The interplay between platelets and coagulation

Cees Weeterings

The interplay between platelets and coagulation

Het samenspel tussen bloedplaatjes en stolling
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge van het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 23 juni 2009 des middags te 12.45 uur

door

Cornelis Weeterings

geboren op 21 januari 1981
te Leerbroek

Promotor: Prof. dr. Ph.G. de Groot

Co-promotor: Dr. J.A. Lisman

CONTENTS

Introduction

The role of GPIb in coagulation

The role of GPIb in platelet adhesion

Discussion

Chapter 1A

Platelet aggregation: involvement of thrombin and fibrin(ogen)

Ton Lisman, Cees Weeterings, and Philip G de Groot

Thrombosis and Haemostasis Laboratory, Department of Haematology, University Medical Centre Utrecht, Utrecht, and Institute of Biomembranes, Utrecht University, Utrecht, The Netherlands

ABSTRACT

Platelets play a key role in hemostasis and thrombosis. The formation of a platelet plug is accompanied by the generation of thrombin, which results in the generation of fibrin required for stabilization of the platelet plug. Platelet plug formation and coagulation are closely linked processes. Thrombin is a potent platelet activator, which proceeds through proteolysis of the protease activated receptors (PARs). Furthermore, thrombin binds glycoprotein Ib(alpha), which amplifies platelet activation by accelerating PAR-1 activation, and possibly also by direct signaling events through glycoprotein Ib(alpha). Moreover, thrombin's specificity towards other substrates changes after binding glycoprotein Ib(alpha). Fibrinogen and fibrin, the end product of the coagulation cascade, are also involved in platelet aggregation. Both fibrinogen and fibrin bind the integrin alpha(IIb)beta(3), and another fibrin receptor involved in platelet aggregation has been postulated. This review will discuss the role of thrombin and fibrin(ogen) in platelet functioning, and will highlight pathways at the crossroad of coagulation and platelet functioning, which are potential targets for antithrombotic therapy.

INTRODUCTION

Blood platelets play a pivotal role in hemostasis and thrombosis, as evidenced by the bleeding tendency of patients with qualitative or quantitative disorders of platelets, and the therapeutic efficacy of anti-platelet drugs for thrombotic manifestations. After vessel wall injury, platelets are recruited to the exposed subendothelial tissue. The collagen fibers from the subendothelium bind the plasma protein von Willebrand factor (vWF), which, once bound to collagen, becomes able to interact with glycoprotein Ib (GPIb) on the platelet surface. The transient GPIb-vWF interactions results in a reduction of velocity of the platelet, which enables firm adhesion to collagen via the platelet collagen receptors alpha(2)beta(1) and GPVI. Alternatively, other platelet receptors can mediate firm adhesion by an interaction with other adhesive ligands. Specifically, alpha(IIb)beta(3) can bind vWF, fibrinogen, vitronectin and thrombospondin, alpha(5)beta(1) can bind fibronectin, alpha(6)beta(1) can bind laminin, and alpha(v)beta(3) can bind vitronectin. Subsequently, platelets are activated by either adhesive proteins such as collagen, or by thrombin generated by the coagulation system (see below). Platelet activation is propagated by stimulatory molecules excreted from the platelets, including thromboxane A2, which is synthesized in the platelet on activation, and by ADP and serotonin, which are released from the dense granules after activation. Platelet activation leads to activation of integrin alpha(IIb)beta(3),
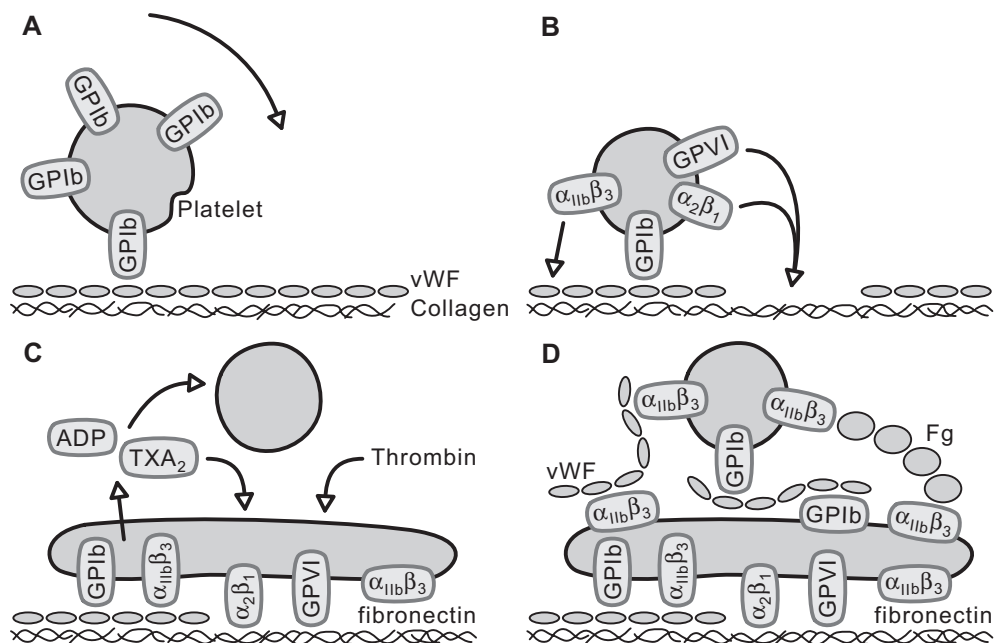

Figure 1 - Schematic representation of platelet adhesion and aggregation under flow conditions.
A) Rolling of platelets over collagen-bound vWF mediated by GPIb. B) Firm attachment mediated by alpha(2)beta(1) and glycoprotein VI (GP VI) binding to collagen, and by alpha(IIb)beta(3) binding to collagen-bound vWF. C) Platelet activation, secretion, and spreading. D) Aggregate formation.

which is than able to bind vWF and fibrinogen. VWF and fibrinogen are able to bind two platelets, which leads to the formation of a platelet aggregate, and thus the formation of a stable platelet plug. The sequential steps of platelet adhesion and aggregation are depicted in figure 1.

Stabilization of the platelet plug proceeds via the formation of fibrin through the coagulation system. Coagulation starts when blood comes in contact with tissue factor, which may be present on subendothelial cells, such as fibroblasts and smooth muscle cells, or may be present in encrypted form in the bloodstream (the so-called blood-borne tissue factor). Through a series of enzymatic reactions, TF-induced coagulation leads to the formation of thrombin, which cleaves fibrinogen to fibrin. Fibrin spontaneously polymerizes and forms an insoluble mesh in between the platelet plug. Thrombin also converts factor XIII to XIIIa, which crosslinks the fibrin clot, resulting in further stabilization of the hemostatic plug.

Platelet plug formation and fibrin formation are closely linked processes, and the true relevance of the interplay between the two may still be underappreciated. It has been known for many years that activated platelets form a surface on which the enzymatic reactions leading to the generation of thrombin can occur. It has also been known for a long time that thrombin is a potent platelet activator, however, the exact mechanisms by which thrombin activates a platelet are still incompletely understood. Finally, some evidence that besides vWF and fibrinogen also other ligands including fibrin can aggregate platelets has appeared in literature.

This paper will review the mechanisms by which thrombin and fibrin(ogen) interact with platelets, and how these interactions contribute to platelet activation and aggregation.

THROMBIN-MEDIATED PLATELET ACTIVATION

Thrombin can interact with a platelet via at least two different receptor classes, the protease activated receptors (PARs) and GPIb(alpha). Thrombin can activate PARs via proteolysis of a single peptide bond, which leads to the generation of a signal inside the platelet. Thrombin binds to, but does not proteolytically cleave GPIb(alpha). Thrombin binding to GPIb(alpha) enhances the activation of PAR-1, results in enhancement of platelet associated coagulation reactions, and cleaves glycoprotein V (GPV), one of the proteins complexed with GPIb(alpha), from the platelet surface. Detailed descriptions of these various interactions of thrombin with the platelet will be described below.

Platelet activation via protease activated receptors
Protease activated receptors were first discovered in 1990 with the cloning of the receptor now known as PAR-1.[1] PARs are seven-transmembrane G-protein-coupled receptors with an unusual way by which they are activated. The PARs

carry their own ligand that remains cryptic until proteolysis of the N-terminal part of the receptor occurs. Proteolysis of the receptor exposes a new N-terminus, which is now able to bind intramolecularly to the body of the receptor, thereby inducing transmembrane signaling. Alternatively, receptor activation may occur in the absence of proteolysis by a peptide comprising the cryptic sequence that binds directly to the body of the receptor.[1] PARs 1, 3, and 4 can be activated by thrombin,[1-3] whereas PAR 2 can be activated by trypsin, tryptase, and by coagulation factors VIIa and Xa.[4-6]
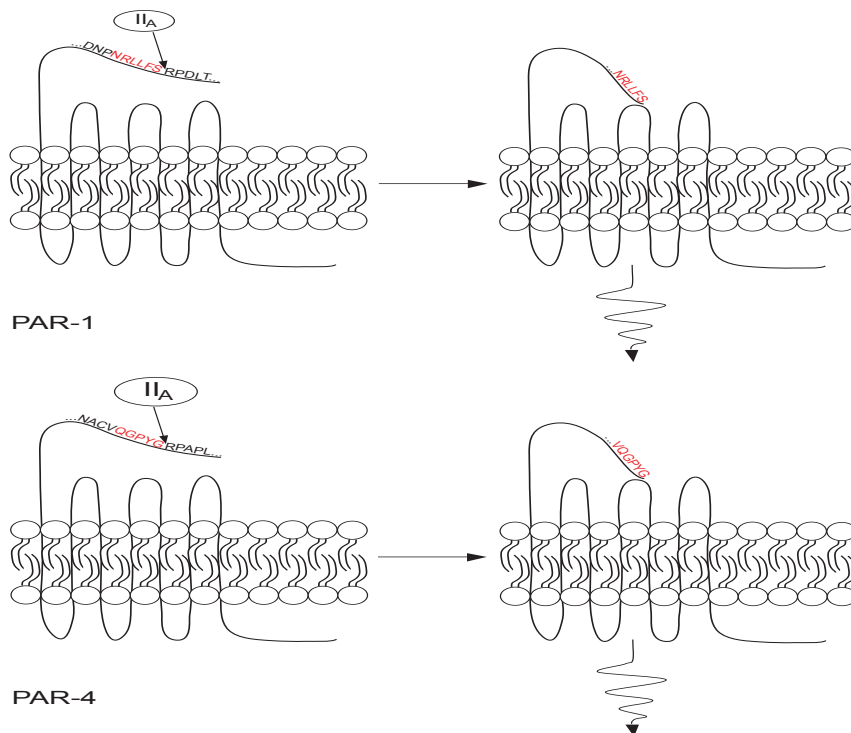
Figure 2. Thrombin-mediated activation of PARs in human platelets. Human platelets express both PAR-1 and −4. Thrombin cleaves at the N-terminal extracellular part of the receptor, thereby exposing a new N-terminus (SFLLRN and GYPGQV, respectively), which binds to the body of the receptor, leading to G-protein-coupled signal transduction.

Thrombin-mediated platelet activation via PARs has been studied extensively in both human and mouse platelets. Interestingly, different isoforms of the PARs are required for activation of human platelets as compared to mouse platelets. Therefore, the activation of human and mouse platelets by thrombin will be discussed separately in the following sections.

Activation of human platelets
Human platelets express PAR-1 and PAR-4, and activation of either is sufficient to

trigger platelet aggregation.[7] However, distinct differences between the mode of platelet activation via PAR-1 and −4 exist. PAR-1 is activated by low concentrations of thrombin. Thrombin binding to PAR-1 is facilitated by the hirudin-like sequence on PAR-1 that binds thrombin exosite 1.[8] PAR-4 lacks a hirudin-like sequence, and therefore requires a 10-100 fold higher thrombin concentration to elicit intracellular signaling.[7] Moreover, when thrombin is bound to GPIb(alpha), the rate of PAR-1 but not −4 activation is enhanced about 5-fold.[9,10] Proteolysis of the receptor by thrombin exposes a new N-terminus, which is now able to bind intramolecularly to the body of the receptor, thereby inducing transmembrane signaling (figure 2). Initiation of signaling occurs via the activation of Gq, G12, and Gi family members (reviewed in [11]), although the involvement of Gi in human platelets has been questioned.[12] This results in the activation of a variety of signaling molecules, including phospholipase C(gamma), phosphatidylinositol-3-kinase, and a number of small G-proteins including Rho, Rac, and Rap1 (reviewed in [13]). This leads to an increase in cytosolic calcium, and the inhibition of generation of cyclic-AMP via
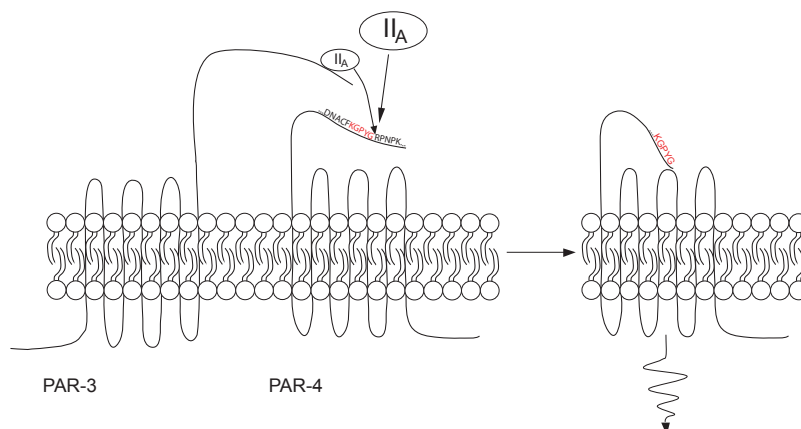
Figure 3. Thrombin-mediated activation of PARs in mouse platelets. Mouse platelets express PAR-3 and −4. PAR-3, however, is not cleaved by thrombin, but rather binds thrombin with high affinity and serves as a docking molecule to present thrombin to PAR-4, which is cleaved by thrombin. PAR-3 is necessary for PAR-4 activation at low concentrations of thrombin. At higher thrombin levels, PAR-4 can be activated without the requirement for PAR-3.

adenylyl cyclase. The signals generated by the PARs are transient. Theoretically, a single thrombin molecule may cleave multiple PARs, and cleavage of a single PAR could result in sustained activation of the receptor. However, since the extent of second messengers generated is directly proportional to the concentration of thrombin,[14] and as final events such as calcium influx are transient,[7] a rapid termination mechanism must occur. It has been demonstrated that the PARs are rapidly phosphorylated and internalized.[15] Part of the receptors are degraded in lysosomes, and part recycle to the cell-surface. The recycled receptors are unable to be stimulated again by the same agonist, and therefore platelets can be desensitized to thrombin, or to either of the thrombin receptor activating peptides

(SFLLRN and GYPGQV, for PAR-1 and −4, respectively). The kinetics of signal termination differ between PAR-1 and −4.[7] The calcium signal elicited by PAR-1 is strong, but declines rapidly, whereas the signal elicited by PAR-4 is more gradual and prolonged. The calcium signal elicited by a high concentration of thrombin shows characteristics of a combined signaling through both PARs, i.e., a rapid rise followed by a slow decay. PAR-1 activation was shown to be the main mediator of exposure of procoagulant phospholipids on the platelet membrane after thrombin stimulation.[16] Apparently, activation of PAR-4 gives a weaker signal, which corresponds to the observation that PAR-4-induced platelet activation is completely dependent on the release of ADP, whereas PAR-1-induced platelet aggregation is only in part dependent on ADP.[17]

It is unclear why two thrombin receptors with distinct function are present on human platelets. It is possible that these two receptors are simply providing redundancy in the complex system of platelet activation. Alternatively, the capacity of platelets to respond to thrombin over a broad concentration range may be important for reasons, which are not yet understood. Also, it might be possible that PAR-4 primarily functions as a mediator of responses to proteases other than thrombin, e.g., cathepsin G[18] or plasmin.[19] Realizing the complexity of thrombin activation of human platelets is crucial in the development of pharmaceuticals inhibiting thrombin-mediated platelet activation (see below).

Activation of murine platelets
In contrast to human platelets, mouse platelets utilize PAR-3 and −4 to generate thrombin signals.[20] PAR-3 is an odd member of the PAR family. In humans, PAR-3 is expressed on a number of cell types but not on platelets, and can generate intracellular signals after cleavage by thrombin.[2] Curiously, however, human PAR-3 is not activated by peptides comprising the tethered ligand sequence. In mice, PAR-3 does not generate intracellular signaling.[20] Instead, it functions as a cofactor for PAR-4 activation, perhaps analogous to the cofactor function of GPIb(alpha) in PAR-1 activation in human platelets. PAR-3 binds thrombin already at relatively low concentrations, and after binding thrombin is presented to PAR-4, which is cleaved and generates intracellular signals (figure 3). At high thrombin concentrations, PAR-4 can be cleaved independently of PAR-3. The relevance of this cofactor function of PAR-3 in murine platelets was demonstrated by PAR-3 knockout mice, whose platelets showed impaired platelet responses to thrombin. Moreover, PAR-3 knockouts had a prolonged bleeding time, and were protected against thrombosis both in a ferric chloride model in the mesenteric vein, as well as in a model of tissue factor-induced pulmonary embolism.[21] Interestingly, the PAR-4 knockout mice, whose platelets lack any response to thrombin, showed a similar prolongation of the bleeding time, and a similar protection against thrombosis in the two mentioned models. This may indicate that in the bleeding time and thrombosis models used, only a limited amount of thrombin is generated which is only able to activate platelets in the presence of both PAR-3 and −4.

Possibilities for therapeutic intervention

Blocking thrombin-mediated platelet activation by PAR-inhibiting pharmacological agents might be an attractive approach to treat (arterial) thrombosis. The use of PAR antagonists as antithrombotic drugs has, at least in theory, some potential advantages over antithrombotic drugs that are currently available in the clinic. One alternative to PAR antagonism would be direct or indirect thrombin inhibition by drugs such as ximelagatran and heparin. A major disadvantage of drugs inhibiting (the generation of) thrombin is their relatively narrow therapeutic window. Even at therapeutic levels, thrombin-inhibiting drugs are inevitably associated with an increased bleeding risk.[22] This phenomenon may be in part explained by the fact that when inhibiting thrombin, also the generation of fibrin, and the anticoagulant protein C system are inhibited, leading to a complex disturbance of the hemostatic balance.

Direct inhibition of PARs selectively ablates the ability of thrombin to activate a platelet, while all the other hemostatic functions of thrombin are preserved. As in mice the absence of one of the PARs does not lead to an overt bleeding tendency, it might be that it is possible to antagonize one or both PARs on human platelets to obtain an antithrombotic state without inducing a severe hemostatic defect. The same argument may hold for the comparison of PAR antagonists with other platelet inhibitors, such as alpha(IIb)beta(3) blockers or P2Y12 antagonists. The latter drugs inhibit platelet function induced by all agonists, while PAR blockers specifically block platelet activation by thrombin, while leaving other platelet activation pathways intact. A potential disadvantage of PAR antagonism as antithrombotic strategy is that platelets contain 2 members of the PAR family, and that it might be necessary to block both to obtain sufficient antithrombotic potential. Furthermore, PARs are also present on other vascular cells (e.g., endothelial cells), and PAR antagonism might result in unwanted side effects as PAR signaling in these cells will also be inhibited. A number of small molecule PAR-1 antagonists have been described (reviewed in [23-25]), and the few in vivo animal studies that have been performed with these compounds suggest that PAR antagonism may have potential as an antithrombotic drug in humans.[26,27]

Thrombin interaction with GPIb(alpha)

GPIb(alpha) has been found to be the high-affinity receptor for thrombin[28,29] and is constitutively expressed in platelets in complex with GPIb(beta), GPIX and GPV.[30] GPIb(alpha) consists of a short cytoplasmic tail, a small transmembrane region and an extracellular fragment that can be cleaved by proteases such as calpain, trypsin, plasmin, and elastase to yield a 135 kD fragment, referred to as glycocalicin. The extracellular portion consists of a 45 kD N-terminal domain (residues 1-282), which is composed of eight leucine-rich repeats and contains the binding sites for many GPIb(alpha) ligands, such as thrombin, factor XI and vWF, a short region (residues 283-302) enriched in negatively charged residues, and a long and highly glycosylated macroglycopeptide stack (residues 303-485), which includes three sulphated tyrosine residues that are essential for the interaction with thrombin.[31]

Although there are about 25000 copies of GPIb(alpha) on the platelet, only 1000-1500 account for the high-affinity binding of thrombin.[32] Part of the GPIb/IX/V-complex has been shown to reside in lipid rafts and this phenomenon may account for the difference between the amount of GPIb(alpha) copies and high affinity receptors on the platelet.[33] It has already been shown that factor XI bound to GPIb(alpha) is localized in lipid rafts of activated platelets.[34]

Crystal structure of thrombin bound to GPIb(alpha)
Thrombin contains two anion-binding exosites referred to as exosite I and exosite II, and a catalytic pocket. Exosite I is important in binding of multiple substrates, including fibrin and fibrinogen and PAR-1, whereas exosite II is also known as the heparin binding site. Recently, site-directed mutagenesis has indicated that many basic exosite II residues are involved in binding to GPIb(alpha).[35,36] Peptide competition studies have revealed that the thrombin-binding domain on GPIb(alpha) is located at the C-terminal part of the 45 kD N-terminal domain. In addition, two groups have published the crystal structure of thrombin bound to the N-terminal part of GPIb(alpha).[37,38] Both groups showed that both exosites of thrombin were involved in binding of GPIb(alpha). Furthermore, both crystal structures showed that GPIb(alpha) has a second binding site for thrombin. Celikel et al. and Dumas et al. both conclude that initial binding of thrombin to GPIb(alpha) is dependent on exosite II, followed by a conformational change in thrombin, which facilitates the binding of thrombin to another GPIb(alpha) molecule via exosite I. However, both crystal structures show differences in the interaction of the second thrombin with GPIb(alpha) and this resulted in different interpretations about the possible consequence of thrombin binding to GPIb(alpha) (figure 4). Celikel et al. postulate binding of one thrombin molecule to two GPIb(alpha) molecules of the same platelet, which induces clustering of GPIb(alpha) and subsequent signaling.[37] In contrast, Dumas et al. suggest binding of one thrombin molecule to two GPIb(alpha) molecules of two different platelets, resulting in possible aggregation of platelets via the thrombin-GPIb(alpha) interaction.[38]

The clustering model suggested by the group of Celikel is in concordance with observations of Ramakrishnan et al, who suggested the clustering of GPIb(alpha) after GPV cleavage by thrombin.[39] However, there is still no solid evidence for the clustering of GPIb(alpha) by thrombin. The aggregation model suggested by the group of Dumas is not supported by other studies. On the contrary, platelets from PAR-4-deficient mice did not aggregate upon treatment with 500 nM thrombin in vitro, suggesting that GPIb alone is not sufficient to aggregate platelets.[40] The exact role of the interaction between thrombin and GPIb(alpha) and its role in platelet activation is thus still a matter of debate and several reviews have tried to elucidate this controversy.[41-43]

Our own research group has recently found that thrombin immobilized on a surface is also able to induce platelet adhesion, activation and aggregate formation under flow conditions, which is mediated by GPIb(alpha) and the activation of PAR-1
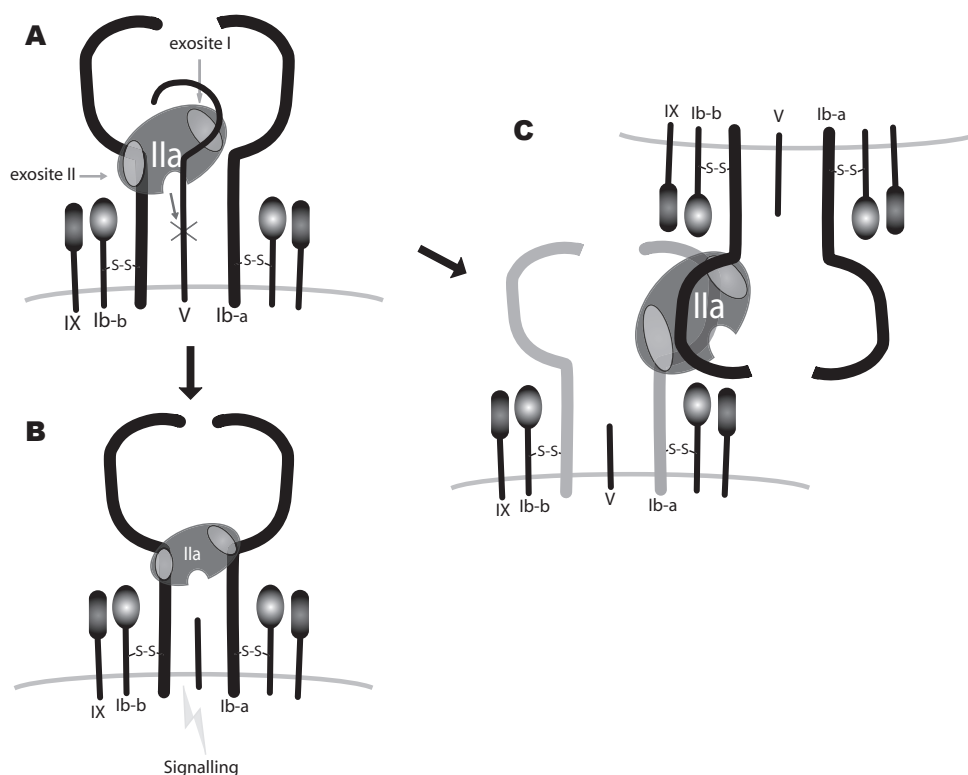
Figure 4. Schematic representation of thrombin binding to GPIb(alpha). (A) Thrombin binds to the tyrosine-sulphated region of GPIb(alpha) via its exosite II. After binding to GPIb(alpha) thrombin is able to cleave GPV, resulting in the ability to bind via exosite I to another GPIbα molecule. The cleavage of GPV is necessary for this clustering as the intact GPV most likely sterically hinders the interaction between the two GPIb(alpha) molecules. From this point on the two crystal structures of Celikel et al. and Dumas et al. suggest opposite consequences. (B) Celikel et al. suggest clustering of GPIb(alpha) on the same platelet via thrombin, which could subsequently lead to intracellular signaling. (C) Dumas et al. suggest an adhesion mechanism, which involves the interaction of thrombin between two GPIb(alpha) molecules on two different platelets, eventually resulting in possible platelet aggregation.

(Weeterings C., Adelmeijer J., Myles T., de Groot Ph.G., Lisman T.; unpublished results). The role of exosite II in this interaction is important, because recombinant thrombins with mutations in exosite II are unable to induce platelet adhesion.

Thrombin binding to GPIb(alpha) causes intracellular signaling
Until recently, not much was known about signaling pathways induced by thrombin binding to GPIb(alpha). The role of GPIb(alpha) in signaling via thrombin seemed unimportant, when the thrombin receptor PAR1 was discovered.[1] Up to date, only a handful of publications describe signaling via GPIb(alpha). The first indication for signaling via GPIb(alpha) upon thrombin binding came from a study from Ramakrishnan et al, which showed that platelets from GPV deficient mice could aggregate upon treatment with DIP-thrombin, a proteolytically inactive form of thrombin.[39] This indicates that the binding of thrombin to GPIb(alpha) induces

intracellular signaling independent of the proteolytic activity of thrombin, and thus independent of PAR-1. However, removal of GPV from the complex is required for signaling to occur, since wild-type platelets failed to respond to DIP-thrombin. Ramakrishnan et al. could completely inhibit this platelet aggregation of GPV null mice by addition of an antagonist of the P2Y12 receptor (Gi-coupled receptor), but not by an antagonist of the P2Y1 receptor (Gq-coupled receptor).[39] This suggests a prominent role for ADP secretion in thrombin-induced signaling upon binding to GPIb(alpha).

In addition, Adam et al. investigated the role of immobilized thrombin in inducing intracellular signaling.[44] They used active site-blocked thrombin to exclude signaling pathways caused by activation of PAR-1. The secretion of ADP and its subsequent binding to its receptor P2Y12 played an important role in signaling. The ADP scavenger apyrase could inhibit platelet adhesion to PPACK-thrombin. Additionally, P2Y1 and P2Y12 antagonists reduced platelet adhesion to immobilized thrombin. Furthermore, signaling events comprised an increased level of PI3-kinase and Src-family members and adhesion to immobilized thrombin was strongly dependent on protein kinase C.[44] However, in contrast to the results of Ramakrishnan et al., Adam et al. showed intracellular signaling upon treatment with active site-blocked thrombin, which is independent of GPV cleavage.

Other studies showed that thrombin binding to GPIb(alpha) results in the release of ATP and mobilization of internal calcium stores.[32,45] Furthermore, thrombin binding to GPIbα induced activation of MEK-1, involved in the MAPK signaling pathway, and of the Rho-kinase p160ROCK, which is involved in platelet shape change, and this was independent of mobilization of internal calcium stores.[46] Furthermore, Dubois et al. observed cleavage of talin, a protein important in anchoring actin filaments to integrins, through a calcium independent mechanism.[46] Thus, shape change and the rearrangement of the platelet cytoskeleton are initiated by the interaction between thrombin and GPIb(alpha).

Consequences of thrombin binding to GPIb(alpha)
The substrate specificity of thrombin substantially changes after binding to GPIb(alpha), both in a prohemostatic and anticoagulant way. The anticoagulant function of this interaction comprises the inability of thrombin to cleave fibrinogen, when thrombin is bound to GPIb(alpha).[47,48] Furthermore, the binding of thrombin to GPIb(alpha) limits the activation of factors V and VIII, thus reducing the rate of thrombin formation.[49] On the other hand, the binding of thrombin to GPIb(alpha) serves a procoagulant role, as it prevents thrombin from binding to thrombomodulin.[32] Furthermore, PAR-1 activation is induced 5-fold upon thrombin binding to GPIb(alpha)[9] and also factor XI activation is accelerated, when both factor XI and thrombin bind to GPIb(alpha).[50] Because thrombin binds to GPIb(alpha) via its heparin-binding site, thrombin bound to GPIb(alpha) is protected against the heparin-accelerated inhibition by anti-thrombin III.[51] As the binding sites on GPIb(alpha) for thrombin and vWF only partially overlap, it has been implied that

binding of thrombin to GPIb(alpha) regulates the affinity of GPIb(alpha) for vWF. A recent study suggested a mechanism through which binding of thrombin to GPIb(alpha) stabilizes platelet-platelet contacts by mediating a tighter association between the vWF A1-domain and GPIb(alpha).[52]

In addition, recent findings (as described in the previous section) indicated that thrombin binding to GPIb(alpha) also causes signaling events. PAR-1 activation was shown to be the main mediator of exposure of procoagulant phospholipids on the platelet membrane after thrombin stimulation, but GPIb(alpha) significantly contributes to this process.[16,53] Thus, therapeutically blocking the interaction between GPIb(alpha) and thrombin could be a useful method in preventing thrombosis, by interfering with the amplification of the coagulation pathway and platelet activation. Already, a couple of monoclonal antibodies, like LJIb-10[32] and VM16d,[54] have been described that specifically inhibit the binding of thrombin to GPIb(alpha), but not the binding of vWF to GPIb(alpha). However, to our knowledge, these antibodies have not yet been tested in relevant (in vivo) thrombosis models.

