg/ml ($p<0.05$ and $p<0.01$, respectively). Clot lysis times dose dependently decreased, with a statistically significant decrease at rFL-TFPI concentrations of 1 and 2 μg/ml ($p<0.01$).

In figure 1C, the effect of PENTA on clotting times and clot lysis times are shown. Clotting times were dose dependently increased, with statistical significance at a concentration of 1.6 anti Xa Units/ml ($p<0.05$) and higher ($p<0.01$). The clot lysis time was dose dependently decreased, with statistical significance at 0.8 anti Xa Units/ml and higher ($p<0.01$).

Effects of the low molecular weight heparin Fragmin on clotting and clot lysis times are shown in figure 1D. Clotting times dose dependently increased upon addition of LMWH. Statistical significance was reached at LMWH concentrations of 0.25 anti Xa U/ml and higher ($p<0.01$). A dose dependent decrease in clot lysis time was observed, which reached statistical significance at LMWH concentrations of 0.125 anti Xa U/ml and higher ($p<0.01$).

The thrombin inhibiting chloromethlyl ketone peptide PPACK dose dependently inhibited clotting time (figure 1E), with statistical significance reached at a concentration of 0.25 μM or higher ($p<0.01$). PPACK had no effect on clot lysis time in the range of concentrations tested.

Hirudin, the other thrombin inhibitor tested, showed similar results as PPACK. Clotting times were significantly prolonged at hirudin levels of 6.125 U/ml or higher ($p<0.01$), whereas no effect on clot lysis time was observed (figure 1F).

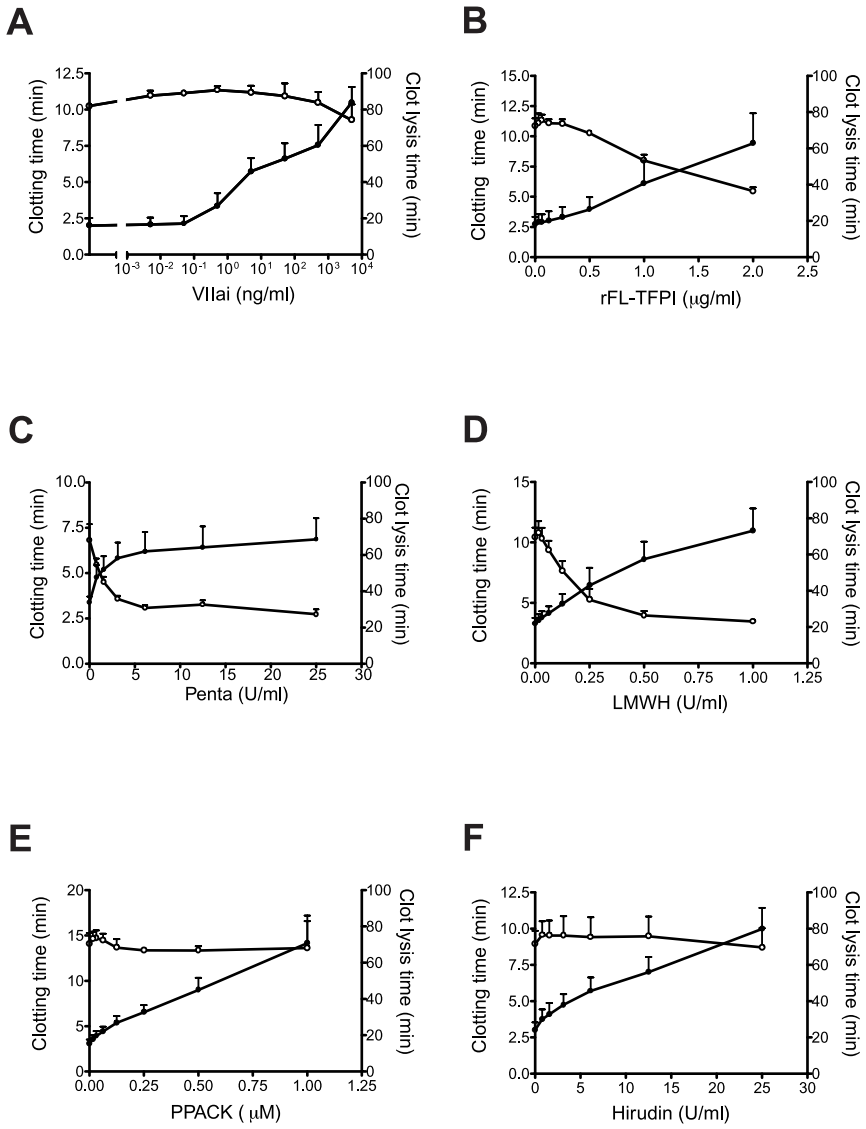

Figure 1. Effects of VIIai (A), rFL-TFPI (B), PENTA (C), LMWH (D), PPACK, and hirudin (E) on clot formation (closed symbols) and clot lysis time (open symbols). Coagulation was initiated in pooled normal plasma by the addition of tissue factor (Innovin, 1:1000), calcium chloride (17 mM), and phospholipid vesicles (10 μM), and fibrinolysis was induced by the addition of tPA (30 U/ml). Clot lysis profiles were measured for increasing concentrations of the different anticoagulants. Shown are the mean values obtained from three independent experiments. Error bars indicate standard deviation.

Endogenous thrombin potential in the presence of anticoagulant drugs

We next tested the 6 anticoagulants using a fixed concentration of each anticoagulant which results in an approximate doubling of coagulation time as derived from clot lysis curves (figure 2A). Under these conditions, clot lysis times are significantly decreased for TFPI, PENTA, and LMWH, but not for VIIai, PPACK, and Hirudin (figure 2B). Similarly, as shown in figure 2C, endogenous thrombin potential (ETP) is decreased by TFPI ($77 \pm 12\%$ of ETP in absence of anticoagulant drug), PENTA ($26 \pm 6\%$), and LMWH ($54 \pm 9\%$), whereas the ETP did not significantly change by VIIai ($110 \pm 17\%$), PPACK (90 ± 30), and hirudin ($98 \pm 14\%$). The thrombin generation curve did show a shift to the right for all anticoagulants, confirming that clot formation was inhibited for all compounds.

Discussion

This in vitro study shows that anticoagulant drugs targeting factor Xa induce enhancement of plasma fibrinolytic potential due to attenuation of TAFI activation. Anticoagulant drugs exclusively inhibiting tissue factor or thrombin do not show an effect on fibrin clot lysis in the assay set-up employed in this study. All drugs tested are potent inhibitors of clot formation, for which only a relatively small amount of thrombin is required. The bulk of the thrombin is generated after formation of the fibrin clot[39,41]. The generation of the thrombin burst after clot formation is required for TAFI activation. Direct inhibition of tissue factor or thrombin apparently fails to prevent or diminish this secondary thrombin generation, and thereby fails to induce hyperfibrinolysis.

The effect of the different anticoagulants on secondary thrombin generation was investigated by measurements of the (extrinsic) endogenous thrombin potential (ETP)[327]. The ETP measures the total amount of 'active' thrombin formed in a plasma sample upon activation. Indeed, as hypothesized from the clot lysis experiments, only the drugs targeting factor Xa resulted in a significant decrease in ETP when concentrations of the drugs were used which were equally potent in delaying clot formation. In these experiments we found an excellent correlation between acceleration of TAFI-dependent clot lysis and the ETP, but it is uncertain whether these two parameters are exchangeable in all situations. It is not yet known how much active thrombin at what stage during clot lysis is required for TAFI activation, and it is therefore conceivable that in certain situations discrepancies between clot-lysis experiments and ETP values may occur.

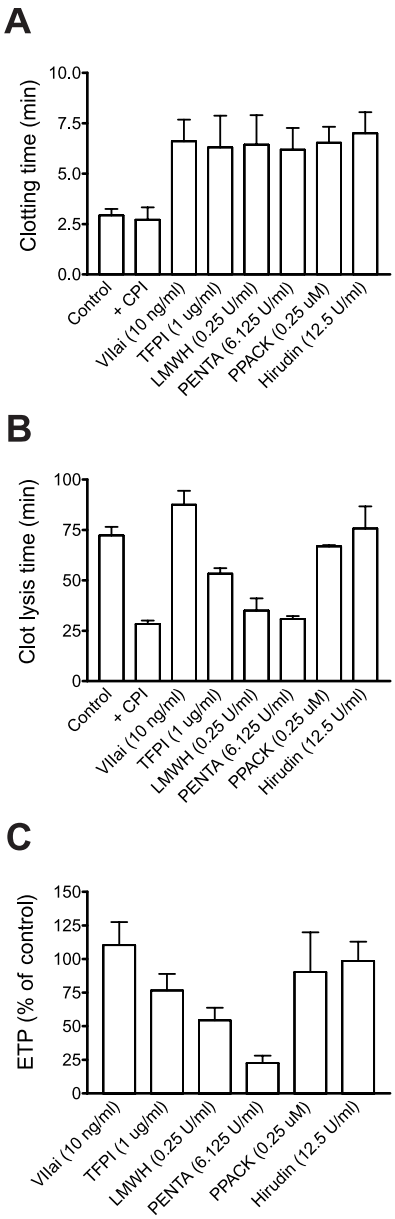

Figure 2. A. From figures 1 A-F, a single concentration of each anticoagulant, which resulted in an approximate equal prolongation of coagulation time, was selected. B. Clots lysis times under these conditions were significantly shortened for TFPI, LMWH, and PENTA, but not for VIIai, PPACK, and hirudin. C. The endogenous thrombin potential was determined for the different anticoagulants at a single concentration. Shown are mean values of three independent experiments. Error bars indicate standard deviation.

Why does only inhibition of factor Xa affect secondary thrombin generation? Inhibition of the TF pathway does not attenuate secondary thrombin generation as this process depends on factor XIa mediated activation of the intrinsic pathway of coagulation[198]. Once a small amount of thrombin is generated via the TF pathway, thrombin amplifies its own generation in a TF-independent manner. The reason why direct thrombin inhibitors fail to inhibit secondary thrombin generation is, at first sight, less obvious. Most likely, the inhibitory activity of direct thrombin inhibitors towards in situ generated thrombin is simply not fast enough to prevent explosive thrombin generation as already suggested by Lindhout et al.[329], who did observe a decrease in thrombin potential upon addition of hirudin to normal plasma, but high concentrations were required. In addition, Mohri and coworkers previously described a complete lack of effect of hirudin on thrombin potential[330].

Although the physiological relevance of TAFI activation and its inhibition by anticoagulant drugs targeting factor Xa remains uncertain, our findings may have clinical consequences. On one hand, induction of hyperfibrinolysis by anticoagulant drugs may be beneficial in certain clinical conditions as prevention or treatment of thrombus formation is accomplished by two mechanisms (i.e., prevention of clot formation and facilitation of clot breakdown). On the other hand, the induction of hyperfibrinolysis could lead to an increased risk of bleeding. This could imply that bleeding episodes induced by factor Xa inhibitors could be managed by antifibrinolytic agents like aprotinin or ε-aminocaproic acid.

In conclusion, we have shown that anticoagulant drugs targeting factor Xa have both anticoagulant and profibrinolytic properties. The clinical relevance of this finding warrants further investigation.

Acknowledgements

The authors like to thank Dr. M. Johannessen and Ms. V.E. Nielsen (Novo Nordisk, Gentofte, Denmark) for their kind assistance in performing the thrombin generation experiments. We would also like to thank Drs. U. Hedner and M. Kjalke (Novo Nordisk, Måløv, Denmark) for their generous gifts of VIIai and rFL-TFPI, and Dr. R. Wallis (Ciba Geigy, Horsham, UK) for the generous gift of recombinant hirudin.

Chapter 7

Recombinant factor VIIa reverses the anticoagulant and profibrinolytic effects of fondaparinux in vitro

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Abstract

Org31540/SR90107A (fondaparinux sodium, PENTA) is a synthetic pentasaccharide, which selectively inhibits factor Xa. PENTA has recently been registered in Europe and the United States for prevention of venous thromboembolism following hip fracture, hip replacement, and knee replacement surgery. However, an antidote in case bleeding complications occur has not yet been established. In this study, we have explored the effects of recombinant factor VIIa on the defects in both clot formation and TAFI-mediated downregulation of fibrinolysis induced by PENTA in vitro. In vitro clot lysis assays were performed in plasma from healthy volunteers to which PENTA was added. Addition of PENTA to pooled normal plasma significantly delayed clot formation whereas clot lysis was significantly enhanced due to decreased activation of thrombin activatable fibrinolysis inhibitor (TAFI). Addition of recombinant factor VIIa corrected the defect in clot formation induced by PENTA, and the defective TAFI activation was partially restored. On addition of PENTA to individual plasma samples of 35 healthy volunteers, a heterogeneous response with respect to clot lysis time was observed. The decrease in clot lysis time on addition of PENTA was correlated with endogenous factor VII levels. In conclusion, recombinant factor VIIa might be a good therapeutic option in patients treated with PENTA who develop bleeding complications, since both clot formation as well as fibrinolytic resistance are improved.

Introduction

The synthetic pentasaccharide Org31540/SR90107A (Fondaparinux sodium, Arixtra, PENTA; Sanofi-Synthelabo, Paris, and NV Organon, Oss, The Netherlands) is a novel, chemically synthesized antithrombotic drug, which exerts its antithrombotic effect by selective inhibition of coagulation factor Xa activity in an antithrombin dependent manner[331]. Recent clinical studies suggested that PENTA has superior qualities over other low-molecular-weight heparins with respect to risk-benefit ratio in preventing venous thromboembolism after hip replacement surgery[332,333], hip-fracture surgery[325], and elective major knee surgery[334], for which PENTA has been recently registered in Europe and the United States. Also, PENTA was shown to be effective for treatment of proximal vein thrombosis[335], as adjunctive therapy in acute myocardial infarction[336], and to prevent abrupt vessel closure during percutaneous transluminal coronary angioplasty[337].

A possible adverse event associated with the administration of heparin-like

anticoagulants is the risk of bleeding[322]. Therapeutical options in patients treated with PENTA who develop bleeding complications are until now limited. We hypothesize that the administration of recombinant factor VIIa (rFVIIa) could reverse the anticoagulant effects of PENTA. rFVIIa has been originally developed for patients with haemophilia A or B with inhibitory antibodies[195]. Recently, however, it has been suggested that rFVIIa could become a general haemostatic agent as it has shown to be effective in arresting bleedings or limit blood loss in various clinical settings[162,169,174,180,338].

In a previous study we have shown that rFVIIa, in addition to its procoagulant properties, can function as an antifibrinolytic agent when added to plasma from patients with severe haemophilia A by means of enhancing the activation of thrombin activatable fibrinolysis inhibitor (TAFI)[215]. As TAFI is activated by high concentrations of thrombin[65], we speculated that PENTA might not only function as an anticoagulant but also as a profibrinolytic agent by attenuating TAFI activation.

In this study, we investigated the effect of rFVIIa on clot formation and on TAFI mediated down-regulation of fibrinolysis in plasma from healthy individuals to which PENTA was added.

Materials and methods

Plasma samples

Pooled normal plasma was obtained by combining plasma from 40 healthy volunteers. Individual plasma samples from 35 healthy laboratory volunteers were also used in this study.

Blood samples were obtained by venipuncture from the antecubital vein into 3.2% sodium citrate (9:1, v/v). To obtain platelet poor plasma, the samples were centrifuged twice at 2000g for 15 minutes. Plasma samples were stored at –80°C until use.

Materials

Recombinant factor VIIa (NovoSeven®) was a generous gift from Novo Nordisk A/S (Bagsvaerd, Denmark). Fondaparinux sodium was from NV Organon (Oss, The Netherlands). Tissue type plasminogen activator (t-PA) was from Chromogenix (Mölndal, Sweden). Recombinant human tissue factor (Innovin) was from Dade Behring GmbH (Marburg, Germany), and carboxypeptidase inhibitor from potato (CPI) was purchased from Calbiochem (La Jolla, CA). Phospholipid vesicles consisting of 40% L-α-dioleoylphosphatidylcholine, 20% L-α-dioleoylphosphatidylserine and 40% L-α-dioleoylphosphatidylethanolamine (all from Sigma, St. Louis, MO) were prepared

according van Wijnen[210]. Total phospholipid content of the vesicles was determined by phosphate analysis according to Rouser[211]. Factor VII, factor V, factor X, and factor II activity levels were determined by a one-stage clotting assay using factor VII deficient plasma from Helena Laboratories (Beaument, TX, USA), and factor V, X and II deficient plasma from Boeringer Mannheim GmbH (Mannheim, Germany). In these assays, thromborel S (Dade-Behring, Mannheim, Germany) was used as tissue factor source. Antithrombin levels were determined using the Coamatic antithrombin kit from Chromogenix (Mölndal, Sweden). TAFI antigen levels were determined by a sandwich-type ELISA as described[206].

Clot lysis assay

Lysis of a tissue factor-induced clot by exogenous t-PA was studied by monitoring changes in turbidity during clot formation and subsequent lysis essentially as described previously[212], except for a change in tissue factor concentration. Fifty microliters of plasma was pipetted into a microtiter plate. Fifty microliters of a mixture containing tissue factor (diluted Innovin, final dilution 1000 times), $CaCl_2$ (final concentration 17 mM), t-PA (final concentration 30 U/ml; 56 ng/ml), and phospholipid vesicles (final concentration 10 μM), diluted in Hepes buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 3 mM $CaCl_2$, 0.1% BSA, pH 7.4) was added. After thorough mixing, turbidity at 405 nm was measured in time at 37°C in a Spectramax 340 kinetic microplate reader (Molecular Devices Corporation, Menlo Park, CA, USA). Coagulation time was defined as the time to reach the midpoint of clear to maximal turbid transition. Clot lysis time was defined as the time from the midpoint of the clear to maximum turbid transition, which characterises clot formation, to the midpoint of the maximum turbid to clear transition, which represents clot lysis. To assess the contribution of TAFI activation to clot lysis time, experiments were performed in which CPI (25 μg/mL), a specific inhibitor of activated TAFI[199], was added to the plasma.

Statistical analysis

Statistical analysis was performed using the GraphPad InStat (San Diego, USA) software package. Differences in clotting time and clot lysis time in pooled normal plasma was determined by standard t-test. Differences in clotting time and clot lysis time in the 35 individual samples were assayed by repeated measures one-way analysis of variance (ANOVA) using the Tukey-Kramer post-test. Statistical significance of linear correlations was determined by Pearson's correlation coefficient. P values <0.05 were considered statistically significant.

Results

PENTA delays clot formation and accelerates clot breakdown in pooled normal plasma
The effect of PENTA in a plasma system in which coagulation was initiated with a diluted prothrombin reagent as tissue factor source was investigated. As shown in figure 1A, PENTA dose-dependently inhibited clot formation in pooled normal plasma (open circles). In the same assay, the effect of PENTA on clot lysis induced by exogenous t-PA was investigated. Figure 1B shows that PENTA dose-dependently decreased clot lysis time (open circles). Simultaneous experiments were performed in which CPI, a specific inhibitor of activated TAFI, was added to the plasma (figure 1B, squares). PENTA only affected clot lysis time in the presence of TAFI activity.

rFVIIa accelerates clot formation and enhances TAFI-mediated down-regulation of fibrinolysis in plasma containing PENTA
On addition of rFVIIa (40 nM) to pooled normal plasma to which PENTA was added, clotting times were significantly lowered over the whole range of PENTA tested (figure 1A, closed circles). Clot lysis time in PENTA-containing plasma was prolongued by rFVIIa (figure 1B closed circles). A significant prolongation of clot lysis time was observed for PENTA levels above 1.4 μg/ml.

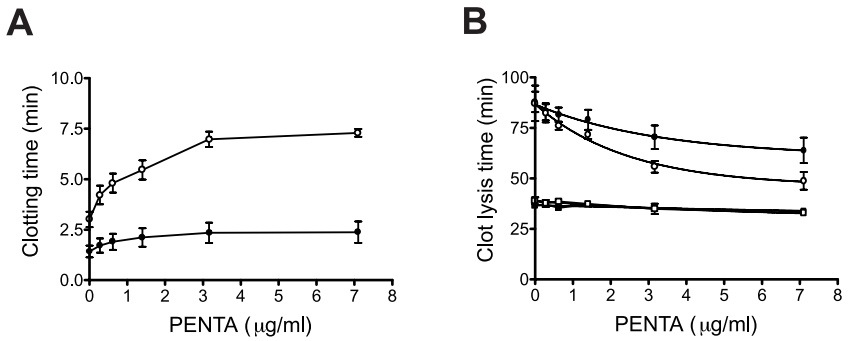

Figure 1. Defective clot formation and TAFI-mediated down-regulation of fibrinolysis induced by PENTA can be improved by rFVIIa. Panel A shows the effect of PENTA on clotting time in absence (open symbols) or presence (closed symbols) of rFVIIa (40 nM). Panel B shows the effect of PENTA on clot lysis time in absence (circles) or presence (squares) of CPI, and in absence (open symbols) or presence (closed symbols) of rFVIIa (40 nM). The mean of 3 independent experiments is shown. Error bars indicate standard deviation.

The profibrinolytic potential of PENTA varies between individuals; effects of rFVIIa

The effect of addition of PENTA (3.5 μg/ml), rFVIIa (40 nM), and a combination of both on clot formation and fibrinolytic potential was investigated in plasma samples from 35 healthy volunteers. We observed that addition of a fixed concentration of PENTA reduced clot lysis time in some, but not all plasma samples. Therefore, we separately analysed samples that gave more than 5% reduction of clot lysis time on addition of 3.5 μg/ml PENTA (high responders; figure 2, panels A and C), and samples

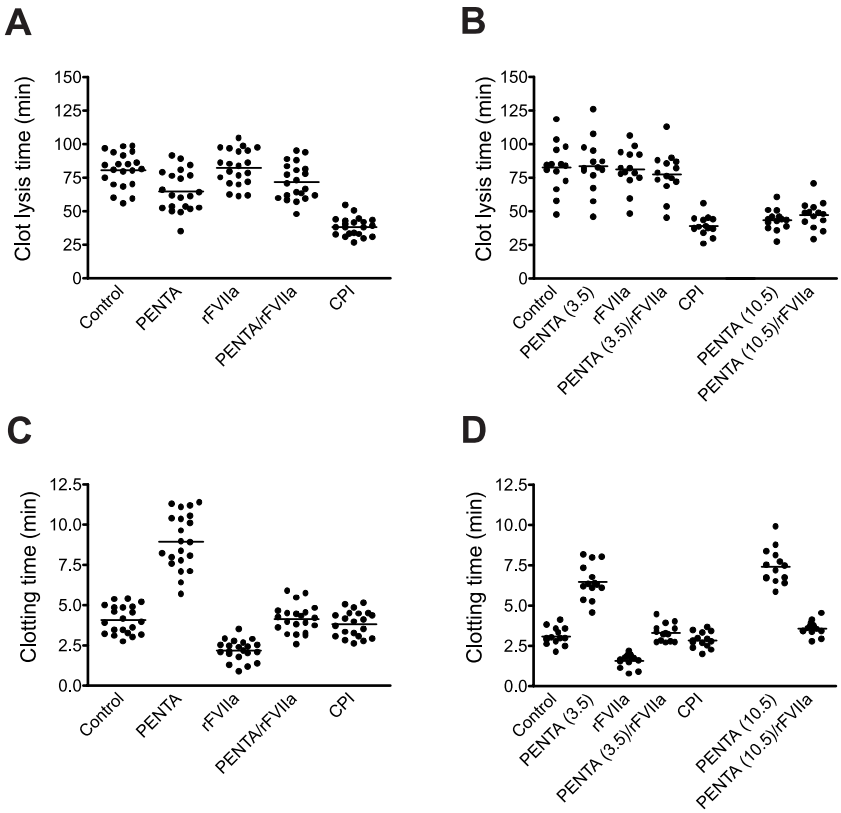

Figure 2. Effect of PENTA (3.5 μg/ml), rFVIIa (40 nM), a combination of PENTA and rFVIIa, and CPI (25 μg/ml) on clot lysis time and clotting time in plasma of 35 healthy volunteers. Panels A and C show clot lysis times and clotting times, respectively, of plasma samples which gave a more than 5% reduction of clot lysis time on addition of PENTA. Panels B and D show clot lysis times and clotting times, respectively, of plasma samples which showed a less than 5% reduction of clot lysis time on addition of PENTA. Panels B and D also show the effect of a high concentration of PENTA (10.5 μg/ml), and of a combination of a high concentration of PENTA in combination with rFVIIa (40 nM) on clot lysis time and clotting time.

that gave less than 5% reduction of clot lysis time on addition of 3.5 μg/ml PENTA (low responders; figure 2, panels B and D).

Figure 2A shows clot lysis times from the high responders. The decrease in clot lysis time on addition of PENTA was statistically significant (Control 80 ± 13 min, PENTA 65 ± 15 min; mean ± SD, $p<0.001$). On addition of rFVIIa to PENTA anticoagulated plasma in this group, a significant increase in clot lysis time was seen (PENTA 65 ± 15 min, PENTA + rFVIIa 72 ± 13 min, $p<0.01$). Addition of rFVIIa in the absence of PENTA had no effect on clot lysis time. On addition of CPI, clot lysis times decreased to 38 ± 7 min, indicating that PENTA only partially decreased TAFI activation.

In plasma samples from the low responders, addition of rFVIIa to plasma containing 3.5 μg/ml PENTA did not change clot lysis time (figure 2B). However, when 10.5 μg/ml PENTA was added to low responder plasma, a decrease in clot lysis time was seen, which could be partially corrected by the addition of rFVIIa (figure 2B, right panel; 10.5 μg/ml PENTA 43 ± 7 min, 10.5 μg/ml PENTA + rFVIIa 47 ± 10, $p=0.02$).

In both groups, addition of rFVIIa significantly decreased clotting time, clotting time increased significantly on addition of PENTA, and a combination of rFVIIa and PENTA resulted in clotting times comparable to the control situation.

On evaluation of clotting times of the two groups we found that in the high responder group, clotting times in the control situation were significantly higher compared to clotting times in the low responder group (4.07 ± 0.86 min vs. 3.08 ± 0.53 min, $p<0.001$). Clotting times were significantly correlated with the percentage of reduction of clot lysis time on addition of PENTA ($r=0.6076$, $p=0.0001$).

Endogenous factor VII levels determine the extent of acceleration of fibrinolysis by PENTA in individual plasma samples

Plasma levels of factors V, X, II, antithrombin and TAFI were not correlated with the percentage of reduction of clot lysis time on addition of PENTA. Also, no correlation was found between plasma levels of factors V, X, II and antithrombin and clotting time in the control situation.

Only plasma levels of factor VII were significantly correlated with the percentage of reduction of clot lysis time on addition of PENTA ($r= -0.4069$, $p=0.015$). Moreover, plasma levels of factor VII were significantly correlated with clotting time in the control situation ($r= -0.3680$, $p=0.03$).

Discussion

Addition of PENTA to plasma from healthy volunteers leads to a delay of tissue factor induced clot formation, and to acceleration of fibrinolysis due to diminished TAFI activation.

The observation that inhibition of coagulation factor X results in inhibition of TAFI activation is in accordance with a previous study by Broze and Higuchi, who showed premature fibrinolysis in plasma deficient in either one of the intrinsic coagulation factors[200]. It was postulated that haemophilia is not only a disorder of coagulation, but that defective TAFI activation might also cause part of the bleeding tendency observed in these patients. Likewise, part of the bleeding diathesis of patients anticoagulated with heparin derivatives, may be due to induction of hyperfibrinolysis as a consequence of defective activation of TAFI.

In this study, we have shown that addition of rFVIIa to plasma containing PENTA not only leads to a profound acceleration of clot formation, but also to an improvement of TAFI-mediated inhibition of fibrinolysis.

Interestingly, the extent of reduction of clot lysis time induced by PENTA varied strongly between samples of healthy individuals. On addition of a fixed concentration of PENTA, accelerated fibrinolysis was seen in some (high responders), but not all (low responders) plasma samples from healthy volunteers, and an effect of rFVIIa on clot lysis time was only observed in those plasma samples in which PENTA accelerated clot lysis. This high responder/low responder phenomenon appeared to be correlated with clotting times in the absence of PENTA (i.e., basal thrombin generating capacity). A larger basal thrombin generating capacity indicated less inhibition of TAFI activation by the fixed concentration of PENTA. The basal clotting time and thus the pro-fibrinolytic capacity of PENTA was correlated to plasma levels of factor VII. Thus, it seems that the capacity of a plasma sample to generate thrombin determines the extent of acceleration of fibrinolysis by PENTA (i.e., higher levels of factor VII increase basal thrombin generating capacity and consequently decrease inhibition of TAFI activation). Therefore, the bleeding risk associated with the administration of PENTA might be increased in patients with a low basal thrombin generating capacity, as this determines the extent of inhibition of TAFI activation by PENTA. On the other hand, PENTA dosage might have to be increased to reach an optimal therapeutic effect in patients with a high basal thrombin generating capacity (for example caused by classical thrombophilic factors such as FV_{Leiden}, $FII_{G20210A}$, and high levels of FVIII).

In conclusion, rFVIIa may be a useful therapeutic option in those instances where patients treated with PENTA develop bleeding complications as both clot formation and fibrinolytic resistance of a clot are improved.

Chapter 8

Recombinant factor VIIa enhances deposition of platelets with congenital or acquired $\alpha_{IIb}\beta_3$ deficiency to endothelial cell matrix and collagen under conditions of flow via tissue factor-independent thrombin generation.

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Submitted for publication

Abstract

A novel approach to treat bleeding episodes in patients with Glanzmann's thrombasthenia (GT) and perhaps also in patients receiving $\alpha_{IIb}\beta_3$-inhibitors, is the administration of recombinant factor VIIa (rFVIIa). The mechanism of action of rFVIIa in these patients is, however, still unclear. We studied the effect of rFVIIa-mediated thrombin formation on adhesion of $\alpha_{IIb}\beta_3$-deficient platelets under flow conditions. Adhesion of $\alpha_{IIb}\beta_3$-deficient platelets to the extracellular matrix (ECM) of stimulated human umbilical vein endothelial cells or to collagen type III was studied using a model system with washed platelets and red cells. When $\alpha_{IIb}\beta_3$-deficient platelets were perfused over the surface at arterial shear rate for 5 minutes, a low surface coverage was observed (GT platelets: mean $\pm$ SEM $37.5 \pm 5.0\%$, normal platelets preincubated with an RGD-containing peptide: $7.4 \pm 2.1\%$). When rFVIIa, together with factors X and II was added to the perfusate, platelet deposition significantly increased (GT platelets: mean $\pm$ SEM $67.0 \pm 4.3\%$, normal platelets preincubated with an RGD-containing peptide $48.2 \pm 2.9\%$). The same effect was observed when normal platelets were pretreated with the commercially available anti-$\alpha_{IIb}\beta_3$ drugs abciximab, eptifibatide, or tirofiban. It was shown that tissue factor-independent thrombin generation (presumably induced by binding of rFVIIa to adhered platelets) was responsible for the increase in platelet deposition. In conclusion, defective adhesion of $\alpha_{IIb}\beta_3$-deficient platelets to ECM can be restored by tissue factor-independent rFVIIa-mediated thrombin formation. The enhanced generation of platelet procoagulant surface facilitates fibrin formation, so that lack of platelet aggregate formation might be compensated for.

Introduction

Platelets play a crucial role in hemostasis and in thrombotic processes. Upon vessel wall injury, platelets adhere to the subendothelium through the interaction of glycoprotein Ib with von Willebrand factor (vWF) bound to subendothelial collagen. Stable adhesion is subsequently accomplished by binding of platelet integrin receptors such as $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_v\beta_3$ to their ligands present in the subendothelium. When stable adhesion is accomplished, secretion and activation of the platelets takes place, followed by aggregation through bridging of vWF or fibrinogen to $\alpha_{IIb}\beta_3$ on two different platelets[11].