Proteolysis of GPV by thrombin
GPV is, like the other members of the GPIb/V/IX complex, a leucine-rich glycoprotein, which is non-covalently associated with the complex.[30] The number of GPV molecules on the platelet surface is approximately 50% of the number of GPIb(alpha) and (beta) and GPIX molecules,[30] and it has therefore been proposed that the basic unit of the complex consist of one GPV and two GPIb(alpha), GPIb(beta), and GPIX molecules. The exact function of GPV in the complex is unknown. Mutations in GPIb(alpha), GPIb(beta), and GPIX associated with a bleeding tendency, referred to as the Bernard-Soulier syndrome, have been described (reviewed in [55]). However, no mutations in GPV associated with a bleeding tendency have thus far been found. It has been suggested that GPV is required for optimal expression of the other components of the complex,[56,57] but it has also been described that cells transfected with GPIb(alpha), GPIb(beta), and GPIX express comparable levels of these proteins in the absence or presence of cotransfection of GPV.[58] Moreover, the GPV knockout mouse expresses similar levels of GPIb(alpha), GPIb(beta), and GPIX compared to wild-type animals.[59,60]

GPV can be proteolysed by thrombin, releasing a 69 kD soluble fragment.[61] The role of GPV proteolysis in platelet physiology is unclear, but the conservation of the thrombin cleavage site in GPV in mice and rats may suggest that it is important.[62] Although GPV proteolysis is linear with respect to thrombin concentration, the extent of platelet activation is not related to the amount of GPV proteolyzed.[63] Maximal platelet activation by thrombin can already occur when less than 1% of the GPV has been cleaved of, which might suggest that proteolysis of GPV is not required for thrombin-induced platelet activation. Renewed interest in GPV proteolysis occurred with the generation of GPV knockout mice. Two groups have independently described generation and phenotyping of GPV knockout mice,

but opposing results have been reported using these two different strains.[59,60] Kahn et al. reported a complete lack of phenotype of their mice.[60] In contrast to GPIb(alpha) knockouts, which showed reduced platelet count with giant platelets comparable to the phenotype observed in human Bernard-Soulier syndrome,[64] platelet size and platelet count in the GPV knockouts were normal, as was the adhesion of GPV-/- platelets to immobilized vWF A1 domain. Moreover, platelet aggregation in response to thrombin and tail bleeding time was not different in GPV null mice compared to controls. Further characterization of these mice by Moog et al. revealed that GPV null mice had defective thrombus formation in vivo and defective collagen induced platelet aggregation in vitro, which was ascribed to the ability of GPV to function as an adhesive receptor for collagen.[65] These results suggest that GPV positively regulates platelet thrombus formation. An opposite conclusion was reached based on the GPV knockout mice created by Ramakrishnan et al.[59] Platelets from their knockout mice exhibited increased responses to thrombin. Even more, platelets from their GPV-/- mice could be fully activated with proteolytically inactive thrombin.[39] The bleeding time in these GPV-/- animals was shortened, as compared to wild type animals, and using intravital microscopy, increased thrombus formation and embolisation could be demonstrated in the GPV knockouts.[66] The precise mechanism by which cleavage of GPV from the complex renders the platelets more reactive is unclear, but it has been suggested that removal of GPV exposes a thrombin receptor function on the remainder of the complex, presumably GPIb(alpha).[39] Thrombin binding to the GPIb/IX complex after GPV removal induces proteolysis-independent signaling, sufficiently robust to lead to full aggregation. This process may be mediated by interplatelet crosslinking of GPIb(alpha) by means of the interaction of a single thrombin molecule with two GPIb(alpha) molecules mediated by both exosite 1 and 2 on thrombin. The thrombin-induced signaling pathways via GPIb(alpha) are incompletely known, but include activation of Src family kinases, protein kinase C, and phosphatidylinositol-3-kinase.[44] It is known that the adaptor protein 14-3-3(zeta), calmodulin, and the cytoskeletal actin binding protein are coupled to the GPIb/V/IX complex, but their role in thrombin-induced signaling via GPIb(alpha) has to our knowledge not been demonstrated.[67]

The development of an ELISA to measure the soluble fragment facilitated in vivo analysis of GPV proteolysis by thrombin.[68] As the release of sGPV from platelets in vitro is proportional to the amount of thrombin, measurement of sGPV in patient plasma would give an estimate of thrombin-mediated platelet activation in vivo. Proof of concept for this was obtained in a rat thrombosis model.[69] Continuous infusion of tissue factor resulted in thrombin generation (as measured by thrombin-antithrombin complexes) and the appearance of soluble GPV in plasma. These effects could be inhibited by hirudin, which confirms the specificity of this novel marker of in vivo platelet activation by thrombin. Subsequent studies have shown elevated levels of soluble GPV in patients with atherosclerosis,[70] myocardial infarction,[71] unstable angina,[72] atrial fibrillation,[73] and stroke.[74] Follow-up studies will be required to assess the informative, diagnostic, and prognostic value of this

novel marker.

PLATELET AGGREGATION - INVOLVEMENT OF FIBRINOGEN AND FIBRIN

Fibrinogen and vWF are traditionally considered to be the two most important proteins capable of inducing platelet aggregation. Both molecules are able to bind alpha(IIb)beta(3) on two different platelets, leading to platelet-platelet interaction. Since both ligands are present in plasma and in platelet alpha-granules, they can compete for alpha(IIb)beta(3), which may lead to incomplete bridge formation, thereby limiting thrombus formation.[75] This incomplete bridge formation occurs when alpha(IIb)beta(3) on one platelet is occupied by fibrinogen, and alpha(IIb)beta(3) on the adjacent platelet has vWF bound to it. Consistent with this hypothesis, larger aggregates are observed under conditions in which either vWF or fibrinogen is absent (von Willebrands disease type III, and afibrinogenemia, respectively), although these aggregates appear less densely packed, and therefore less stable.[75,76] It appears, however, that other proteins are also able to induce alpha(IIb)beta(3)-dependent platelet aggregation, since mice lacking both vWF and fibrinogen are still able to form platelet thrombi.[77] Possible ligand candidates for alpha(IIb)beta(3)-dependent aggregation other than fibrinogen and vWF are fibronectin,[78] vitronectin,[79] and CD40 ligand (CD154).[80]

Fibrinogen-mediated platelet aggregation

Fibrinogen is a homodimeric molecule of alpha, beta, and gamma chains. Fibrinogen-mediated platelet aggregation proceeds via the dodecapeptide sequence HHLGGAKQADVG at the carboxy-terminal end of the fibrinogen gamma chain (residues 400-411).[81] Previously, also two RGD sequences in the fibrinogen alpha chain have been postulated to bind alpha(IIb)beta(3) (residues 95-97 and 572-574). RGD is a consensus binding sequence for integrins, which was identified using synthetic peptides (reviewed in [82]). However, although RGD-containing peptides or RGD peptidomimetics are able to block fibrinogen binding to alpha(IIb)beta(3),[83] the fibrinogen RGD sequences are not directly involved in the interaction with alpha(IIb)beta(3), as genetically engineered fibrinogen variants lacking the RGD sequences are still able to interact normally with alpha(IIb)beta(3).[84] Recombinant fibrinogens with extensions or truncations of the gamma-chain completely abolish the interaction of fibrinogen with platelets.[85,86] The absolute requirement for the carboxy-terminal dodecapeptide sequences does, however, not exclude the participation of other domains in fibrinogen in its interaction with alpha(IIb)beta(3).

In fact, several studies have suggested that the dodecapeptide sequence is essential but not sufficient to mediate fibrinogen binding to alpha(IIb)beta(3). The sequences 316-322 in the gamma-chain, and 15-42 in the beta chain have been shown to be involved in high affinity binding of fibrinogen with platelets.[87,88]
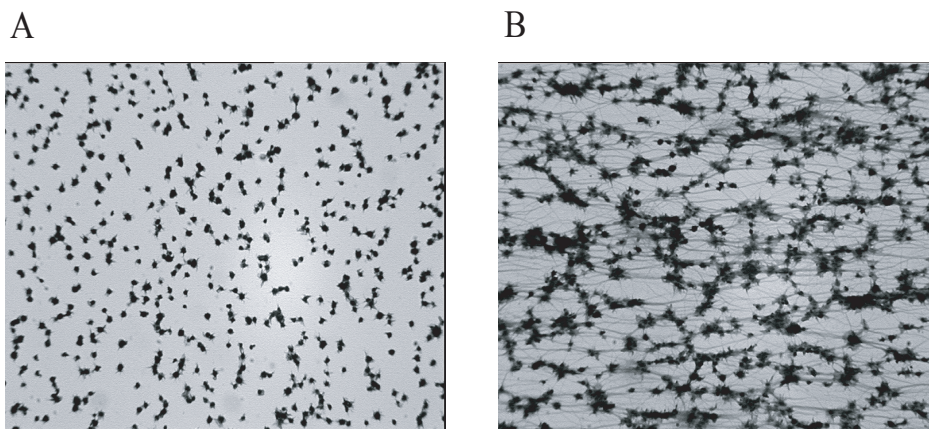
A   B

Figure 5. Platelet aggregation independent of alpha(IIb)beta(3) via polymerizing fibrin. In this experiment, the adhesion of platelets treated with an alpha(IIb)beta(3) inhibitor to collagen was studied under flow conditions in absence (panel A) or presence (B) of rFVIIa. In absence of thrombin generation via rFVIIa, perfusion of alpha(IIb)beta(3)-inhibited blood over collagen resulted in adhesion of platelets, but not platelet-platelet contacts or generation of fibrin was observed. In contrast, after addition of rFVIIa, extensive fibrin deposition and the formation of platelet aggregates was observed. This research was originally published in Blood. Ton Lisman, Jelle Adelmeijer, Harry F. G. Heijnen, Philip G. de Groot, Recombinant factor VIIa restores aggregation of alpha(IIb)beta(3)-deficient platelets via tissue factor-independent fibrin generation [98] with permission form © the American Society of Hematology

Fibrin-mediated platelet aggregation

Similar to fibrinogen, also fibrin is able to bridge platelets via alpha(IIb)beta(3). The platelet-fibrin interaction can also be inhibited by RGD-containing peptides and peptides comprising the dodeca-sequence.[89] However, compared to the inhibition of the fibrinogen-platelet interaction, higher concentrations of these peptides are required for full inhibition. This may indicate that after the fibrinogen to fibrin conversion other platelet binding epitopes become involved. The interaction of fibrin with alpha(IIb)beta(3) on the activated platelet also results in clot retraction, but curiously the RGD and dodecapeptide sequences in fibrin are not involved in this process.[90] Rather, the gamma chain sequences 316-322 and 370-383 have been reported to be essential for clot retraction.[91,92]

Although it is traditionally believed that platelet aggregation proceeds via alpha(IIb)beta(3), as platelets from patients lacking this receptor (i.e., patients with Glanzmann Thrombasthenia), fail to aggregate in response to all agonists, indications for a platelet aggregation pathway independently of this receptor exist. A complete lack of platelet-platelet interaction in GT patients, as observed in aggregation experiments in platelet rich plasma (PRP), might not fully reflect the in vivo defect of these patients. Studies with washed platelets indicate that alpha(IIb)beta(3)-deficient platelets are able to aggregate through polymerizing fibrin. The interaction of alpha(IIb)beta(3)-deficient platelets with polymerizing fibrin and the aggregation of platelets from a patient completely lacking alpha(IIb) and beta(3) was already reported in 1981 and 1989, respectively, but these observations were given little attention.[93,94] More recently, Soslau and coworkers

provided more extensive evidence that alpha(IIb)beta(3)-deficient platelets are indeed able to aggregate through polymerizing fibrin via a mechanism requiring platelet activation mediated by thrombin bound to GPIb.[45,95] In these experiments, intact platelet functions in terms of signal transduction were mandatory for alpha(IIb)beta(3)-independent platelet aggregation and an unidentified platelet receptor for polymerizing fibrin was postulated. In contrast, Jarvis et al. showed that also fixed platelets are able to aggregate through polymerizing fibrin, and therefore these authors concluded that alpha(IIb)beta(3)-independent platelet aggregation is merely a consequence of trapping of platelets into a fibrin network.[96] However, immunoprecipitation studies of alpha(IIb)beta(3)-independent platelet aggregates suggested the existence of one or more specific fibrin receptors on the platelet surface.[97] Also, in our laboratory, we have shown that platelets are able to aggregate in vitro independent of alpha(IIb)beta(3) by a process which depends on the formation of polymerizing fibrin.[98] One of the key experiments demonstrating alpha(IIb)beta(3)-independent platelet aggregation under conditions of flow is shown in figure 5. Studies using blockers of platelet signal transduction pathways, and electron microscopy studies suggest alpha(IIb)beta(3)-independent aggregation via polymerizing fibrin to be mediated by a specific receptor for fibrin, which only binds fibrin after platelet activation.

Clinical evidence for the existence of alpha(IIb)beta(3)-independent aggregation has emerged from studies in which patients with GT received recombinant factor VIIa (rFVIIa, NovoSeven). rFVIIa, which stimulates thrombin and fibrin formation at the site of injury, was effective during bleeding episodes and controlled hemostasis during surgery in a substantial number of GT patients.[99] We showed that the efficacy of rFVIIa in these patients can be explained by enhancement of thrombin and fibrin generation at the site of injury, facilitating alpha(IIb)beta(3)-independent adhesion and aggregation, thereby compensating the lack of alpha(IIb)beta(3)-dependent adhesion and aggregation. The mechanisms behind alpha(IIb)beta(3)-independent platelet aggregation and adhesion are poorly understood. It has been shown that thrombin binding to GPIb, as well as thrombin- or collagen-mediated activation of the platelet are crucial for this process,[45,98] but the reason for this is unclear. Possibly, thrombin binding to GPIb initiates signaling events through GPIb and/or the protease activated receptor 1 (PAR-1) leading to alpha(IIb)beta(3)-independent aggregation. Alternatively, thrombin binding to GPIb might be essential for localizing thrombin to the platelet surface to generate fibrin required for aggregation in situ.

PERSPECTIVE

In this paper, novel insights in the mechanisms by which thrombin and its downstream product fibrin are involved in platelet activation and aggregation have been described. Evidently, the interplay between platelets and the coagulation system is significant for the formation of a hemostatic plug or a pathological

thrombus. It is therefore conceivable that intervention on the crossroad of platelets and coagulation may be an important novel strategy in the treatment of thrombosis. The inhibition of specific steps in thrombus formation may have advantages over currently used strategies such as platelet inhibiting drugs, or drugs interfering with thrombin generation, as these drugs have a limited therapeutical window due to bleeding complications, which can probably be ascribed to an overall inhibition of hemostatic functions (e.g., overall inhibition of thrombin generation by anticoagulants, overall inhibition of platelet aggregation by alpha(IIb)beta(3) blocking drugs, etc). The inhibition of specific individual processes may have the advantage that as overall hemostatic capacity is better preserved, these types of drugs may be sufficiently effective as antithrombotics, while resulting in less bleeding problems.

It has to be noted that the relative importance of a number of these thrombin or fibrin-related processes is incompletely known. Whereas the inhibition of PARs appears to be an effective antithrombotic strategy, it is unknown if inhibition of one of the two PARs on human platelets is sufficient to obtain a strong enough antithrombotic potential in humans. The significance of the interaction of thrombin with GPIb and its associated signaling events for induction of hemostasis is not known. Inhibition of thrombin binding to GPIb will also result in partial inhibition of several other hemostatic processes including PAR-1 activation, and thrombin generation via factor XI, both of which are mediated by GPIb. Moreover, inhibition of thrombin binding to GPIb may also in part promote hemostasis, as thrombin bound to GPIb loses the ability to cleave fibrinogen to fibrin. Although it has been suggested that selective targeting of thrombin-mediated proteolysis of GPV could be explored as an antithrombotic strategy, the role of GPV proteolysis in platelet function is still debated, and awaits further investigations. Finally, the significance of fibrin-mediated platelet aggregation independent of alpha(IIb)beta(3) is at present not known. Although partial inhibition of the platelet-fibrin interaction could be of benefit in treatment of thrombosis, it might be that this strategy results in formation of unstable thrombi with the potential to embolise and cause problems in a different part of the vasculature.

In conclusion, although exciting new concepts in hemostasis regarding the interplay between platelets and fibrin formation have emerged, both by generation of animal models, and state of the art biochemical work, a lot remains to be learned on the significance of these processes for thrombosis and hemostasis, and on the potential of interfering drugs as an antithrombotic strategy.

REFERENCES

1. Vu TK, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 1991;64:1057-1068.

2. Ishihara H, Connolly AJ, Zeng D et al. Protease-activated receptor 3 is a second thrombin receptor in humans. Nature 1997;386:502-506.

3. Xu WF, Andersen H, Whitmore TE et al. Cloning and characterization of human protease-activated receptor 4. Proc.Natl.Acad.Sci.U.S.A 1998;95:6642-6646.

4. Nystedt S, Emilsson K, Larsson AK, Strombeck B, Sundelin J. Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. Eur.J.Biochem. 1995;232:84-89.

5. Molino M, Barnathan ES, Numerof R et al. Interactions of mast cell tryptase with thrombin receptors and PAR-2. J.Biol.Chem. 1997;272:4043-4049.

6. Camerer E, Huang W, Coughlin SR. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. Proc.Natl.Acad. Sci.U.S.A 2000;97:5255-5260.

7. Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J.Clin. Invest 1999;103:879-887.

8. Ishii K, Gerszten R, Zheng YW et al. Determinants of thrombin receptor cleavage. Receptor domains involved, specificity, and role of the P3 aspartate. J.Biol.Chem. 1995;270:16435-16440.

9. De Candia E, Hall SW, Rutella S et al. Binding of thrombin to glycoprotein Ib accelerates the hydrolysis of Par-1 on intact platelets. J.Biol.Chem. 2001;276:4692-4698.

10. Adam F, Verbeuren TJ, Fauchere JL, Guillin MC, Jandrot-Perrus M. Thrombin-induced platelet PAR4 activation: role of glycoprotein Ib and ADP. J.Thromb.Haemost. 2003;1:798-804.

11. Coughlin SR. Thrombin signalling and protease-activated receptors. Nature 2000;407:258-264.

12. Kim S, Foster C, Lecchi A et al. Protease-activated receptors 1 and 4 do not stimulate G(i) signaling pathways in the absence of secreted ADP and cause human platelet aggregation independently of G(i) signaling. Blood 2002;99:3629-3636.

13. Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-activated receptors. Pharmacol.Rev. 2001;53:245-282.

14. Ishii K, Hein L, Kobilka B, Coughlin SR. Kinetics of thrombin receptor cleavage on intact cells. Relation to signaling. J.Biol.Chem. 1993;268:9780-9786.

15. Hoxie JA, Ahuja M, Belmonte E et al. Internalization and recycling of activated thrombin receptors. J.Biol.Chem. 1993;268:13756-13763.

16. Andersen H, Greenberg DL, Fujikawa K et al. Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. Proc.Natl. Acad.Sci.U.S.A 1999;96:11189-11193.

17. Chung AW, Jurasz P, Hollenberg MD, Radomski MW. Mechanisms of action of proteinase-activated receptor agonists on human platelets. Br.J.Pharmacol. 2002;135:1123-1132.

18. Sambrano GR, Huang W, Faruqi T et al. Cathepsin G activates protease-activated receptor-4 in human platelets. J.Biol. Chem. 2000;275:6819-6823.

19. Quinton TM, Kim S, Derian CK, Jin J, Kunapuli SP. Plasmin-mediated activation of platelets occurs by cleavage of protease-activated receptor 4. J.Biol. Chem. 2004;279:18434-18439.

20. Kahn ML, Zheng YW, Huang W et al. A dual thrombin receptor system for platelet activation. Nature 1998;394:690-694.

21. Weiss EJ, Hamilton JR, Lease KE, Coughlin SR. Protection against thrombosis in mice lacking PAR3. Blood 2002;100:3240-3244.

22. Levine MN, Raskob G, Landefeld S, Kearon C. Hemorrhagic complications of anticoagulant treatment. Chest 2001;119:108S-121S.

23. Selnick HG, Barrow JC, Nantermet PG, Connolly TM. Non-peptidic small-molecule antagonists of the human platelet thrombin receptor PAR-1. Curr.Med.Chem.Cardiovasc. Hematol.Agents 2003;1:47-59.

24. Damiano BP, Derian CK, Maryanoff BE, Zhang HC, Gordon PA. RWJ-58259: a selective antagonist of protease activated receptor-1. Cardiovasc.Drug Rev. 2003;21:313-326.

25. Ahn HS, Chackalamannil S, Boykow G, Graziano MP, Foster C. Development of proteinase-activated receptor 1 antagonists as therapeutic agents for thrombosis, restenosis and inflammatory diseases. Curr.Pharm.Des 2003;9:2349-2365.

26. Derian CK, Damiano BP, Addo MF et al. Blockade of the thrombin receptor protease-activated receptor-1 with a small-molecule antagonist prevents thrombus formation and vascular occlusion in nonhuman primates. J.Pharmacol.Exp.Ther. 2003;304:855-861.

27. Andrade-Gordon P, Derian CK, Maryanoff BE et al. Administration of a potent antagonist of protease-activated receptor-1 (PAR-1) attenuates vascular restenosis following balloon angioplasty in rats. J.Pharmacol.Exp.Ther. 2001;298:34-42.

28. Okumura T, Hasitz M, Jamieson GA. Platelet glycocalicin. Interaction with thrombin and role as thrombin receptor of the platelet surface. J.Biol.Chem. 1978;253:3435-3443.

29. Jamieson GA, Okumura T. Reduced thrombin binding and aggregation in Bernard-Soulier platelets. J.Clin.Invest 1978;61:861-864.

30. Modderman PW, Admiraal LG, Sonnenberg A, von dem Borne AE. Glycoproteins V and Ib-IX form a noncovalent complex in the platelet membrane. J.Biol. Chem. 1992;267:364-369.

31. Marchese P, Murata M, Mazzucato M, et al. Identification of three tyrosine residues of glycoprotein Ib alpha with distinct roles in von Willebrand factor and alpha-thrombin binding. J. Biol. Chem. 1995;270:9571-9578.

32. De Marco L, Mazzucato M, Masotti A, Fenton JW, Ruggeri ZM. Function of glycoprotein Ib alpha in platelet activation induced by alpha-thrombin. J.Biol.Chem. 1991;266:23776-23783.

33. Shrimpton CN, Borthakur G, Larrucea S et al. Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation. J.Exp.Med. 2002;196:1057-1066.

34. Baglia FA, Shrimpton CN, Lopez JA, Walsh PN. The glycoprotein Ib-IX-V complex mediates

localization of factor XI to lipid rafts on the platelet membrane. J.Biol.Chem. 2003;278:21744-21750.

35. Li CQ, Vindigni A, Sadler JE, Wardell MR. Platelet glycoprotein Ib alpha binds to thrombin anion-binding exosite II inducing allosteric changes in the activity of thrombin. J.Biol. Chem. 2001;276:6161-6168.

36. De Cristofaro R, De Candia E, Landolfi R, Rutella S, Hall SW. Structural and functional mapping of the thrombin domain involved in the binding to the platelet glycoprotein Ib. Biochemistry 2001;40:13268-13273.

37. Celikel R, McClintock RA, Roberts JR et al. Modulation of alpha-thrombin function by distinct interactions with platelet glycoprotein Ibalpha. Science 2003;301:218-221.

38. Dumas JJ, Kumar R, Seehra J, Somers WS, Mosyak L. Crystal structure of the GpIbalpha-thrombin complex essential for platelet aggregation. Science 2003;301:222-226.

39. Ramakrishnan V, DeGuzman F, Bao M et al. A thrombin receptor function for platelet glycoprotein Ib-IX unmasked by cleavage of glycoprotein V. Proc.Natl.Acad.Sci.U.S.A 2001;98:1823-1828.

40. Sambrano GR, Weiss EJ, Zheng YW, Huang W, Coughlin SR. Role of thrombin signalling in platelets in haemostasis and thrombosis. Nature 2001;413:74-78.

41. Adam F, Bouton MC, Huisse MG, Jandrot-Perrus M. Thrombin interaction with platelet membrane glycoprotein Ib alpha. Trends Mol.Med. 2003;9:461-464.

42. Sadler JE. Structural biology. A menage a trois in two configurations. Science 2003;301:177-179.

43. Vanhoorelbeke K, Ulrichts H, Romijn RA, Huizinga EG, Deckmyn H. The GPIbalpha-thrombin interaction: far from crystal clear. Trends Mol.Med. 2004;10:33-39.

44. Adam F, Guillin MC, Jandrot-Perrus M. Glycoprotein Ib-mediated platelet activation. A signalling pathway triggered by thrombin. Eur.J.Biochem. 2003;270:2959-2970.

45. Soslau G, Class R, Morgan DA et al. Unique pathway of thrombin-induced platelet aggregation mediated by glycoprotein Ib. J.Biol.Chem. 2001;276:21173-21183.

46. Dubois C, Steiner B, Kieffer N, Reigner SC. Thrombin binding to GPIbalpha induces platelet aggregation and fibrin clot retraction supported by resting alphaIIbbeta3 interaction with polymerized fibrin. Thromb. Haemost. 2003;89:853-865.

47. Jandrot-Perrus M, Clemetson KJ, Huisse MG, Guillin MC. Thrombin interaction with platelet glycoprotein Ib: effect of glycocalicin on thrombin specificity. Blood 1992;80:2781-2786.

48. De Marco L, Mazzucato M, Masotti A, Ruggeri ZM. Localization and characterization of an alpha-thrombin-binding site on platelet glycoprotein Ib alpha. J.Biol.Chem. 1994;269:6478-6484.

49. Esmon CT, Lollar P. Involvement of thrombin anion-binding exosites 1 and 2 in the activation of factor V and factor VIII. J.Biol.Chem. 1996;271:13882-13887.

50. Baglia FA, Badellino KO, Li CQ, Lopez JA, Walsh PN. Factor XI binding to the platelet glycoprotein Ib-IX-V complex promotes factor XI activation by thrombin. J.Biol.Chem. 2002;277:1662-1668.

51. Becker DL, Fredenburgh JC, Stafford AR, Weitz JI. Exosites 1 and 2 are essential for protection of fibrin-bound thrombin from heparin-catalyzed inhibition by antithrombin and heparin cofactor II. J.Biol.Chem. 1999;274:6226-6233.

52. Mendolicchio GL, Celikel R, Varughese KI, Savage B, Ruggeri ZM. Modulation of von Willebrand Factor A1 domain-mediated platelet aggregation/agglutination by alpha-thrombin.[abstract]. Blood 2004;104:182aa.

53. Dormann D, Clemetson KJ, Kehrel BE. The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity. Blood 2000;96:2469-2478.

54. Mazurov AV, Vinogradov DV, Vlasik TN et al. Characterization of an antiglycoprotein Ib monoclonal antibody that specifically inhibits platelet-thrombin interaction. Thromb. Res. 1991;62:673-684.

55. Lopez JA, Andrews RK, fshar-Kharghan V, Berndt MC. Bernard-Soulier syndrome. Blood 1998;91:4397-4418.

56. Meyer SC, Fox JE. Interaction of platelet glycoprotein V with glycoprotein Ib-IX regulates expression of the glycoproteins and binding of von Willebrand factor to glycoprotein Ib-IX in transfected cells. J.Biol.Chem. 1995;270:14693-14699.

57. Calverley DC, Yagi M, Stray SM, Roth GJ. Human platelet glycoprotein V: its role in enhancing expression of the glycoprotein Ib receptor. Blood 1995;86:1361-1367.

58. Li CQ, Dong JF, Lanza F et al. Expression of platelet glycoprotein (GP) V in heterologous cells and evidence for its association with GP Ib alpha in forming a GP Ib-IX-V complex on the cell surface. J.Biol.Chem. 1995;270:16302-16307.

59. Ramakrishnan V, Reeves PS, DeGuzman F et al. Increased thrombin responsiveness in platelets from mice lacking glycoprotein V. Proc.Natl.Acad. Sci.U.S.A 1999;96:13336-13341.

60. Kahn ML, Diacovo TG, Bainton DF et al. Glycoprotein V-deficient platelets have undiminished thrombin responsiveness and Do not exhibit a Bernard-Soulier phenotype. Blood 1999;94:4112-4121.

61. Berndt MC, Phillips DR. Purification and preliminary physicochemical characterization of human platelet membrane glycoprotein V. J.Biol.Chem. 1981;256:59-65.

62. Ravanat C, Morales M, Azorsa DO et al. Gene cloning of rat and mouse platelet glycoprotein V: identification of megakaryocyte-specific promoters and demonstration of functional thrombin cleavage. Blood 1997;89:3253-3262.

63. McGowan EB, Ding A, Detwiler TC. Correlation of thrombin-induced glycoprotein V hydrolysis and platelet activation. J.Biol.Chem. 1983;258:11243-11248.

64. Ware J, Russell S, Ruggeri ZM. Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. Proc.Natl. Acad.Sci.U.S.A 2000;97:2803-2808.

65. Moog S, Mangin P, Lenain N et al. Platelet glycoprotein V binds to collagen and participates in platelet adhesion and aggregation. Blood 2001;98:1038-1046.

66. Ni H, Ramakrishnan V, Ruggeri ZM et al. Increased thrombogenesis and embolus formation in mice lacking glycoprotein V. Blood 2001;98:368-373.

67. Canobbio I, Balduini C, Torti M. Signalling through the platelet glycoprotein Ib-V-IX complex. Cell Signal. 2004;16:1329-

1344.

68. Azorsa DO, Moog S, Ravanat C et al. Measurement of GPV released by activated platelets using a sensitive immunocapture ELISA--its use to follow platelet storage in transfusion. Thromb.Haemost. 1999;81:131-138.