The importance of $\alpha_{IIb}\beta_3$ in platelet functioning is demonstrated by the bleeding

tendency of patients with Glanzmann's thrombasthenia (GT), who have a congenital qualitative or quantitative defect in this receptor[339]. Also, inhibition of $\alpha_{IIb}\beta_3$ by anti-platelet drugs such as abciximab, eptifibatide, and tirofiban is of proven benefit to patients suffering from acute coronary artery disease[340]. Although these $\alpha_{IIb}\beta_3$-antagonists are highly effective in preventing reocclusion after thrombolysis, stenting, or angioplasty, administration of these compounds also induces bleeding in a significant number of patients[341].

Traditionally, platelet concentrates are administered to patients with GT during bleeding episodes, or prophylactically during surgery. However, platelet concentrates carry the risk of alloimmunisation to human leukocyte antigens or to $\alpha_{IIb}\beta_3$, making further administration of donor platelets ineffective. Bleeding complications in patients receiving anti-$\alpha_{IIb}\beta_3$ drugs may be controlled simply by withdrawing infusion of the drug. Due to the relatively short half-life of these compounds[340], the induced platelet defect is rapidly reversed. When urgent bleeding problems occur, platelet transfusion has been shown to reverse abciximab's inhibitory effect. However, in case of eptifibatide and tirofiban, which are dosed in a way the peak concentration is very high relative to the amount of $\alpha_{IIb}\beta_3$ molecules present in circulation, transfused platelets are also inhibited rapidly after infusion by free circulating drug[340].

A novel approach to treat patients with GT during bleeding episodes or surgery is the administration of recombinant factor VIIa (rFVIIa, NovoSeven®, Bagsværd, Denmark)[162,342]. rFVIIa was originally developed for the treatment of inhibitor-complicated haemophilia A and B[145,343]. Currently, novel indications for rFVIIa, including its use in patients with liver disease[169,293], thrombocytopenia[344] and platelet function defects[162,163], and in patients without coagulation disorders who are bleeding as a result of extensive surgery or major trauma[174,180], are explored in clinical trials. The use of rFVIIa in patients with GT appears to be safe and effective, although randomised controlled clinical trials have not been performed in this small patient group. The apparent success of rFVIIa in GT may possibly be translated to patients who suffer from uncontrollable bleeding as a consequence of administration of anti-$\alpha_{IIb}\beta_3$ drugs. A single case in which rFVIIa was used for bleeding management of a patient treated with tirofiban has recently been reported[18].

The mechanism of action of rFVIIa in platelet related bleeding disorders is still a matter of debate. To explain the efficacy of rFVIIa in haemophilia, both tissue factor-dependent and independent enhancement of thrombin generation has been suggested to play a role[182,183,189,215]. At first sight, the efficacy of rFVIIa in platelet-related bleeding disorders is curious, because of the presence of a fully competent coagulation system in

these patients. However, it has been suggested that enhancement of thrombin generation may enhance recruitment of defective platelets to the site of injury as well as enhance fibrin deposition, thereby compensating for the platelet defect[190]. Thrombin has multiple actions on platelets, which are, at present, not fully understood. It has been proposed that the glycoprotein Ib/V/IX complex is an important thrombin receptor on platelets[345,346]. Thrombin binding to this complex initiates signalling events by enhancing activation of the classical thrombin receptor (PAR-1)[17], and it has been proposed to facilitate the cleavage of glycoprotein V from the complex, resulting in a hyperresponsive platelet[347,348]. Also, thrombin binding to GPIb appears essential for thrombin-mediated induction of platelet procoagulant activity[346]. Furthermore, thrombin is able to activate the low affinity thrombin receptor (PAR-4)[349], but whether binding to the glycoprotein Ib/V/IX complex also enhances PAR-4 cleavage is not known.

In this study we have generated a model to study the effect of rFVIIa-mediated thrombin generation on platelet adhesion under flow conditions in a model system using $\alpha_{IIb}\beta_3$-inhibited platelets. Using a model system of isolated platelets and red cells, and purified clotting factors, we show an enhancement of platelet adhesion to subendothelial material. Important denominators in thrombin-mediated enhancement of platelet deposition in our system were subsequently investigated.

Materials and methods

Proteins, antibodies, and anti-$\alpha_{IIb}\beta_3$ drugs

rFVIIa (NovoSeven®), a goat polyclonal inhibitory antibody against tissue factor, and a monoclonal antibody against factor VIIa were generous gifts from Dr. U. Hedner (Novo Nordisk, Måløv, Denmark). Factor X was purified from fresh frozen plasma by immunoaffinity chromatography followed by Q-sepharose chromatography as previously described[350] or purchased from Kordia B.V. (Leiden, The Netherlands). Prothrombin was purified from fresh frozen plasma according to Koedam et al.[351]. Collagen type III was from Sigma (St. Louis, Mo). Recombinant hirudin was a generous gift from R. Wallis (Ciba Geigy, Horsham, UK). Recombinant annexin V was a generous gift from Dr. W.L. van Heerde (University Medical Centre St. Radboud, Nijmegen, the Netherlands).

Inhibitory antibodies against glycoprotein Ib (AK-2, ascites fluid) and von Willebrand factor (RAG-35, ascites fluid) were generous gifts from Dr. M. Berndt (Baker Institute, Melbourne, Australia) and Dr. J.A. van Mourik (CLB, Amsterdam, The Netherlands),

respectively. Fab fragments of a monoclonal antibody, which specifically inhibits thrombin binding to glycoprotein Ib (LJIb-10) were a generous gift from Dr. Z.M. Ruggeri (The Scripps Research Institute, La Jolla, CA). Fluorescein isothiocyanate (FITC) labeled goat anti mouse IgG was purchased from Calbiochem (La Jolla, CA).
The RGD containing peptide D-arginyl-glycyl-L-aspartyl-L-tryptophane (dRGDW) was generously provided by Dr. J. Bouchaudon (Rhône Poulenc Rorer, Chemistry Department, Centre de Recherche de Vitry, Vitry sur Seine, France).
Abciximab (ReoPro) was from Centocor (Malvern, PA). Eptifibatide (Integrilin) was purchased from COR Therapeutics (South San Francisco, CA). Tirofiban (Aggrastat) was from Merck (White House Station, NJ).

Cell culture
Human umbilical vein endothelial cells were isolated and grown to confluence as described[352]. Cells of the second passage were seeded on gelatin coated thermanox coverslips. The cells were stimulated overnight with phorbol myristate acetate (PMA, Sigma, St. Louis, MO; 20 ng/ml final concentration) for 16 hours. After stimulation, the endothelial cell matrix was isolated by removing the cells with 0.1 mol/L NH_4OH for 15 minutes at room temperature, and subsequently the matrices were washed 3 times with phosphate buffered saline (PBS, 10 mmol/L phosphate buffer, 150 mmol/L NaCl, pH=7.4).

Collagen coated surfaces
Collagen type III was solubilized in 50 mM acetic acid and sprayed on thermanox or glass coverslips (the latter were used for immunofluorescence studies) using a retouching airbrush (Badger model 100, Badger Brush Co, Franklin Park, Il.) at a density of 30 $\mu g/cm^2$. After the spraying procedure, coverslips were blocked for 1 hour at room temperature with 4% human albumin in PBS.

Blood collection
Blood was drawn from healthy volunteers who denied ingestion of aspirin or other NSAID's for the preceding 10 days into 1/10 volume 3.4% sodium citrate. For selected experiments, blood from six unrelated patients with type 1 GT was used.

Perfusion studies

Perfusions were carried out in a single pass perfusion chamber as described previously[353]. Perfusions were carried out with reconstituted blood, which was prepared as follows. Platelet rich plasma (PRP) was prepared from whole blood by centrifugation (10 minutes at 200g at room temperature). The PRP was acidified by addition of 1/10 volume of ACD (2.5% trisodiumcitrate, 1.5% citric acid, and 2% D-glucose), and the platelets were spun down (500g, 15 min). The platelet pellet was resuspended in HEPES-Tyrode buffer (137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgCl_2$, 5 mM d-glucose, pH= 7.35). Prostacyclin (PGI_2, 10 ng/ml) was added to prevent platelet activation during the subsequent washing step. Platelets were spun down and resuspended in a small volume of HEPES-Tyrode buffer. The platelets were diluted in human albumin solution (HAS; 4% human albumin, 4 mM KCl, 124 mM NaCl, 20 mM $NaHCO_3$, 2 mM Na_2SO_4, 1.5 mM $MgCl_2$, 5 mM d-glucose, pH 7.35). Red cells were washed twice with 0.9% NaCl containing 5 mM d-glucose (2000g, 5 min), and finally cells were packed (2000g, 15 min).

Platelets were mixed with red cells to obtain reconstituted blood containing 200.000 platelets/μl and a haematocrit of 40%. The reconstituted blood was preincubated with buffer or clotting factors for 5 minutes at 37°C and perfused for 5 minutes at a shear rate of 1600 s^{-1}. After perfusion, slides were washed with HEPES buffer (10 mM Hepes, 150 mM NaCl, pH=7.35) and fixed in 0.5% glutaraldehyde in PBS. Subsequently, slides were dehydrated in methanol and stained with May-Grünwald and Giemsa as described previously[354]. Platelet adhesion was evaluated using computer-assisted analysis with OPTIMAS 6.0 software (DVS, Breda, The Netherlands), and was expressed as the percentage of the surface covered with platelets.

Immunofluorescence microscopy

To investigate direct binding of rFVIIa to platelets adhered under flow conditions, washed platelets and red cells were perfused over a collagen-coated surface (5 min at a shear rate of 1600 s^{-1}) in presence and absence of rFVIIa (1.2 μg/ml) and calcium chloride (5 mM). After perfusion, the coverslips were washed with HEPES buffer containing 5 mM calcium chloride and fixed with 3% paraformaldehyde and 0.002% glutardialdehyde in PBS. Subsequently, coverslips were washed with PBS, and blocked with 1% BSA, 0.1% glycine in PBS for 10 minutes at room temperature. Coverslips were incubated with a monoclonal anti FVII antibody (10 μg/ml in PBS) for 45 minutes at 37°C. After washing and subsequent blocking, coverslips were incubated with FITC-labeled goat anti mouse IgG (1:20 diluted in PBS) for 45 minutes at 37°C. After washing, coverslips were mounted

in Mowiol 40-80 containing 0.1% paraphenylenediamine. Bound rFVIIa was visualized using confocal laser scanning microscopy using Leica TCS 4D (Heidelberg, Germany) equipment.

Statistical analysis

Statistical analysis was performed using the GraphPad InStat (San Diego, USA) software package. Comparison of mean surface coverage values were undertaken by standard Student's t-test. $P<0.05$ was considered statistically significant.

Results

Enhancement of deposition of $\alpha_{IIb}\beta_3$-deficient platelets to PMA-stimulated endothelial cell matrix (PMA-ECM) upon addition of coagulation factors VIIa, X, and II

Washed platelets and red cells obtained from 6 unrelated patients with type I GT were perfused over PMA-ECM at 1600 s^{-1} for 5 minutes. In figure 1A, the morphological appearance of a typical experiment performed with GT platelets to PMA-ECM is presented. The surface coverage was $37.5 \pm 5.0\%$. (mean $\pm$ SEM of 16 coverslips; with 4 patients experiments were performed in triplicate, with the other 2 patients, experiments were performed in duplicate). No platelet aggregates were observed. Upon addition of a thrombin-generating system, consisting of rFVIIa (1.2 μg/ml), factor X (10 μg/ml), and

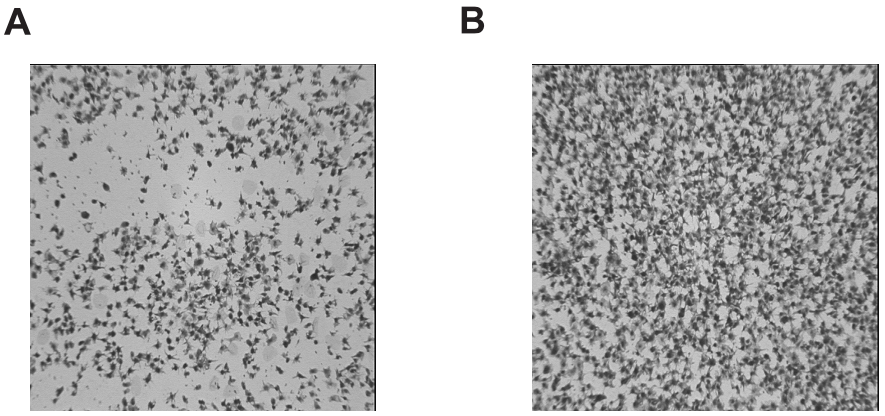

Figure 1. The effect of coagulation factors VIIa, X, and II on adhesion of platelets from patients with type I GT to the subendothelial matrix of PMA-stimulated endothelial cells under flow conditions. Washed platelets and red cells (200.000 platelets/μl; haematocrit 40%) were perfused over PMA-ECM for 5 minutes at 1600 s^{-1} in absence (panel A) or presence (panel B) of a thrombin-generating system (1.2 μg/ml rFVIIa, 10 μg factor X, 20 ng/ml prothrombin, 3 mM $CaCl_2$). Shown is a typical example from one GT patient.

prothrombin (20 ng/ml), a significant increase in platelet adhesion was observed ($67.0 \pm 4.3\%$; mean $\pm$ SEM of 16 coverslips, $p<0.0001$, figure 1B shows a typical example of the morphology). Platelet aggregates were still not formed.

Enhancement of platelet deposition to PMA-ECM in a model for GT upon addition of coagulation factors VIIa, X and II.

In order to study the mechanism by which addition of coagulation factors VIIa, X, and II lead to a significant increase in adhesion of $\alpha_{IIb}\beta_3$ deficient platelets to PMA-ECM, a model system for GT was created. Perfusion experiments were performed with platelets from healthy volunteers pretreated with a peptide containing the RGD sequence in order to block ligand binding to $\alpha_{IIb}\beta_3$. In absence of the RGD-containing peptide, perfusion of washed platelets and red cells over PMA-ECM at 1600 s^{-1} resulted in a high surface coverage ($69.2 \pm 3.0\%$, mean $\pm$ SEM of 6 coverslips), and extensive aggregate formation was observed (figure 2A). Addition of the dRGDW peptide (200 μM, final concentration in platelet suspension) resulted in an extensive reduction in platelet adhesion ($7.4 \pm 2.1\%$; mean $\pm$ SEM of 18 coverslips), whereas aggregate formation was completely absent (figure 2B). Upon addition of purified coagulation factors VIIa, X, and II to $\alpha_{IIb}\beta_3$-inhibited platelets, a significant enhancement of platelet adhesion was observed ($48.2 \pm 2.9\%$; mean $\pm$ SEM of 18 coverslips, $p<0.0001$, figure 2C). When only rFVIIa was added to the perfusate, no increase in platelet adhesion was observed (data not shown). The morphologic appearance of GT platelets adhered to PMA-ECM was similar to that observed in the dRGDW-model, although the

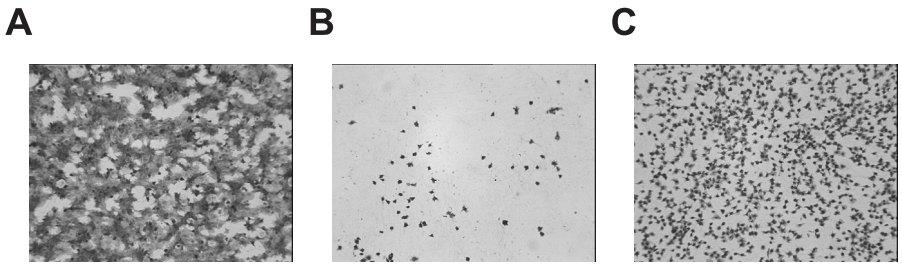

Figure 2. Development of a model system for adhesion of thrombasthenic platelets to subendothelial structures under flow conditions and the effect of the thrombin generating system. Washed normal platelets and red cells (200.000 platelets/μl; haematocrit 40%) were perfused over PMA-ECM for 5 minutes at 1600 s^{-1} in absence (panel A) or presence (panels B and C) of an RGD-containing peptide (rRGDW; 200 μM). Panel C shows the effect of the thrombin-generating system (1.2 μg/ml rFVIIa, 10 μg factor X, 20 ng/ml prothrombin, 3 mM $CaCl_2$) on adhesion of RGD-inhibited platelets.

surface coverage observed with GT platelets was slightly higher than that of dRGDW-inhibited normal platelets.

Enhancement of deposition of platelets treated with $\alpha_{IIb}\beta_3$ -blocking drugs to PMA-ECM upon addition of coagulation factors VIIa, X, and II.

When normal platelets were pretreated with the registered anti-$\alpha_{IIb}\beta_3$ drugs abciximab (10 μg/ml), eptifibatide (10 μg/ml), and tirofiban (1 μg/ml), a similar morphological pattern of adhesion was seen compared to the studies using dRGDW to block $\alpha_{IIb}\beta_3$. As shown in table 1, a low adhesion was observed when platelets treated with an anti-$\alpha_{IIb}\beta_3$ drug were perfused over PMA-ECM. Upon addition of the thrombin-generating system, platelet deposition was significantly enhanced.

Additions	Surface coverage (% ± SEM)
Abciximab	5.6 ± 1.2 (n=6)
Abciximab + VIIa/X/II	46.8 ± 1.9* (n=6)
Eptifibatide	13.0 ± 3.0 (n=6)
Eptifibatide + VIIa/X/II	46.3 ± 1.0* (n=6)
Tirofiban	7.9 ± 1.8 (n=6)
Tirofiban + VIIa/X/II	57.9 ± 1.4* (n=6)

Table 1. Effects of VIIa-mediated thrombin generation on platelet adhesion to PMA-ECM in the presence of $\alpha_{IIb}\beta_3$-blocking drugs. Washed platelets were preincubated with abciximab, eptifibatide or tirofiban for 30 minutes at room temperature. After addition of washed red cells, platelets were perfused over PMA-ECM for 5 minutes at a shear rate of 1600 s^{-1}. * $p<0.001$ vs drug in the absence of VIIa/X/II. n indicates the number of evaluated coverslips.

The generation of thrombin independently of tissue factor results in enhancement of platelet deposition in the GT model

When hirudin (5 U/ml) was added to a dRGWD-inhibited platelet suspension together with the thrombin generating system, the increase in platelet deposition to PMA-ECM induced by the thrombin generating system was completely abolished (table 2). Similarly, addition of annexin V (50 μg/ml) to dRGDW-inhibited platelets completely abrogated the enhancement in adhesion induced by the thrombin generating system. However, when all tissue factor activity in the system was neutralized by an inhibitory polyclonal antibody (500 μg/ml in PBS, both the PMA-ECM as well as the platelet suspension were treated for 45 minutes at room temperature), addition of the thrombin generating system still enhanced platelet deposition to the same extent as in

the absence of tissue factor blockade (table 2). The inhibitory capacity of the antibody was demonstrated using a standard prothrombin time assay and by a factor Xa generation assay on PMA-ECM. Preincubation of the tissue factor source with the antibody prolonged the prothrombin time from 12 to over 200 seconds, and preincubation of PMA-ECM with the antibody completely abolished rFVIIa-induced Xa generation in a static assay.

Additions	Surface coverage (% ± SEM)
None	7.4 ± 2.1* (n=18)
+ VIIa/X/II	48.2 ± 2.9 (n=18)
+ VIIa/X/II + Hirudin	6.7 ± 0.9* (n=7)
+ VIIa/X/II + Annexin V	10.1 ± 2.0* (n=6)
+ VIIa/X/II + anti-TF	58.8 ± 4.1 (n=8)

Table 2. Tissue factor-independent thrombin generation on procoagulant surface enhances deposition of $\alpha_{IIb}\beta_3$-inhibited platelets to PMA-ECM under flow conditions. Washed platelets and red cells were preincubated with the RGD containing peptide dRGDW and perfused over PMA-ECM at a shear rate of 1600 s^{-1} for 5 minutes. Hirudin or annexin V was added to the perfusate right before perfusion. Both the platelet suspension and the surface were preincubated with the anti-TF antibody for 45 minutes at room temperature. * $p<0.001$ vs addition of VIIa/X/II. n indicates the number of evaluated coverslips.

Thrombin-mediated enhancement of platelet deposition in the GT model is dependent on the GPIb-vWF interaction and on thrombin binding to GPIb

When the platelets were pretreated with an inhibitory antibody against either GPIb or vWF (45 min at room temperature, both antibodies were used in a dilution of 1:250), the enhancement in adhesion induced by the thrombin generating system was completely inhibited (table 3).

If platelets were pretreated with Fab fragments of an antibody which specifically blocks the binding of thrombin to GPIb (LJIb-10; 50 μg/ml), the enhancement of platelet adhesion induced by the thrombin generating system was completely abolished (table 3). The antibody did not affect platelet adhesion to purified vWF (not shown).

Additions	Surface coverage (% ± SEM)
None	7.4 ± 2.1* (n=18)
+ VIIa/X/II	48.2 ± 2.9 (n=18)
+ VIIa/X/II + anti GPIb	6.7 ± 1.5* (n=8)
+ VIIa/X/II + anti vWF	2.5 ± 1.3* (n=4)
+ VIIa/X/II + LJIb-10	8.3 ± 1.1* (n=7)

Table 3. Increased adhesion of $\alpha_{IIb}\beta_3$-inhibited platelets to PMA-ECM by VIIa mediated thrombin generation is dependent on the GPIb-vWF interaction and on thrombin binding to GPIb. Washed platelets and red cells were preincubated with the RGD containing peptide dRGDW and perfused over PMA-ECM at a shear rate of 1600 s^{-1} for 5 minutes. Platelets were preincubated with antibodies against GPIb, vWF, or the thrombin binding site on GPIb (LJIb-10) for 45 minutes at room temperature. * $p<0.001$ vs addition of VIIa/X/II. n indicates the number of evaluated coverslips.

Tissue factor-independent thrombin generation enhances platelet deposition to collagen type III upon $\alpha_{IIb}\beta_3$ blockade

To confirm that rFVIIa is able to generate thrombin independently of tissue factor in our model, experiments were performed using a tissue factor-poor adhesive surface. Platelet adhesion to collagen (type III) in presence of dRGDW was low (6.4 ± 2.0%, mean ± SEM of 12 coverslips), presumably due to the absence of vWF in the perfusate, which is necessary for reduction of the velocity of the platelets. Aggregate formation was completely absent (figure 3A). Upon addition of the thrombin-generating system to

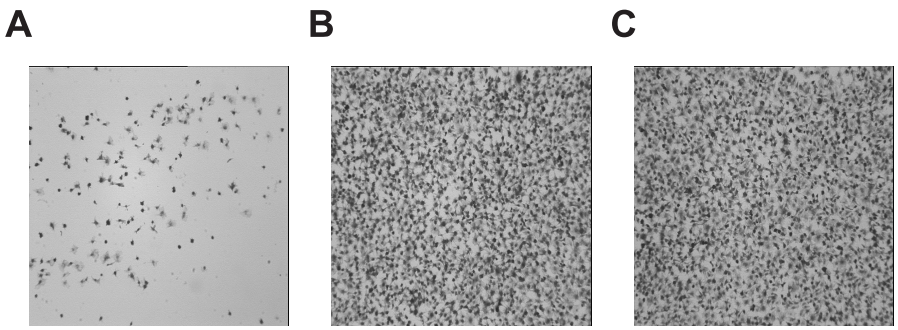

Figure 3. Adhesion of $\alpha_{IIb}\beta_3$-inhibited platelets to collagen type III under flow conditions; increased adhesion by the thrombin generating system is independent of tissue factor. Washed normal platelets and red cells (200.000 platelets/μl; haematocrit 40%) were perfused over collagen type III for 5 minutes at 1600 s^{-1} in presence of an RGD-containing peptide (rRGDW; 200 μM) in the absence (panel A) or presence (panels B and C) of the thrombin-generating system (1.2 μg/ml rFVIIa, 10 μg factor X, 20 ng/ml prothrombin, 3 mM $CaCl_2$). In panel C both the surface and the platelet suspension was preincubated with inhibitory tissue factor IgG.

$\alpha_{IIb}\beta_3$-inhibited platelets, the adhesion to collagen was significantly increased ($57.0 \pm 1.4\%$, mean $\pm$ SEM of 12 coverslips, $p<0.001$, figure 3B). Pretreatment of both the surface and the platelet suspension with anti-tissue factor IgG (500 μg/ml) did not abolish the enhanced platelet deposition induced by the thrombin-generating system (54.7 ± 1.3, mean $\pm$ SEM of 12 coverslips, figure 3C), confirming that the thrombin generation also occurred independently of tissue factor.

rFVIIa binds to collagen-adhered platelets under flow conditions

To investigate direct binding of rFVIIa to platelets adhered and activated under flow conditions, perfusion experiments were performed in which washed platelets and red cells were perfused over a collagen-coated surface in presence and absence of rFVIIa and calcium chloride. After perfusion, platelets were fixed and bound rFVIIa was visualized using immunofluorescence. As shown in figure 4, intense staining for rFVIIa was observed. No staining was observed when rFVIIa or the primary antibody (not shown) was omitted. Also, no fluorescence was observed when annexin V or EDTA was added to the perfusate (data not shown). Addition of an inhibitory antibody against tissue factor did not affect the signal (not shown).

Tissue factor-independent thrombin generation is associated with the release of glycoprotein V from the platelet surface

Thrombin binding to GPIb has been proposed to facilitate the release of a soluble fragment of GPV from the platelet surface. To investigate whether thrombin

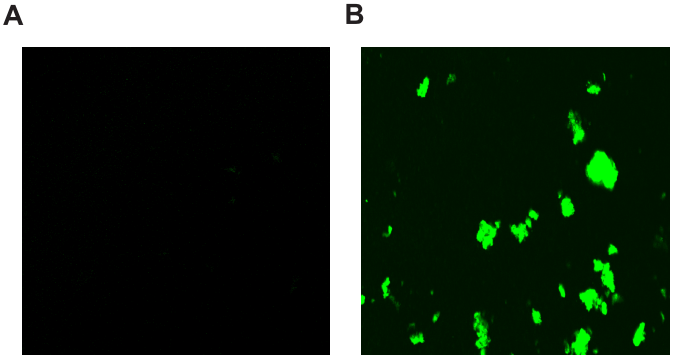

Figure 4. Binding of rFVIIa to collagen-adhered platelets under flow conditions. Washed platelets and red cells were perfused over a collagen coated glass coverslip in absence (panel A) or presence (panel B) of rFVIIa (1.2 μg/ml). After perfusion, coverslips were fixed, and bound rFVIIa was visualized by confocal laser scanning fluorescence microscopy using a monoclonal antibody against factor VII followed by a FITC-labelled secondary antibody.

generation in our system also results in the release of GPV, we performed perfusion experiments with washed platelets and red cells over a collagen-coated surface in the presence of RGD, and in the presence or absence of purified factors VIIa, X, and II. The perfusate was collected in 50 mM EDTA (1/10, v/v), cells were removed by centrifugation, and soluble GPV was measured in the supernatant by ELISA (asserachrom soluble GPV, Diagnostica Stago, Asnieres, France). Upon addition of the thrombin-generating system, a significant increase in soluble GPV was found in the supernatant as shown in figure 5 (control: 56 ± 7 ng/ml, mean $\pm$ SEM of four independent experiments in duplicate, in presence of rFVIIa/X/II 116 ± 14 ng/ml, $p=0.002$).

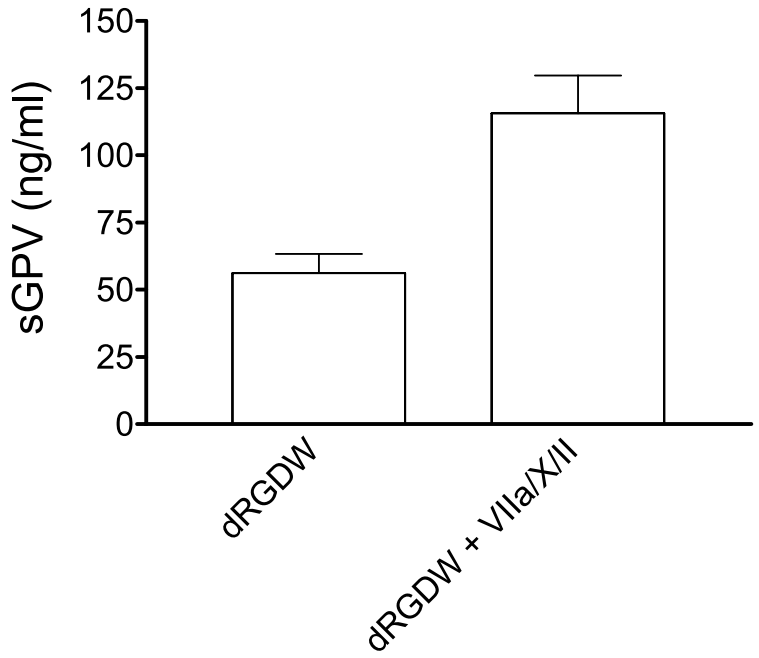

Figure 5. Tissue factor-independent thrombin generation is associated with release of a soluble fragment of GPV from the platelet surface. Washed normal platelets and red cells (200.000 platelets/µl; haematocrit 40%) were perfused over collagen type III for 5 minutes at 1600 s^{-1} in presence of an RGD-containing peptide (rRGDW; 200 µM) in the absence or presence of the thrombin-generating system (1.2 µg/ml rFVIIa, 10 µg factor X, 20 ng/ml prothrombin, 3 mM $CaCl_2$). The perfusate was collected in EDTA (1/10, v/v), cells were removed by centrifugation, and soluble glycoprotein V was measured in the supernatant. Shown is the mean of 4 independent experiments performed in duplicate. Error bars indicate SEM.