69. Ravanat C, Freund M, Mangin P et al. GPV is a marker of in vivo platelet activation--study in a rat thrombosis model. Thromb. Haemost. 2000;83:327-333.

70. Blann AD, Lanza F, Galajda P et al. Increased platelet glycoprotein V levels in patients with coronary and peripheral atherosclerosis--the influence of aspirin and cigarette smoking. Thromb.Haemost. 2001;86:777-783.

71. Aleil B, Mossard JM, Wiesel ML, Lanza F, Cazenave JP. Increased plasma levels of soluble platelet glycoprotein V in patients with acute myocardial infarction. J.Thromb.Haemost. 2003;1:1846-1847.

72. Morel O, Hugel B, Jesel L et al. Circulating procoagulant microparticles and soluble GPV in myocardial infarction treated by primary percutaneous transluminal coronary angioplasty. A possible role for GPIIb-IIIa antagonists. J.Thromb.Haemost. 2004;2:1118-1126.

73. Kamath S, Blann AD, Chin BS et al. A study of platelet activation in atrial fibrillation and the effects of antithrombotic therapy. Eur. Heart J. 2002;23:1788-1795.

74. Wolff V, Aleil B, Giroud M et al. Soluble platelet glycoprotein V is a marker of thrombosis in patients with ischemic stroke. Stroke 2005;36:e17-e19.

75. Wu YP, Vink T, Schiphorst M et al. Platelet thrombus formation on collagen at high shear rates is mediated by von Willebrand factor-glycoprotein Ib interaction and inhibited by von Willebrand factor-glycoprotein IIb/IIIa interaction. Arterioscler.Thromb.Vasc.Biol. 2000;20:1661-1667.

76. Remijn JA, Wu YP, IJsseldijk MJ et al. Absence of fibrinogen in afibrinogenemia results in large but loosely packed thrombi under flow conditions. Thromb. Haemost. 2001;85:736-742.

77. Ni H, Denis CV, Subbarao S et al. Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. J.Clin.Invest 2000;106:385-392.

78. Ni H, Yuen PS, Papalia JM et al. Plasma fibronectin promotes thrombus growth and stability in injured arterioles. Proc.Natl. Acad.Sci.U.S.A 2003;100:2415-2419.

79. Wu YP, Bloemendal HJ, Voest EE et al. Fibrin-incorporated vitronectin is involved in platelet adhesion and thrombus formation through homotypic interactions with platelet-associated vitronectin. Blood 2004;104:1034-1041.

80. Andre P, Prasad KS, Denis CV et al. CD40L stabilizes arterial thrombi by a beta3 integrin-dependent mechanism. Nat. Med. 2002;8:247-252.

81. Kloczewiak M, Timmons S, Hawiger J. Localization of a site interacting with human platelet receptor on carboxy-terminal segment of human fibrinogen gamma chain. Biochem.Biophys.Res.Commun. 1982;107:181-187.

82. Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion: RGD and integrins. Science 1987;238:491-497.

83. Coller BS, Anderson K, Weisman HF. New antiplatelet agents: platelet GPIIb/IIIa antagonists. Thromb.Haemost. 1995;74:302-308.

84. Farrell DH, Thiagarajan P, Chung DW, Davie EW. Role of fibrinogen alpha and gamma chain sites in platelet aggregation. Proc.Natl.Acad. Sci.U.S.A 1992;89:10729-10732.

85. Zaidi TN, McIntire LV, Farrell DH, Thiagarajan P. Adhesion of platelets to surface-bound fibrinogen under flow. Blood 1996;88:2967-2972.

86. Hettasch JM, Bolyard MG, Lord ST. The residues AGDV of recombinant gamma chains of human fibrinogen must be carboxy-terminal to support human platelet aggregation. Thromb.Haemost. 1992;68:701-706.

87. Chen CS, Chou SH, Thiagarajan P. Fibrin(ogen) peptide B beta 15-42 inhibits platelet aggregation and fibrinogen binding to activated platelets. Biochemistry 1988;27:6121-6126.

88. Remijn JA, IJsseldijk MJ, van Hemel BM et al. Reduced platelet adhesion in flowing blood to fibrinogen by alterations in segment gamma316-322, part of the fibrin-specific region. Br.J.Haematol. 2002;117:650-657.

89. Briet E, Peters M. The incidence of inhibitors in hemophilia A and the induction of immune tolerance. Adv.Exp.Med.Biol. 2001;489:89-97.

90. Rooney MM, Farrell DH, van Hemel BM, de Groot PG, Lord ST. The contribution of the three hypothesized integrin-binding sites in fibrinogen to platelet-mediated clot retraction. Blood 1998;92:2374-2381.

91. Remijn JA, IJsseldijk MJ, de Groot PG. Role of the fibrinogen gamma-chain sequence gamma316-322 in platelet-mediated clot retraction. J.Thromb.Haemost. 2003;1:2245-2246.

92. Podolnikova NP, Yakubenko VP, Volkov GL, Plow EF, Ugarova TP. Identification of a novel binding site for platelet integrins alpha IIb beta 3 (GPIIbIIIa) and alpha 5 beta 1 in the gamma C-domain of fibrinogen. J.Biol. Chem. 2003;278:32251-32258.

93. Niewiarowski S, Levy-Toledano S, Caen JP. Platelet interaction with polymerizing fibrin in Glanzmann's thrombasthenia. Thromb.Res. 1981;23:457-463.

94. McGregor L, Hanss M, Sayegh A et al. Aggregation to thrombin and collagen of platelets from a Glanzmann thrombasthenic patient lacking glycoproteins IIb and IIIa. Thromb.Haemost. 1989;62:962-967.

95. Soslau G, Favero M. The GPIb-thrombin pathway: evidence for a novel role of fibrin in platelet aggregation. J.Thromb. Haemost. 2004;2:522-524.

96. Jarvis GE, Atkinson BT, Frampton J, Watson SP. Thrombin-induced conversion of fibrinogen to fibrin results in rapid platelet trapping which is not dependent on platelet activation or GPIb. Br.J.Pharmacol. 2003;138:574-583.

97. Osdoit S, Rosa JP. Polymeric fibrin interacts with platelets independently from integrin alphaIIbbeta3. [abstract]. Blood 2001;98:518aa.

98. Lisman T, Adelmeijer J, Heijnen HF, de Groot PG. Recombinant factor VIIa restores aggregation of alphaIIbbeta3-deficient platelets via tissue factor-independent fibrin generation. Blood 2004;103:1720-1727.

99. Poon MC, d'Oiron R, Hann I et al. Use of recombinant factor VIIa (NovoSeven) in patients with Glanzmann thrombasthenia. Semin. Hematol. 2001;38:21-25.

Chapter 1B

Tissue Factor-Independent Effects of Recombinant Factor VIIa on Hemostasis

Cees Weeterings [1], Ton Lisman [1,2], Philip G de Groot [1]

From [1] the Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, the Netherlands; [2] Surgical Research Laboratory, Department of Surgery, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands

In the early 1970s factor (F) VIII concentrates became available for replacement therapy for patients with hemophilia A.[1] This was a major step forward in the management of these patients but, unfortunately, treatment was complicated by the development of inhibitory antibodies against FVIII in about 25% of treated patients treated.[2] The initial management of patients with hemophilia complicated by inhibitors involved the use of prothrombin complex concentrates, but this treatment was not very effective and it was associated with thromboembolic complications.[3] A much more efficient way to treat inhibitor patients was the administration of plasma-derived FVIIa.[4] Based on the initial success achieved with plasma-derived FVIIa, recombinant FVIIa (rFVIIa; NovoSeven®, Novo Nordisk, Bagsværd, Denmark) was produced in sufficient quantities and further developed into a drug approved worldwide for the treatment of hemophilia patients with inhibitors. The administration of rFVIIa has been shown to be a safe and effective treatment for bleeding episodes and surgical bleeds in this patient population.[5]

Following the encouraging results derived from rFVIIa use in patients with hemophilia A or B with inhibitors, studies describing its successful use in a diversity of other congenital and acquired bleeding disorders were published.[6,7] Furthermore, patients with various platelet-related bleeding disorders have been successfully treated with rFVIIa, and it has been suggested that rFVIIa could become a universal haemostatic agent.[8] However, the molecular mechanisms responsible for the haemostatic efficacy of rFVIIa in platelet-related bleeding disorders are poorly understood.

Mechanism of action of rFVIIa: general concepts

Initial ideas about the mechanism of action of rFVIIa were simple and straightforward.[9] Proteolytically inactive by itself, FVIIa needed the binding to tissue factor (TF) in order to become enzymatically active. Following administration, rFVIIa was thought to bind to TF that became exposed to the circulation after injury to a vessel wall. This binding would result in increased thrombin generation localised at the site of injury. The importance of TF in this process was highlighted by studies of non-coagulopathic chimpanzees infused with rFVIIa; such studies showed that increases in F1+2 levels, the activation peptide of prothrombin, could be neutralised by co-infusion with an antibody against TF, pointing to an essential role of TF in the procoagulant effects of rFVIIa.[10]

The enhanced local thrombin concentration induced by the binding of rFVIIa to TF would have the following consequences: increased fibrin deposition; a changed fibrin structure; increased fibrin stability due to activation of thrombin-activatable fibrinolysis inhibitor (TAFI) and FXIII; and increased platelet activation. Together, these factors explained the efficacy of rFVIIa. The extremely low incidence of thrombotic complications associated with rFVIIa treatment was explained by the need for rFVIIa to bind to TF before it could become enzymatically active.

Mechanism of action of rFVIIa in platelet disorders

rFVIIa increases platelet adhesion and aggregation

It is still generally accepted that a TF-dependent mechanism contributes strongly to the beneficial effects of rFVIIa in hemophilia patients. It is questionable, however, whether the mechanism of action of rFVIIa in platelet related bleeding disorders is entirely based on TF-dependent enhancement of thrombin generation. Relatively high concentrations of rFVIIa are required for the hemostatic efficacy of rFVIIa in clinical applications other than hemophilia.[11] The dissociation constant (K_d) for rFVIIa for binding to TF is around 0.5 nM,[12] and the plasma concentrations of rFVIIa required for efficacy in platelet-related bleeding disorders are at least one order of magnitude higher than this. Hoffman and colleagues were the first to show that rFVIIa could activate FX on monocytes or platelets independently of TF.[13,14] It has also been shown that rFVIIa can bind directly to activated platelets, which are known not to express TF.[13] Based on these observations, our laboratory has also that TF-independent thrombin generation is responsible for increased platelet deposition onto collagen after addition of rFVIIa shown to an in-vitro flow model. rFVIIa restored the decreased adhesion observed with thrombocytopenic blood,[15] and we further demonstrated that rFVIIa restored not only platelet adhesion but also platelet aggregate formation in the blood of patients with Glanzmann's thrombasthenia (GT).[16] The restoration of platelet adhesion and platelet-platelet interaction cannot be inhibited by an antibody against TF but is dependent on both thrombin generation and the exposure of anionic phospholipids on the platelet surface. These findings suggest that rFVIIa can be an effective therapeutic agent in functional platelet disorders in the absence of exposed TF.

Glanzmann's thrombasthenia and the role of αIIbβ3

Platelets circulate in blood in a quiescent state, but when they encounter a rupture in the vessel wall, they adhere, become activated and interact with each other, resulting in a platelet aggregate that seals the damaged vessel.[17] Adhesion of platelets strongly depends on the binding of the platelet receptor complex glycoprotein (GP)Ib-IX-V to von Willebrand factor, which is first adsorbed from the plasma to collagen at the site of injury. The subsequent platelet-platelet interaction is, under normal physiological conditions, completely dependent on the presence of the integrin GPIIb:IIIa (αIIbβ3). Following platelet activation, αIIbβ3 undergoes a conformational change, allowing the binding of fibrinogen or von Willebrand factor from the surrounding plasma. Both molecules are composed of identical parts, allowing them to bind to two different platelets at the same time. Fibrinogen and von Willebrand factor represent the "glue" of a platelet aggregate.

Patients with GT have a congenital deficiency of αIIbβ3 and, as a consequence, defective platelet-platelet interactions. rFVIIa has shown efficacy in the treatment of patients with GT, and this is supported by in vitro whole blood flow experiments showing that rFVIIa can restore the deficient platelet adhesion found in these individuals.[16,18] Encouragingly, our own in vitro observations support in vivo results from GT patients treated with rFVIIa, but the in vitro findings left us with two

unresolved questions: First, how can platelets form aggregates in the absence of αIIbβ3? Second, what are the molecular mechanisms responsible for the controlled enhancement of thrombin generation at the site of vascular injury in the absence of TF?

αIIbβ3-independent platelet aggregation

A complete lack of platelet aggregation is observed when in vitro classic aggregation experiments are performed using GT platelets. Whether or not the observed loss of capacity to aggregate in vitro is an absolute reflection of the in vivo situation in GT patients is uncertain. As early as the 1980s, it was reported that αIIbβ3-deficient platelets can aggregate through polymerised fibrin.;[19,20] more recently, Soslau et al extended these forgotten studies and showed that platelets can indeed aggregate in vitro in a αIIbβ3-independent manner via a mechanism that requires polymerized fibrin and thrombin.[21] Inhibition of platelet activation prevents αIIbβ3-independent aggregation, indicating that the process involves more than simply the trapping of platelets in a fibrin network, as suggested by others.[22]

When we used washed platelets and purified clotting factors in our laboratory, we found that addition of rFVIIa could induce aggregation of αIIbβ3-deficient platelets and that this aggregation was dependent on the formation of fibrin, the induction of thrombin generation, and the activation of platelets.[23] Subsequent electron microscopic analysis of confirmed that the observed aggregation was not just the consequence of platelets trapped in a fibrin network. Instead, when platelets become activated, they express a receptor for polymerized fibrin on their surface; although the identity of this receptor has yet to be established, its presence might explain the efficacy of rFVIIa in treating bleeding episodes in patients with GT. Administration of high amounts of rFVIIa to a patient with GT results in enough thrombin generation at the site of vascular injury to allow a αIIbβ3-independent platelet aggregation, which, in turn, is sufficient to replace the platelet aggregation that would normally occur through fibrinogen and αIIbβ3.

The same mechanism may also account, in part, for the mode of action of rFVIIa in other indications. rF VIIa can bind directly to activated platelets with a K_d of around 90 nM. This is not really surprising, because activated platelets expose anionic phospholipids on their surface, and a classic characteristic of vitamin K-dependent proteins (such as rFVIIa) is their binding to negatively charges phospholipids.[24] It has been shown in a purified system that rFVIIa, in the presence of anionic phospholipids and Ca^{2+}, is able to activate FX, though at a much slower rate than in the presence of TF.[25] However, it is unlikely that the presence of anionic phospholipids alone could explain the TF-independent effects of rFVIIa, as rFVIIa is only minimally effective in activating FX under these conditions.

A possible role of GPIb in rFVIIa-mediated activation of platelets

In view of this observation, we hypothesized that there must be a receptor on platelets that binds rFVIIa and makes it a more efficient enzyme. Following publication of reports showing that platelet receptor GPIb can bind different clotting factors, such as FII, FXI and FXII,[26] we examined the binding of rFVIIa

to GPIbα. We showed that that rFVIIa can bind to a purified plasma fragment of GPIbα, named glycocalicin, with a Kd of approximately 80 nM.[27] In addition, Chinese hamster Ovary (CHO) cells transfected with the GPIb-IX complex can bind to rFVIIa while wild-type CHO cells cannot. In additional experiments, we found that rFVIIa binds to GPIb expressed on activated platelets, but hardly to GPIb on non-stimulated platelets. Furthermore, the binding of rFVIIa to activated platelets also resulted in increased thrombin generation on the surface of the platelet, independently of TF. These results demonstrate that GPIb on the surface of platelets can mediate rFVIIa-dependent thrombin formation on the platelet surface.

Why rFVIIa can bind only to GPIb on the surface of activated platelets and not to GPIb on the surface of quiescent platelets is unknown. Perhaps platelet activation induces a conformational change in the GPIb-IX-V complex that results in the exposure of a hidden binding site for rVIIa[26,28] (Figure 1). However, it is also possible that the lipid microenvironment of GPIb changes during platelets activation, and
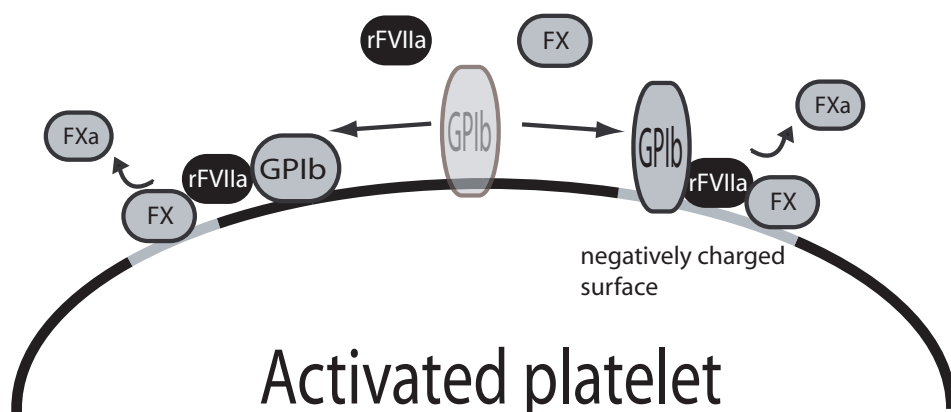

Figure 1 - Schematic representation of the possible mechanisms of action of rFVIIa on the platelet surface. F, factor; GP, glycoprotein; rFVIIa, recombinant activated factor VII.

that the specific combination of lipids and GPIb is responsible for the binding of rVIIa.[29] Alternatively, there is an entropy measure that must be considered in any binding reaction. rFVIIa bound to anionic phospholipids has less mobility than rFVIIa in plasma. This loss of freedom represents unfavorable entropy and must be overcome by favorable energy effects caused by the interaction between rFVIIa and anionic phospholipids. It is possible that the energy profit produced by the binding of rFVIIa to GPIb is insufficient to compensate for the loss of entropy. When rFVIIa binds first to anionic phospholipids, the entropy of the system decreases and the binding of rFVIIa to GPIb might become thermodynamically favorable. More experiments are necessary to understand the role of platelets and platelet receptors in the efficacy of rFVIIa in platelet-related bleeding disorders.

Summary

Enhancement of thrombin generation by rFVIIa at the site of vessel injury results in a variety of effects that lead to production of a more stable clot. It is obvious that binding of rFVIIa to TF is the dominant mechanism responsible for the increased thrombin generation, but there is now also ample evidence that rVIIa is able to enhance local thrombin generation in the absence of tissue factor. This TF-independent effect of rFVIIa is dependent on the presence of (activated) platelets, and the effects cannot be explained by the presence of anionic phospholipids alone – specific platelet receptors are involved. This mechanism contributes to enhanced rFVIIa-induced clot formation in patients with platelet-related bleeding disorders. Further study is required to determine whether - and to what degree – these TF-independent effects of rFVIIa play a role in other congenital or acquired bleeding disorders.

REFERENCES

1. Key NS, Negrier C. Coagulation factor concentrates: past, present, and future. Lancet 2007;370:439-448.

2. Hoyer LW. Factor VIII inhibitors Curr. Opin. Hematol. 1995;2:365-371.

3. Köhler M. Thrombogenicity of prothrombin complex concentrates. Thromb. Res. 1999;95(4 Suppl 1):S13-S17.

4. Hedner U, Kisiel W. Use of human factor VIIa in the treatment of two hemophilia A patients with high-titer inhibitors. J. Clin. Invest 1983;71:1836-1841.

5. Shapiro AD, Gilchrist GS, Hoots WK, Cooper HA, Gastineau DA. Prospective, randomised trial of two doses of rFVIIa (NovoSeven) in haemophilia patients with inhibitors undergoing surgery. Thromb. Haemost. 1998;80:773-778.

6. Poon MC. The evidence for the use of recombinant human activated factor VII in the treatment of bleeding patients with quantitative and qualitative platelet disorders. Transfus. Med. Rev. 2007;21:223-236.

7. Hedner U. Mechanism of action of factor VIIa in the treatment of coagulopathies. Semin. Thromb. Hemost. 2006;32 Suppl 1:77-85.

8. Mannucci PM, Levi M. Prevention and treatment of major blood loss. N. Engl. J. Med. 2007;356:2301-2311.

9. Lisman T, de Groot PG. Mechanism of action of recombinant factor VIIa. J. Thromb. Haemost. 2003;1:1138-1139.

10. ten Cate H, Bauer KA, Levi M, et al. The activation of factor X and prothrombin by recombinant factor VIIa in vivo is mediated by tissue factor. J. Clin. Invest 1993;92:1207-1212.

11. Hedner U. Dosing and monitoring NovoSeven treatment. Haemostasis. 1996;26 Suppl 1:102-108.

12. Björquist P, Boström S. Determination of the kinetic constants of tissue factor/factor VII/factor VIIA and antithrombin/heparin using surface plasmon resonance. Thromb. Res. 1997;85:225-236.

13. Hoffman M, Monroe DM, Roberts HR. Human monocytes support factor X activation by factor VIIa, independent of tissue factor: implications for the therapeutic mechanism of high-dose factor VIIa in hemophilia. Blood 1994;83:38-42.

14. Monroe DM, Hoffman M, Oliver JA, Roberts HR. Platelet activity of high-dose factor VIIa is independent of tissue factor. Br. J. Haematol. 1997;99:542-547.

15. Lisman T, Adelmeijer J, Cauwenberghs S, van Pampus ECM, Heemskerk JWM, de Groot PG. Recombinant factor VIIa enhances platelet adhesion and activation under flow conditions at normal and reduced platelet count. J. Thromb. Haemost. 2005;3:742-751.

16. Lisman T, Moschatsis S, Adelmeijer J, Nieuwenhuis HK, de Groot PG. Recombinant factor VIIa enhances deposition of platelets with congenital or acquired αIIbβ3 deficiency to endothelial cell matrix and collagen under conditions of flow via a tissue factor-independent thrombin generation. Blood 2003;101:1864-1870.

17. Ruggeri ZM, Mendolicchio GL. Adhesion mechanisms in platelet function. Circ. Res. 2007;100:1673-1685.

18. Poon MC, D'Oiron R, Von Depka M, et al. Prophylactic and therapeutic recombinant factor VIIa administration to patients with Glanzmann's thrombasthenia: results of an international survey. J. Thromb. Haemost. 2004;2:1096-1103.

19. Niewiarowski S, Levy-Toledano S, Caen JP. Platelet interaction with polymerizing fibrin in Glanzmann's thrombasthenia Thromb. Res. 1981;23:457-463.

20. McGregor L, Hanss M, Sayegh A, et al. Aggregation to thrombin and collagen of platelets from a Glanzmann thrombasthenic patient lacking glycoproteins IIb and IIIa. Thromb. Haemost. 1989;62:962-967.

21. Soslau G, Class R, Morgan DA, et al. Unique pathway of thrombin-induced platelet aggregation mediated by glycoprotein Ib. J. Biol. Chem. 2001;276:21173-21183.

22. Jarvis GE, Atkinson BT, Frampton J, Watson SP. Thrombin-induced conversion of fibrinogen to fibrin results in rapid platelet trapping which is not dependent on platelet activation or GPIb. Br. J. Pharmacol. 2003;138:574-583.

23. Lisman T, Adelmeijer J, Heijnen HFG, de Groot PG. Recombinant factor VIIa restores aggregation of αIIbβ3-deficient platelets via tissue factor independent fibrin formation. Blood 2004;103:1720-1727.

24. Monroe DM, Hoffman M, Roberts HR. Platelets and thrombin generation. Arterioscler. Thromb. Vasc. Biol. 2002;22:1381-1389.

25. Bom VJ, Bertina RM. The contributions of Ca^{2+}, phospholipids and tissue-factor apoprotein to the activation of human blood-coagulation factor X by activated factor VII Biochem. J. 1990;265:327-336.

26. Clemetson KJ. A short history of platelet glycoprotein Ib complex. Thromb. Haemost. 2007;98:63-68.

27. Weeterings C, Adelmeijer J, de Groot PG, Lisman T. Glycoprotein Ib alpha contributes to tissue factor-independent thrombin generation by recombinant factor VIIa on the activated platelet surface. J. Thromb .Haemost. 2007;5 Supplement 2:O-S-042.

28. Lisman T, Weeterings C, de Groot PG. Platelet aggregation: involvement of thrombin and fibrin(ogen). Front. Biosci. 2005;10:2504-2517.

29. Baglia FA, Shrimpton CN, López JA, Walsh PN. The glycoprotein Ib-IX-V complex mediates localization of factor XI to lipid rafts on the platelet membrane. J. Biol. Chem. 2003;278:21744-21750.

Chapter 2

Aim of this thesis

General introduction

Upon vascular damage, subendothelial matrix proteins become exposed to flowing blood and platelets adhere to the exposed subendothelium to prevent excessive bleeding. The first part of this thesis discussed platelet plug formation and the role thrombin and fibrin(ogen) play in this process (Chapter 1A). Thrombin can be generated via the extrinsic or the intrinsic pathway of coagulation. The extrinsic pathway of coagulation is initiated when tissue factor (TF) becomes exposed to flowing blood and via a series of activation steps leads to the generation of thrombin and conversion of fibrinogen to fibrin. The intrinsic pathway of coagulation, also referred to as the contact activation pathway, involves the activation of factor (F) XII by non-physiological surfaces such as kaolin or glass, which leads to the generation of activated FXI and via subsequent series of activation steps again resulting in the generation of thrombin and subsequent fibrin formation (Figure 1).
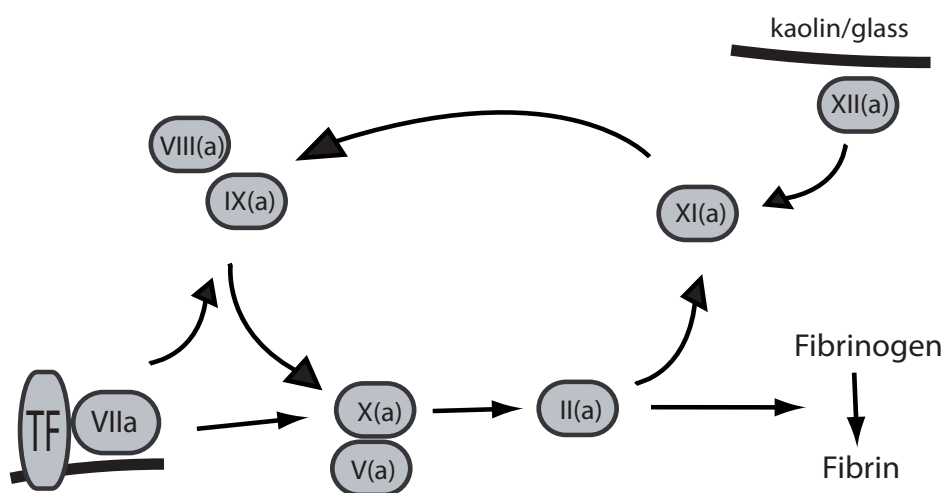
Figure 1 – Simplified model of coagulation.

Recombinant factor VIIa (rFVIIa; Novoseven, Novo Nordisk, Bagsværd, Denmark) was originally developed for patients with haemophilia A and B, whose treatment was complicated by the presence of inhibitors against FVIII and FIX, respectively. Infusion of rFVIIa into those patients results in an increased thrombin generation, mediated by TF-dependent as well as TF-independent coagulation. The second part of the introduction discussed the mechanism of action of rFVIIa in more detail (Chapter 1B).

Aim of this thesis

In this thesis, the interaction of coagulation factors with platelet surfaces will be discussed. The main focus will be on the role glycoprotein Ibα (GPIbα) plays in this process. The consequences of ligand binding to GPIbα on the function of these ligands and the role of GPIbα as an adhesive receptor will be described.

The first part of the thesis deals with the consequences of interaction of coagulation factors with the activated platelet on the process of coagulation itself. In chapter 3, the role of GPIbα in the mechanism of action of rFVIIa is studied. The molecular interaction between rFVIIa and GPIbα and the effect of the interaction with GPIbα on thrombin generation mediated by rFVIIa has been investigated. In chapter 4, platelet adhesion to FIX(a) has been examined under static conditions as well as under conditions of flow. Furthermore, the effect of GPIbα on FIXa-mediated FXa-generation has been investigated.

In the second part of the thesis, the consequences of ligand binding to GPIbα on platelet adhesion will be discussed. In chapter 5, the interaction between thrombin and GPIbα has been investigated, by studying platelet adhesion to immobilized thrombin. The contribution of the different exosites of thrombin has been examined by using several thrombin mutants. Furthermore, platelet adhesion to thrombin bound to fibrin has been investigated. Chapter 6 examines the interaction between FXII and von Willebrand factor and describes a new role for FXII in thrombus formation, which has been studied using an in vitro flow model. Chapter 7 deals with the platelet activating properties of SSL5, a small protein secreted by S. aureus. More specific, the effect of SSL5 on platelets has been investigated using platelet aggregometry and platelet adhesion studies to immobilized SSL5. Furthermore, platelet receptors that are involved in this process have been studied. In chapter 8, the role of GPIbα and the activated platelet in coagulation will be discussed in a broader perspective.

Chapter 3

The Glycoprotein Ib-IX-V complex contributes to tissue factor-independent thrombin generation by recombinant factor VIIa on the activated platelet surface

Cees Weeterings [1,2], Philip G de Groot [1,2], Jelle Adelmeijer [3], Ton Lisman [1,2,3]

From [1] the Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, The Netherlands; [2] the Institute of Biomembranes, Utrecht University, Utrecht, The Netherlands and [3] Surgical Research Laboratory, Department of Surgery, University Medical Center Groningen, University of Groningen, The Netherlands

ABSTRACT

Several lines of evidence suggest that recombinant factor VIIa (rFVIIa) is able to activate factor X on an activated platelet, in a tissue factor (TF)-independent manner. We hypothesized that besides the anionic surface, a receptor on the activated platelet surface is involved in this process.

Here, we showed that, in an ELISA set-up, a purified extracellular fragment of GPIbα bound to immobilized rFVIIa. Surface plasmon resonance established an affinity constant (K_d) of approximately 20 nM for this interaction. In addition, CHO cells transfected with the GPIb-IX-V complex could adhere to immobilized rFVIIa, whereas wild-type CHO cells could not. Furthermore, platelets stimulated with a combination of collagen and thrombin adhered to immobilized rFVIIa under static conditions. Platelet adhesion was inhibited by treatment with O-sialoglycoprotein endopeptidase, which specifically cleaves GPIbα from the platelet surface. In addition, rFVIIa-mediated thrombin generation on the activated platelet surface was inhibited by cleaving GPIbα from its surface.