Discussion

This study shows that rFVIIa-mediated thrombin generation profoundly enhances deposition of platelets with a congenital or drug-induced defect in the integrin $\alpha_{IIb}\beta_3$ to the extracellular matrix of cultured human umbilical vein endothelial cells and to purified collagen type III. As the enhancement in platelet deposition by the thrombin generating system (coagulation factors VIIa, X and II) seems independent of tissue factor, but dependent on the presence of procoagulant surface, we propose a model in which thrombin is generated via activation of factor X by rFVIIa bound to adhered platelets. The generated factor Xa subsequently assembles into a prothrombinase complex, of which the factor Va in our assay set-up is presumably secreted by already adhered platelets. Finally, the in situ generated thrombin binds to GPIb on platelets, which are either in suspension or rolling on the surface, resulting in platelet activation. This thrombin-mediated activation of platelets somehow results in enhanced platelet deposition. The exact mechanism, which is responsible for the thrombin induced enhancement of platelet deposition is at present not clear, but might involve PAR-1 mediated activation, and/or cleavage of glycoprotein V from the GPIb/V/IX complex[347]. A tissue factor-independent enhancement of thrombin generation by rFVIIa involving binding to activated platelets or monocytes has been proposed to explain efficacy of rFVIIa in haemophilia as well as other haemostatic disorders in which rFVIIa has been shown beneficial[188-190]. Previously, it was generally accepted that rFVIIa exerts its haemostatic effect in a tissue factor-dependent manner[182,183]. In this study we now show that rFVIIa is able to generate thrombin on platelets adhered to a collagen surface under flow conditions without requirement for tissue factor, by binding directly to the platelet surface, supporting the TF independent mechanism of rFVIIa as suggested by Monroe et al[189]. A mechanistic explanation for the efficacy of rFVIIa in patients with GT is still lacking. Our study suggests that a local enhancement of thrombin generation, either in a tissue factor dependent or independent manner, may be able to increase adhesion of $\alpha_{IIb}\beta_3$-deficient platelets. Although enhancement of adhesion still does not result in a stable platelet plug, due to the absence of $\alpha_{IIb}\beta_3$-mediated platelet-platelet interaction, it does provide an increase of procoagulant surface at the site of injury. The increased procoagulant surface facilitates a further enhancement of thrombin generation and subsequent fibrin formation. This enhancement in fibrin deposition might compensate for the lack of aggregate formation. The assay set-up employed in this study features some simplifications that require explanation. Firstly, in this study we compare adhesion of $\alpha_{IIb}\beta_3$-deficient platelets in

absence or presence of rFVIIa-mediated thrombin generation. In order to translate this experiment into the in vivo situation, one must realise that if a patient with $\alpha_{IIb}\beta_3$ deficiency receives rFVIIa, an increase in thrombin generation at the site of injury will take place (in contrast to the on/off situation in our experimental set-up), and that the increase in adhesion is probably more subtle compared to the herein shown in vitro experiment. Secondly, our thrombin generating system is simplified, as it does not contain inhibitors of thrombin generation (TFPI, antithrombin and the protein C system), and factors VIII, IX, and XI are lacking. Due to this simplification, dose-response experiments with rFVIIa will not provide physiologically relevant information. Finally, as no fibrinogen was added to the reconstituted blood, the interplay between platelet deposition and fibrin formation was not studied. Studying fibrin formation to a tissue factor-rich surface is accompanied by a number of technical difficulties. In case of perfusion of recalcified blood, thrombin generation is already initiated before perfusion by activation of the contact activation system. Moreover, fibrin deposition does not halt when the blood has left the tissue factor-rich surface, resulting in obstruction of the perfusion chamber with macroscopic clots. Using low molecular weight heparin anticoagulated blood, which is commonly used to study fibrin deposition under flow conditions[355], endogenous factor VII is partially activated by factor XIIa generated by the contact system, which is not blocked by LMWH (T. Lisman, unpublished observations), resulting in an underestimation of the effect of exogenous added rFVIIa. However, Galan et al. recently reported improvement of both platelet deposition and fibrin formation under flow conditions using low molecular weight heparin-anticoagulated blood from patients with GT, which corresponds to the hypothesized mechanism of action put forward in our current study[356].

In conclusion, in this study we show that a tissue factor-independent generation of thrombin via rFVIIa profoundly increases adhesion of $\alpha_{IIb}\beta_3$-deficient platelets under flow conditions in vitro. We hypothesize that administration of rFVIIa to patients with GT results in an increased platelet adhesion at the site of injury, followed by an enhanced deposition of fibrin. The increased fibrin formation might compensate for the lack of aggregation and therefore explain the therapeutic efficacy of rFVIIa in GT. We speculate that rFVIIa may also be an effective therapeutic agent for patients who are bleeding as a consequence of treatment with anti-$\alpha_{IIb}\beta_3$ drugs, with a mechanism of action comparable to that proposed here for GT patients.

Acknowledgements

The authors like to thank Dr. U. Hedner for the generous gifts of rFVIIa, and the antibodies against TF and VIIa. We would like to thank Dr. R. Wallis for the generous gift of recombinant hirudin, and Dr. W.L. van Heerde for Annexin V. Dr. M Berndt, Dr. J.A. van Mourik, and Dr. Z.M. Ruggeri are gratefully acknowledged for their gifts of antibodies against glycoprotein Ib, vWF, and the thrombin binding site on glycoprotein Ib, respectively. We thank Dr. J. Bouchaudon for the gift of dRGDW. Finally we are grateful to Dr. M. Peters (Academic Medical Centre Amsterdam, The Netherlands), and Dr. M.C. Kappers-Klunne (University Hospital Dijkzigt, Rotterdam, The Netherlands) for their kind assistance with the patient studies.

Chapter 9

General discussion

The development of rFVIIa for treatment of patients with inhibitor-complicated haemophilia has been a major step forward in controlling bleeding episodes or providing haemostasis during interventional procedures[195]. Traditional haemostatic agents used in these patients, i.e., (activated) prothrombin complex concentrates (aPCC's and PCC's) and porcine FVIII, have disadvantages, which are not encountered with the use of rFVIIa. rFVIIa showed significantly higher efficacy rates compared to PCC's[137,146-148,150,357,358], and undesirable side effects, in particular thromboembolic complications[133], are much lower. In fact, thromboembolic complications associated with rFVIIa use are extremely rare. A major complication of the use of porcine FVIII is the development of inhibitory antibodies[96]. Antibody formation in haemophilia patients treated with rFVIIa has not yet been reported. Finally, as rFVIIa is a recombinant product, which does not contain albumin or other stabilizing proteins, the risk of viral transmission (e.g., HIV or hepatites) is extremely low[143].

The high efficacy rate and virtual absence of serious side effects of rFVIIa in patients with haemophilia in combination with concepts on the general mechanism of action of the drug, supported subsequent use of rFVIIa in patients with other haemostatic disorders, including patients with liver cirrhosis[168,169,295], patients with platelet-related bleeding disorders[162,163,344], patients with bleedings induced by anticoagulant drugs[18,172], and patients with uncontrollable hemorrhage in absence of preexisting coagulopathy[176,179]. Although the efficacy of rFVIIa in these novel indications has not yet been thoroughly established, the clinical data available so far, are encouraging. rFVIIa has been proposed to be a universal haemostatic drug, although efficacy, safety, and cost-effectiveness remains to be proven for indications outside haemophilia. The major safety concern remains the risk of thrombotic complications, especially in older patients with established risk factors for arteriothrombotic disease, and in patients with (non-overt) DIC.

The precise molecular mechanisms, by which rFVIIa provides haemostasis in haemophilia or in the novel indications, are at present unknown. Unravelling these mechanisms might provide a rationale for dosing schedules for the different indications. Moreover, a better understanding could rationalize a selection of patients that will benefit from treatment. Next to evaluation of a possible procoagulant effect, a risk assessment for a specific type of bleeding in patients with a specific type of coagulopathy might be possible if more information on the mechanism of action would be available. In this chapter, the contribution of this thesis to knowledge on mechanism of action of rFVIIa in its different applications will be discussed. The information obtained in this thesis will be combined with existing literature to formulate a general hypothesis on the action of rFVIIa in vivo.

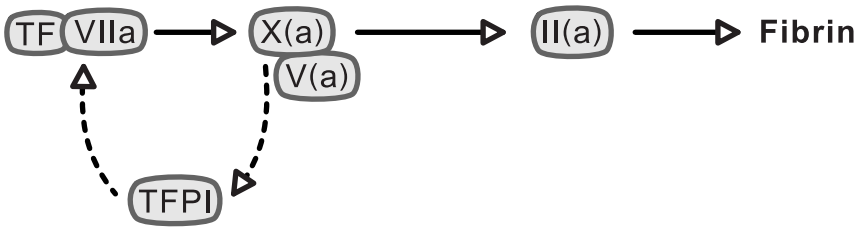

Figure 1. Coagulation in severe haemophilia A. In factor VIII or IX deficiency, clot formation is delayed due to a lack of tenase activity. Moreover, as factor XI-mediated secondary thrombin generation does not take place as a result of absence of tenase activity, TAFI activation does not occur (see figure 5 in chapter 1 for comparison).

Antifibrinolytic properties of rFVIIa in haemophilia

Previous studies on the mechanism of action of rFVIIa in haemophilia focussed on the effect on thrombin generation or clot formation[183,184,189]. As it was previously hypothesized that the bleeding diathesis in haemophilia not only is a consequence of defective clot formation, but also of premature breakdown of the clot due to defective TAFI activation (figure 1)[200,201], experiments to investigate a possible antifibrinolytic effect of rFVIIa were initiated. In chapter 2 of this thesis we showed that rFVIIa is able to protect plasma clots from patients with haemophilia from premature fibrinolysis, specifically by inducing activation of TAFI (figure 2). In our assay set-up,

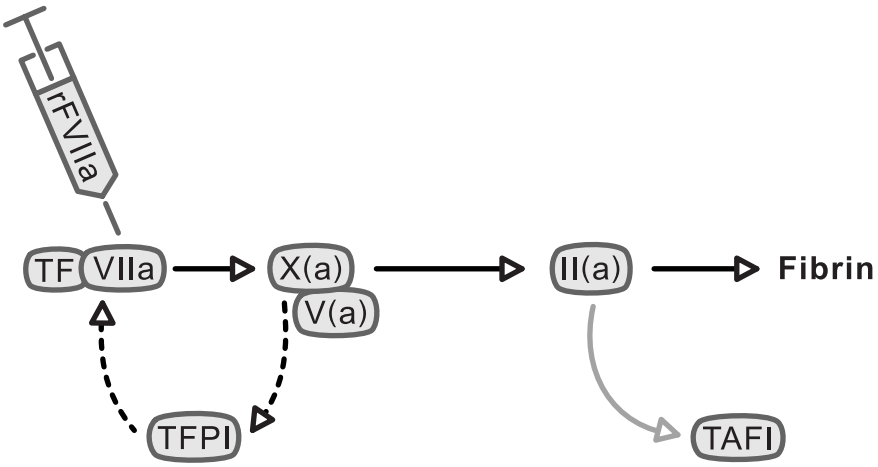

Figure 2. High dose rFVIIa restores TAFI activation in plasma from patients with severe haemophilia A by enhancing thrombin generation required for TAFI activation.

approximately 10 times more rFVIIa was required for inhibition of fibrinolysis compared to the amount of rFVIIa required for acceleration of clot formation. This relatively weak antifibrinolytic potential of rFVIIa in haemophilia might explain the high plasma levels of rFVIIa required for effective haemostasis.

However, whether defective TAFI activation actually contributes to the bleeding diathesis of haemophiliacs is still unclear. Even more, the relevance of TAFI for normal or pathological haemostasis is not yet known. Animal models suggest a role for TAFI activation in thrombosis; inhibition of TAFI significantly accelerated thrombolysis in different models[311,312,326]. These experiments, however, were all performed using a non-specific carboxypeptidase inhibitor, so effects ascribed to TAFI inhibition could also be caused by inhibition of other (perhaps unknown) carboxypeptidases. Furthermore, a lack of phenotype of the TAFI knockout mouse[105], and the absence of identified patients with an isolated TAFI deficiency hamper elucidation of a role for TAFI in vivo.

Suitable animal models to test the hypothesis on haemophilia being a defect in the fibrinolytic system rather than a defect in clot formation are, to our knowledge, still lacking. Moreover, it is clear that differences between murine knockout models and human disorders exist, both for coagulation defects and for defects in the fibrinolytic system. It would, however, be interesting to see whether cross-breeding mice with a defects in one of the intrinsic coagulation factors with the TAFI knockout would lead to an aggravation of the bleeding diathesis of the single knockout.

If the antifibrinolytic effect of rFVIIa has any relevance in vivo, important consequences for dosing may arise. Firstly, the dosage of rFVIIa required for effective (long-term) haemostasis may be dependent on where the injury is localized. The amounts of tissue factor and thrombomodulin expressed at the site of injury, as well as the local fibrinolytic activity of the tissue may be important in the amount of rFVIIa required for cessation or prevention of bleedings. The presence of relatively high amounts of TF might reduce plasma levels of rFVIIa required for effective haemostasis, as the antifibrinolytic potential of rFVIIa has been shown to increase with increasing TF. The role of thrombomodulin expression on the antifibrinolytic potential of rFVIIa is more complicated. The presence of thrombomodulin may either enhance or inhibit TAFI activation, presumably depending on its density on the endothelial cell surface[67]. It has been demonstrated in vitro that the thrombin-thrombomodulin complex has antifibrinolytic properties at low concentrations by enhancing TAFI activation. At higher concentrations, however, thrombin-thrombomodulin preferably activates protein C, which results in acceleration of fibrinolysis by decreasing thrombin

generation required for TAFI activation[67]. Thus, the amount of rFVIIa required for downregulation of fibrinolysis in a specific area of the vasculature may also depend on the pro- or antifibrinolytic state of thrombomodulin near the site of injury. Finally, the fibrinolytic potential of the tissue is probably important with respect to the dose of rFVIIa required to induce haemostasis. Indeed, preliminary clinical data suggested that higher plasma levels of rFVIIa are required to treat patients with bleedings in the oral cavity (in which a high fibrinolytic activity is present). It was shown that treatment of oral cavity bleeding by continuous infusion had a very low efficacy rate compared to treatment by repeated bolus injections[359]. As lower peak levels are reached using continuous infusion, enhancement of TAFI activation might be insufficient, with rebleeding as a consequence.

Lack of antifibrinolytic effect of rFVIIa in patients with cirrhosis or during liver transplantation

After demonstrating an antifibrinolytic effect of rFVIIa in plasma from patients with haemophilia, we hypothesized that a similar mechanism could also apply in plasma from patients with liver failure. As patients with liver failure have decreased levels of all procoagulant proteins (except for factor VIII), we hypothesized both primary (i.e., the thrombin required for clot formation) and secondary thrombin generation (i.e., the thrombin generated after formation of the fibrin clot, which is required for TAFI activation) to be impaired, similarly to that observed in plasma completely deficient in either one of the intrinsic coagulation factors. Moreover, a hyperfibrinolytic state is said to be present in at least part of the patients with stable cirrhosis[268], and development of hyperfibrinolysis is observed during liver transplantation[285].
However, using the clot lysis assay described in this thesis, we could not observe accelerated fibrinolysis in plasma samples from patients with stable cirrhosis (chapter 4). Even though the levels of both the thrombin generating proteins as well as TAFI were significantly decreased, TAFI activation appeared normal as both clot lysis time and clot lysis ratio (i.e., ratio of clot lysis time in absence and presence of a specific inhibitor of activated TAFI) were not significantly different from those found in healthy volunteers. The deficiencies in antifibrinolytic proteins induced by cirrhosis are probably compensated for by deficiencies in profibrinolytic proteins. TAFI activation can proceed relatively normal in plasma from patients with cirrhosis because the defect in thrombin generating capacity is compensated for by a reduced thrombin inhibition (e.g., by antithrombin).

Development of hyperfibrinolysis was seen in samples taken during orthotopic liver transplantation. However, this hyperfibrinolytic state was not caused by defective TAFI activation. At the end of surgery, a transient hypofibrinolytic state was detected, presumably a consequence of massive PAI-1 release. If this temporary hypofibrinolytic state contributes to post-operative thrombotic complications (e.g., portal vein thrombosis) is not known.

TAFI activation thus appeared already optimal in plasma from patients with stable cirrhosis and in samples taken during OLT, implicating that sufficient 'free' or 'active' thrombin is generated. Consequently, addition of rFVIIa to cirrhotic plasma did not have an effect on clot lysis time (chapter 5). Measurements of thrombin conversion in time showed that rFVIIa only accelerated primary thrombin generation (i.e., the small amount of thrombin required for clot formation), but that the total amount of thrombin converted during the assay is already maximal in the absence of rFVIIa. In contrast, in haemophilic plasma both primary and secondary thrombin generation are enhanced by rFVIIa (unpublished results).

According to the in vitro data obtained in this thesis, the prohaemostatic properties of rFVIIa in patients with liver disease can not be explained by an effect on the fibrinolytic system. Rather, enhancement of clot formation and platelet adhesion by rFVIIa could lead to a more stable haemostatic plug in these patients. As patients with liver failure have both a qualitative and quantitative defect in platelet function (see chapter 3), it is conceivable that enhancement of thrombin generation would lead to enhancement of platelet adhesion, similar to the mechanisms described in chapter 8 (see also below). The increased procoagulant surface would subsequently result in enhancement of fibrin deposition.