In summary, three lines of evidence showed that rFVIIa interacts with the GPIb-IX-V complex, and this interaction enhanced TF-independent thrombin generation mediated by rFVIIa on the activated platelet surface. The rFVIIa-GPIbα interaction could contribute to cessation of bleeding after administration of rFVIIa to patients with bleeding disorders.

INTRODUCTION

Recombinant factor VIIa (rFVIIa) was originally developed for the treatment of hemophilia A and B patients, who have developed inhibitory antibodies against factor VIII and IX as a result of treatment with FVIII or FIX concentrates.[1] Nowadays, it has also been registered for use in patients with factor VII-deficiency, acquired hemophilia and inhibitor-complicated Glanzmann's thrombasthenia. Its mechanism of action is thought to involve the local enhancement of thrombin generation at the site of vessel wall damage. Enhancement of thrombin generation will result in enhanced fibrin formation, as well as changes in fibrin structure that will result in a clot that is better protected against fibrinolysis.[2] Improved clot stability is also achieved through increased activation of thrombin-activatable fibrinolysis inhibitor (TAFI).[3] Finally, enhanced thrombin generation results in an acceleration of platelet activation, which will facilitate induction of hemostasis in two ways. First, platelet activation directly contributes to formation of the hemostatic plug. Second, activation of platelets results in an increase in thrombin generation, as platelet activation will expose procoagulant phospholipids on the platelet surface.

It has been suggested that enhancement of thrombin generation by rFVIIa is solely dependent on the presence of tissue factor (TF).[4,5] However, different independent experiments have shown that the effect of rFVIIa can proceed via TF-dependent[3,6] as well as TF–independent[7-9] pathways, and it has been postulated that both mechanisms are operative in vivo.[10]

Although the mechanisms through which rFVIIa exerts its activity have been studied extensively, there are still some unresolved questions. The now widely used standard dose of rFVIIa (90 µg/kg) results in plasma levels that by far exceed the affinity constant (K_d) for TF binding, and it has not yet been fully clarified why these high plasma concentrations of rFVIIa are required for induction of hemostasis. Furthermore, even higher doses of rFVIIa (up to 270 µg/kg) appear more efficient compared with the regular dose in treatment of inhibitor-complicated hemophilia.[11-13] In addition, relatively high doses are thought to be required for induction of hemostasis in other indications (eg, treatment of platelet-related bleeding disorders).[14-16]

It is unclear why such high doses of rFVIIa are required for the induction of hemostasis, as TF is thought to be saturated with rFVIIa at much lower doses than achieved with the regular dose of rFVIIa.[17] A possible explanation for the requirement of higher doses than initially anticipated is the inhibitory effect of zymogen factor VII on TF-FVIIa initiated coagulation.[6] An alternative explanation is that TF-independent thrombin generation accounts for the clinical efficacy of high-dose rFVIIa, since the affinity of rFVIIa for the platelet surface is much lower than for TF. This latter hypothesis is supported by observations with rFVIIa variants with substantially increased TF-independent activity, but unaltered TF-dependent activity compared with wild-type rFVIIa. These rFVIIa variants were shown to be much more potent in a murine model of hemophilia compared with wild-type rFVIIa, showing that TF-independent thrombin generation accounts, at least for a

substantial part, for the hemostatic efficacy of rFVIIa.[18]

Activated platelets have been shown to play an important role in the controlled enhancement of thrombin generation. rFVIIa can directly bind to negatively charged phospholipids exposed on activated platelets and this supports thrombin generation independently of TF.[9,19] The activation of factor X (FX) on anionic phospholipids is a very inefficient process and we hypothesized that, besides negatively charged phospholipids, there might be a role for a receptor on the activated platelet surface in rFVIIa-mediated thrombin generation. A possible candidate is TF, which has been reported to be present on the platelet surface after platelet activation. Several groups described the presence of TF pre-mRNA in platelets,[20,21] whereas others have reported that TF can be taken up by platelets after interaction with TF-containing microparticles.[22] Previous experiments by our group showed that rFVIIa increased platelet deposition onto collagen, and this increased platelet deposition could not be inhibited by antibodies that inhibit TF.[7] Therefore we hypothesized that another platelet receptor is involved in the binding of rFVIIa, and we focused on the GPIb-IX-V complex because it is one of the most abundant receptors on platelets. The GPIb-IX-V complex was originally identified as the receptor for von Willebrand factor, but recent research has shown that it can also serve as a receptor for other proteins, including thrombin,[23] factor XI,[24] factor XIIa,[25] high molecular weight kininogen,[26] and (activated) protein C.[27] This led to our hypothesis that GPIbα is involved in the mechanism of action of rFVIIa.

In this study, we have investigated whether rFVIIa and GPIbα can interact with each other. Furthermore, we examined the role of GPIbα in the interaction of platelets and CHO cells transfected with the GPIb-IX-V complex to immobilized rFVIIa. Finally, we investigated whether the interaction between GPIbα and rFVIIa affects thrombin generation on the activated platelet surface.

METHODS

Materials

Soluble recombinant tissue factor (residues 1-290, sTF), tissue factor pathway inhibitor, rFVIIa, recombinant factor VII, a neutralizing antibody against factor VIII, and an inhibitory antibody against TF were a generous gift from Dr. M. Kjalke (Haemostasis Biology, Novo Nordisk, Denmark). The PAR1-agonist peptide SFLLRN was from Bachem (Bubendorf, Switzerland). Annexin A5 was a kind gift of Dr. C. Reutelingsperger (Maastricht, the Netherlands). Thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Factor X (FX) was purified from fresh-frozen plasma by immunoaffinity chromatography followed by Q-sepharose chromatography as described previously.[28] Human coagulation factors V, prothrombin, and anti-thrombin III (ATIII) were purchased from Haematologic Technologies Inc (Essex Junction, VT). S2238 was purchased from Chromogenix (Milan, Italy). Pefabloc Xa and Pefachrome Xa were from Pentapharm (Basel, Switzerland). O-sialoglycoprotein endopeptidase was purchased from Cedarlane

Laboratories (Hornby, Ontario, Canada). The Technothrombin TGA kit was obtained from Technoclone Ltd (Surrey, United Kingdom). Glycocalicin (GC) was purified as described previously.[29] Fully sulphated wild-type recombinant GPIbα, comprising residues 1-290, was produced and purified as described previously.[30] Essentially fatty acid free bovine serum albumin (BSA) and p-nitrophenyl phosphate substrate (PNP) were obtained from Sigma-Aldrich (St Louis, MO). All other chemicals used in the experiments were of analytical grade.

Platelet preparation

Blood from healthy volunteers, who claimed not to have used aspirin or other nonsteroidal anti-inflammatory drugs for the preceding 10 days, was drawn into one-tenth volume of 3.4% sodium citrate. Washed platelets were prepared as described previously.[7] The blood was centrifuged at 200g for 15 min at room temperature. The platelet-rich plasma (PRP) was removed and acidified by addition of one-tenth volume of ACD (2.5% trisodium citrate, 1.5% citric acid, and 2% D-glucose). Platelets were centrifuged (500g, 15 min) and the platelet pellet was resuspended in Hepes-Tyrode buffer at pH 6.5 (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgSO_4$, 5 mM D-glucose). Prostacyclin (PGI_2, 10 ng/ml) was added to prevent platelet activation during the subsequent washing step. Platelets were centrifuged (500g, 15 min) and resuspended in a small volume of HEPES-Tyrode buffer. Platelet suspension was further diluted in HEPES-Tyrode buffer at pH 7.35 to a platelet count of 200 x 10^9/L (200.000/µl). GPIbα-depleted platelets were prepared by treating the platelets for 30 min at 37 °C with 80 µg/ml O-sialoglycoprotein endopeptidase (OSE). Proteolysis of GPIb was measured by investigating binding of a GPIbα-specific antibody (AN51, Dako, Glostrup, Denmark) to platelets by FACS analysis. OSE-treatment reduced GPIbα expression on platelets to less than 5% (supplemental Figure 1).

Cell culture

Wild-type Chinese Hamster Ovary (CHO) cells and CHO cells stably expressing the GPIb-IX-V (a generous gift of Dr J.A. Lopez (Seattle, Washington)) were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium with 10% fetal calf serum in the presence of penicillin and streptomycin. Cells were subjected to selection by using G418 as described previously.[31]

Binding studies

rFVIIa was immobilized for 2 hrs at 37ºC on a Costar 96-wells plate at the indicated concentrations. After blocking the wells with 2% BSA for at least 30 min, the wells were incubated with 2 µg/ml GC for 90 min at 22ºC. Bound GC was detected with an in house rabbit polyclonal antibody against GPIb, followed by a peroxidase-labeled swine-anti-rabbit antibody detected with TMB peroxidise substrate solution (Tebu-Bio, Heerhugowaard, the Netherlands). Results were obtained by measuring optical density at 450 nm on a SpectroMax Reader (Molecular Devices, Wokingham, United Kingdom).

For cell adhesion experiments, rFVIIa was immobilized for 2 hrs at 37ºC on an Immulon-2B flatbottom microtiter plate (Dynatech Laboratories Inc, Chantilly, VA) and subsequently blocked with 2% BSA for at least 30 min. Washed platelets (200.000/µl in HEPES-Tyrode's pH 7.35) or CHO cells (resuspended at 106 cells per ml in DMEM:F-12, 0.5% BSA, 1 mM CaCl$_2$, 25 µM ZnCl$_2$) were allowed to adhere for 60 min at 37ºC. After extensive washing with Tris-buffered Saline (TBS), intrinsic phosphatase activity was measured using PNP (3 mg/ml dissolved in 50 mM acetic acid, 1% Triton X-100, pH 5.0) and after 30 min the reaction was stopped with 1 M NaOH. Optical density was measured at 405 nm.

Surface plasmon resonance analysis

Binding studies were performed using a BIAcore2000 biosensor system (Biacore AB, Uppsala, Sweden) and surface plasmon resonance (SPR) analysis was done as described before.[32] rFVIIa or factor X were immobilized on a CM5-sensor chip, using the amine-coupling kit as prescribed by the supplier. A control channel was routinely activated and blocked in the absence of protein. Binding to coated channels was corrected for binding to non-coated channels. SPR analysis was performed in HEPES (25 mM NaCl, 125 mM HEPES, 3 mM CaCl$_2$, pH 7.4) buffer with a flow rate of 5 µl/min. Regeneration of the sensor chip surface was performed by incubating with 10 mM TDOC in Tris pH 9.0 for 2 min at a flow rate of 5 µl/min.

Thrombin generation

Thrombin generation experiments were performed as described previously by Monroe et al.[9] In short, isolated platelets were stimulated with a combination of convulxin (100 ng/ml) and SFLLRN (100 µM) or vehicle. Platelets were mixed with plasma concentrations of prothrombin (1.2 µM), factor X (135 nM), factor V (7 µg/ml), factor VII (10 nM), antithrombin (2.5 µM) and TFPI (tissue factor pathway inhibitor, 0.1 µg/ml). Thrombin generation was initiated with indicated concentrations of rFVIIa and at indicated time points small samples were taken and added to thrombin substrate solution (1 mM EDTA, 50 µM Pefabloc Xa and 0.5 mM S2238). After 25 minutes, the reaction was stopped by addition of 50% acetic acid. Optical density was measured at 405 nM. Thrombin activity was calculated by comparing the optical density to a standard range of thrombin. Thrombin activity curves were fitted using a modified Gaussian equation as described previously.[33]
For thrombin generation in plasma, isolated washed platelets were pretreated with OSE (80 µg/ml) or vehicle, before stimulation with a combination of convulxin (100 nM) and SFLLRN (100 µM). Next, platelets were reconstituted in 'artificial' hemophilic plasma (pooled normal plasma, to which a neutralizing antibody against FVIII was added (final concentration, 10 Bethesda units) as described previously,[34] and thrombin generation was initiated by 25 or 100 nM rFVIIa. The formation of thrombin was monitored in time using the Technothrombin TGA kit, an assay based on a fluorescent thrombin substrate, according to the instructions of the manufacturer.

Statistical analysis

Statistical analysis was performed using the GraphPad InStat (San Diego, CA) software package. Statistical differences in binding and adhesion were analysed by Student's t-test or standard one-way analysis of variance (ANOVA). Statistical differences in thrombin generation were analyzed by paired Student's t-test. P values less than 0.05 were considered statistically significant.
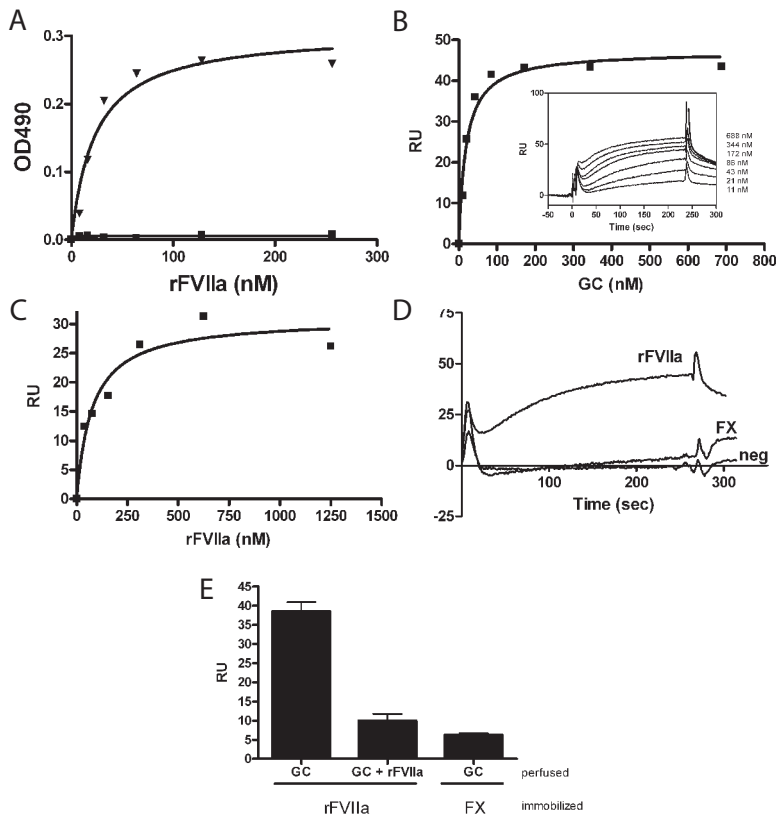
RESULTS

Figure 1 – Glycocalicin interacts with rFVIIa. (A) rFVIIa was immobilized on a microtiter plate at concentrations indicated. Subsequently, wells were incubated with GC (5 µg/ml) or vehicle and bound GC was detected by using a polyclonal antibody against GPIb (2 µg/ml). (B) rFVIIa (3400 RU) was immobilized on a CM5 sensor chip and binding of GC was investigated by surface plasmon resonance. After adjusting for binding to a blanc channel, the response of GC at equilibrium was determined and plotted against the concentration applied. Inset shows representative surface plasmon resonance traces of indicated concentrations of GC. (C) GC (1100 RU) was immobilized on a CM5 sensor chip and binding of rFVIIa was investigated by surface plasmon resonance. (D) Representative traces of GC (67 nM) binding to a rFVIIa-coated channel, a channel coated with factor X (FX, 5400 RU) or an uncoated channel (neg). (E) Binding of GC (67 nM) to rFVIIa was investigated in the absence or presence of 2 µM rFVIIa. Representatives of at least 3 experiments are shown.

Glycocalicin binds to immobilized rFVIIa

To investigate the interaction between rFVIIa and GPIbα, we tested the binding of the purified extracellular fragment of GPIbα, glycocalicin (GC), to immobilized rFVIIa in an ELISA setup. As shown in Figure 1A, GC readily bound to immobilized rFVIIa in a concentration dependent manner. Binding of GC to rFVIIa was further investigated using surface plasmon resonance. rFVIIa was coated to a CM5 sensor chip via amine-coupling with a maximum adsorption of 3400 RU and perfused with different concentrations of GC. Nonlinear regression analysis resulted in a affinity constant (K_d) of 19.5 ± 1.4 nM (Mean ± SD, figure 1B). Immobilized rFVIIa showed only minimal binding to factor X (Figure 1D). A recombinant, truncated form of GPIbα, comprising residues 1 to 290, did not bind to immobilized rFVIIa (data not shown). In addition, when a CM5 sensor chip was coated with GC, rFVIIa bound in a dose-dependent manner with a calculated K_d of 78 ± 24 nM (Mean ± SD, figure 1C). The interaction of GC with immobilized rFVIIa was abrogated by addition of rFVIIa in solution, confirming the specificity of the interaction (Figure 1E).

CHO cells expressing the GPIb/IX/V-complex interact with immobilized rFVIIa

To further investigate the interaction between rFVIIa and GPIbα, we allowed mock-transfected CHO cells (CHO-wt) as well as CHO cells expressing the GPIb-IX-V complex (CHO-Ib) to adhere to immobilized rFVIIa under static conditions. Expression of GPIb was confirmed by FACS analysis (Supplemental Figure 2). As shown in Figure 2, CHO-Ib cells interacted with immobilized rFVIIa whereas CHO-wt did not, suggesting a specific interaction between rFVIIa and the GPIb-IX-V complex. However, pretreatment of the CHO-Ib cells with OSE, which removed all GPIbα from the cells (which was confirmed by flow cytometry), did not affect adhesion to rFVIIa.
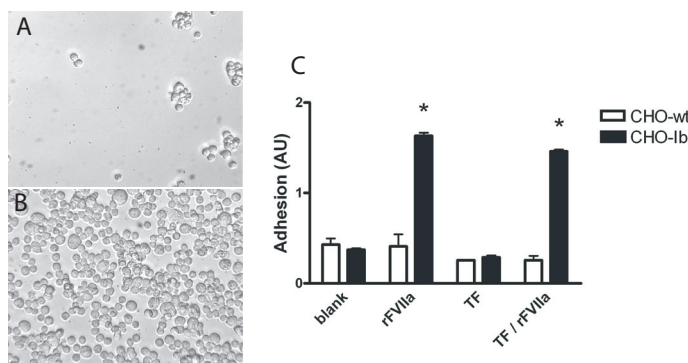

Figure 2 – CHO cells transfected with the GPIb-IX-V complex bind to immobilized rFVIIa. (A,B) mock-transfected CHO cells (CHO-wt, 1 x 10⁶ cells/ml; A) and CHO cells transfected with the GPIb-IX-V complex (CHO-Ib, 1 x 10⁶ cells/ml; B) were allowed to adhere under static conditions to immobilized rFVIIa (10 μg/ml) for 60 min at 37°C. After gentle washing, adhesion was visualized by light transmission microscopy (original magnification, 400x). (C) CHO-wt and CHO-Ib (1 x 10⁶ cells/ml) were allowed to adhere under static conditions for 60 min at 37°C to immobilized rFVIIa (10 μg/ml) or recombinant soluble tissue factor (TF; 10 μg/ml). Subsequently, TF-immobilized wells were blocked with 2% BSA, before incubation with rFVIIa or vehicle ("TF/rFVIIa" and "TF", respectively). Adhesion was quantified by measuring the intrinsic phosphatase activity and optical density is depicted as arbitrary units of adhesion (AU). Images and graph are representative of at least 3 independent experiments performed in triplicate. Error bars indicate standard deviation. *: P<0.01 as compared to blank.

TF did not interfere in the interaction between the GPIb-IX-V complex expressed on CHO cells and immobilized rFVIIa, as adhesion of CHO-Ib cells to immobilized rFVIIa was not affected by an inhibitory antibody against TF or by preincubation of immobilized rFVIIa with sTF (data not shown). Furthermore, CHO-Ib cells readily adhered to rFVIIa in complex with immobilized sTF (Figure 2C). Again, this shows that the binding of rFVIIa to TF does not interfere with the interaction between rFVIIa and the GPIb-IX-V complex.

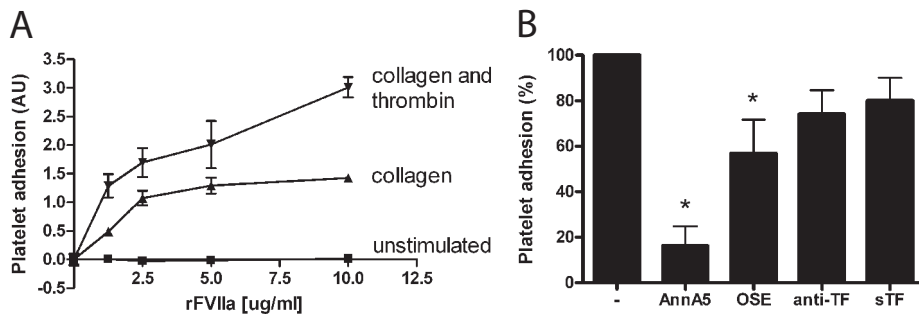## Proteolysis of GPIbα reduces adhesion of activated platelets to immobilized rFVIIa

Figure 3 – Platelet adhesion to immobilized rFVIIa is dependent on negatively charged phospholipids and GPIbα. (A) Washed platelets were stimulated with collagen (4 µg/ml) or a combination of collagen (4 µg/ml) and thrombin (1 U/ml). Subsequently, platelets were allowed to adhere under static conditions for 60 min at 37°C to immobilized rFVIIa at the concentrations indicated. (B) Washed platelets were pretreated with Annexin A5 (30 µg/ml, AnnA5), OSE (80 µg/ml), an inhibitory antibody against TF (0.5 mg/ml, anti-TF), sTF (10 µg/ml) or vehicle (-) before stimulation with a combination of collagen (4 µg/ml) and thrombin (1 U/ml) and subsequently allowed to adhere to rFVIIa as mentioned above. Adhesion of stimulated platelets to rFVIIa (5 µg/ml) is indicated as 100% platelet adhesion. Graph shows mean platelet adhesion of at least 2 independent experiments performed in triplicate. *: P<0.05 as compared to control.

To investigate whether the interaction between rFVIIa and GPIbα is sufficiently strong to bind platelets, washed platelets were allowed to adhere to immobilized rFVIIa under static conditions. Nonstimulated platelets did not bind to immobilized rFVIIa to a substantial extent. However, upon stimulation with collagen (4 µg/ml) or a combination of collagen (4 µg/ml) and thrombin (1 U/ml) for 15 min, platelet adhesion was substantially increased compared with nonstimulated platelets (Figure 3A). Adhesion was dependent on negatively charged phospholipids, as the addition of Annexin A5 decreased adhesion of activated platelets to rFVIIa (Figure 3B). Subsequently, we pretreated platelets with O-sialoglycoprotein endopeptidase (OSE), which cleaves the extracellular portion of GPIbα. When platelets were pretreated with OSE (80 µg/ml, 30 min, 37°C), adhesion to immobilized rFVIIa was inhibited by approximately 40% (Figure 3B), suggesting an important role for GPIbα in the interaction of platelets with rFVIIa. Platelet adhesion in the presence of an inhibitory antibody against TF was only slightly decreased, suggesting only a minor role for platelet-exposed TF (Figure 3B).

Proteolyis of GPIbα reduces thrombin formation on activated platelets by rFVIIa

To study whether GPIbα plays a role in the generation of thrombin on the activated platelet surface, we investigated thrombin generation under hemophilic conditions using a modification of the cell-based model as described by Monroe et al.[9] In the setup used in our experiments, coagulation was started by high-dose rFVIIa in the absence of a TF-bearing cell, in combination with platelet activation by SFLLRN/ convulxin. The initial slope of the curve was taken as a measure for the rate of thrombin generation. As shown in Figure 4, pretreatment of the platelets with OSE showed small but significant inhibition of thrombin generation in the presence of 100 nM rFVIIa, whereas it showed more pronounced inhibition in the presence of 25 nM rFVIIa (100 nM: 2.99 ± 0.79 vs 3.47 ± 0.64, $p<0.01$; 25 nM: 0.99 ± 0.36 vs 1.39 ± 0.56, $p<0.05$ [mean $\pm$ SD, n=5], respectively). However, this inhibition was only observed in 5 out of 10 donors tested. The donors that did not respond to OSE-treatment, showed comparable peak thrombin generation compared with control. The cause of this donor variability remains to be elucidated.

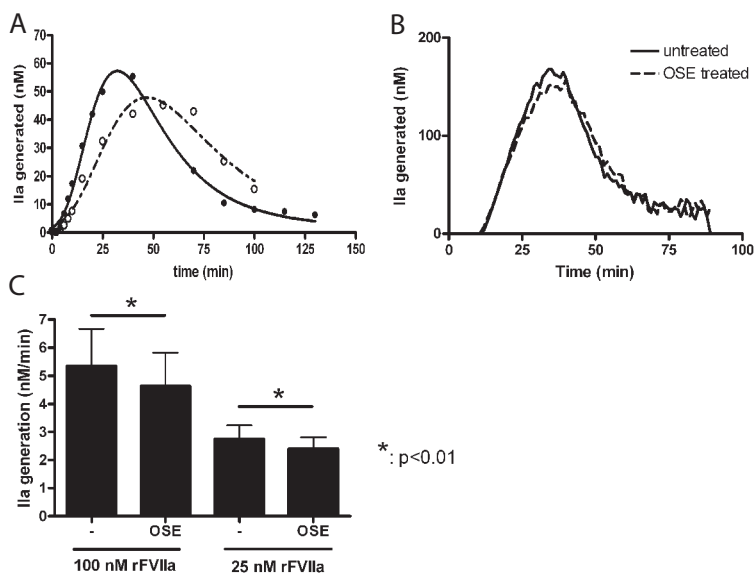Furthermore, in the absence of rFVIIa, hardly any thrombin generation was

Figure 4 – GPIbα accelerates rFVIIa-mediated thrombin generation. Platelets were pretreated with OSE (160 µg/ml, dotted line, open circles) or vehicle (straight line, closed circles) for 30 min at 37°C and subsequently stimulated with a combination of convulxin (100 ng/ml) and SFLLRN (100 µM). (A) Next, thrombin generation was followed in time after addition of 25 nM VIIa in the presence of plasma concentrations of coagulation factors VII, X, II, V and Ca^{2+}, and inhibitors TFPI and antithrombin III. Samples were taken at indicated time points and the amount of thrombin generated was calculated. (B) Platelets were reconstituted in 'artificial' hemophilic plasma (final concentration 200.000/µl) and coagulation was started by the addition of 25 or 100 nM rFVIIa. The formation of thrombin was followed in time using a commercially available thrombin generation assay. (C) Quantification of the initial slope of plasma-based thrombin generation curves as shown in (B). Shown are mean values from thrombin generation curves generated from 8 different donors in which thrombin generation curves were generated in 'artificial' hemophilic plasma with 25 or 100 nM of rFVIIa in absence or presence of OSE pretreatment. Error bars indicate standard deviations.

observed during the investigated time period (data not shown). The area under the curve, a measure for total thrombin generation, was similar comparing treated versus non-treated platelets, indicating that GPIbα does not influence the total amount of FX converted to FXa, but only affects the rate of conversion of FX into factor Xa.

To study the effect of GPIb in thrombin generation in a more physiological model, we used a commercially available thrombin generation assay, in which we reconstituted platelets in 'artificial' hemophilic plasma. Again, the initial slope of the curve was taken as a measure for the rate of thrombin generation. As shown in Figure 4B, thrombin generation was significantly inhibited upon treatment with OSE as compared to controls (100 nM rFVIIa: 4.64 ± 1.19 vs 5.34 ± 1.33, p<0.01; 25 nM rFVIIa: 2.40 ± 0.41 vs 2.75 ± 0.48, p<0.05 [mean ± SD, n=8], respectively).

DISCUSSION

In this study, three lines of evidence show that rFVIIa is able to interact with the GPIb-IX-V complex. First, we showed that rFVIIa and purified GC interact with each other, both in an ELISA setup as well as in surface plasmon resonance analysis. Furthermore, CHO cells transfected with the GPIb-IX-V complex were able to adhere to immobilized rFVIIa, whereas wild-type cells were not. Finally, we demonstrated that, upon activation, platelets were able to adhere to immobilized rFVIIa in a GPIbα-dependent manner. Furthermore, the formation of thrombin on the activated platelet surface was reduced upon proteolysis of GPIbα. Taken together, these observations suggest that the interaction of rFVIIa with GPIbα plays an important role in TF-independent enhancement of thrombin generation by rFVIIa on the activated platelet surface. Surprisingly, proteolysis of GPIbα did affect platelet adhesion but not adhesion of transfected CHO cells to immobilized rFVIIa. At least in the CHO cell system it thus appears that other components of the GPIb-IX-V complex are involved in the interaction with rFVIIa. At present, tools to investigate the interaction of rFVIIa with other components of the complex are not available, and the role of the other subunits in the interaction remains to be established. However, because rFVIIa-mediated platelet adhesion and thrombin generation on the activated platelet are affected by specific proteolysis of GPIbα, we are convinced that GPIbα is the main physiological receptor for rFVIIa within the GPIb-IX-V complex.

Activated platelets are thought to play an important role in enhancing thrombin generation when the initial exposed TF of the vessel wall is covered with platelets and is not accessible anymore for plasma FVIIa. First, it has been shown that TF is incorporated in the growing thrombus by recruiting TF-containing microvesicles in a mechanism dependent on P-selectin and PSGL-1.[22,35] Second, activation of platelets results in exposure of negatively charged phospholipids, which is important in the propagation of coagulation. We propose a third mechanism by which platelets can propagate secondary hemostasis: GPIbα expressed on activated platelets
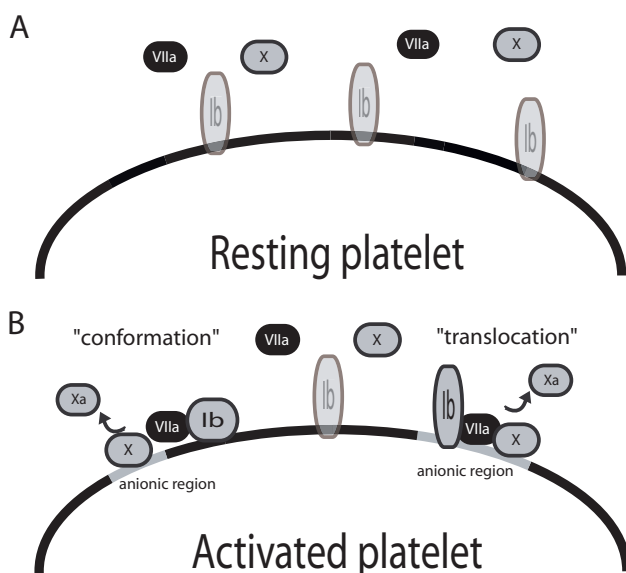
Figure 5 – Hypothetical model for the role of GPIbα in the mechanism of action of rFVIIa. (A) On the resting platelet, the interaction between rFVIIa and the GPIb-IX-V complex (depicted as 'Ib') does not occur, as the resting platelet does not express negatively charged phospholipids, and presumably because the GPIb-IX-V complex is not in a rFVIIa-binding membrane environment. (B) After activation of the platelet, rFVIIa is able to interact with the GPIb-IX-V complex. This interaction is critically dependent on the negatively charged surface, but may also involve translocation of the GPIb-IX-V complex to an anionic, or lipid raft region of the platelet surface. Alternatively, the interaction might require a conformational change with the GPIb-IX-V complex. The interaction of rFVIIa with GPIb result in acceleration of Xa generation.

acts in localizing rFVIIa on the platelets surface, thereby enhancing its enzymatic efficacy.

rFVIIa interacts with purified GC, but a recombinant soluble form of GPIbα, consisting of residues 1 to 290, does not interact with rFVIIa, whereas recombinant soluble GPIbα does bind both von Willebrand factor and thrombin, two other ligands for GPIbα. This suggests that the glycosylated stack of the GPIbα molecule plays a role in the interaction with rFVIIa or that GC has a different conformation compared to recombinant soluble GPIbα. Furthermore, rFVIIa does not bind to resting platelets, while GPIbα is abundantly present on the surface of resting platelets. In addition, CHO-cells expressing the GPIb-IX-V complex readily adhere to immobilized rFVIIa without the need for prior activation. An interesting explanation for these contradictory results could be that GPIbα needs to be located in the proper lipid microenvironment. Alternatively, platelet activation could result in a conformational change within the GPIb-IX-V complex, which changes the complex into a rFVIIa-binding conformation. Apparently, the GPIb-IX-V complex expressed on the CHO cells already has the sufficient characteristics necessary for binding of rFVIIa. In platelets, after activation, the lipid microenvironment of GPIbα could change, and the specific combination of lipids and GPIbα could be responsible for the binding of rFVIIa. In this way, activation of the platelets could serve two roles: (1) exposure of negatively charged phospholipids at the platelet surface, which is a prerequisite for rFVIIa binding to platelets; and (2) translocation of GPIbα to these negatively charged regions, which provides the perfect surface for the propagation of coagulation or an activation-induced conformational change in the complex (Figure 5). In this way, rFVIIa can interact with the negatively charged

surface via its Gla domain and (perhaps simultaneously) with GPIbα via another part of the molecule, in which the interaction with GPIbα accelerates propagation of coagulation. However, other explanations for the discrepancy observed between the two cell types cannot be ruled out; therefore future experiments will have to prove this model system.

Recent reports have suggested the presence of TF on activated platelets. However, it is improbable that in our model TF is involved in the interaction of rFVIIa with platelets. As shown by Monroe et al,[9] inhibitory antibodies against TF showed no reduction in thrombin generation on the activated platelet surface. In addition, in our model a delay in thrombin generation was observed after cleavage of GPIbα, irrespective of whether TF is involved or not. Furthermore, we have shown that the binding of CHO-Ib cells to immobilized rFVIIa is not influenced by the presence of TF. Panes et al[21] reported the colocalization of GPIbα and TF on the activated platelet surface, which leaves the possibility that both GPIbα and TF interact with rFVIIa at the same time, possibly serving different roles in the mechanism of action of rFVIIa.

Our results clearly show that rFVIIa can interact with GPIbα. The most important cellular receptor for rFVIIa is TF, but besides TF, several receptors have been reported to interact with rFVIIa. The TF/rFVIIa-complex is able to bind and signal via a protease-activated receptor, presumably PAR-2 (reviewed by Riewald and Ruf[36]). Furthermore, recent reports show that the endothelial protein C receptor is able to bind rFVIIa at the endothelial cell surface.[37-40] However, the effect of binding of rFVIIa to these receptors on procoagulant activity of rFVIIa is still a matter of debate, as the groups show contradictory results on activation of protein C and generation of FXa. It remains to be elucidated if and how all these receptors interplay in the mechanism of action of rFVIIa.

Recently, Tranholm et al reported a variant of rFVIIa with improved TF-independent, but similar TF-dependent thrombin generation capacity compared with wild-type rFVIIa.[18] An interesting application of the results presented in this manuscript would be to construct a new variant that has an improved interaction with GPIbα, which can ultimately lead to an enhanced efficacy of rFVIIa. This could improve current therapy, because lower doses of rFVIIa or less frequent administration of rFVIIa would be necessary, which would obviously benefit the patient.

In conclusion, our findings suggest an important role for GPIbα in the mechanism of action of rFVIIa; therefore the development of rFVIIa variants with an improved binding to GPIbα could ultimately lead to improved therapy for hemophilia patients.

REFERENCES

1. Hedner U. Recombinant activated factor VII as a universal haemostatic agent. Blood Coagul.Fibrinolysis 1998;9 Suppl 1:S147-S152.

2. He S, Blomback M, Jacobsson EG, Hedner U. The role of recombinant factor VIIa (FVIIa) in fibrin structure in the absence of FVIII/FIX. J.Thromb. Haemost. 2003;1:1215-1219.

3. Lisman T, Mosnier LO, Lambert T et al. Inhibition of fibrinolysis by recombinant factor VIIa in plasma from patients with severe hemophilia A. Blood 2002;99:175-179.

4. Butenas S, Brummel KE, Branda RF, Paradis SG, Mann KG. Mechanism of factor VIIa-dependent coagulation in hemophilia blood. Blood 2002;99:923-930.

5. Butenas S, Brummel KE, Bouchard BA, Mann KG. How factor VIIa works in hemophilia. J.Thromb.Haemost. 2003;1:1158-1160.

6. van 't Veer C, Mann KG. The regulation of the factor VII-dependent coagulation pathway: rationale for the effectiveness of recombinant factor VIIa in refractory bleeding disorders. Semin. Thromb.Hemost. 2000;26:367-372.

7. Lisman T, Moschatsis S, Adelmeijer J, Nieuwenhuis HK, de Groot PG. 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. Blood 2003;101:1864-1870.

8. Hoffman M, Monroe DM, Roberts HR. Human monocytes support factor X activation by factor VIIa, independent of tissue factor: implications for the therapeutic mechanism of high-dose factor VIIa in hemophilia. Blood 1994;83:38-42.

9. Monroe DM, Hoffman M, Oliver JA, Roberts HR. Platelet activity of high-dose factor VIIa is independent of tissue factor. Br.J.Haematol. 1997;99:542-547.

10. Lisman T, de Groot PG. Mechanism of action of recombinant factor VIIa. J.Thromb.Haemost. 2003;1:1138-1139.

11. Cooper HA, Jones CP, Campion E, Roberts HR, Hedner U. Rationale for the use of high dose rFVIIa in a high-titre inhibitor patient with haemophilia B during major orthopaedic procedures. Haemophilia. 2001;7:517-522.

12. Kenet G, Lubetsky A, Luboshitz J, Martinowitz U. A new approach to treatment of bleeding episodes in young hemophilia patients: a single bolus megadose of recombinant activated factor VII (NovoSeven). J.Thromb. Haemost. 2003;1:450-455.

13. Santagostino E, Mancuso ME, Rocino A et al. A prospective randomized trial of high and standard dosages of recombinant factor VIIa for treatment of hemarthroses in hemophiliacs with inhibitors. J.Thromb.Haemost. 2006;4:367-371.

14. Monroe DM, Hoffman M, Oliver JA, Roberts HR. A possible mechanism of action of activated factor VII independent of tissue factor. Blood Coagul.Fibrinolysis 1998;9 Suppl 1:S15-S20.

15. Kjalke M, Ezban M, Monroe DM et al. High-dose factor VIIa increases initial thrombin generation and mediates faster platelet activation in thrombocytopenia-like conditions in a cell-based model system. Br.J.Haematol. 2001;114:114-120.

16. Roberts HR, Monroe DM, White GC. The use of recombinant factor VIIa in the treatment of bleeding disorders. Blood 2004;104:3858-3864.

17. Hedner U, Erhardtsen E. Potential role for rFVIIa in transfusion medicine. Transfusion 2002;42:114-124.

18. Tranholm M, Kristensen K, Kristensen AT et al. Improved hemostasis with superactive analogs of factor VIIa in a mouse model of hemophilia A. Blood 2003;102:3615-3620.

19. Kjalke M, Kjellev S, Rojkjaer R. Preferential localization of recombinant factor VIIa to platelets acti-vated with a combination of thrombin and a GPVI receptor agonist. J.Thromb.Haemost. 2007

20. Schwertz H, Tolley ND, Foulks JM et al. Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenicity of human platelets. J.Exp.Med. 2006;203:2433-2440.

21. Panes O, Matus V, Saez CG et al. Human platelets synthesize and express functional tissue factor. Blood 2007;109:5242-5250.

22. Del Conde I, Shrimpton CN, Thiagarajan P, Lopez JA. Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. Blood 2005;106:1604-1611.

23. Harmon JT, Jamieson GA. The glycocalicin portion of platelet glycoprotein Ib expresses both high and moderate affinity receptor sites for thrombin. A soluble radioreceptor assay for the interaction of thrombin with platelets. J.Biol.Chem. 1986;261:13224-13229.

24. Baglia FA, Badellino KO, Li CQ, Lopez JA, Walsh PN. Factor XI binding to the platelet glycoprotein Ib-IX-V complex promotes factor XI activation by thrombin. J.Biol.Chem. 2002;277:1662-1668.

25. Bradford HN, Pixley RA, Colman RW. Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. J.Biol.Chem. 2000;275:22756-22763.

26. Joseph K, Nakazawa Y, Bahou WF, Ghebrehiwet B, Kaplan AP. Platelet glycoprotein Ib: a zinc-dependent binding protein for the heavy chain of high-molecular-weight kininogen. Mol.Med. 1999;5:555-563.

27. White TC, Berny MA, Fernández JA, Griffin JH, Gruber A, McCarty OJ. Glycoprotein Ib medaties platelet recruitment, activation and aggregation on immobilized protein C and activated protein C under shear. J. Thromb. Haemost. 2007;5 Suppl 2: O-M-030.

28. Hackeng TM, van 't Veer C, Meijers JC, Bouma BN. Human protein S inhibits prothrombinase complex activity on endothelial cells and platelets via direct interactions with factors Va and Xa. J.Biol. Chem. 1994;269:21051-21058.

29. Weeterings V, Adelmeijer J, Myles T, de Groot PG, Lisman T. Glycoprotein Ibalpha-mediated platelet adhesion and aggregation to immobilized thrombin under conditions of flow. Arterioscler.Thromb.Vasc. Biol. 2006;26:670-675.

30. Huizinga EG, Tsuji S, Romijn RA et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. Science 2002;297:1176-1179.

31. Lopez JA, Leung B, Reynolds CC, Li CQ, Fox JE. Efficient plasma membrane expression of a functional platelet glycoprotein Ib-IX complex requires the presence of its

three subunits. J.Biol.Chem. 1992;267:12851-12859.

32. Lenting PJ, Neels JG, van den Berg BM et al. The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. J.Biol.Chem. 1999;274:23734-23739.

33. Allen GA, Wolberg AS, Oliver JA et al. Impact of procoagulant concentration on rate, peak and total thrombin generation in a model system. J.Thromb. Haemost. 2004;2:402-413.

34. Lisman T, de Groot PG, Lambert T, Rojkjaer R, Persson E. Enhanced in vitro procoagulant and antifibrinolytic potential of superactive variants of

recombinant factor VIIa in severe hemophilia A. J.Thromb. Haemost. 2003;1:2175-2178.

35. Giesen PL, Rauch U, Bohrmann B et al. Blood-borne tissue factor: another view of thrombosis. Proc.Natl.Acad. Sci.U.S.A 1999;96:2311-2315.

36. Riewald M, Ruf W. Orchestration of coagulation protease signaling by tissue factor. Trends Cardiovasc.Med. 2002;12:149-154.

37. Preston RJ, Ajzner E, Razzari C et al. Multifunctional specificity of the protein C/activated protein C Gla domain. J.Biol. Chem. 2006;281:28850-28857.

38. Ghosh S, Pendurthi UR, Steinoe A, Esmon CT, Rao

LV. Endothelial cell protein C receptor acts as a cellular receptor for factor VIIa on endothelium. J.Biol.Chem. 2007;282:11849-11857.

39. Lopez-Sagaseta J, Montes R, Puy C et al. Binding of factor VIIa to the endothelial cell protein C receptor reduces its coagulant activity. J.Thromb. Haemost. 2007;5:1817-1824.

40. Steinoe A, Sorensen BB, Stennicke HR. Recombinant activated human factor VII (FVIIA) binds to the endothelial protein C receptor (EPCR) on human umbilical vein endothelial cells (HUVEC). J. Thromb. Haemost. 2007;5 Suppl 2: P-T-015.
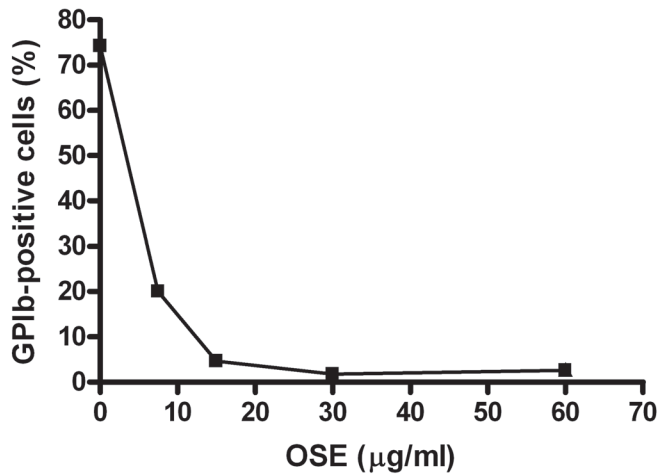
SUPPLEMENTAL DATA

Figure 1 – Cleavage of GPIbα from the platelet surface by OSE. Isolated platelets were treated for 30 min at 37°C with vehicle or OSE at indicated concentrations. Proteolysis of GPIb was measured by investigating binding of a GPIbα-specific antibody (AN51, Dako, Glostrup, Denmark) to platelets by FACS analysis.
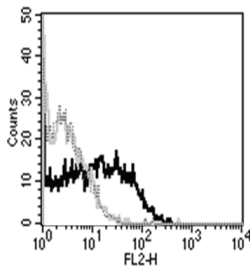

Figure 2 – GPIbα expression on CHO cells. Wild-type CHO cells (CHO-wt) and CHO cells transfected with the GPIb-complex (CHO-Ib) were analysed for the expression of GPIb. Binding of an antibody specific for GPIbα (AN51. Dako, Glostrup, Denmark) to CHO-Ib (black line) was compared to CHO-wt (grey line) and isotype control (grey dotted line) as expressed by fluorescence intensity (FL2-H).

Chapter 4

Glycoprotein Ibα modulates factor IXa-mediated factor Xa-generation on the activated platelet surface

Cees Weeterings [1], Philip G de Groot [1], Ton Lisman [1,2]

From [1] the Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, the Netherlands; [2] Surgical Research Laboratory, Department of Surgery, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands

ABSTRACT

Introduction - The platelet surface plays a pivotal role in coagulation. Besides providing a negatively charged phospholipid surface, platelet receptors have been shown to interact with coagulation factors which could influence thrombin generation on the activated platelet surface.

Methods & Results - To investigate the interaction of factor IX (FIX) with platelets, we allowed platelets to adhere to immobilized FIX or activated FIX (FIXa). Unstimulated platelets adhered to FIXa and only weakly to FIX. After stimulation with SFLLRN or collagen, platelet adhesion to FIX and FIXa was significantly increased. The addition of Annexin A5 only slightly decreased platelet adhesion to FIX(a), confirming previous data showing that FIX(a) can bind to platelets independently of a negatively charged phospholipid surface. Proteolysis of GPIb-alpha by O-sialoglycoprotein endopeptidase profoundly reduced platelet adhesion to FIX(a), and CHO cells expressing the GPIb/IX/V complex bound immobilized FIX, indicating GPIb-alpha may serve as the FIX receptor on platelets. Furthermore, platelets pretreated with SFLLRN adhered to immobilized FIXa under conditions of flow, showing that the interaction between platelets and FIX(a) was sufficiently strong to resist shear forces. In addition, proteolysis of GPIb-alpha from the activated platelet surface resulted in accelerated FIXa-mediated FXa-generation. Addition of rFVIIa inhibited platelet adhesion to FIX(a) under static conditions, suggesting the binding sites for rFVIIa and FIX(a) on GPIb-alpha overlap. Furthermore, active-site inactivated rFVIIa (rFVIIai) inhibited FIXa-mediated factor Xa-generation on activated platelets.

Conclusion - Taken together, these results show that FIX(a) interacts with GPIb-alpha on the activated platelet surface under static conditions and under conditions of flow. The interaction of FIX(a) with GPIb-alpha modulates FXa generation on the activated platelet surface.

INTRODUCTION

The process of coagulation is vital in controlling of bleeding at the site of vascular injury. The extrinsic pathway of coagulation is initiated when tissue factor becomes exposed to flowing blood and, via a series of activation steps, leads to the conversion of fibrinogen into fibrin by thrombin. The intrinsic pathway of coagulation, referred to as the contact activation pathway, involves the activation of factor XII by non-physiological surfaces such as kaolin or glass, which leads to the generation of activated factor XI (FXIa) and, via subsequent series of activation steps, results again in the formation of fibrin. The deficiency of one of the coagulation factors in both systems, with the exception of factor XII, leads to a bleeding diathesis, indicating the essential role of coagulation in hemostasis.

Platelets have been described to play a pivotal role in supporting coagulation reactions. The procoagulant nature of platelets has been mostly attributed to negatively charged phospholipids, which become exposed after platelet activation.[1-4] Although the traditional belief is that negatively charged phospholipids on activated platelets are required and sufficient for platelet-mediated coagulation reactions, more and more evidence has accumulated indicating that, in addition to negatively charged lipids, platelet receptors could modulate coagulation reactions as well. We have recently identified glycoprotein Ibα (GPIbα) as a platelet receptor for coagulation factor VIIa, and we have shown that absence of GPIbα reduces tissue factor-independent thrombin generation by pharmacological doses of rFVIIa.[5] Also, coagulation factor XI and (activated) protein C have been shown to interact with GPIbα, although the consequences of these interactions for coagulation are at present unclear.[6-8]

The hypothesis that negatively charged lipids are necessary but not sufficient for platelet-mediated coagulation is in part based on observations showing that binding of some coagulation proteins to platelets is qualitatively different from their binding to synthetic lipid vesicles. In particular, binding of coagulation factor IX(a) to platelets is substantially different from the interaction with negatively charged phospholipid vesicles. Not only is the binding of FIXa to platelets of much higher affinity as compared to the interaction with lipid vesicles (2-3 nM vs > 500 nM, respectively), also the structural requirements are different. Binding of FIXa to lipid vesicles is critically dependent on the presence of its Gla domain, whereas des-Gla FIXa binds to platelets with similar affinity as compared to full-length FIXa.[9,10] These observations are consistent with the presence of a platelet-binding protein for FIX(a).

As part of a systematic study in which we were interested in the interactions of coagulation factor with GPIbα, we investigated the interaction of FIX(a) with platelets, and the role of GPIbα therein. Furthermore, we investigated the effect of GPIbα on FIXa-mediated FXa-generation on the activated platelet surface.

METHODS

Materials

Convulxin was obtained from Alexis Biochemicals (San Diego, CA). The PAR1-agonist peptide SFLLRN was from Bachem (Bubendorf, Switzerland). The RGD-containing peptide D-arginyl-glycyl-L-aspartyl-L-tryptophane (dRGDW) was synthesized at the Department of Membrane Enzymology, Faculty of Chemistry (University of Utrecht, The Netherlands). O-sialoglycoprotein endopeptidase was obtained from Cedarlane Laboratories (Hornby, ON). Collagen type III and ionomycin were from Sigma (St Louis, MO). RAP was produced as described previously.[11] Annexin A5 was a kind gift of Dr. C. Reutelingsperger (Maastricht, the Netherlands). rFVIIa and rFVIIai were a generous gift from Drs M. Kjalke and P.K. Holm (Haemostasis Biology, Novo Nordisk, Malov, Denmark). Plasma-derived factor IXa-beta was from Enzyme Research Labs (South Bend, IN). Plasma-derived factor VIII and factor IX were from Sanquin BV (Amsterdam, the Netherlands). Factor X was purified from fresh-frozen plasma by immunoaffinity chromatography followed by Q-Sepharose chromatography as described previously.[12] Pefachrome Xa was purchased from Pentapharm (Basel, Switzerland). Essentially fatty acid free bovine serum albumin (BSA) was obtained from Sigma-Aldrich (St Louis, MO).

Platelet preparation

Blood from healthy volunteers, who claimed not to have used aspirin or other nonsteroidal anti-inflammatory drugs for the preceding 10 days, was drawn into one-tenth volume of 3.4% sodium citrate. This was done with the approval from the Institutional Review Board from the University Medical Center Utrecht (Utrecht, the Netherlands) and in accordance with the Declaration of Helsinki. Washed platelets were prepared as described previously.[13] For static adhesion experiments, platelets were further diluted in HEPES-Tyrode buffer at pH 7.35 (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgSO_4$, 5 mM D-glucose) to a final platelet count of 200×10^9/L. For flow experiments, washed 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 $CaCl_2$, 5 mM D-glucose, pH 7.35) to a final platelet count of 333×10^9/L. GPIbα-depleted platelets were prepared by treating the platelets for 30 minutes at 37°C with 30 μg/ml OSE. Proteolysis of GPIbα was monitored by assessing binding of a GPIbα-specific antibody (AN51, Dako, Glostrup, Denmark) to platelets by FACS analysis. OSE-treatment reduced GPIbα expression on platelets to less than 5%.[5]

Platelet adhesion

For platelet adhesion experiments, FIX or FIXa was immobilized for 2 hrs at 37ºC on an Immulon-2B flatbottom microtiter plate (Dynatech Laboratories Inc, Chantilly, VA), which was subsequently blocked with 2% BSA for at least 30 min. Washed platelets (200.000/μl in HEPES-Tyrode's pH 7.35) were allowed to adhere for 60 min at 37ºC. After extensive washing with Tris-buffered Saline (TBS, 10

mM Tris, 150 mM NaCl, pH 7.4), intrinsic phosphatase activity was measured using p-nitrophenyl phosphate (3 mg/ml dissolved in 50 mM acetic acid, 1% Triton X-100, pH 5.0) and after 30 min the reaction was stopped with 1 M NaOH. Optical density was measured at 405 nm.

Perfusion studies

Perfusions were carried out in a single-pass perfusion chamber as described previously.[14] Coverslips were coated with factor IX or factor IXa (5 µg/ml) as described previously.[13] Reconstituted blood was prepared as described previously[13] and was subsequently perfused over the coated coverslips for 5 minutes at a
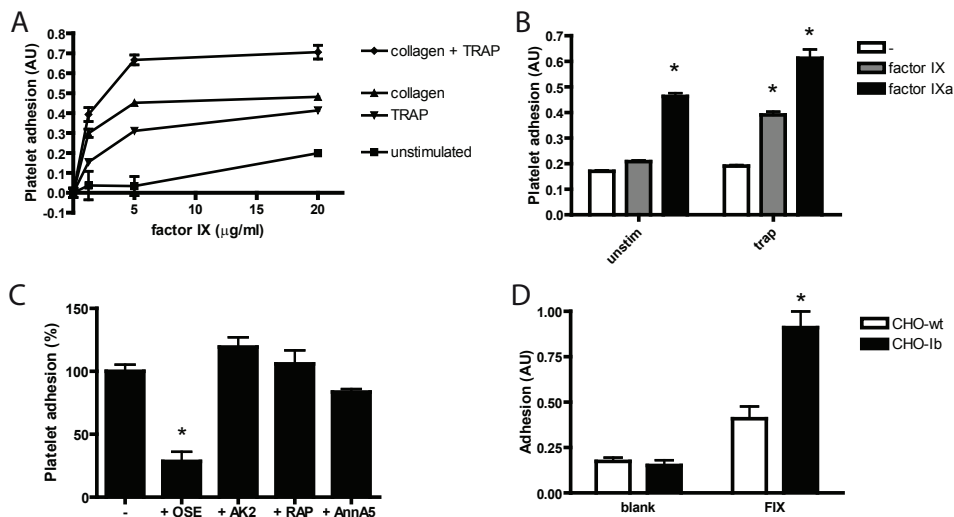

Figure 1 – Activated washed platelets adhere to immobilized factor IX. (A) Factor IX was immobilized on a microtiter plate at concentrations indicated. Isolated washed platelets were stimulated with SFLLRN (TRAP, 15 µM, 15 min at 37°C), collagen (4 µg/ml, 15 min at 37°C), a combination of TRAP and collagen, or vehicle and subsequently allowed to adhere under static conditions to immobilized factor IX for 60 min at 37°C. Platelet adhesion was quantified by measuring the intrinsic phosphatase activity and optical density is depicted as arbitrary units of adhesion (AU). Graphs show data from a single experiment performed in triplicate, which are representative for at least 2 independent experiments. (B) Isolated washed platelets were stimulated with TRAP or vehicle (unstim) and subsequently allowed to adhere to vehicle, immobilized factor IX (2.5 µg/ml) or factor IXa (2.5 µg/ml). Platelet adhesion was quantified by measuring the intrinsic phosphatase activity and optical density is depicted as arbitrary units of adhesion (AU). Graphs show data from a single experiment performed in triplicate, which are representative for at least 2 independent experiments. *: P< 0.05 compared to control (-). Error bars indicate standard error of the mean. (C) Isolated washed platelets were pretreated with O-sialoglycoprotein endopeptidase (OSE, 30 µg/ml, 30 min at 37°C) where indicated, before stimulation with a combination of collagen and TRAP. Subsequently, platelets were allowed to adhere to immobilized factor IX (2.5 µg/ml) in the absence (-) or presence of receptor-associated protein (RAP, 35 µg/ml), Annexin A5 (AnnA5, 30 µg/ml) or AK2 (10 µg/ml). Adhesion of stimulated platelets to FIX in the absence of competitors is indicated as 100% platelet adhesion. Graphs show mean platelet adhesion from at least 2 independent experiments performed in triplicate. *: P< 0.05 compared to control. Error bars indicate standard error of the mean. (D) CHO-wt and CHO-Ib (10⁶ cells/ml) were allowed to adhere under static conditions for 60 minutes at 37°C to vehicle (blank) or immobilized FIX (5 µg/ml). Adhesion was quantified by measuring the intrinsic phosphatase activity and optical density is depicted as arbitrary units of adhesion (AU). Graphs show mean adhesion from at least 3 independent experiments performed in triplicate. *: P< 0.05 compared to CHO-wt. Error bars indicate standard error of the mean.

constant flow rate of 300 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 phosphate buffered saline (PBS). Subsequently, slides were dehydrated in methanol and stained with May-Grünwald and Giemsa as described previously.[15] Next, the slides were examined using conventional light microscopy with a 40/1.00 PL APO oil immersion lens on a Leitz Diaplan (Carl Zeiss MicroImaging GmbH, Gottingen, Germany). Platelet adhesion was evaluated using computer-assisted analysis with OPTIMAS 6.2 software (Dutch Vision Systems [DVS], Breda, The Netherlands). The total surface area stained with May-Grünwald and Giemsa was calculated and expressed as the percentage of the surface covered with platelets.

CHO-cell adhesion

Wild-type Chinese Hamster Ovary (CHO) cells and CHO cells stably expressing the GPIb-IX-V (a generous gift of Dr J.A. Lopez (Seattle, Washington)) were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium with 10% fetal calf serum in the presence of penicillin and streptomycin. Cells were subjected to selection by using G418 as described previously.[16]

For cell adhesion experiments, FIX was immobilized for 2 hrs at 37ºC on an Immulon-2B flatbottom microtiter plate (Dynatech Laboratories Inc, Chantilly, VA) and subsequently blocked with 2% BSA for at least 30 min. Washed platelets (200.000/µl in HEPES-Tyrode's pH 7.35) or CHO cells (resuspended at 10^6 cells per ml in DMEM:F-12, 0.5% BSA) were allowed to adhere for 60 min at 37ºC. After extensive washing with Tris-buffered Saline (TBS), intrinsic phosphatase activity was measured using p-nitrophenyl phosphate (3 mg/ml dissolved in 50 mM acetic acid, 1% Triton X-100, pH 5.0) and after 30 min the reaction was stopped with 1 M NaOH. Optical density was measured at 405 nm.

Factor Xa generation

Isolated washed platelets were pretreated with OSE (30 µg/ml) or vehicle for 30 min at 37°C. Subsequently, platelets (200.000/µl) were stimulated with a combination of convulxin (100 ng/ml) and SFLLRN (15 µM), ionomycin (5 µg/ml) or vehicle. Next, 30 µl of a 2x concentrated mixture of coagulation factors X (10 µg/ml, final), VIII (1 U/ml, final) and IXa (2.5 µg/ml, final) in HEPES-buffer (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, 3 mM $CaCl_2$, 0.1% BSA), in the presence or absence of rFVIIai (20 µg/ml, final), was added to 30 µl of stimulated platelets. Subsequently, FXa generation was measured in time by addition of 15 µl pefachrome Xa (0.4 mM final concentration) at 405 nm on a Spectromax Reader (Molecular Devices, Wokingham, United Kingdom).