Enhancement of platelet deposition and activation by rFVIIa, however, could lead to enhanced secretion of PAI-1 and α_2-antiplasmin from platelet α-granules[68,69], thereby downregulating the fibrinolytic system. This possible antifibrinolytic effect of rFVIIa is obviously not picked up in our plasma-based assay. Also, a lack of effect of rFVIIa on TAFI activation in our assay does not necessarily mean that rFVIIa does not enhance TAFI activation in vivo. The presence of thrombomodulin, flow, (activated) platelets, etc, could very well influence the effect of rFVIIa on TAFI activation in vivo. Very little is known about TAFI activation in vivo, and more sophisticated assays (e.g., an assay to measure plasma levels of the TAFI activation peptide, which could be a measure for TAFI activation) will be required to get more insight into this phenomenon.

rFVIIa as a potential haemostatic drug in treatment of bleedings induced by anticoagulant drugs

One of the most important complications of antithrombotic drugs designed to inhibit the coagulation system, is the development of bleeding complications[322]. It is traditionally believed that the bleeding diathesis induced by anticoagulant drugs is a consequence of inhibition of clot formation. In chapter 6 we have shown that anticoagulant drugs completely or partially directed against factor Xa, not only inhibit clot formation, but also accelerate clot breakdown by inhibition of TAFI activation in vitro. Whether this phenomenon has any in vivo relevance is still unknown, but would it indeed have physiological relevance, important consequences arise.

On one hand, anticoagulant drugs targeting factor Xa might be more potent in prevention or treatment of thrombosis in comparison with thrombin inhibitors or TF antagonists, as Xa inhibitors induce both an anticoagulant and a profibrinolytic effect. On the other hand, anticoagulant drugs, which inhibit Xa, might induce a more severe bleeding tendency for the same reason. The selective Xa inhibitor PENTA, has been shown to be more effective in preventing venous thromboembolism following knee[334], hip replacement[332,333], or hip fracture[325] surgery compared to enoxaparin (a low molecular weight heparin, which inhibits both factor Xa and thrombin). One study reported an increased number of major bleeds when PENTA was used[334], whereas the other studies did not detect a significant difference in bleeding episodes between PENTA and enoxaparin. A meta analysis of these 4 randomised double-blind studies showed that major bleeding occurred more frequently in the fondaparinux-treated group ($p=0.008$)[360]. However, the incidence of bleeding episodes leading to death or reoperation, or occuring in a critical organ did not differ between fondaparinux and enoxaparin.

We investigated the effect of rFVIIa on coagulation and clot lysis in plasma containing PENTA (Chapter 7). rFVIIa was able to completely reverse the anticoagulant effect of PENTA. Interestingly, the profibrinolytic effect induced by PENTA was only partially reversed by rFVIIa. Even at supratherapeutic levels rFVIIa did not completely reverse clot lysis in PENTA containing plasma (unpublished data). This is in contrast with the situation in factor VIII deficient plasma, in which addition of sufficient rFVIIa normalized clot lysis time (i.e., clot lysis time in factor VIII deficient plasma with high dose rFVIIa equalled clot lysis time in the same plasma with 1 U/ml of purified factor VIII). The reason why PENTA is still able to (partially) inhibit secondary but not primary thrombin generation in the presence of rFVIIa is unclear. Either the

concentration of Xa required for secondary thrombin generation is much higher than that required for clot formation, or inhibition of Xa by PENTA is, in the presence of rFVIIa, simply not fast enough to delay clot formation.
Our results, as well as data from a phase I PENTA/rFVIIa interaction trial (Dr. M. Levi, personal communication) indicate that rFVIIa may be effective in reversing the antithrombotic effects of PENTA in case bleeding complications occur. However, would the induction of hyperfibrinolysis by PENTA have physiological relevance, adjunctive treatment with antifibrinolytic drugs might be considered as rFVIIa only partially reverses this antifibrinolytic effect.

Enhancement of platelet deposition by rFVIIa-mediated thrombin generation

The mechanism by which rFVIIa induces haemostasis in patients with qualitative or quantitative platelet disorders is poorly understood. As these patients have an intact coagulation system, it is curious at first sight how enhancement of extrinsic coagulation by rFVIIa could assist haemostasis. However, the traditional view of platelet function and clot formation seen as more or less separate processes is out of date. First of all, thrombin generated by the coagulation system is involved in platelet activation. Furthermore, platelet adhesion to fibrin[361] as well as a possible role of polymerizing fibrin in platelet activation and aggregation[362] may have physiological relevance. Finally, the expression of procoagulant surface by platelets seems to play a pivotal role in the propagation of thrombin generation; cell surfaces presumed to be involved in the initiation of coagulation (e.g., fibroblasts and smooth muscle cells) do not provide a suitable surface for tenase and prothrombinase-mediated large-scale thrombin generation[49].
Experiments presented in chapter 8 of this thesis have revealed an important role of in-situ generated thrombin for adhesion of platelets with congenital or acquired $\alpha_{IIb}\beta_3$ deficiency. The generation of a small amount of thrombin profoundly enhances the deposition of $\alpha_{IIb}\beta_3$-deficient platelets to subendothelial matrix of cultured endothelial cells and to collagen under flow conditions. In fact, we have also shown in the same model system that also normal platelets show increased platelet deposition (in this case reflected by the formation of more extensive platelet aggregates) to subendothelial matrix or to collagen. Furthermore, platelet adhesion and activation (relected by platelet spreading) to a fibrinogen surface was significantly enhanced by the generation of thrombin. Increased adhesion and aggregate formation to subendothelial matrix was also observed under conditions mimicking thrombocytopenia (all unpublished results).

We hypothesize that platelets from patients with liver failure will respond to rFVIIa mediated thrombin formation as well, but this hypothesis has not yet been tested. These results are in accordance with results published by Diaz-Ricart et al., who observed that thrombin increased adhesion of both normal and $\alpha_{IIb}\beta_3$-inhibited platelets to denuded rabbit aorta segments under flow conditions[363]. In these experiments, thrombin was either preincubated with the surface, or added to the perfusate, with similar results.

In our experiments, in which the thrombin was generated via rFVIIa and factor X, it appeared that the presence of tissue factor was not mandatory for thrombin generation. Supported by our data and by experiments published by Monroe et al., who observed direct binding of rFVIIa to platelets in suspension[189], we postulate the following general model for enhancement of platelet adhesion under flow conditions by rFVIIa-mediated thrombin generation. First, a platelet adheres to subendothelial structures (e.g., collagen), becomes activated, and consequently expresses procoagulant surface. rFVIIa can subsequently bind to this already adhered platelet, and activates factor X on the platelet surface. The formed factor Xa subsequently assembles into a prothrombinase complex resulting in the generation of thrombin. This thrombin formed on the platelet surface can result in activation of platelets either from suspension, or rolling on the surface (figure 3). This activation proceeds via thrombin binding to GPIb, and may involve cleavage of GPV and/or PAR-1 activation[17,348]. Enhancement of platelet adhesion results in an increase in the expression of procoagulant surface, and this may facilitate further (large-scale) thrombin generation and subsequent fibrin formation. Enhancement of thrombin generation by

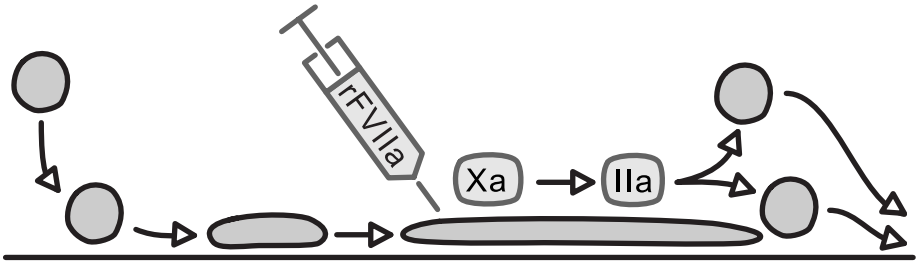

Figure 3. A model for enhancement of platelet deposition by rFVIIa-mediated thrombin generation. rFVIIa binds to already adhered platelets expressing anionic phospholipids and converts factor X to Xa. Factor Xa subsequently activates prothrombin, and the thrombin formed activates platelets, which are either rolling on the surface, or in suspension. Platelet activation by in situ formed thrombin renders the platelet more reactive towards the surface via an incompletely understood mechanism, but might involve cleavage of GPV from the GPIb/V/IX complex and/or PAR-1 activation.

an increased procoagulant surface might also result in enhanced TAFI activation.
Thus, rFVIIa enhances thrombin generation, leading to increased platelet deposition, and consequently increased fibrin deposition, resulting in a more stable haemostatic plug. This mechanism potentially applies to patients with normal platelets, and to patients with all acquired and drug-induced platelet disorders (e.g., GT, uremia, cirrhosis, $\alpha_{IIb}\beta_3$-inhibitors) with only one exception, which is GPIb deficiency. The scarce clinical evidence available suggests that also in BSS rFVIIa has haemostatic efficacy, and if this is true, a different mechanism than the one described above would be applicable.
Platelets deficient in GPIb have a strongly reduced thrombin binding capacity and are also less responsive to thrombin[364]. Enhanced thrombin generation by rFVIIa will thus not lead to an enhanced activation as seen with platelets on which the GPIb/V/IX complex is present. Moreover, platelets deficient in GPIb poorly adhere to subendothelial structures[365], thereby impairing the expression of procoagulant surface at the site of injury. It might be that in BSS, platelets are 'trapped' in a developing fibrin network by an $\alpha_{IIb}\beta_3$-mediated mechanism. Although the development of procoagulant activity in GPIb deficient platelets may be impaired, rFVIIa-mediated enhancement of thrombin generation could be possible on BSS platelets, as these platelets already express procoagulant surface under resting conditions[366]. Enhancement of thrombin generation would again lead to enhancement of fibrin deposition and/or improvement of fibrin structure, thereby inducing haemostasis in the BSS patient.

General model for the mechanism of action of rFVIIa

A number of variables concerning the effects of rFVIIa in vivo in enhancing the haemostatic potential are still unknown, especially with respect to structure and composition of the haemostatic plug, and its resistance against the fibrinolytic system. Both changes in fibrin structure, as well as changes in fibrin-platelet interaction potentially contribute to stability of the haemostatic plug. However, sufficient in vitro data are available to postulate a general model for the mechanism of action of rFVIIa. The central player in this model is thrombin. It is now generally accepted that rFVIIa administration to any patient (with or without defects in the haemostatic system) will enhance thrombin formation at the site of injury. The exact mechanism by which this thrombin is generated is still a matter of debate; the mechanism, which seems most plausible, taken experimental data available at present, will be discussed below (see also figure 4). The mechanisms by which enhancement of thrombin generation leads to the formation of a more stable haemostatic plug (and consequently the induction of 'long

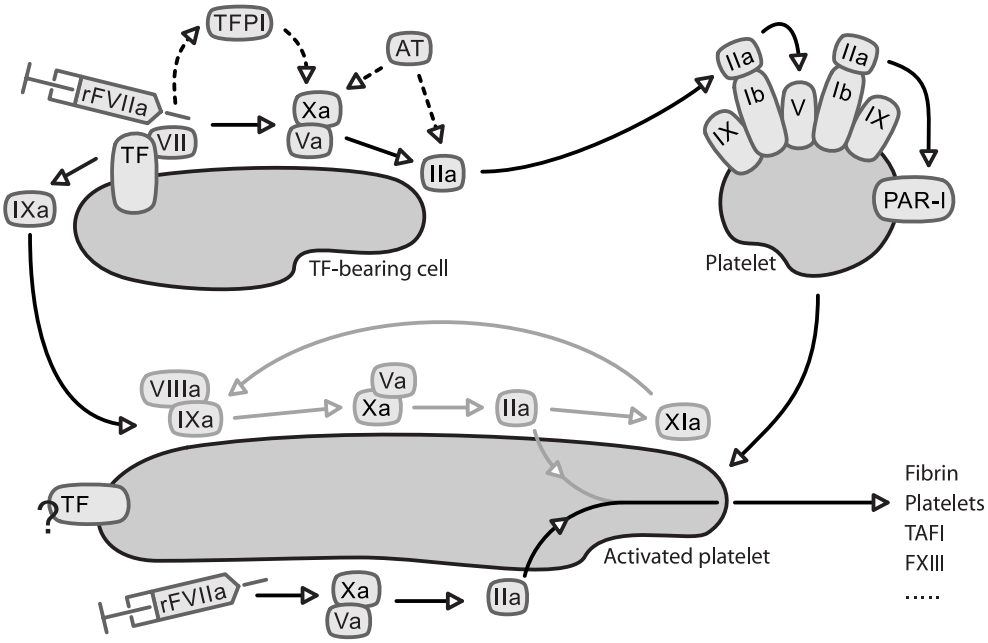

Figure 4. A general model for the mechanism of action of rFVIIa. rFVIIa may enhance initiation of coagulation on the TF-bearing cell by competing with endogenous zymogen factor VII for TF. A small amount of thrombin formed on the TF-bearing cell can activate platelets; thrombin binding to GPIb accelerates PAR-1 activation and results in release of sGPV from the complex. Factor IXa formed on the tissue factor-bearing cell can transfer to the activated platelet, and start generating large amounts of thrombin. In case of haemophilia, this pathway is not active (grey arrows). Large-scale thrombin generation on the activated platelet can also occur by direct binding of rFVIIa to the platelet. Without the requirement for TF, platelet-bound rFVIIa can activate factor X. rFVIIa-mediated enhancement of thrombin generation on the activated platelet by platelet-associated TF might also occur. The enhancement of large-scale thrombin generation will affect platelet activation, fibrin formation, TAFI activation, factor XIII activation and other processes.

term' haemostasis) are incompletely known, but possible targets will be discussed in this section.

Enhancement of thrombin generation by rFVIIa may be accomplished by tissue factor-dependent[182,183] or -independent[189] mechanisms, or perhaps both. rFVIIa may enhance initiation of coagulation on a tissue factor-bearing cell (e.g., a fibroblast or a smooth muscle cell). rFVIIa competes with endogenous zymogen factor VII for binding to TF[183], thereby enhancing Xa and IXa generation on the TF-bearing cell. A relatively small amount of thrombin generated on the TF-bearing cell subsequently leads to platelet activation, accompanied by the expression of procoagulant surface, suitable for large-scale thrombin generation. Enhancement of initiation of coagulation may

already result in enhancement of platelet deposition, and consequently enhancement of expression of procoagulant surface.

As the membrane environment on the TF-bearing cell is not suitable for large-scale thrombin generation, the action has to move towards the activated platelet. Factors Xa and IIa are probably not able to diffuse to the activated platelet surface. Factor Xa will be inhibited by TFPI, and both factor Xa and IIa are in solution highly susceptible to inhibition by antithrombin[49]. Factor IXa, however, is in solution not rapidly inhibited, and is thus able to transfer to the activated platelet surface to generate large amounts of thrombin. Thrombin generation on the platelet surface is further amplified by the activation of factor XI[367]. It has been shown that factor XI binding to GPIb enhances its activation by thrombin, and it is thus conceivable that GPIb-bound thrombin is responsible for factor XI activation[368].

A variation on this model involves the possible transfer of 'blood borne' tissue factor to platelets[25,369]. If during the haemostatic response encrypted TF is transferred from leukocytes to platelets, the platelet (or, more precisely, the 'platelet-TF hybrid') can be the surface on which both initiation and propagation of thrombin formation takes place.

An alternative pathway of thrombin generation by rFVIIa, which may be relevant especially in case of factor VIII or IX deficiency, is the TF-independent thrombin generation on activated platelets[189]. rFVIIa has been shown to bind with weak affinity to activated platelets where it can activate factor X, without the requirement for TF. In case of haemophilia, large-scale thrombin generation on platelets is absent, because only factor IXa can transfer from the TF-bearing cell to the platelet, so in absence of intrinsic tenase activity, platelet thrombin generation is absent. It has been hypothesized that TF-independent thrombin generation is completely responsible for the haemostatic efficacy of rFVIIa in haemophilia, and that the same mechanism would also apply in patients with other haemostatic disorders[190].

Enhancement of thrombin generation by rFVIIa potentially results in a variety of events, which lead to a more stable haemostatic plug.

1) Changes in fibrin structure. It has been shown that the amount and the rate of thrombin generation influence the structure (e.g., thickness) of the fibrin fibres[191,192]. Increased thrombin generation might lead to a more stable, less porous fibrin clot[193], better suitable to resist shear forces in the circulation, and less susceptible to fibrinolysis.
2) Enhanced or more rapid activation of factor XIII could lead to enhanced or more efficient fibrin cross-linking, thereby contributing to clot stability and resistance to breakdown by the fibrinolytic system[40].

3) Enhanced activation of TAFI could directly contribute to downregulation of the fibrinolytic system (this thesis).
4) More extensive platelet activation could lead to a more densely packed platelet plug. Also, the expression of procoagulant surface, either by an increased platelet number at the site of injury, or by a stronger activation signal due to the higher concentrations of thrombin generated, could lead to further enhancement of thrombin generation (chapter 8 of this thesis). Finally, increased platelet activation could result in an increased secretion or excretion of clot stabilizing factors such as PAI-1, and α_2-antiplasmin, and FXIII.

Which of these mechanisms contribute to enhancement of clot stability by rFVIIa in vivo, and to what extent, remains to be elucidated. Future studies will need to focus on structure, composition, and stability of haemostatic plugs in physiological relevant models. Models as described in this thesis provide potentially relevant information, but have the limitation that the interaction between platelets and the coagulation system are not included. Thus, extensions of the currently used models could be employed to get closer to the in vivo situation. In vitro models should incorporate shear stress, as this plays a significant role in platelet-vessel wall, and platelet-fibrin interaction. Animal models might also be valuable in investigating physiologically important determinants of clot stability.

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Samenvatting

Haemostase

Haemostase is het proces wat ervoor zorgt dat een beschadiging in een bloedvat snel en effectief wordt hersteld om overtollig bloedverlies te voorkomen. Het haemostatische proces is in normale omstandigheden strikt gereguleerd. Een haemostatisch systeem dat uit balans raakt, kan aanleiding geven tot bloedingen of trombose.
In geval van vaatwandbeschadiging treden een aantal, aan elkaar gekoppelde processen op, die resulteren in afsluiting van het bloedvat. Allereerst trekt het bloedvat samen, waardoor de hoeveelheid bloed die door het vat stroomt verminderd wordt. Vervolgens hechten bloedplaatjes, die in rustende toestand circuleren in het bloed, aan eiwitten die vlak onder de wand van een bloedvat zitten (zoals collageen). Gehechte bloedplaatjes worden geactiveerd waardoor deze bloedplaatjes weer andere bloedplaatjes kunnen binden. Zo ontstaat een prop van bloedplaatjes die voor een eerste afdichting van het bloedvat zorgt. De bloedplaatjesprop alleen is echter niet voldoende. Versterking van de plaatjesprop door een fibrinenetwerk is noodzakelijk voor permanente afsluiting van de vaatwandbeschadiging. De eerste aanzet tot vorming van een fibrine stolsel is het in contact komen van het eiwit tissue factor (TF) met de bloedstroom. Tissue factor is een eiwit dat op een groot aantal celtypen aanwezig is, behalve op celtypen binnen een bloedvat (zoals endotheelcellen). Als tissue factor in contact komt met het bloed, bindt dit eiwit het proenzym factor VII. Als factor VII geactiveerd wordt (tot factor VIIa), is het tissue factor-VIIa complex in staat andere stollingseiwitten te activeren, uiteindelijk leidend tot de vorming van het enzym trombine. Trombine is vervolgens in staat het plasma eiwit fibrinogeen te knippen tot fibrine, dat spontaan polymeriseert tot een onoplosbare vorm. Naast het knippen van fibrinogeen is trombine in staat bloedplaatjes te activeren, activeert het factor XIII wat vervolgens het fibrinenetwerk versterkt door vertakkingen aan te brengen, en activeert het TAFI (thrombin activatable fibrinolysis inhibitor door trombine te activeren fibrinolyseremmer). Geactiveerd TAFI is betrokken bij de bescherming van het fibrine netwerk tegen afbraak. Afbraak van fibrine verloopt via een proces dat fibrinolyse genoemd wordt. Als reactie op de vaatwandbeschadiging, stoot het nabijgelegen endotheel het enzym tPA uit. tPA kan het plasma eiwit plasminogeen activeren tot plasmine, dat in staat is het fibrinestolsel af te breken. De activatie van plasminogeen is het effectiefst wanneer tPA en plasminogeen gebonden zijn aan fibrine. Geactiveerd TAFI verwijdert de bindingsplaatsen voor tPA en plasminogeen van fibrine en remt zo de fibrine afbraak.

Afwijkingen in de haemostase leidend tot een bloedingsneiging

Veel eiwitten betrokken bij het haemostase proces zijn ontdekt doordat patiënten die zo'n eiwit missen door een erfelijk defect, een bloedings- of trombosneiging hebben. Ook kunnen veranderingen in de haemostase die leiden tot bloedingen of trombose verworven worden. Dit gaat meestal gepaard met een ziekte die niet gerelateerd aan de haemostase is, of is de oorzaak van het gebruik van medicijnen die direct of indirect de bloedstolling beïnvloeden

In mensen komen vele erfelijke afwijkingen in bloedplaatjes, het proces van fibrinevorming, of de fibrinolyse die gepaard gaan met een bloedingsneiging voor. Sommige daarvan zijn bijzonder zeldzaam, terwijl andere erfelijke afwijkingen veel vaker voorkomen. De ziekten die van belang zijn in dit proefschrift zullen hieronder kort worden besproken.

Een bekende bloederziekte is haemofilie. Patiënten met haemofilie missen stollingsfactor VIII (haemofilie A) of IX (haemofilie B) welke betrokken zijn bij het proces van trombinevorming. Deze patiënten hebben afhankelijk van de ernst van de ziekte (hoeveel actief eiwit ze nog aanmaken) een milde tot ernstige bloedingsneiging, die zich vooral uit als spier- en gewrichtsbloedingen, nabloeden na kleine ingrepen zoals kiezen trekken, en ernstig bloedverlies bij trauma of operaties. Behandeling van haemofilie bestaat uit het teruggeven van de missende stollingsfactor. Vroeger werd dit eiwit gezuiverd uit plasma van bloeddonoren, tegenwoordig zijn ook producten op de markt die verkregen zijn via recombinant DNA technieken. Het nadeel van producten verkregen uit humaan materiaal is de kans op besmetting met virussen zoals het HIV virus en hepatitis B en C. Behandeling van haemofilie met gezuiverde stollingsfactoren wordt vaak bemoeilijkt doordat het lichaam antistoffen tegen de missende factor gaat aanmaken. De getransfundeerde factor is immers een lichaamsvreemde stof voor de haemofilie patiënt. Na het ontwikkelen van antistoffen is het geven van gezuiverd factor VIII of IX zinloos, omdat deze meteen door de antistof geneutraliseerd wordt.

Een zeldzame afwijking aan de bloedplaatjes staat bekend als Glanzmann's Thrombasthenia (GT). Patiënten met deze afwijking missen een receptor ($\alpha_{IIb}\beta_3$) op het bloedplaatje. Deze receptor is betrokken bij zowel de hechting van het plaatje aan de adhesieve eiwitten buiten het vat, als bij het hechten van de bloedplaatjes aan elkaar. Patiënten met GT hebben een ernstige bloedingsneiging gekarakteriseerd door neusbloedingen, blauwe plekken, tandvlees bloedingen, overvloedig menstrueren, gastrointestinale bloedingen, en ernstig bloedverlies bij trauma of operaties. Behandeling van GT bestaat uit het transfunderen van gezonde bloedplaatjes, of het

geven van synthetische medicijnen zoals DDAVP of remmers van de fibrinolyse zoals trasylol. Het nadeel van bloedplaatjestransfusie is dat antistoffen tegen zowel de missende receptor als tegen HLA determinanten op het plaatje (de exacte samenstelling van deze eiwitten is bij ieder mens anders) ontwikkeld kunnen worden. Ook hier is weer het transfunderen van bloedplaatjes na het ontwikkelen van antistoffen zinloos, en moet een alternatieve behandeling gezocht worden.
Een zeer complexe verworven bloedingsneiging wordt gevonden bij patiënten met chronisch of acuut leverlijden. Deze patiënten hebben afwijkingen in bloedplaatjes aantal en functie, trombine en fibrinevorming, en in het fibrinolysesysteem. Dit komt onder meer omdat bijna alle eiwitten die betrokken zijn bij de haemostase gemaakt worden in de lever. Deze patiënten kunnen bloeden na kleine ingrepen zoals een leverbiopsie of een kiesextractie. Bij grotere chirurgische ingrepen (zoals een levertransplantatie) treedt vaak ernstig bloedverlies op, dat gecorrigeerd moet worden door transfusie van bloedproducten.
Patiënten met trombose krijgen vaak medicijnen die haemostatische processen remmen (antitrombotica) of stolselafbraak stimuleren (fibrinolytica). Remmers van bloedplaatjes, zoals aspirine (wat plaatjes activatie remt) of medicijnen die $\alpha_{IIb}\beta_3$ remmen, remmers van trombinevorming, en stimulators van de fibrinolyse worden toegepast bij preventie en/of behandeling van trombose. Het nadeel van deze medicijnen is dat ze terwijl ze trombose voorkomen, ook een bloedingsneiging induceren.

Recombinant stollingsfactor VIIa (rFVIIa) – een doekje voor het bloeden

Geactiveerd bloedstollingsfactor VII gezuiverd uit humaan plasma werd in 1983 voor het eerst toegepast bij een patiënt met haemofilie A die een operatie moest ondergaan. Deze patiënt kon vanwege de aanwezigheid van antistoffen tegen factor VIII niet met factor VIII concentraat behandeld worden. Het idee was, dat door infusie van geactiveerd factor VII in hoge concentratie trombinevorming gestimuleerd zou kunnen worden, en dat het stollingsdefect veroorzaakt door de factor VIII of IX deficiëntie daardoor gecompenseerd zou worden. Deze en de daaropvolgende behandelingen met plasma factor VIIa waren succesvol. Echter, het was niet realistisch om een groot aantal patiënten met plasma gezuiverd factor VIIa te gaan behandelen omdat humaan plasma maar erg weinig factor VII bevat, en de zuivering bovendien een lage opbrengst gaf. Daarom werd besloten factor VIIa als recombinant eiwit te gaan produceren, wat mogelijk werd toen het gen voor dit eiwit in 1986 gekloneerd werd. Klinische trials met rFVIIa lieten zien dat haemofilie patiënten met antistoffen veilig en effectief behandeld

konden worden tijdens bloedingen of chirurgische ingrepen. rFVIIa werd in 1996 in Europa geregistreerd als behandelmethode voor patiënten met haemofilie en antistoffen tegen de missende factor.
Het gegeven dat rFVIIa bij haemofilie patiënten veilig en effectief trombinegeneratie lijkt te verhogen opende de weg naar nieuwe toepassingen. Patiënten met bloedplaatjes-afwijkingen (zoals GT), leverziekte, en patiënten behandeld met antitrombotica lijken veilig en effectief met rFVIIa behandeld te kunnen worden tijdens bloedingen. Bovendien lijkt rFVIIa ook bloedverlies tijdens levertransplantaties, en tijdens chirurgische ingrepen bij patiënten zonder stollingsafwijking te kunnen verminderen. Als laatste lijkt rFVIIa een zeer effectief middel om oncontroleerbare bloedingen te stelpen bij patiënten zonder stollingsafwijking (bijvoorbeeld bloedingen ten gevolge van trauma). rFVIIa lijkt dus een soort universeel middel om bloedingen te stoppen of voorkomen of om bloedverlies tegen te gaan.
Hoewel de haemostatische effecten van rFVIIa worden toegeschreven aan versterking van trombinegeneratie op de plaats van de vaatwandbeschadiging, is over het exacte mechanisme dat voor die versterkte trombinevorming zorgt, als over hoe die versterkte trombinevorming nou precies bijdraagt tot het induceren van permanente haemostase nog weinig bekend. Ook wordt niet goed begrepen waarom zeer hoge concentraties rFVIIa nodig zijn voor het induceren van haemostase.
Een van de discussies in de literatuur is of het versterken van de trombinevorming door rFVIIa via een TF afhankelijk of onafhankelijk mechanisme verloopt. Als rFVIIa zou werken via een TF afhankelijk mechanisme, is niet geheel duidelijk waarom zulke hoge concentraties nodig zijn voor het induceren van haemostase. De affiniteit van VIIa voor TF is namelijk bijzonder groot, en TF zou al verzadigd met rFVIIa moeten zijn bij veel lagere concentraties. Er is echter voorgesteld dat deze hoge rFVIIa concentraties nodig zijn, omdat rFVIIa competitie aangaat met niet geactiveerd factor VII. Een TF onafhankelijk mechanisme gaat uit van directe binding van rFVIIa aan geactiveerde bloedplaatjes, waarop vervolgens trombinevorming plaatsvindt. De affiniteit van rFVIIa voor geactiveerde plaatjes is vele malen lager dan de affiniteit voor TF, en dit zou de reden zijn voor de hoge rFVIIa concentraties nodig voor het stelpen van bloedingen.
Het doel van dit proefschrift was meer inzicht te krijgen over het werkings-mechanisme van rFVIIa in de verschillende ziektebeelden.

rFVIIa en haemofilie

In het eerste deel van dit proefschrift is gekeken naar de consequenties van versterkte trombinevorming door rFVIIa in plasma van patiënten met haemofilie A. Het was al bekend dat rFVIIa in haemofilieplasma de vorming van een fibrinestolsel kon versnellen. In hoofdstuk 2 is gekeken naar de invloed van rFVIIa op de afbraak van het fibrinestolsel door het fibrinolytisch systeem. Eerder is beschreven dat haemofilie wellicht niet alleen een probleem van vorming van het fibrinestolsel veroorzaakt, maar dat dit stolsel wellicht ook vatbaarder is voor afbraak door het fibrinolytisch systeem omdat TAFI activatie verminderd of afwezig is. In hoofdstuk 2 laten we zien dat rFVIIa niet alleen de stolselvorming versneld, maar dat ook de afbraak van het stolsel geremd wordt door versterking van TAFI activatie. Er is echter veel meer rFVIIa nodig voor maximale TAFI activatie dan voor versnelling van de stolselvorming. Dit zou mede een verklaring kunnen zijn waarom hoge rFVIIa concentraties noodzakelijk zijn om permanente haemostase te induceren in deze patiëntengroep.

rFVIIa en leverziekten

Nadat een antifibrinolytisch effect van rFVIIa in plasma van haemofilie patiënten gevonden was, was de volgende hypothese dat ditzelfde mechanisme ook op zou kunnen gaan in plasma van patiënten met leverziekten. In hoofdstuk 3 is een literatuuroverzicht van de haemostatische afwijkingen in patiënten met leverziekten gegeven. Een van de aspecten die beschreven is in de literatuur is het optreden van hyperfibrinolyse (versnelde stolselafbraak) in een deel van die patiënten. Omdat TAFI een vrij recent ontdekt eiwit is, is nog weinig bekend over de rol van TAFI in patiënten met leverziekten. In hoofdstuk 4 is een studie beschreven over dit onderwerp. Zoals verwacht vonden we verlaagde TAFI concentraties in patiënten met stabiele levercirrhose (TAFI wordt net als vele andere haemostatische eiwitten gemaakt in de lever). Echter, wanneer gekeken werd naar stolselafbraak in plasma van deze patiënten werd geen hyperfibrinolyse gevonden. Wij vonden dat verlaagde concentraties van eiwitten die de fibrinolyse remmen (zoals TAFI) gecompenseerd werden door lage concentraties van eiwitten die de fibrinolyse stimuleren. De discrepantie tussen onze bevindingen en de in eerdere literatuur beschreven resultaten zijn waarschijnlijk te wijten aan verschillen in de experimentele opzet.
Vervolgens is in hoofdstuk 5 het effect van rFVIIa op stolselvorming en stolselafbraak in plasma van patiënten met stabiele levercirrhose en in plasma van patiënten die een

levertransplantatie ondergaan bekeken. In tegenstelling tot de situatie in haemofilieplasma, heeft toevoeging van rFVIIa aan plasma van patiënten met leverlijden geen effect op de fibrinolyse, waarschijnlijk omdat al maximale bescherming tegen stolselafbraak aanwezig is. De effectiviteit van rFVIIa in leverziekten is dus waarschijnlijk toe te schrijven aan versnelling van stolselvorming.