Statistical analysis

Statistical analysis was performed using the GraphPad InStat (San Diego, CA) software package. Statistical differences in binding and adhesion were analyzed by Student's t-test or standard one-way analysis of variance (ANOVA). P values less than 0.05 were considered statistically significant.
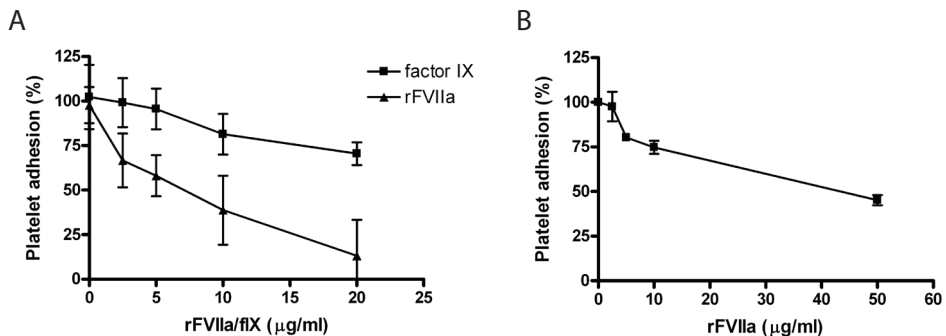
Figure 2 – Static platelet adhesion to immobilized FIX is inhibited by FIX or rFVIIa in solution.
Isolated washed platelets were stimulated with a combination of SFLLRN (15 µM, 15 min at 37°C) and collagen (4 µg/ml, 15 min at 37°C). Subsequently, activated platelets were allowed to adhere under static conditions to (A) immobilized factor IX (1.25 µg/ml) for 60 min at 37°C in the presence of indicated concentrations of factor IX or rFVIIa in solution or to (B) immobilized factor IXa (1.25 µg/ml) for 60 min at 37°C in the presence of indicated concentrations of rFVIIa in solution. Adhesion was quantified by measuring the intrinsic phosphatase activity and optical density is depicted as arbitrary units of adhesion (AU). Adhesion of stimulated platelets to immobilized factor IX was indicated as 100% adhesion. Graph shows mean platelet adhesion of at least 2 independent experiments performed in triplicate

RESULTS

Activated platelets adhere to FIX(a) under static conditions

To investigate the interaction of platelets with FIX, we initially used static adhesion assays in which isolated platelets were allowed to adhere to wells coated with increasing concentrations of FIX. As shown in Figure 1A, non-stimulated platelets hardly adhered to immobilized FIX. Upon stimulation with the thrombin receptor activating peptide SFLLRN (TRAP, 15 µM), collagen (4 µg/ml), or a combination of collagen and TRAP, increasing platelet deposition with increasing concentrations of immobilized FIX was observed. Whereas non-stimulated platelets hardly adhered to FIX, platelets did adhere to FIXa (Figure 1B) without prior stimulation. Again, after stimulation of platelets with TRAP, adhesion to FIXa increased.

Proteolysis of the extracellular part of GPIbα by OSE reduced platelet adhesion to FIX substantially (Figure 1C). In contrast, an antibody which abrogates VWF binding to GPIbα (AK2, 10 µg/ml), did not affect platelet adhesion to FIX (Figure 1C). Inhibition of ligand binding to the integrin αIIbβ3, by addition of the peptide dRGDW, also reduced adhesion to FIX substantially, but this was rather due to the loss of small aggregate formation than loss of primary adhesion as evidenced by light microscopy (data not shown). Whereas apoER2' is involved in the interaction with FXI and platelets,[8] inhibition of apoER2' by receptor-associated protein (RAP) did not have an effect on platelet adhesion to FIX (Figure 1C).

To investigate the role of negatively charged phospholipids, platelets were allowed to adhere to FIXa in the presence of Annexin A5 (30 µg/ml). As shown in Figure 1C, the addition of Annexin A5 had no effect on platelet adhesion to FIXa.

To further investigate the interaction between FIX and GPIbα, we allowed mock-

transfected CHO cells (CHO-wt) and CHO cells expressing the GPIb-IX-V complex (CHO-Ib) to adhere to immobilized FIX under static conditions. As shown in Figure 1D, CHO-Ib cells, but not CHO-wt cells, interacted with immobilized FIX, suggesting a specific interaction between FIX and the GPIb-IX-V complex.

Competition of platelet binding to FIX with rFVIIa

To further examine specificity of the interaction between FIX and platelets, platelets were allowed to adhere to immobilized FIX in the presence of FIX in solution. As shown in figure 2A, increasing concentrations of FIX in solution inhibited platelet adhesion to immobilized FIX. However, inhibition was only ~25% at 20 µg/ml FIX in solution. Previously we showed rFVIIa, which is homologous to FIX, to interact with GPIbα. As shown in Figure 2A, platelet adhesion to immobilized FIX was significantly inhibited in the presence of rFVIIa in solution. Also, rFVIIa dose-dependently inhibited platelet adhesion to immobilized FIXa, but inhibition of platelet adhesion to immobilized FIXa was profoundly less efficient as compared to inhibition of platelet adhesion to FIX (Figure 2B).

Activated platelets adhere to FIXa under conditions of flow

To investigate whether the interaction between activated platelets and FIXa is sufficiently strong to resist shear forces, we examined platelet adhesion to immobilized FIXa in a perfusion model. Reconstituted blood (a mixture of 60% washed platelets in a 4% human albumin solution and 40% red blood cells) was perfused over a coverslip coated with FIXa for 5 minutes at 300 s^{-1}. As shown in Figure 3A, non-stimulated platelets hardly adhered to FIXa. Upon stimulation of
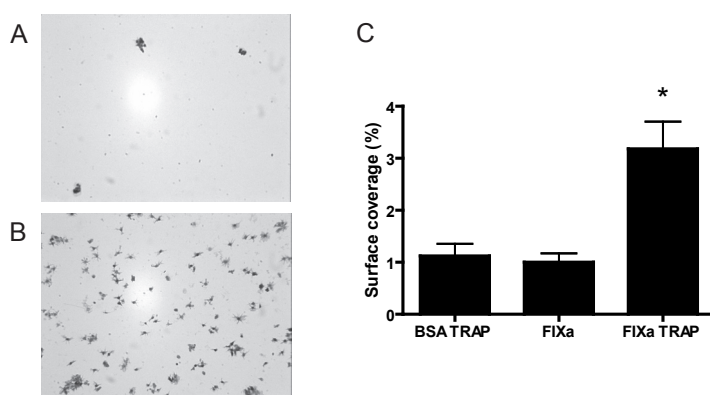

Figure 3 – Platelets adhere to factor IX(a) under conditions of flow. Isolated washed platelets were pretreated with SFLLRN (TRAP, 15 min, 37°C, panel A) or vehicle (panel B). Subsequently, reconstituted blood (washed platelets in a 4% human albumin solution and isolated red cells, 40% hematocrit, 200.000 platelets/µl) was perfused over a coverslip coated with 5 µg/ml factor IXa at a shear rate of 300 s^{-1} for 5 min at 37°C using a single-pass perfusion chamber. After perfusion, coverslips were fixed and stained with May-Grünwald/Giemsa and examined by light microscopy. Representative images of at least 3 independent experiments performed in triplicate are shown. (C) Graph shows mean surface coverage of at least 3 independent experiments performed in triplicate. *: P< 0.05 compared to control. Error bars indicate standard error of the mean.

platelets with SFLLRN platelets readily adhered to FIXa (Figure 3B), whereas they did not interact with an uncoated coverslip (Figure 3C).

FXa generation by FIXa on the activated platelet surface is inhibited by GPIbα

To investigate potential consequences of the interaction of FIX(a) with GPIbα, we investigated FIXa-mediated FXa-generation on the platelet surface in a purified system. Non-stimulated platelets generated little FXa after addition of FIXa and FVIII, as shown in Figure 4A. Upon stimulation with a combination of convulxin (100 nM) and SFLLRN (15 μM) for 15 minutes at 37°C, or the calcium ionophore, ionomycin, profound FXa generation via FIXa was observed over time (Figure 4). When platelets were pretreated with OSE to remove GPIbα and subsequently stimulated, FIXa-mediated FXa-generation was increased as compared to non-treated stimulated platelets (Figure 4).
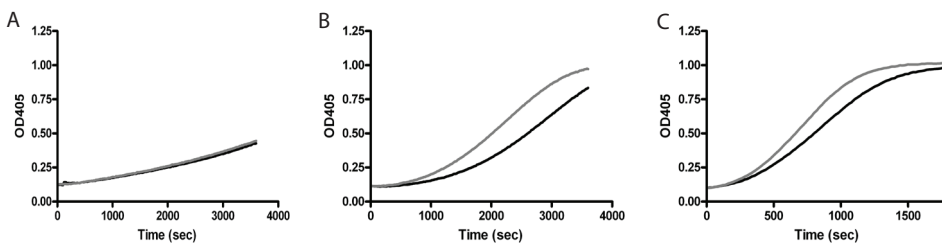

Figure 4 – Proteolysis of GPIbα accelerates FIXa-mediated FXa generation. Isolated washed platelets were pretreated with OSE (30 μg/ml, grey lines) or vehicle (black lines) for 30 min at 37°C and subsequently treated with (A) vehicle, (B) a combination of convulxin (100 ng/ml) and SFLLRN (15 μM), or (C) ionomycin (5 μg/ml). Next, a mixture of coagulation factors X (5 μg/ml), VIII (1 U/ml) and IXa (1.25 μg/ml) in HEPES-buffer was added to washed platelets. Subsequently, FXa generation was followed in time by measuring the conversion of the chromogenic FXa substrate pefachrome Xa. Graphs show results of a single experiment representative for at least 3 independent experiments.

FXa generation by FIXa on the platelet surface is inhibited by rFVIIai

Data shown above suggest that FIX(a) and rFVIIa bind to similar sites within GPIbα. Interestingly, the interaction of rFVIIa with GPIbα potentiates coagulant capacity, whereas the interaction of FIX(a) with GPIbα inhibits coagulant capacity. Therefore, we examined the effect of proteolytically inactive rFVIIa (rFVIIai) on FIXa-mediated FXa-generation. As shown in Figure 5A, rFVIIai inhibited FXa-generation by FIXa on platelets stimulated with a combination of convulxin and SFLLRN, whereas rFVIIai only had a minor effect on FIXa-mediated FXa generation on platelets stimulated with ionomycin. To investigate the contribution of GPIbα on FXa-generation in the presence of rFVIIai, washed platelets were pretreated with OSE and FIXa-mediated Xa generation was assessed in presence or absence of rFVIIai. As shown in figure 5B, the inhibitory effect of rFVIIai on FXa-generation by FIXa was absent when GPIbα had been removed from the platelet surface.
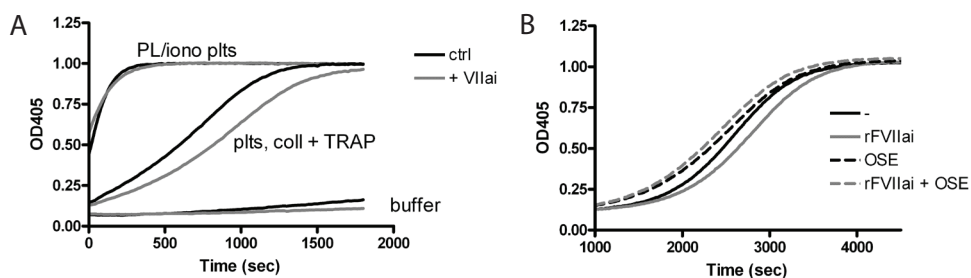
Figure 5 – rFVIIai inhibits FIXa-mediated factor Xa generation. (A) Isolated washed platelets were activated with vehicle, a combination of collagen (4 µg/ml) and TRAP (15 µM) or ionomycin (5 µg/ml). Next, a mixture of coagulation factors X (5 µg/ml), factor VIII (1 U/ml) and factor IXa (1.25 µg/ml), in the presence or absence of rFVIIai (20 µg/ml), was added to washed platelets. Subsequently, FXa generation was followed in time by measuring the conversion of the chromogenic FXa substrate pefachrome Xa. (B) Isolated washed platelets were pretreated with OSE (30 µg/ml, 30 min, 37°C) or vehicle and subsequently activated with a combination of collagen and TRAP. Factor Xa generation was measured in the presence or absence of rFVIIai (20 µg/ml) as described above.

DISCUSSION

The contribution of the platelet surface to coagulation has been a topic of interest for many years. Although initially it was thought that the negatively charged phospholipid surface provided by the activated platelet was required and sufficient to support coagulation, a substantial amount of evidence indicates a role for protein cofactors in localizing coagulation factors to the platelet surface.[5,17,18] In previous studies, it was already shown that enzymatically or chemically modified FIX, not (functionally) expressing its Gla-domain, could still interact with platelets, with an almost similar affinity as wild-type FIX, indicating only a minor role for negatively charged phospholipids.[10] A specific platelet receptor for FIX was suggested to account for this difference in affinity. Here, we identify GPIbα as a receptor for FIX and FIXa on the activated platelet surface. The interaction between GPIbα and FIXa is sufficiently strong to resist shear forces, as demonstrated by the ability of activated platelets to adhere to immobilized FIXa. Furthermore, GPIbα modulates the generation of activated FX mediated by FIXa.

Our results add to the suggestion that GPIbα is a multiligand receptor on the platelet surface which actively participates in regulation of thrombin generation, since not only FVIIa and IX(a), but also factor XI, thrombin, and (activated) protein C (APC) interact with the receptor. A role for GPIbα in regulation of FXI activation has been demonstrated but was recently challenged. No data on regulation of protein C functionality by GPIbα is available, but FVa inactivation by protein C was shown to proceed via strikingly different kinetics on platelets, endothelial cells, and lipid vesicles. On platelets FVa was more resistant to APC as compared to lipid vesicles, which may indicate APC reactivity is modulated by the platelet, possibly by GPIbα.[19] These combined data indicate that the role of GPIbα in regulation of thrombin generation on the platelet surface is presumably complex, and the relative contribution of the interaction of each of the mentioned coagulation factors

to thrombin generation in a complex system needs to be investigated in future studies.

The exact binding site on GPIbα for FIX has yet to be identified, but our results suggest that the glycosylated stack of GPIbα is involved, since binding of FIX(a) to platelets could be competed by rFVIIa, which was previously shown by us to presumably bind to the glycosylated stack. Furthermore, an antibody directed against the VWF-binding site on GPIbα, which is located in the N-terminal leucine rich repeat region, did not inhibit platelet adhesion to FIX.

There is a discrepancy between FXa-generation after treatment with OSE and FXa-generation in the presence of rFVIIai. If rFVIIai would only compete with FIXa for the binding site to GPIbα, an increase in FIXa-mediated FXa-generation is to be expected, because in that situation the inhibition of FIXa via binding to GPIbα is abrogated. An explanation for this discrepancy could be that rFVIIai, as a potent anticoagulant, inhibits the activation of FX by FIXa via a direct interaction with FX. However, future experiments will have to prove this hypothesis.

We have previously shown that GPIbα accelerates tissue factor-independent thrombin generation by pharmacological doses of rFVIIa in a model of hemophilia. In contrast, FXa-generation by FIXa is inhibited when GPIbα is present on the platelet surface. Therefore, the interaction between FIX and GPIbα could be an interesting therapeutic target. The development of recombinant FIX mutants with a decreased binding capacity to FIX might improve treatment of hemophilia B patients. Otherwise, directly inhibiting the interaction between FIX and GPIbα in combination with FIX administration might improve FIX efficacy. However, because our results show that GPIbα could have an important role in regulating FIX activity, targeting the GPIbα-binding capacity of FIX could result in thrombotic complications and therefore should be investigated carefully.

In conclusion, GPIbα contributes to the interaction between FIX and platelets. Whereas proteolysis of GPIbα results in loss of platelet adhesion, FXa-generation on the activated platelet surface is accelerated. In this way, GPIbα acts as an important modulator of coagulation on the activated platelet surface.

Acknowledgements

REFERENCES

1. Bevers EM, Comfurius P, Zwaal RF. Changes in membrane phospholipid distribution during platelet activation. Biochim. Biophys.Acta 1983;736:57-66.

2. Marcus AJ. The role of lipids in blood coagulation. Adv.Lipid Res. 1966;4:1-37.

3. Rosing J, Bevers EM, Comfurius P et al. Impaired factor X and prothrombin activation associated with decreased phospholipid exposure in platelets from a patient with a bleeding disorder. Blood 1985;65:1557-1561.

4. Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. Thromb. Haemost. 2002;88:186-193.

5. Weeterings C, de Groot PG, Adelmeijer J, Lisman T. The glycoprotein Ib-IX-V complex contributes to tissue factor-independent thrombin generation by recombinant factor VIIa on the activated platelet surface. Blood 2008;112:3227-3233.

6. Baglia FA, Shrimpton CN, Emsley J et al. Factor XI interacts with the leucine-rich repeats of glycoprotein Ibalpha on the activated platelet. J.Biol. Chem. 2004;279:49323-49329.

7. Baglia FA, Badellino KO, Li CQ, Lopez JA, Walsh PN. Factor XI binding to the platelet glycoprotein Ib-IX-V complex promotes factor XI activation by thrombin. J.Biol.Chem. 2002;277:1662-1668.

8. White TC, Berny MA, Tucker EI et al. Protein C supports platelet binding and activation under flow: role of glycoprotein Ib and apolipoprotein E receptor 2. J.Thromb.Haemost. 2008;6:995-1002.

9. Jones ME, Griffith MJ, Monroe DM, Roberts HR, Lentz BR. Comparison of lipid binding and kinetic properties of normal, variant, and gamma-carboxyglutamic acid modified human factor IX and factor IXa. Biochemistry 1985;24:8064-8069.

10. Rawala-Sheikh R, Ahmad SS, Monroe DM, Roberts HR, Walsh PN. Role of gamma-carboxyglutamic acid residues in the binding of factor IXa to platelets and in factor-X activation. Blood 1992;79:398-405.

11. Bu G, Schwartz AL. RAP, a novel type of ER chaperone. Trends Cell Biol. 1998;8(7):272-6.

12. Hackeng TM, van ', V, Meijers JC, Bouma BN. Human protein S inhibits prothrombinase complex activity on endothelial cells and platelets via direct interactions with factors Va and Xa. J.Biol.Chem. 1994;269:21051-21058.

13. Weeterings C, Adelmeijer J, Myles T, de Groot PG, Lisman T. Glycoprotein Ibalpha-mediated platelet adhesion and aggregation to immobilized thrombin under conditions of flow. Arterioscler.Thromb.Vasc. Biol. 2006;26:670-675.

14. Sixma JJ, de Groot PG, van ZH, IJsseldijk M. A new perfusion chamber to detect platelet adhesion using a small volume of blood. Thromb.Res. 1998;92:S43-S46.

15. Sakariassen KS, Aarts PA, de Groot PG, Houdijk WP, Sixma JJ. A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components. J.Lab Clin.Med. 1983;102:522-535.

16. Lopez JA, Leung B, Reynolds CC, Li CQ, Fox JE. Efficient plasma membrane expression of a functional platelet glycoprotein Ib-IX complex requires the presence of its three subunits. J.Biol.Chem. 1992;267:12851-12859.

17. London F, Ahmad SS, Walsh PN. Annexin V inhibition of factor IXa-catalyzed factor X activation on human platelets and on negatively-charged phospholipid vesicles. Biochemistry 1996;35:16886-16897.

18. Monroe DM, Hoffman M, Roberts HR. Platelets and thrombin generation. Arterioscler.Thromb.Vasc.Biol. 2002;22:1381-1389.

19. Camire RM, Kalafatis M, Simioni P, Girolami A, Tracy PB. Platelet-derived factor Va/Va Leiden cofactor activities are sustained on the surface of activated platelets despite the presence of activated protein C. Blood 1998;91:2818-2829.

Chapter 5

Glycoprotein Ibα-mediated platelet adhesion and aggregation to immobilized thrombin under conditions of flow

Cees Weeterings [1,2], Jelle Adelmeijer [1], Timothy Myles [3], Philip G de Groot [1,2], Ton Lisman [1,2]

From [1] the Thrombosis and Haemostasis Laboratory, Department of Haematology, University Medical Centre Utrecht, the Netherlands; and [2] the Institute of Biomembranes, Utrecht University, Utrecht, the Netherlands; and [3] the Division of Hematology, Department of Medicine, Stanford University School of Medicine, Stanford, CA

ABSTRACT

Objectives - Thrombin interacts with platelets via the protease-activated receptors (PARs) 1 and 4, and via glycoprotein Ibα (GPIbα). Recently, it was shown that platelets are able to adhere to immobilized thrombin under static conditions via GPIbα.

Methods and Results - Here, we show that platelets are also able to adhere to and form stable aggregates on immobilized thrombin under conditions of flow. Adhesion and aggregation to thrombin was dependent on the interaction with GPIbα, as addition of glycocalicin or an antibody blocking the interaction between thrombin and GPIbα inhibited platelet adhesion. Additionally, platelet adhesion to recombinant thrombin mutants, which are unable to bind GPIbα, was severely suppressed. Furthermore, platelet adhesion to thrombin was dependent on activation of PARs, and partly on granule secretion and thromboxane-A2 synthesis. Immobilization of thrombin on a fibrin network resulted in substantially increased adhesion compared to fibrin alone. The adhesion to fibrin alone was completely abolished by addition of dRGDW, whereas fibrin-bound thrombin still showed substantial platelet adhesion in the presence of dRGDW, indicating that fibrin-bound thrombin is able to directly capture platelets under flow.

Discussion - These results indicate that platelets are able to adhere to thrombin under flow conditions, which is dependent on the interaction with GPIbα.

INTRODUCTION

Thrombin has a central role in hemostasis. It activates platelets, cleaves fibrinogen into fibrin and activates factor XIII. Furthermore, thrombin enhances coagulation by activating factors V, VIII, and XI, but it also inhibits coagulation by activating protein C, and attenuates fibrinolysis by activating thrombin activatable fibrinolysis inhibitor (TAFI). Upon clot formation, thrombin is immobilized to the fibrin clot[1] and this binding to fibrin could be important in localizing thrombin to the site of vascular injury. Fibrin-bound thrombin is protected against inactivation by the heparin-antithrombin complex,[2,3] but the active site still remains accessible, as fibrin-bound thrombin is still capable of cleaving fibrinogen and activating factor XI.[4]

Thrombin can activate platelets via the protease-activated receptors (PARs) PAR1 and PAR4, which are generally assumed to account for the moderate- and low-affinity binding sites for thrombin, respectively.[5] GPIbα is described to be the high-affinity receptor for thrombin.[6,7] GPIb consists of two subunits, GPIbα and GPIbβ, and is expressed in platelets as a complex with GPIX and GPV in a 2:2:2:1 stoichiometry. However, there are ≈25000 copies of GPIbα on the platelet surface, but only a small number (≈100-1000) appear to be involved in the high-affinity binding of thrombin.[8] The localization of the GPIb-IX-V complex in rafts has proved to be important in platelet activation by von Willebrand Factor (vWF),[9] and it has been postulated that raft association may also account for the difference in high-affinity binding sites for thrombin and GPIbα copies on the platelet.[10]

Thrombin contains two anion binding sites or exosites referred to as exosite I and exosite II, a catalytic pocket and a Na^+ binding site. Exosite I is important in the binding of multiple substrates, including fibrin and fibrinogen[11] and PAR1,[12] whereas exosite II is referred to as the heparin binding site.[13] The catalytic pocket is responsible for the actual cleavage of the substrates and the amount of Na^+ bound to the Na^+ binding site regulates the affinity of thrombin for its substrates (reviewed by Di Cera[14]). Recently, site-directed mutagenesis has indicated the involvement of many basic exosite II residues in GPIbα binding.[15,16] In addition, the crystal structures of thrombin bound to GPIbα reported by Celikel et al[17] and Dumas et al[18] revealed the importance of both exosites of GPIbα in the binding of thrombin. Although there were many discrepancies between the two structures, which resulted in fundamentally different functional interpretations, both structures showed that two thrombin molecules can interact with a single GPIbα molecule, one via exosite I and the other via exosite II. It was proposed that the first thrombin molecule binds via its exosite II to GPIbα, which is followed by a conformational change in GPIbα, after which a second thrombin molecule can bind via its exosite I to a different location on GPIbα. The interpretation of Celikel involved dimerisation of two GPIbα molecules on the same platelet via thrombin, whereas Dumas described the possibility of aggregation of platelets via two GPIbα molecules bridged by thrombin (reviewed by Sadler[19]).

It was previously shown that platelets are able to adhere to immobilized thrombin under static conditions.[20] However, it is unclear whether this interaction is sufficiently

strong to resist shear forces. In this study, we investigated whether platelets could interact with immobilized thrombin under flow conditions. Furthermore, the role of fibrin-bound thrombin is not yet completely understood and in this study we investigated whether fibrin-bound thrombin contributes to platelet adhesion. We show that thrombin immobilized either directly on a glass coverslip or on fibrin induces platelet adhesion and aggregate formation under flow conditions, which is dependent on its interaction with GPIbα.

METHODS

Materials

Purified plasma-derived thrombin was kindly provided by Dr. W. Kisiel (University of New Mexico Health Sciences Center, Albuquerque, NM). Recombinant thrombin mutants were generated and purified as described previously.[21] Thrombin and fibrinogen used for generating fibrin-coated coverslips were purchased from Kordia Life Sciences (Leiden, the Netherlands). The RGD-containing peptide D-arginyl-glycyl-L-aspartyl-L-tryptophane (dRGDW) was synthesized at the Department of Membrane Enzymology, Faculty of Chemistry (University of Utrecht, the Netherlands). A rabbit polyclonal inhibitory antibody against the protease-activated receptor 1 (PAR1) was a generous gift of Dr. D. C. Foster (Cytokine Biology, ZymoGenetics, Seattle, WA). Fab fragments of a monoclonal antibody, which specifically inhibits thrombin binding to GPIb (LJIb-10), were a generous gift from Dr Z. M. Ruggeri (The Scripps Research Institute, La Jolla, CA). An antibody against vWF (RAG-35, ascites fluid) that inhibits the binding of vWF to GPIb was a generous gift of Dr J.A. van Mourik (CLB, Amsterdam, the Netherlands). PPACK [D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone] and the PAR1-agonist peptide SFLLRN were from Bachem (Bubendorf, Switzerland). The PAR4-agonist peptide GYPGQV was synthesized by GenScript (Piscataway, NJ). The P2Y1 antagonist adenosine-3',5'-diphosphate (A3P5P) was purchased from Sigma-Aldrich Chemicals BV (Zwijndrecht, the Netherlands) and the P2Y12 antagonist AR-C69931MX was a generous gift from AstraZeneca (Loughborough, United Kingdom). The thromboxane-receptor antagonist SQ30741 was kindly provided by Bristol-Meyers-Squibb (Maarssen, The Netherlands). Nk, a GPIbα-cleaving metalloproteinase, was purified from cobra (Naja kaouthia) venom (Sigma, St. Louis, MO) using the method previously described for mocarhagin[22] and was a generous gift of Dr R.K. Andrews (Monash University, Clayton, Australia). Essentially fatty acid free bovine serum albumin (BSA) and mepacrine were obtained from Sigma-Aldrich (St Louis, MO). ImmunO Human Albumin Fraction V was purchased from MP Biomedicals Inc (Eschwege, Germany). All other chemicals used in the experiments were of analytical grade.

Purification of glycocalicin

Glycocalicin was purified from fresh-frozen plasma by immuno-affinity chromatography using an in house mouse monoclonal antibody against human

GPIbα coupled to CNBr-activated Sepharose 4B (5 mg antibody/ml column) (Pharmacia, Uppsala, Sweden), followed by affinity chromatography on Wheat-germ agglutinin-agarose (Fluka Chemie, Buchs, Switzerland). The purified glycocalicin appeared as a single band on SDS-PAGE and concentration was measured by using a BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Platelet preparation

Blood from healthy volunteers, who claimed not to have used aspirin or other nonsteroidal anti-inflammatory drugs for the preceding 10 days, was drawn into one-tenth volume of 3.4% sodium citrate. Washed platelets were prepared as described previously.[23] The blood was centrifuged at 200g for 15 min at room temperature. The platelet-rich plasma (PRP) was removed and acidified by addition of one-tenth volume of ACD (2.5% trisodium citrate, 1.5% citric acid, and 2% D-glucose). Platelets were centrifuged (500g, 15 min) and the platelet pellet was resuspended in Hepes-Tyrode buffer at pH 6.5 (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgCl_2$, 5 mM D-glucose). Prostacyclin (PGI_2, 10 ng/ml) was added to prevent platelet activation during the subsequent washing step. Platelets were centrifuged (500g, 15 min) 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 $CaCl_2$, 5 mM D-glucose, pH 7.35) to a platelet count of 333 x 10^9/L (333.000/µl). Red blood cells were obtained by centrifuging blood at 200g for 10 min at room temperature. PRP was removed and used for platelet isolation. The pellet was centrifuged (2000g, 10 min) and resuspended in 0.9% NaCl containing 5 mM D-glucose. The obtained red blood cells were washed twice with 0.9% NaCl containing 5 mM D-glucose, and finally cells were packed (2000g, 15 min). For perfusion experiments, a mixture of 40% red blood cells and 60% washed platelets was prepared. Consequently, the reconstituted blood had a platelet count of 200 x 10^9/L (200.000/µl) and a hematocrit of 40%.

GPIb-depleted platelets were prepared by using the cobra venom Nk. Like mocarhagin, Nk cleaves GPIbα to release a ~45-kDa N-terminal fragment, and inhibits von Willebrand factor binding to washed platelets (R. Andrews, personal communication). Washed platelets (diluted in HEPES-Tyrode buffer, pH 7.4) were incubated for 30 min at 37ºC with 5 µg/ml Nk or vehicle in the presence of 1 mM Ca^{2+}. Nk-activity was stopped by adding 5 mM EDTA. After incubation, platelets were centrifuged (500g, 15 min) in the presence of prostacyclin (PGI_2, 10 ng/ml) and the platelet pellet was resuspended in a small volume of HEPES-Tyrode at pH 6.5 and diluted in human albumin solution as described above.

Coating of the coverslips

For end-point perfusion experiments, glass coverslips (Menzel Gläser 18x18 mm) were cleaned overnight with 100% alcohol and rinsed with distilled water before coating.
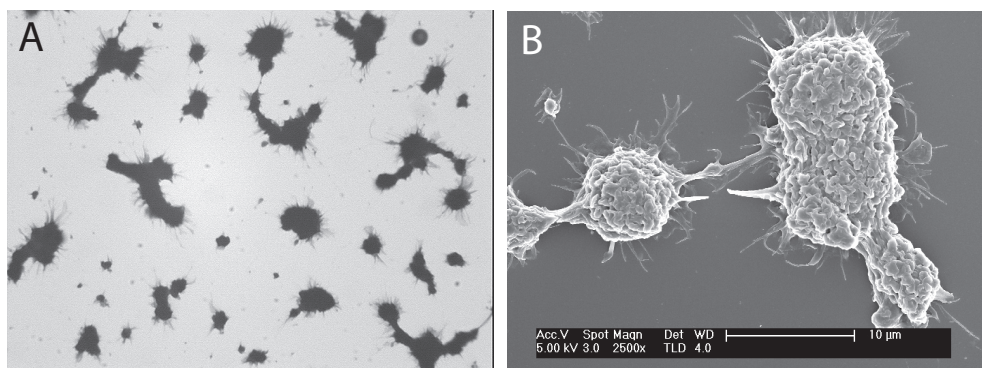
Figure 1 – Platelet adhesion to immobilized thrombin. Reconstituted blood was perfused over a coverslip coated with 25 µg/ml thrombin at a shear rate of 300 s⁻¹ for 5 min at 37ºC using a single-pass perfusion chamber. After perfusion, coverslips were fixed and stained with May-Grünwald/Giemsa and examined by light microscopy (original magnification 400x; (A), or coverslips were fixed and examined by Scanning Electron Microscopy (original magnification 2500x; B). Representative images of at least 6 independent experiments are shown.