rFVIIa en anticoagulantia

In hoofdstuk 6 is gekeken naar de effecten van medicijnen die fibrinegeneratie remmen (door stolfactoren te remmen) op de fibrinolyse. Van al deze medicijnen zijn de remmende effecten op stolselvorming algemeen geaccepteerd, maar er is nooit eerder onderzoek gedaan naar het effect op TAFI activatie (en dus bescherming tegen stolselafbraak). Er werd gevonden dat de medicijnen die mede gericht zijn tegen geactiveerd stollingsfactor X (zoals de zeer veel gebruikte heparine-achtigen) maar niet degenen die TF of trombine remmen de fibrinolyse stimuleren. De stimulatie van fibrinolyse door factor Xa remmers wordt waarschijnlijk veroorzaakt doordat deze medicijnen niet alleen de kleine hoeveelheid trombine nodig voor de vorming van een fibrinestolsel vertragen, maar ook de aanmaak van grote hoeveelheden trombine die nodig is voor TAFI activatie tegengaan.
In hoofdstuk 7 is gekeken naar de effecten van een nieuw antitromboticum, fondaparinux, een heparine derivaat dat specifiek factor Xa remt. Dit nieuwe medicijn lijkt superieur ten opzichte van traditionele heparine-achtigen wat betreft effectiviteit bij het voorkomen van diep veneuze trombose na operaties. Echter, er is nog geen goede behandelmethode voor patiënten die bloedingen krijgen ten gevolge van behandeling met fondaparinux. Een mogelijk effectieve therapie voor patiënten behandeld met fondaparinux die een bloeding krijgen is de toediening van rFVIIa. Daarom werden de effecten van rFVIIa op stolselvorming en stolselafbraak in plasma waaraan fondaparinux was toegevoegd onderzocht. Fondaparinux remt dus de stolselvorming en stimuleert stolselafbraak door TAFI activatie te remmen. rFVIIa gaat de remming van stolselvorming door fondaparinux volledig tegen. De stimulatie van stolselafbraak door fondaparinux wordt door rFVIIa gedeeltelijk gecompenseerd. rFVIIa zou dus mogelijk een goede therapie zijn voor patiënten die bloedingen ontwikkelen nadat ze met fondaparinux behandeld zijn.

rFVIIa en $\alpha_{IIb}\beta_3$ deficiëntie

Nadat in het grootste deel van dit proefschrift de effecten van rFVIIa op stolselvorming en stolselbescherming door middel van TAFI activatie zijn bekeken, wordt in hoofdstuk 8 ingegaan op de effecten van trombine gevormd via rFVIIa op bloedplaatjesadhesie onder stromingscondities. Bloedplaatjes van patiënten met GT hechten niet aan elkaar en slecht aan adhesieve eiwitten die zich vlak onder de bloedvatwand bevinden. Als geïsoleerde bloedplaatjes van GT patiënten gemengd met rode bloedcellen over de extracellulaire matrix van gekweekte endotheelcellen geïsoleerd uit humane navelstrengen werden geleid, was de adhesie van deze plaatjes aan het oppervlak erg laag. Als nu aan de bloedplaatjes rFVIIa en de stolfactoren X en II werden toegevoegd, nam de adhesie aanzienlijk toe. Hetzelfde werd gezien als remmers van $\alpha_{IIb}\beta_3$ (o.a. remmers die ook als antithromboticum worden toegepast) werden gebruikt. Ook de adhesie van $\alpha_{IIb}\beta_3$-deficiënte plaatjes aan collageen nam aanzienlijk toe wanneer de stolfactoren werden toegevoegd. We hebben laten zien dat TF onafhankelijke trombine vorming verantwoordelijk is voor deze toename in adhesie. Deze trombine wordt gevormd doordat rFVIIa bindt aan al geadhereerde plaatjes, factor X activeert, en vervolgens protrombine geactiveerd wordt. Het gevormde trombine activeert plaatjes die in oplossing zijn, of die over het oppervlak rollen, waardoor ze zich permanent aan het oppervlak hechten.
De toename in adhesie alleen kan nog niet de therapeutische efficiëntie van rFVIIa bij GT patiënten verklaren. De bloedplaatjes verkleven immers nog steeds niet aan elkaar, en er kan nog steeds geen stabiele plaatjesprop worden gevormd. Echter, de geactiveerde bloedplaatjes vormen een oppervlak voor verdere trombinevorming, wat resulteert in een versterking van fibrinegeneratie. De versterkte fibrinevorming door rFVIIa zou dus het plaatjesdefect in GT kunnen compenseren.

Conclusies van dit proefschrift

De in dit proefschrift beschreven studies geven meer inzicht in het werkingsmechanisme van rFVIIa in verschillende ziektenbeelden. Allereerst lijkt het erop dat in sommige situaties rFVIIa niet alleen maar bijdraagt tot een snellere stolselvorming, maar dat rFVIIa via versterkte TAFI activatie het stolsel beschermt tegen afbraak door het fibrinolytisch systeem. Dit mechanisme is tot nu toe alleen nog maar onderzocht in laboratoriummodellen. Of dit mechanisme in het menselijk lichaam ook van toepassing is, is vooralsnog onduidelijk. Bovendien is het belang van TAFI in de fysiologie en

pathofysiologie van de bloedstolling nog onduidelijk. Wellicht dat dierexperimentele modellen meer duidelijkheid over deze vragen zouden kunnen geven.

Ook suggereren studies beschreven in dit proefschrift dat versterking van trombinegeneratie via rFVIIa tot een versterking van adhesie van bloedplaatjes aan de beschadigde vaatwand kan leiden, en dat de verhoogde depositie van bloedplaatjes meehelpt verdere trombine- en fibrinegeneratie te stimuleren. Het is voorstelbaar dat versterking van trombinegeneratie door rFVIIa in elk ziektebeeld leidt tot versterking van plaatjesdepositie, versterking van fibrinevorming, en versterking van TAFI activatie. Onderzoek in de toekomst zal deze hypothese en het belang van deze verschillende mechanismen moeten onderzoeken. Experimenten met proefdieren lijken onvermijdelijk om een beter inzicht te krijgen in structuur en samenstelling van een haemostatische prop, gemaakt in af- of aanwezigheid van rFVIIa, in vivo.

List of publications

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Dankwoord

Het is af! Vier jaar vol leren, pipetteren, schrijven, reizen, brainstormen, eten, drinken, en meer van het soort onzin dat je als AIO doet, zitten er op. Het proefschrift wat uit deze 4 jaar voortgekomen is, is het resultaat van veel samenwerkingsverbanden en ik ben erg veel mensen dankbaar voor hun bijdrage, waarover zo meer.
AIO ben je 24 uur per dag. Ik moet daarom ook een aantal mensen bedanken voor de 'buitenschoolse activiteiten' van de afgelopen 4 jaar.

Maar eerst dus de mensen die een bijdrage aan de inhoud van dit boekje hebben geleverd.

Allereerst mijn begeleiders Prof. Dr. Ph.G. de Groot en Dr. H.K. Nieuwenhuis. Beste Flip, de grootste bijdrage was van jou. Erg bedankt voor alle vrijheid, wijsheid, en eigenwijsheid (fles wijn verwedden om de uitkomst van een experiment.....). De open deur mentaliteit en het feit dat je niet 'baas' maar 'begeleider' was, heeft mij erg gemotiveerd gehouden. Ik hoop dat ik je nog vaker lastig mag vallen!
Beste Karel, met jouw kritische blik bracht je (nog meer) klinische relevantie in mijn artikelen, en zorgde je ervoor dat ik niet al te snel tevreden was.

Degene die ervoor gezorgd heeft dat ik in gespreid bed dit onderzoek heb kunnen doen is Pim Tijburg. Beste Pim, erg bedankt voor je onmetelijke vertrouwen, je in mijn ogen soms wat premature enthousiasme na een enkel experiment, onze vele discussies over wetenschap, leven en toekomst, de inhoud van je adressenboek (veel essentiele samenwerkingen zijn toch bij jou begonnen), nakijken van manuscripten, en natuurlijk de afdeling secundaire arbeidsvoorwaarden.

Jelle, behalve dat je erg veel gepipetteerd, geperfundeerd, en gefluoriseerd hebt (en veel resultaten zijn in dit boekje terug te vinden!), ben je ook een perfecte collega en borrelkoning!

Sultana, topstudent! Jij hebt in je eentje de fundering voor hoofdstuk 8 gelegd. Ik vond het erg leuk om je te begeleiden.

Joost Meijers, jouw belangrijkste bijdrage aan dit boekje is toch wel je suggestie om eens wat lysis assays met VIIa te gaan doen geweest. Ook bedankt voor je interesse, vertrouwen ('hoe kan dit nou briljant zijn als ik er niet bij sta ?!') en voor je 'belangeloze' bijdrage aan allerhande 'klusjes' (vragen/eiwitten/plasma's).

Laurent Mosnier, erg bedankt voor 3 artikelen samen, en voor je hulp met alles wat met TAFI te maken heeft.

Frank Leebeek, wat begon als een obscuur transport van wat plasma samples van het Dijkzigt naar het UMC, heeft uiteindelijk geleid tot 3 publicaties. Bedankt voor je enthousiasme, en je kennis over stolling en leverziekten. Er zijn nog te veel ideeen, gemaakt in verloren uurtjes op vliegvelden, blijven liggen. Wellicht nog iets voor de toekomst.

Harry Janssen, bedankt voor het leveren (no pun intended) van de plasmasamples voor hoofdstuk 4, en voor je positieve houding ten opzichte van het manuscript.

Karina Meijer en Jan van der Meer, bedankt voor het uitwisselen van plasma en labdata van de levertransplantatiestudie.

Mies Kappers-Klunne en Marjolein Peters wil ik bedanken voor de hulp in mijn zoektocht naar Glanzmann plaatjes.

Evelien Mauser-Bunschoten ben ik erkentelijk voor het uitwisselen van plasma en klinische data wat betreft rFVIIa en haemofilie.

Rob Fijnheer, voor grappen & grollen, en voor je hulp met het verzamelen van haemofilie plasma's in het begin.

Thierry Lambert, I'm very happy that you were willing to share your plasma samples, with chapter 2 of this thesis as a result.

Daniëlle Horbach, hier is ie dan! Jij bent degene geweest die mij enthousiast heeft gemaakt voor het werken aan stolling (toen ik nog jong en onschuldig was.....).

It was a privilege to work on this project, which was both financially and scientifically sponsored by Novo Nordisk. I am really happy with the support and enthousiasm I got from a lot of people from Novo Nordisk.

First of all I have to thank Yves Laurian. Dear Yves, your enthousiasm for our work, your knowledge on NovoSeven in platelet related bleeding disorders, and all your

efforts to provide us with materials to perform the best possible research have been a great help.

Roger Houben, bedankt voor je interesse, en voor in meer of mindere mate zinvolle gesprekken aan bar en telefoon.

Marianne Baak, erg bedankt voor al die kleine dingen die het allemaal weer iets makkelijker voor me maakten.

Charles Pyke, Egon Persson, Rasmus Røjkjær, Marianne Kjalke, and Ulla Hedner, thanks for giving me the opportunity to work in your lab, and for the exchange of knowledge and materials the past 4 years. Your hospitality during my stay has been great – however, I could have done without the 'schnapps'.....
Dear Ulla, thanks for your enthousiasm – it's an honour that you're part of my thesis review committee.

Britt Sørensen, you've been a great 'roommate'. Annette Danielsen, thanks for getting me started in the lab, and for cleaning up after me. Also a big thank you to the rest of the partygirls!

Ditte Tornehave, many thanks for introducing me to the world of histology. Steen Kryger, thanks for your help – unfortunately the 'bloody' tails turned out to be a more difficult task than anticipated.

Marie Johannessen and Vinnie Nielsen, how a couple of days work can make it into a thesis! Thanks!

Tsja, en dan de afdeling koffie leuten, borrelen, congresvermaak, weekendvermaak. Behalve van radio 3, moet het vermaak toch van collega's en aanverwante figuren komen.

Allereerst alle borrel/karaoke/cocktail figuren. Geheel in willekeurige volgorde: Klitje (maandagochtend vermaak, waarissienou?, snelle fenotypering GT patienten, dure dranksmaak), Jelle (bar type, barbara type, kwam toen 2 uur vroeger werken.), Wessie (wodka held, Mick Dundee zonder borsthaar), Elsje (preprovoor de spiegel dansen, veel te weinig foute orgelhits gedaan.), Lucio (Red Bull.....vleugels.....feessie), Kurt

(Barcardi by the gallon), Cora (komt altijd.....maar hoe laat?), Chantal (don't ever call me Tonnie again!), Beef (voor dia's & vroeger), Rob (rechtop, ontzettend bedankt voor 'lysis analyzer' en de rest van de ongein), Larry (waar blijft die bierkoelkast nou?), Edje (vreemde eend, breezah, wah blijf me thrui?), Achmed & labinhoud (jong geleerd, oud gedronken, Agnes ff pauze), Crucell (Glendah.....Maarten.....enzo), Ingrid (hoofdstuk apart).

Andere labbewoners, ex-labbewoners, studenten, het was een feest. Tinus, bedankt voor prikken, ouwehoeren & koffie. Marion voor compromitterende foto's op het www.

Lab routine Bep, bedankt voor hulp & vermaak. Yke, afreageer babbelbox, grijze wijze!

Mijn paranimfen. Janine, a.k.a. Moniek!, voorkant n=3, slap ouwehoeren, rock'n'roll life, always forbidden, onbegrijpelijke electronische onderwerpen wie met onbekende regelmaat verschijnen.
Tom B, ouwe vos, main man! Fijne gedachte dat jij je in dat pak moet hijsen. Nu nog die ene hit!

Geert, andere ouwe vos. Bedankt voor plaatjes & illustraties, bebop sessies, nachttrein avondturen.

Theo Lisman, ontzettend bedankt voor lay-out en drukken van dit boekje.

Mijn opa (laudatio non olet) en oma (koffie zwart, 2 koekjes).

Mijn ouders voor het mij laten doen waar ik zin in had.

Het is af. Nu maar een winterslaap.

Curriculum Vitae

Ton Lisman werd geboren op 10 maart 1976 te Arnhem. Hij volgde zijn middelbare schoolopleiding aan het Nederrijn College te Arnhem en de openbare scholengemeenschap Echnaton te Almere, alwaar in 1994 het VWO diploma behaald werd. In datzelfde jaar werd aangevangen met de studie scheikunde aan de Universiteit Utrecht. In 1998 werd het doctoraal diploma behaald met als bijvak Haematologie (Dr. D.A. Horbach, Dr. J.C.M. Meijers en Dr. Ph.G. de Groot, Faculteit Geneeskunde, Universiteit Utrecht) en als hoofdvak Biochemie van Lipiden (Dr. Y.C.M. de Hingh en Prof. Dr. J.A.F. op den Kamp, Faculteit Scheikunde, Universiteit Utrecht). Van januari 1999 tot en met december 2002 was de schrijver van dit proefschrift werkzaam als assistent in opleiding bij de vakgroep Haematologie van het Universitair Medisch Centrum Utrecht onder begeleiding van Prof. Dr. Ph. G. de Groot en Dr. H.K. Nieuwenhuis. In deze periode werd 5 maanden lang onderzoek verricht bij de onderzoeksgroep Vascular Biochemistry van Novo Nordisk A/S in Måløv, Denemarken onder begeleiding van Dr. R. Røjkjær en Dr. E. Persson. De resultaten van het vierjarig promotieonderzoek zijn beschreven in dit proefschrift.