Coating was performed by incubating the coverslips with 100 µl thrombin or thrombin mutant (25 µg/ml) in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 8.0) overnight at 4ºC, followed by incubation with 2% BSA in phosphate-buffered saline (PBS; 10 mM phosphate buffer, 150 mM NaCl, pH 7.4) for 30 min to block unoccupied sites on the coverslip. For real time perfusion studies, the same coating procedure was used, but larger glass coverslips (Menzel Gläser 24x60 mm) were used. These coverslips were incubated with 300 µl thrombin. To obtain fibrin-coated coverslips, fibrinogen (100 µg/ml) was mixed with 0.15 µg/ml thrombin (Kordia Life Sciences, Leiden, the Netherlands) and immediately sprayed onto glass coverslips with a retouching airbrush. The coverslips were incubated for 30 min at 37ºC and afterwards, the coverslips were blocked for at least 30 min at room temperature with 2% BSA in PBS. Possible residual thrombin used to obtain fibrin was shown not to have an effect on platelet adhesion to fibrin under flow conditions as demonstrated by Hantgan et al[24] and confirmed in Figure 5 of this manuscript. Subsequently, fibrin-coated coverslips were incubated with thrombin or thrombin mutant (25 µg/ml) in TBS (pH 8.0) overnight at 4ºC, followed by incubation with 2% BSA in PBS for 30 min.

Perfusion studies

Perfusions were carried out in a single-pass perfusion chamber as described previously.[25] Reconstituted blood was perfused over the coated coverslips for 5 min at a constant flow rate. 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 or prepared for Scanning Electron Microscopy as described below. Subsequently, slides were dehydrated in methanol and stained with May-Grünwald and Giemsa as described previously.[26] Next, the slides were examined using light microscopy. Platelet adhesion was evaluated using computer-assisted analysis with OPTIMAS 6.0 software (Dutch Vision Systems [DVS], Breda, The Netherlands) and was

expressed as the percentage of the surface covered with platelets.

Real time perfusion studies

Real time perfusions were carried out in a single-pass perfusion chamber consisting of a silicon sheet gasket that maintained a flow path height of 0.125 mm and a width of 2 mm.[27,28] Platelets were labeled prior to perfusion for 15 min with 10 μM mepacrine, a concentration known not to interfere with the functional properties of platelets.[29] Reconstituted blood was perfused over immobilized thrombin for 5 min at a constant flow rate. Platelet interaction with the surface was continuously monitored by a fluorescence microscope (Orthoplan Flu. Leica, Germany) equipped with a LI-low-light-level CCD camera (Lambert Instruments, Leuting-wolde, the Netherlands) and a Pioneer DVD-recorder.

Scanning Electron Microscopy

For Scanning Electron Microscopy (SEM) examination, slides were washed with HEPES buffer and fixed in 2% glutaraldehyde in PBS. After washing with distilled water, slides were dehydrated with increasing concentrations of ethanol (80% - 100%) and subsequently treated with hexomethyldisylazane (Fluka Chemie, Buchs, Switzerland). Slides were embedded on a stub in carbon glue. Samples were coated with a thin layer of Platinum/Palladium using an Emitech K-575X Sputter Coater and were examined by SEM (XL30 SFEG, Philips, Eindhoven, the Netherlands).

Statistical analysis

Statistical analysis was performed using the GraphPad InStat (San Diego, CA) software package. Statistical differences in surface coverage were analysed by standard one-way analysis of variance (ANOVA) using Dunnett multiple comparison test. P values less than 0.05 were considered statistically significant.

RESULTS

Platelet adhesion and aggregate formation to immobilized thrombin under conditions of flow

To investigate whether thrombin immobilized on a surface is able to interact with platelets under conditions of flow, reconstituted blood was perfused over immobilized thrombin for 5 min at a shear rate of 300 s^{-1}. Real time perfusion experiments showed rapid adhesion of single platelets to the surface, followed by the formation of large aggregates at sites of primary platelet adhesion (for movie capture, please see http://atvb.ahajournals.org). The aggregates were stable and embolization only occurred sporadically. Figure 1A shows a microscopic picture of platelet adhesion and aggregate formation to immobilized thrombin after 5 min of perfusion at a shear rate of 300 s^{-1}. Scanning Electron Microscope analysis supports our observations that large, stable aggregates were formed on top of initially spread platelets (Figure 1B). Platelet adhesion to coverslips that were

coated only with blocking buffer (BSA) was virtually absent (Figure 2). In contrast to platelet adhesion to vWF,[30] platelets did not roll on immobilized thrombin prior to firm adhesion, but rather attached instantly. Perfusion experiments using a range of shear rates ($100\ s^{-1}$ to $4000\ s^{-1}$), showed platelet adhesion at all shear rates tested, with optimal adhesion at a shear rate of $300\ s^{-1}$ (data not shown). For further perfusion experiments, a shear rate of $300\ s^{-1}$ was used, which is comparable to venous shear rates.
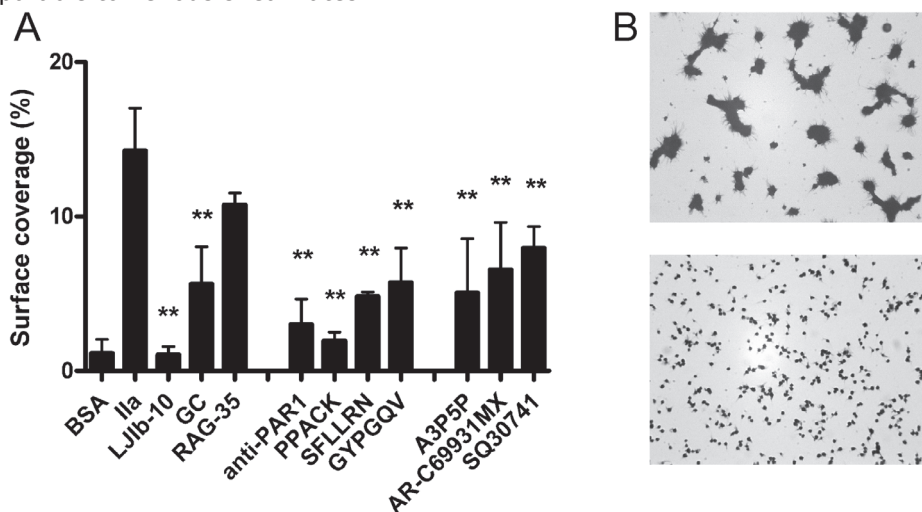

Figure 2 – Determinants of platelet adhesion to immobilized thrombin. (A) Reconstituted blood was perfused over immobilized thrombin (IIa) in the presence of agents interfering with thrombin binding to GPIbα (left section), platelet activation via PARs (mid section) or other platelet receptors (right section). After perfusion, coverslips were stained with May-Grünwald/Giemsa and examined by light microscopy. Graph shows mean surface coverage of at least 3 independent experiments performed in triplicate. **: P<0.01. Error bars indicate standard deviation. (B) Reconstituted blood was perfused over immobilized thrombin in the absence (top) or presence (bottom) of dRGDW (200 μM). After perfusion, coverslips were fixed and stained with May-Grünwald/Giemsa and examined by light microscopy (original magnification 400x). Representative images of at least 3 independent experiments are shown.

Platelet adhesion to immobilized thrombin is mediated by GPIbα

To investigate whether GPIbα is involved in platelet adhesion to immobilized thrombin, we perfused reconstituted blood over immobilized thrombin in the presence of an antibody directed against the thrombin-binding site of GPIbα (LJIb-10, 100 μg/ml). As shown in Figure 2A, surface coverage is substantially and significantly reduced upon addition of LJIb-10. Also, addition of glycocalicin (GC, 50 μg/ml), a proteolytic fragment of the extracellular domain of GPIbα, inhibited platelet adhesion to immobilized thrombin (Figure 2A). Platelet adhesion to immobilized thrombin was not dependent on the GPIb-vWF interaction, as an antibody against vWF (RAG-35, used in a dilution of 1:250), interfering with the interaction of vWF with GPIbα, did not show a reduction in platelet adhesion (Figure 2A).

Platelet adhesion to immobilized thrombin requires activation of PARs, secretion of ADP and thromboxane A2 synthesis

Next, we investigated the role of PAR1 and PAR4 in platelet adhesion to immobilized thrombin. Platelet adhesion to immobilized thrombin could be blocked by addition of an inhibitory antibody against PAR1 (Figure 2A). Platelets desensitized for either PAR1 or PAR4 with the PAR1 activating peptide SFLLRN (15 µM, 30 min, 37ºC) or the PAR4 activating peptide GYPGQV (1 mM, 30 min, 37ºC) had a reduced capacity to adhere to immobilized thrombin (Figure 2A). Furthermore, platelet adhesion to thrombin was almost completely abolished, when thrombin was preincubated for 30 min at 37ºC with 50 µM PPACK, which blocks the active site of thrombin.

Inhibitors of the ADP-receptors P2Y1 (A3P5P, 300 µM) and P2Y12 (AR-C69931MX, 1 mM) partly inhibited platelet adhesion and aggregate formation to immobilized thrombin (Figure 2A). Also, the thromboxane-receptor analog SQ30741 (10 µM) partly inhibited platelet aggregation to immobilized thrombin. Platelet adhesion to immobilized thrombin did not involve the integrin $\alpha IIb\beta 3$, as platelets pretreated with dRGDW (200 µM), a peptide which blocks ligand binding to $\alpha IIb\beta 3$, readily adhered to thrombin. Although in the presence of dRGDW aggregate formation was absent, spreading features such as pseudopod formation and filopodia extension were present (Figure 2B).

Platelet adhesion to immobilized thrombin is dependent on the interaction between thrombin exosite II and GPIbα

To investigate the interaction between thrombin's exosites and the role of GPIbα in the adhesion of platelets to immobilized thrombin, we performed perfusion experiments using the recombinant thrombin (exosite II) mutants R98A and R89A/
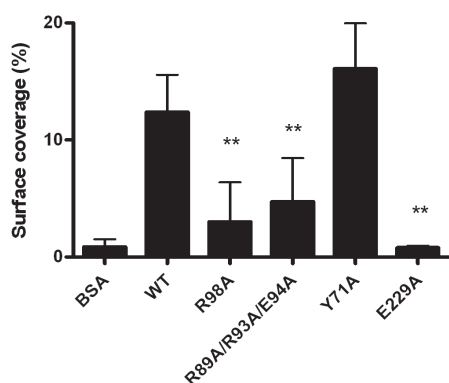
Figure 3 – Platelet adhesion to immobilized thrombin is dependent on exosite II. Reconstituted blood was perfused for 5 min at a shear rate of 300 s^{-1} over a coverslip coated with 25 µg/ml wild-type (WT) thrombin or 25 µg/ml thrombin mutant. After perfusion, coverslips were stained with May-Grünwald/Giemsa and examined by light microscopy. Graph shows mean surface coverage of at least 3 independent experiments performed in triplicate. **: $P<0.01$. Error bars indicate standard deviation.

R93A/E94A, which were previously shown to lack the ability to bind GPIbα.[31] When reconstituted blood was perfused over these recombinant thrombin mutants, platelet adhesion was almost completely absent, whereas platelet adhesion and aggregation to recombinant wild-type thrombin was similar to that observed using plasma-derived thrombin (Figure 3). Also, platelet adhesion to a recombinant

thrombin with a mutation in the Na$^+$ binding site (E229A) was abolished. Y71A, which has a reduced binding capacity for exosite I ligands such as fibrinogen,[11] supported platelet adhesion and aggregation comparable to wild-type thrombin (Figure 3).

To investigate whether the decrease in platelet adhesion is caused by a reduced potential to activate platelets, platelets were aggregated in suspension with the recombinant thrombin mutants. Details of these experiments will be published elsewhere (Myles et al, unpublished data, 2005). The platelet aggregatory potential of Y71A (EC50=4.3±0.1 nM), R98A (EC50=9.7±0.3 nM) and R89A/R93A/E94A (EC50=3.0±0.4 nM) was moderately reduced compared to wild-type (EC50=1.2±0.2 nM). The observation that Y71A and R89A/R93A/E94A have a similar EC50 for aggregation in suspension, but that only R89A/R93A/E94A has a substantially decreased response to immobilized thrombin under flow, indicates that flow-mediated adhesion requires exosite II but not exosite I. However, E229A showed an EC50-value of 39.9±0.1 nM, again indicating that the so-called slow form of thrombin has a severely impaired capacity to activate PAR1.

Fibrin-bound thrombin contributes to platelet adhesion and aggregate formation

Subsequently, we investigated platelet adhesion to immobilized fibrin and fibrin-bound thrombin. Reconstituted blood was perfused for 5 min at a shear rate of 300 s^{-1} over fibrin-coated coverslips, which were incubated with thrombin (25 µg/ml) or vehicle. Platelets readily adhered to fibrin as shown in Figure 4A. Platelet
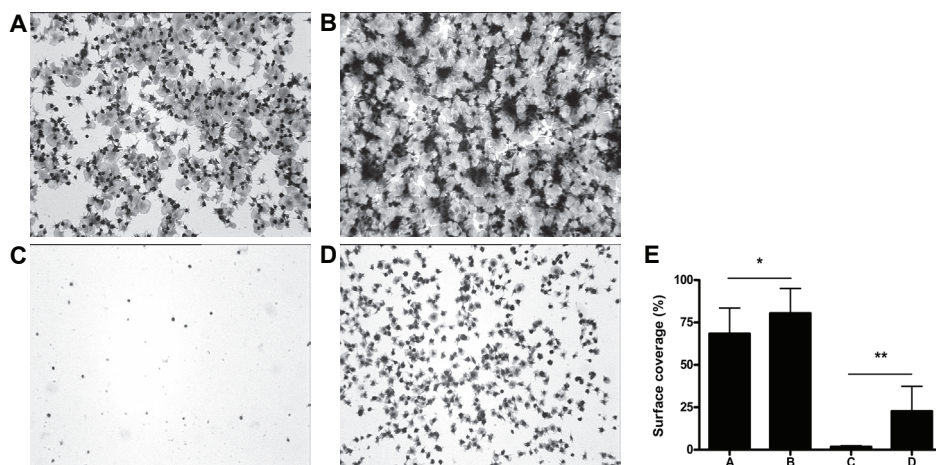
Figure 4 – Fibrin-bound thrombin contributes to platelet adhesion and aggregation. Reconstituted blood was perfused for 5 min at shear rate of 300 s^{-1} over a fibrin-coated coverslip in the absence (panel A) or presence (panel C) of dRGDW (200 µM), or over a fibrin-coated coverslip, which was incubated with 25 µg/ml thrombin in the absence (panel B) or presence (panel D) of dRGDW (200 µM). After perfusion, coverslips were stained with May-Grünwald/Giemsa and examined by light microscopy (original magnification 400x). (E) Surface coverage results of A through D. Graph shows mean surface coverage of at least 3 independent experiments performed in triplicate. *: P<0.05; **: P<0.01. Error bars indicate standard deviation.

adhesion and aggregate formation substantially increased on fibrin with bound thrombin compared to fibrin alone as shown in Figure 4B and increased with increasing thrombin concentrations with half-maximum effect obtained at 15 µg/ml thrombin and maximum effect reached at 50 µg/ml thrombin (data not shown). Platelet adhesion to fibrin is fully dependent on αIIbβ3 and therefore after addition of dRGDW (200 µM) adhesion was abolished (Figure 4C). However, Figure 4D shows that in the presence of dRGDW platelets did adhere to fibrin-bound thrombin, indicating that fibrin-bound thrombin is able to directly bind platelets. Figure 4E shows the surface coverage results of Figure 4A through 4D.

To study whether platelet adhesion to fibrin with thrombin is also dependent on GPIbα, platelets were pretreated with the snake venom Nk (5 µg/ml), which sheds GPIbα from the platelet surface. Platelet adhesion to fibrin alone is partially inhibited by Nk, indicating the involvement of GPIbα in adhesion to fibrin (Figure 5), which is in agreement with experiments performed by Hantgan et al, who showed that platelet adhesion to fibrin is in part dependent on GPIbα.[32] The increase in adhesion of fibrin-bound thrombin was inhibited completely by Nk, as seen in Figure 5. Furthermore, PPACK and an inhibitory antibody against PAR1 inhibited the increase in surface coverage obtained by exposing the fibrin to thrombin (Figure 5).

Recombinant thrombin mutants could also increase adhesion to fibrin as compared to wild-type thrombin (Supplemental Figure 1). However, only wild-type thrombin was capable of inducing large aggregates, whereas immobilization of thrombin mutants only increased surface coverage. The thrombin mutant E229A could not contribute to platelet adhesion to fibrin, but also did not respond in aggregation experiments. In the presence of dRGDW, platelet adhesion to thrombin mutants R98A, R89A/R93A/E94A and E229A was significantly reduced compared to wild-
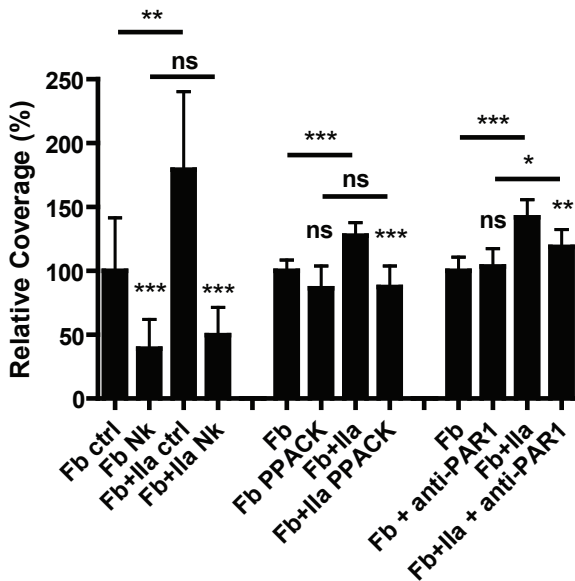
Figure 5 – Platelet adhesion to fibrin-bound thrombin is dependent on GPIbα and PAR1. Reconstituted blood was perfused over fibrin-bound thrombin (Fb+IIa) or fibrin alone (Fb) after pretreatment with Nk (left section), PPACK (mid section) or an inhibitory antibody against PAR1 (right section). After perfusion, coverslips were stained with May-Grünwald/Giemsa and examined by light microscopy. Graph shows mean surface coverage of at least 3 independent experiments performed in triplicate. Adhesion to fibrin alone is indicated as 100% relative coverage. **: $P<0.01$; ***: $P<0.001$; ns indicates not significant. Error bars indicate standard deviation.

type, indicating that platelet adhesion to fibrin-bound thrombin also requires binding of GPIbα and activation of PARs (Supplemental Figure 1). Also, Y71A failed to induce platelet adhesion on fibrin in the presence of dRGDW. In the presence of dRGDW, platelet adhesion to the recombinant thrombin mutants was not significantly increased compared to fibrin alone.

DISCUSSION

This study shows that thrombin immobilized on a coverslip or on fibrin is able to capture platelets under conditions of flow. The capacity of thrombin to function as a platelet adhesive protein has not been recognized previously. Perfusion of reconstituted blood over immobilized thrombin resulted in rapid platelet adhesion and the formation of large, stable aggregates. Platelet adhesion was shown to be dependent on GPIbα and the proteolytic activity of thrombin. We propose the following sequence of events leading to the formation of a stable aggregate when thrombin is immobilized on a surface. Immobilized thrombin is able to capture platelets from flowing blood via GPIbα. Subsequently, intracellular signaling occurs in response to thrombin binding to GPIbα and activation of PAR1 and PAR4, resulting in the formation of thromboxane A2 and secretion of ADP and the activation of αIIbβ3. These processes are responsible for the stable adhesion to thrombin and the formation of aggregates.

Activation of αIIbβ3 is not mandatory for primary platelet adhesion to thrombin, as platelets readily adhere in the presence of dRGDW, whereas signal transduction via PARs, thromboxane A2, and ADP is required for primary adhesion. These processes result in inside-out signaling to GPIbα[33] or relocation of GPIbα into lipid rafts,[9] which we hypothesize to be required for a firm GPIbα-thrombin interaction. However, we cannot exclude that other (unknown) receptors contribute to stable platelet adhesion to thrombin.

Platelet adhesion to immobilized thrombin is dependent on the interaction between thrombin exosite II and GPIbα on the platelet surface. This is demonstrated by the fact that antibodies against the thrombin binding site on GPIbα inhibit platelet adhesion to immobilized thrombin. Furthermore, recombinant thrombins with mutations in exosite II, which virtually abolish the interaction with GPIbα, did not induce platelet adhesion and aggregate formation when immobilized directly on a coverslip, whereas the exosite I mutant does support adhesion. Taken together, these results provide strong evidence that exosite II is essential for platelet adhesion to immobilized thrombin mediated by GPIbα and that the interaction of GPIbα with exosite I apparently is not required or capable of inducing platelet adhesion under flow conditions. This is in correspondence with the observations of Celikel et al,[17] who reported that thrombin first binds to GPIbα via exosite II, after which a second molecule can bind via exosite I.

Although we show that thrombin can act as a platelet adhesive protein, thrombin is usually not present as a surface-bound protein, but functions in hemostasis primarily as a soluble protein. Nevertheless, upon clot formation thrombin is immobilized to

the fibrin clot[1] and this binding to fibrin may be important in localizing thrombin to the site of vascular injury. Our results show that when thrombin is bound to fibrin, platelet adhesion and aggregate formation are substantially enhanced. Although relatively high concentrations of thrombin are required for this process, it would make sense to believe that local thrombin concentrations bound to fibrin could rise to high levels and thereby contribute in the post-recruitment of platelets to the fibrin-clot. Also, in the presence of dRGDW, which completely blocks adhesion to fibrin, platelets readily adhere to fibrin-bound thrombin. This suggests that fibrin-bound thrombin not only increases platelet adhesion and aggregation by enhancement of platelet activation mediated by PARs, but also is able to directly capture platelets via GPIbα. This is further demonstrated by the experiments shown in Figure 5, which show that the increase in platelet adhesion to fibrin with bound thrombin is abolished when platelets are depleted from GPIbα after pretreatment with Nk.

When bound to fibrin, exosite II mutants contributed to platelet adhesion in the absence of dRGDW, which most likely reflects enhancement of platelet activation via PARs. As these exosite mutants could not initiate platelet adhesion in the presence of dRGDW, we conclude that also fibrin-bound thrombin is able to capture platelets via GPIbα. Although Y71A has a reduced binding capacity for fibrin, it can still contribute to platelet adhesion to fibrin. However, it is unable to induce platelet adhesion in the presence of dRGDW. It appears that in our experimental setup the amount of Y71A, which has bound to fibrin, still has the potential to activate PARs and contribute to platelet adhesion to fibrin, but is present in insufficient amounts to directly capture platelets via GPIbα.

It is important to note that when thrombin is immobilized on a surface, probably thrombin's exosites are not both available for ligand binding. Whether the fibrin-bound thrombin is bound to fibrin via exosite I or exosite II is still a matter of debate. Extensive reviews have been dealing with this controversy (Huntington,[34] Mosesson,[35] and Lane[36]). Although from the results in the present study we cannot confirm the exact mechanism of how thrombin is bound to fibrin, the fact that adhesion of GPIbα-depleted platelets to fibrin-bound thrombin is strongly diminished compared to control platelets, combined with the observation that platelet adhesion to thrombin itself is mediated by thrombin exosite II, suggests that thrombin is bound to fibrin via exosite I and contributes to platelet adhesion via a GPIbα-dependent interaction with exosite II. Although exosite I interacts with fibrin, and thrombin also interacts via exosite I with PAR1, PAR1 can still be hydrolysed by fibrin-bound thrombin. This seems contradictory, but Myles et al already described that the ability of thrombin mutants to activate PAR1 or clot fibrinogen differ profoundly, indicating the involvement of different exosite I residues in PAR1 activation and fibrin(ogen) binding.[12]

In conclusion, these experiments show that immobilized thrombin can act as an adhesive surface and is able to directly capture and activate platelets under flow conditions. This platelet adhesion is dependent on the interaction of thrombin with GPIbα, the activation of PARs and the secretion of ADP and thromboxane A2. Platelet adhesion to fibrin-bound thrombin could be a novel target for new

antithrombotic drugs, which could now more specifically interfere with the action of thrombin on platelets on the actual site of thrombosis.

REFERENCES

1. Nilsen DW, Brosstad F, Kierulf P, Godal HC. Binding of various thrombin fractions to fibrin and the influence of AT-III on their adsorption. Thromb.Haemost. 1986;55:352-356.
2. Weitz JI, Hudoba M, Massel D, Maraganore J, Hirsh J. Clot-bound thrombin is protected from inhibition by heparin-antithrombin III but is susceptible to inactivation by antithrombin III-independent inhibitors. J.Clin.Invest 1990;86:385-391.
3. Becker DL, Fredenburgh JC, Stafford AR, Weitz JI. Exosites 1 and 2 are essential for protection of fibrin-bound thrombin from heparin-catalyzed inhibition by antithrombin and heparin cofactor II. J.Biol.Chem. 1999;274:6226-6233.
4. dem Borne PA, Meijers JC, Bouma BN. Effect of heparin on the activation of factor XI by fibrin-bound thrombin. Thromb. Haemost. 1996;76:347-353.
5. Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J.Clin. Invest 1999;103:879-887.
6. Okumura T, Hasitz M, Jamieson GA. Platelet glycocalicin. Interaction with thrombin and role as thrombin receptor of the platelet surface. J.Biol.Chem. 1978;253:3435-3443.
7. Harmon JT, Jamieson GA. The glycocalicin portion of platelet glycoprotein Ib expresses both high and moderate affinity receptor sites for thrombin. A soluble radioreceptor assay for the interaction of thrombin with platelets. J.Biol.Chem. 1986;261:13224-13229.
8. De Marco L, Mazzucato M, Masotti A, Fenton JW, Ruggeri ZM. Function of glycoprotein Ib alpha in platelet activation induced by alpha-thrombin. J.Biol.Chem. 1991;266:23776-23783.
9. Shrimpton CN, Borthakur G, Larrucea S et al. Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation. J.Exp.Med. 2002;196:1057-1066.
10. Vanhoorelbeke K, Ulrichts H, Romijn RA, Huizinga EG, Deckmyn H. The GPIbalpha-thrombin interaction: far from crystal clear. Trends Mol.Med. 2004;10:33-39.
11. Hall SW, Gibbs CS, Leung LL.

Identification of critical residues on thrombin mediating its interaction with fibrin. Thromb. Haemost. 2001;86:1466-1474.
12. Myles T, Le Bonniec BF, Stone SR. The dual role of thrombin's anion-binding exosite-I in the recognition and cleavage of the protease-activated receptor 1. Eur.J.Biochem. 2001;268:70-77.
13. Sheehan JP, Sadler JE. Molecular mapping of the heparin-binding exosite of thrombin. Proc.Natl.Acad. Sci.U.S.A 1994;91:5518-5522.
14. Di Cera E. Thrombin interactions. Chest 2003;124:11S-17S.
15. Li CQ, Vindigni A, Sadler JE, Wardell MR. Platelet glycoprotein Ib alpha binds to thrombin anion-binding exosite II inducing allosteric changes in the activity of thrombin. J.Biol. Chem. 2001;276:6161-6168.
16. De Cristofaro R, De Candia E, Landolfi R, Rutella S, Hall SW. Structural and functional mapping of the thrombin domain involved in the binding to the platelet glycoprotein Ib. Biochemistry 2001;40:13268-13273.
17. Celikel R, McClintock RA, Roberts JR et al. Modulation of alpha-thrombin function by distinct interactions with platelet glycoprotein Ibalpha. Science 2003;301:218-221.
18. Dumas JJ, Kumar R, Seehra J, Somers WS, Mosyak L. Crystal structure of the GpIbalpha-thrombin complex essential for platelet aggregation. Science 2003;301:222-226.
19. Sadler JE. Structural biology. A menage a trois in two configurations. Science 2003;301:177-179.
20. Adam F, Guillin MC, Jandrot-Perrus M. Glycoprotein Ib-mediated platelet activation. A signalling pathway triggered by thrombin. Eur.J.Biochem. 2003;270:2959-2970.
21. Tsiang M, Jain AK, Dunn KE et al. Functional mapping of the surface residues of human thrombin. J.Biol.Chem. 1995;270:16854-16863.
22. Ward CM, Andrews RK, Smith AI, Berndt MC. Mocarhagin, a novel cobra venom metalloproteinase, cleaves the platelet von Willebrand factor receptor glycoprotein Ibalpha. Identification of the sulfated tyrosine/anionic sequence Tyr-276-Glu-282 of glycoprotein Ibalpha as a binding site for von Willebrand factor and alpha-thrombin. Biochemistry

1996;35:4929-4938.
23. Lisman T, Moschatsis S, Adelmeijer J, Nieuwenhuis HK, de Groot PG. 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. Blood 2003;101:1864-1870.
24. Hantgan RR, Endenburg SC, Sixma JJ, de Groot PG. Evidence that fibrin alpha-chain RGDX sequences are not required for platelet adhesion in flowing whole blood. Blood 1995;86:1001-1009.
25. Sixma JJ, de Groot PG, van Zanten H, IJsseldijk M. A new perfusion chamber to detect platelet adhesion using a small volume of blood. Thromb.Res. 1998;92:S43-S46.
26. Sakariassen KS, Aarts PA, de Groot PG, Houdijk WP, Sixma JJ. A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components. J.Lab Clin.Med. 1983;102:522-535.
27. Remijn JA, Wu YP, Ijsseldijk MJ et al. Absence of fibrinogen in afibrinogenemia results in large but loosely packed thrombi under flow conditions. Thromb. Haemost. 2001;85:736-742.
28. Van Os E, Wu YP, Pouwels JG et al. Thrombopoietin increases platelet adhesion under flow and decreases rolling. Br.J.Haematol. 2003;121:482-490.
29. Alevriadou BR, Moake JL, Turner NA et al. Real-time analysis of shear-dependent thrombus formation and its blockade by inhibitors of von Willebrand factor binding to platelets. Blood 1993;81:1263-1276.
30. Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell 1996;84:289-297.
31. Yun TH, Baglia FA, Myles T et al. Thrombin activation of factor XI on activated platelets requires the interaction of factor XI and platelet glycoprotein Ib alpha with thrombin anion-binding exosites I and II, respectively. J.Biol.Chem. 2003;278:48112-48119.
32. Hantgan RR, Hindriks G, Taylor RG, Sixma JJ, de Groot PG.

81

Glycoprotein Ib, von Willebrand factor, and glycoprotein IIb:IIIa are all involved in platelet adhesion to fibrin in flowing whole blood. Blood 1990;76:345-353.

33. Englund GD, Bodnar RJ, Li Z, Ruggeri ZM, Du X. Regulation of von Willebrand factor binding to the platelet glycoprotein Ib-IX by a membrane skeleton-dependent inside-out signal. J.Biol.Chem. 2001;276:16952-16959.

34. Huntington JA. Molecular recognition mechanisms of thrombin. J.Thromb.Haemost. 2005;3:1861-1872.

35. Mosesson MW. Fibrinogen and fibrin structure and functions. J.Thromb.Haemost. 2005;3:1894-1904.

36. Lane DA, Philippou H, Huntington JA. Directing thrombin. Blood 2005;106:2605-2612.

SUPPLEMENTAL DATA
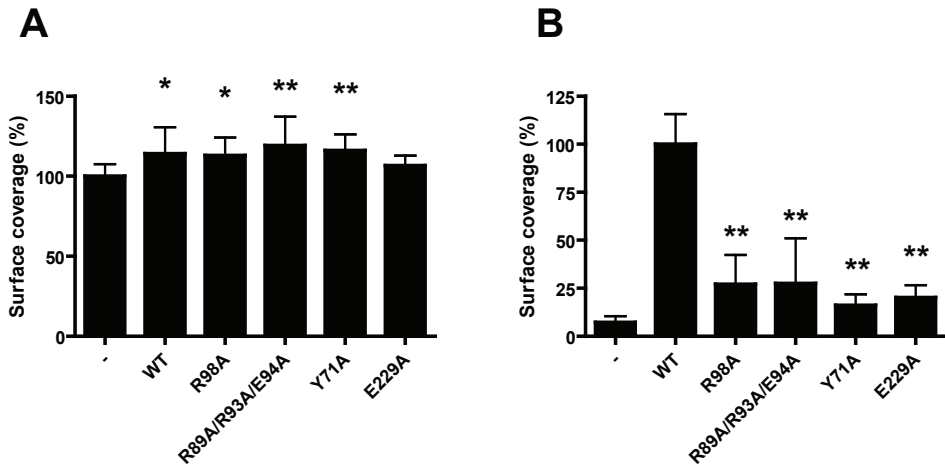
Figure 1 - Platelet adhesion and aggregation to thrombin mutants bound to fibrin. Fibrin-coated coverslips were incubated with 25 μg/ml wild-type (WT) thrombin or 25 μg/ml thrombin mutant. Reconstituted blood was perfused for 5 min at a shear rate of 300 s^{-1} in the absence (A) of presence (B) of dRGDW (200 μM). After perfusion, coverslips were stained with May-Grünwald/Giemsa and examined by light microscopy. Graph shows relative mean surface coverage of at least 3 independent experiments performed in triplicate. Statistical significance is compared to fibrin control (A, indicated as (-)) or wild-type thrombin (B, indicated as (WT)). *: P<0.05, **: P<0.01. Error bars indicate standard deviation.

Chapter 6

Factor XIIa promotes platelet adhesion to von Willebrand Factor

Cees Weeterings [1] *, Coen Maas [1] *, Suzanne Kroon [1], Ton Lisman [2], Philip G de Groot [1]

From [1] the Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, the Netherlands; [2] Surgical Research Laboratory, Department of Surgery, University Medical Center Groningen, the Netherlands
* authors contributed equally to this work

ABSTRACT

Introduction - Factor XII (FXII) has long been established as an important coagulation factor in vitro, but its role in vivo remains elusive. Recent studies have shown that FXII can be activated by unfolded proteins (Maas et al, J Clin Invest. 2008 Sep;118(9):3208-18), but physiologic examples of such proteins remain to be identified. Von Willebrand Factor (VWF) has been shown to unfold upon shear stress, by binding to surfaces such as collagen, or by in vitro modulators such as ristocetin, and we hypothesized that unfolded VWF becomes able to bind and activate FXII.

Methods & Results – Surface plasmon resonance studies revealed that FXII interacts with immobilized VWF with an affinity constant (K_d) of 113 ± 29 nM (mean ± SD). FXII bound to immobilized recombinant A1-domain of VWF, but did not interact with immobilized recombinant A2-domain, suggesting that the A1-domain is responsible for the interaction with VWF. Immobilized VWF was shown to activate FXII in vitro as measured indirectly by the generation of kallikrein. Subsequently, we examined the effect of activated FXII (FXIIa) on the functional properties of VWF. FXIIa inhibited ristocetin-induced platelet agglutination, suggesting a potential overlap in binding sites on VWF for ristocetin and FXIIa. Furthermore, preincubation of VWF with FXIIa, but not FXII, increased platelet deposition to VWF, both under static conditions and under conditions of flow.

Conclusion – FXII interacts with VWF and this interaction results in increased platelet deposition to VWF in vitro. This interaction might play an important role in thrombus formation in vivo and therefore the design of drugs which inhibit the interaction between VWF and FXII could be an interesting therapeutic approach.

INTRODUCTION

Upon vascular damage, subendothelial matrix proteins become exposed to flowing blood and platelets adhere to the exposed subendothelium to prevent excessive bleeding. Platelet adhesion under high shear stress is a multistep process, in which VWF plays a crucial role. Initial adhesion of platelets with the exposed subendothelium requires the interaction of VWF with subendothelial collagen. The subsequent interaction between platelet glycoprotein Ib-alpha (GPIbα) and collagen-bound VWF results in rolling and tethering of the platelet. In this way, platelet velocity is reduced, after which stable platelet adhesion can occur via the collagen receptors GPVI and α2β1.

The process of coagulation is vital in controlling bleeding at the site of vascular injury. The extrinsic pathway of coagulation initiates when tissue factor becomes exposed to flowing blood and via a series of activation steps leads to the generation of thrombin which converts fibrinogen to fibrin. The intrinsic pathway of coagulation, also referred to as the contact activation pathway, involves the activation of factor XII by non-physiological surfaces such as kaolin or glass, which leads to the generation of activated factor XI (FXIa) and via subsequent series of activation steps again resulting in the formation of fibrin. The deficiency of one of the coagulation factors in either the intrinsic or extrinsic coagulation pathway leads to a bleeding diathesis, indicating the essential role of coagulation in hemostasis. However, humans deficient in FXII do not have a bleeding diathesis. Thus, although FXII is important for coagulation in vitro, its relevance in vivo remains elusive.

Renné and coworkers used FXII-deficient mice to show a contribution of FXII in thrombus formation.[1] Although FXII-deficient mice did not suffer from spontaneous or injury-related bleeding, they did show reduced pathological thrombus formation upon vascular injury, which was restored upon infusion with FXII.

VWF has been shown to change its conformation from a globular protein to a stretched form upon binding to an artificial surface or collagen under the influence of shear flow,[2,3] a conformational change that is essential for its interaction with platelet receptor GPIbα. Furthermore, in vitro modulators such as the antibiotic ristocetin or the snake venom botrocetin can alter the conformation of VWF and induce VWF-dependent agglutination of platelets. This conformational change is crucial for the binding of platelets to VWF, as the A1-domain of VWF is not accessible for GPIbα when VWF is in its globular form. In a recent study, our group showed that unfolded proteins can induce the activation of factor XII.[4] We hypothesized that when VWF unfolds, it would become capable of binding and activating FXII.

In this study, we showed a direct interaction between FXII and VWF using surface plasmon resonance and identified the A1-domain of VWF as the major domain responsible for this interaction. Furthermore, we tested whether FXII is activated upon exposure to VWF. Finally, we investigated the consequences of FXII interaction with VWF on platelet adhesion to VWF under static conditions and under conditions of flow.

METHODS

Materials

FXII and prekallikrein (PK) was purchased from EMD Biosciences (Gibbstown, NJ). FXIIa was obtained from Enzyme Research Labs (South Bend, IN). Plasma-derived von Willebrand Factor (VWF) was purified from VWF/factor VIII concentrate (Haemate-P; Behringerwerke AG, Marburg, Germany) as described previously.[5] Expression and purification of recombinant VWF/A1, VWF/A1-R543Q and VWF/A2 were performed as described previously.[6] Ristocetin was purchased from DiaMed (Cressier sur Morat, Switzerland). Chromozym PK was obtained from Roche Applied Sciences (Indianapolis, IN). Essentially fatty acid free bovine serum albumin (BSA) was obtained from Sigma-Aldrich (St Louis, MO).

FXII activation assay

The activation of FXII was measured by conversion of the chromogenic substrate Chromozym PK by kallikrein, the formation of which is dependent on FXIIa. VWF or vehicle was immobilized on Costar 2595 microtiter plates and the reaction was started by addition of Chromozym PK (0.3 mM, final concentration) and $ZnCl_2$ (5.8 µM, final concentration) in the presence of 7.7 nM PK and 0.97 nM FXII in HEPES-buffered saline (HBS, 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 137 mM NaCl, 4 mM KCl, pH 7.4). Conversion of Chromozym PK was followed in time at 405 nm and 37°C in a SpectraMax 340 microplate reader (Molecular Devices). In the absence of FXII, no conversion of the chromogenic substrate was observed.

Surface plasmon resonance analysis

Binding studies were performed using a BIAcore2000 biosensor system (Biacore AB, Uppsala, Sweden) and surface plasmon resonance (SPR) analysis was done as described before.[7] Recombinant VWF or VWF-domains were immobilized on a CM5-sensor chip, using the amine-coupling kit as prescribed by the supplier. A control channel was routinely activated and blocked in the absence of protein. Binding to coated channels was corrected for binding to non-coated channels. SPR analysis was performed in HEPES buffer (25 mM NaCl, 125 mM HEPES, 0.01% Tween-20, pH 7.4) with a flow rate of 30 µl/min. Regeneration of the sensor chip surface was performed by incubating with 10 mM TDOC (taurodeoxycholic acid) in Tris, pH 9.0, for 2 minutes at a flow rate of 30 µl/min.

Platelet preparation

Blood from healthy volunteers, who claimed not to have used aspirin or other nonsteroidal anti-inflammatory drugs for the preceding 10 days, was drawn into one-tenth volume of 3.4% sodium citrate. This was done with the approval from the Institutional Review Board from the University Medical Center Utrecht (Utrecht, the Netherlands) and in accordance with the Declaration of Helsinki. Washed platelets were prepared as described previously.[8] The blood was centrifuged at 200g for 15 minutes at room temperature. The platelet-rich plasma (PRP) was

removed and acidified by addition of one-tenth volume of ACD (2.5% trisodium citrate, 1.5% citric acid, and 2% D-glucose). Platelets were spun down (500g, 15 minutes) and the platelet pellet was resuspended in HEPES-Tyrode buffer at pH 6.5 (10 mM HEPES, 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgSO_4$, 5 mM D-glucose). Prostacyclin (PGI_2, 10 ng/ml) was added to prevent platelet activation during the subsequent washing step. Platelets were spun down (500g, 15 minutes) and resuspended in a small volume of HEPES-Tyrode buffer (pH 6.5). Platelet suspension was further diluted in HEPES-Tyrode buffer at pH 7.35 to a platelet count of 200 x 10^9/L (200.000/µl).

Ristocetin-induced platelet agglutination

Platelet agglutination was measured after addition of 0.2 mg/ml ristocetin using an optical aggregometer (Chrono-Log Corporation, Haverford, PA).[9] Aggregations were performed at 37°C with a sample stir speed of 900 rpm.

Platelet adhesion

For platelet adhesion experiments, VWF (5 µg/ml) was immobilized for 2 hrs at 37ºC on an Immulon-2B flatbottom microtiter plate (Dynatech Laboratories Inc, Chantilly, VA) and subsequently blocked with 2% BSA for at least 30 min. Subsequently, VWF was incubated with FXIIa (10 µg/ml) or vehicle for 60 min at 37ºC. Washed platelets (200.000/µl in HEPES-Tyrode's pH 7.35) were allowed to adhere for 60 min at 37ºC. After extensive washing with Tris-buffered Saline (TBS), intrinsic phosphatase activity was measured using p-nitrophenyl phosphate (PNP, 3 mg/ml dissolved in 50 mM acetic acid, 1% Triton X-100, pH 5.0) and after 30 min the reaction was stopped with 1 M NaOH. Optical density was measured at 405 nm.

For visualization of static platelet adhesion, coverslips were coated with VWF (5 µg/ml) for 2 hrs at 37ºC and subsequently blocked with 2% BSA for at least 30 min. Subsequently, VWF was incubated with FXIIa (10 µg/ml) or vehicle for 2 hrs at 37ºC. Washed platelets (200.000/µl in HEPES-Tyrode's pH 7.35) were allowed to adhere for 60 min at 37 ºC. After extensive washing with TBS, coverslips were fixed and stained with May-Grünwald and Giemsa as described previously. Next, coverslips were examined using conventional light microscopy with a 40/1.00 PL APO oil immersion lens on a Leitz Diaplan (Carl Zeiss MicroImaging GmbH, Gottingen, Germany). Images were acquired using a JAI-CCD camera (Copenhagen, Denmark) coupled to a matrox frame grabber (Matrox Electronic Systems, Quebec, QC) using OPTIMAS 6.2 software (Optimas, Seattle, WA).

Perfusion studies

Perfusions were carried out in a single-pass perfusion chamber as described previously.[10] Coverslips were coated with VWF (5 µg/ml) for 2 hrs at 37ºC and subsequently blocked with 2% BSA for at least 30 min. Next, VWF was incubated with FXIIa (10 µg/ml) or vehicle for 60 min at 37ºC. Subsequently, reconstituted blood was perfused over the coated coverslips for 5 minutes at a constant flow rate. After perfusion, coverslips were washed with HEPES buffer (10 mM HEPES,
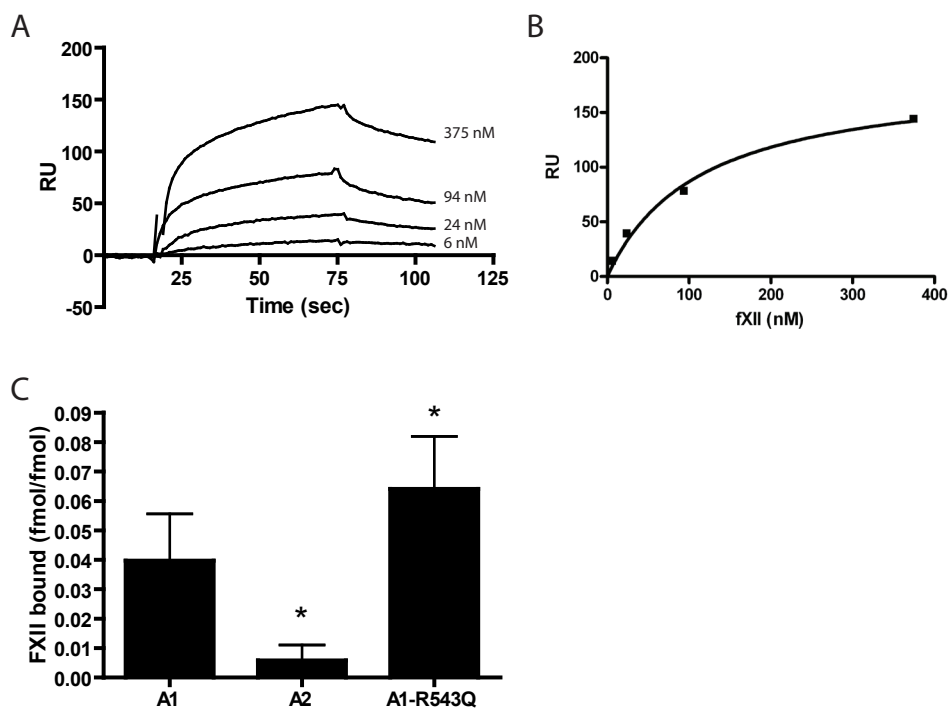
Figure 1 – FXII directly interacts with immobilized VWF. (A) VWF (2400 RU) was immobilized on a CM5 sensor chip and binding of FXII was investigated by surface plasmon resonance. Graph shows representative surface plasmon resonance traces of indicated concentrations of FXII. (B) After adjusting for binding to a blanc channel, the response of FXII at equilibrium was determined and plotted against the concentration applied. Graph shows data from a single experiment representative for at least 3 independent experiments. (C) VWF/A1, VWF/A2 or VWF/A1-R543Q was immobilized on a CM5 sensor chip and binding of FXII (375 nM) was investigated by surface plasmon resonance. After adjusting for binding to a blank channel, the response of FXII at equilibrium was determined, and the amount of bound FXII per fmol immobilized VWF domain was calculated. Graph shows mean data of 3 experiments. *: P< 0.05 compared to binding to A1-domain. Error bars indicate standard deviation.

150 mM NaCl, pH 7.35) and fixed in 0.5% glutaraldehyde in PBS. Coverslips were dehydrated in methanol and stained with May-Grünwald and Giemsa as described previously.[11] Next, coverslips were examined using conventional light microscopy with a 40/1.00 PL APO oil immersion lens on a Leitz Diaplan (Carl Zeiss MicroImaging GmbH, Gottingen, Germany). Platelet adhesion was evaluated using computer-assisted analysis with OPTIMAS 6.2 software. The total surface area stained with May-Grünwald/Giemsa was calculated and expressed as the percentage of the surface covered with platelets. Images were acquired using a JAI-CCD camera (Copenhagen, Denmark) coupled to a matrox frame grabber (Matrox Electronic Systems, Quebec, QC) using OPTIMAS 6.2 software.

Statistical analysis
Statistical analysis was performed using the GraphPad InStat (San Diego, CA) software package. Statistical differences in binding and adhesion were analyzed

by Student's t-test or standard one-way analysis of variance (ANOVA). P values less than 0.05 were considered statistically significant.

RESULTS

Factor XII interacts with Von Willebrand Factor

The interaction between factor XII and unfolded VWF was investigated using surface plasmon resonance. Recombinant full-length VWF was immobilized on a CM5 censor chip and perfused with different concentrations of FXII (Figure 1A). Non-linear regression analysis resulted in an affinity constant (K_d) of 113 ± 29 nM (Mean ± SD, Figure 1B). In contrast, recombinant full-length VWF did not interact with immobilized FXII (data not shown). As shown in Figure 1C, FXII specifically interacted with a recombinant A1-domain of VWF, whereas it did not interact with a recombinant A2-domain. Substitution of an arginine to a glutamine at position 543 of the A1-domain (A1-R543Q), which is associated with type 2B von Willebrand disease and has an increased binding capacity to GPIb,[12] showed increased FXII binding capacity compared to wild-type A1 (Figure 1C).
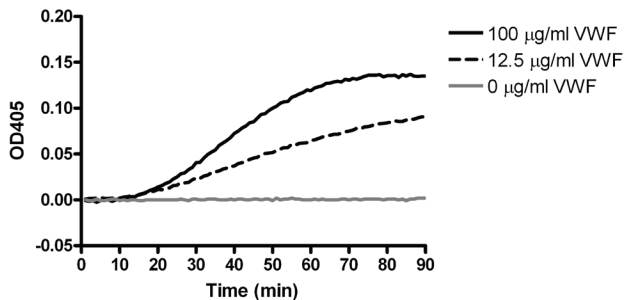

Figure 2 – FXII is activated by immobilized VWF. VWF or vehicle was immobilized on a microtiter plate at the concentrations indicated. Next, FXIIa-dependent kallikrein generation was measured by a chromogenic assay. Graph shows data of a single experiment, which is representative for at least 3 independent experiments.

Factor XII is activated by immobilized von Willebrand factor

We subsequently investigated whether FXII is activated by unfolded von Willebrand factor. FXII-dependent kallikrein generation was induced by VWF. VWF was immobilized on a microtiter plate at concentrations indicated and FXII-activity was monitored in time by the generation of active kallikrein from PK, using a chromogenic assay. As shown in Figure 2, VWF immobilized on a microtiter plate was able to induce FXII-dependent kallikrein generation in a concentration dependent manner. Control experiments in which FXII was omitted, showed no detectable kallikrein generation (data not shown).

FXIIa inhibits ristocetin-induced platelet agglutination

To investigate the effect of FXIIa on the function of VWF, VWF-dependent platelet agglutination was studied. FXIIa was added to isolated washed platelets at concentrations indicated in the presence of 10 µg/ml VWF and platelet agglutination was initiated by addition of a suboptimal dose of ristocetin (0.2 mg/
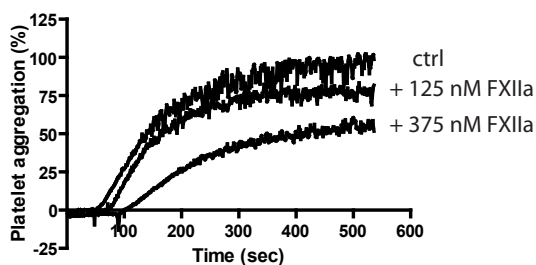
Figure 3 – FXIIa inhibits ristocetin-induced platelet agglutination. Isolated washed platelets (200.000/µl) were isolated and preincubated with FXIIa at the concentrations indicated. VWF-dependent platelet agglutination was initiated by 0.2 mg/ml ristocetin in the presence of 10 µg/ml VWF. Platelet agglutination was monitored using standard suspension aggregometry at 37°C at a stir speed of 900 rpm. Graph shows results of a single experiment, representative for at least 3 independent experiments.

ml). As shown in Figure 3, FXIIa dose-dependently inhibited ristocetin-induced platelet agglutination. In contrast, FXII had no effect on ristocetin-induced platelet agglutination (data not shown).

FXIIa increases platelet adhesion to VWF

To investigate the effect of FXIIa on platelet adhesion to VWF under static conditions, VWF was immobilized on a microtitre plate or a coverslip (5 µg/ml) and subsequently preincubated with FXIIa (125 nM) before allowing washed platelets to adhere. As shown in Figure 4, FXIIa did not increase platelet adhesion to VWF. When platelets were pretreated with dRGDW, which blocks ligand binding to αIIbβ3, platelet adhesion to VWF was reduced and only single platelets were observed. In the presence of dRGDW, FXIIa substantially increased platelet adhesion. Preincubation of VWF with FXII did not affect platelet adhesion to VWF in the presence or absence of dRGDW.

To investigate the effect of FXIIa on platelet adhesion to VWF under conditions of flow, we preincubated VWF with FXIIa (125 nM) or vehicle before perfusing with
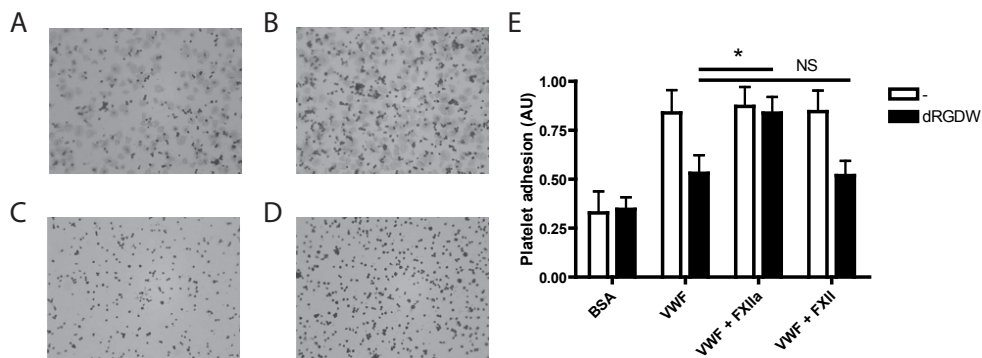
Figure 4 – FXIIa increases platelet adhesion to immobilized VWF under static conditions. Isolated washed platelets were allowed to adhere under static conditions for 60 minutes at 37°C to immobilized VWF (5 µg/ml) in the absence (A) or presence (C) of dRGDW (200 µM), or to immobilized VWF, which was preincubated with 125 nM FXIIa in the absence (B) or presence (D) of dRGDW (200 µM). After gentle washing, coverslips were fixed and stained with May-Grünwald/Giemsa and examined by light microscopy; original magnification 400x. (E) Static platelet adhesion was quantified by measuring the intrinsic phosphatase activity, and optical density is depicted as arbitrary units of adhesion (AU). Graph shows mean surface coverage of at least 3 independent experiments performed in triplicate. *: P< 0.05, NS: not significant. Error bars indicate standard deviation.

reconstituted blood (a mixture of washed platelets in human albumin solution and isolated red cells, 40% hematocrit, 200.000 platelets/µl) for 3 minutes at 300 s^{-1}. As shown in Figure 5, FXIIa substantially increased platelet adhesion to VWF. This was not due to platelet adhesion directly to FXIIa, as platelets did not adhere to a coverslip coated with FXIIa alone (data not shown). When platelets were preincubated with FXIIa before perfusing with reconstituted blood over VWF, platelet adhesion to VWF was increased in a similar fashion as when VWF was preincubated with FXIIa (data not shown). Increased platelet deposition to VWF was not observed when platelets or VWF were preincubated with FXII (data not shown).

DISCUSSION

This study shows that coagulation factor XII interacts with unfolded VWF, which results in FXIIa generation. Furthermore, we showed FXIIa to enhance platelet adhesion to VWF both in static adhesion experiments and in experiments under conditions of flow. These mechanisms may be relevant for regulation of thrombus formation, and supports recent literature that suggests a function for FXII in vivo.

For years, FXII was thought not to play a significant role in vivo because individuals with a complete FXII deficiency do not have a bleeding tendency, in contrast to individuals with deficiencies in downstream components of the intrinsic coagulation pathway. Recently, a function for FXII in vivo was reported by Renné and coworkers.[1,13] Using FXII deficient mice, they showed that FXII is important
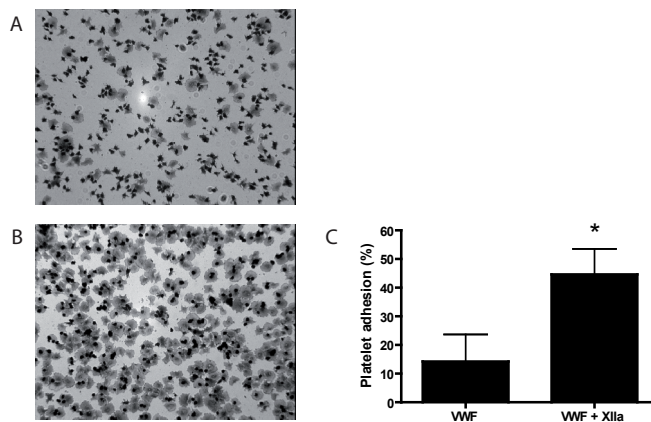
Figure 5 – FXIIa increases platelet adhesion to VWF under conditions of flow. Reconstituted blood (washed platelets in a 4% human albumin solution and isolated red cells, 40% hematocrit, 200.000 platelets/µl) was perfused over VWF coated coverslips incubated with vehicle (A) or 125 nM FXIIa (B) at a shear rate of 300 s^{-1} for 5 min at 37ºC using a single-pass perfusion chamber. After perfusion, coverslips were fixed and stained with May-Grünwald/Giemsa and examined by light microscopy. Representative images of at least 3 independent experiments performed in triplicate are shown. (C) Graph shows mean surface coverage of at least 2 independent experiments performed in triplicate. *: P< 0.05 compared to VWF. Error bars indicate standard deviation.

in pathologic thrombus formation but not in physiological hemostasis. It is not directly evident how our results can be reconciled with the observations by Renné and coworkers, as both in physiological and pathological thrombus formation unfolded VWF is assumed to be involved.

Several epidemiological studies have dealt with the role of FXII levels on the risk of thrombosis. A large case-control study by Doggen et al[14] showed that low levels of FXII increased the risk of thrombosis (thus individuals with high FXII levels have decreased risk of thrombosis). This is in contrast to what may be expected from the data from the group of Renné as well as our experimental data, which show increased platelet deposition to VWF in the presence of FXIIa. The difference between experimental and epidemiological data may be explained by the fact that FXII fulfills several functions. Besides its function in coagulation, activated FXII can activate kallikrein and thereby play an important role in inflammation, fibrinolysis and blood pressure regulation. It has therefore been speculated that the protective effect of FXII on the risk of thrombosis might not be related to its function in coagulation, but rather to its effect on the kallikrein-kinin system.[4]

Further clinical data show a positive correlation between VWF and FXIIa plasma levels in healthy individuals, which might suggest that VWF-dependent FXIIa generation also occurs in vivo.[15] This is supported by elevated FXIIa levels found during pregnancy, which is also accompanied by elevated levels of VWF.[16] VWF-dependent XIIa generation would also be expected to accompany clinical situations in which acute VWF unfolding takes place, for example during myocardial infactions. However, literature on this is conflicting. No FXII activation was observed in a study including 150 patients with acute coronary syndromes.[17] On the other hand, other studies have shown elevated levels of FXIIa in these patients.[16,18]

As previous studies by our group have shown, FXII can be activated by unfolded proteins.[4] VWF has been shown to require a conformational change to become in a GPIbα-binding state. When VWF is in its globular form, the A1-domain is shielded from binding to GPIbα. It has been reported that the D'-D3-domain flanking the A1-domain is responsible for shielding of the A1-domain,[19] but another group described the A2-domain to interact with the A1-domain in such a way that GPIbα is unable to interact with VWF.[20] In this study, we show that FXII can interact with the A1-domain of VWF, which explains the necessity of unfolding of VWF before FXII is able to interact. Question arises whether FXII and GPIbα are interacting in the same region of VWF, and whether this interaction influences the interaction between VWF and GPIbα. Binding studies using recombinant VWF-domains show that the interaction between FXII and the A1-domain is increased, when a VWD type 2B mutation is introduced (A1-R543Q). Moreover, binding of FXII and GPIbα to VWF do not seem to exclude each other, as FXIIa increases platelet deposition to VWF, which is dependent on GPIbα (Figure 5).

FXIIa has already been shown to interact with GPIbα[21] and together with the results we show here, this could imply that FXIIa is able to form a ternary complex with VWF and GPIbα. An interesting hypothesis would be that GPIbα-bound FXIIa increases the affinity of GPIbα for VWF, a model proposed earlier for GPIbα-bound thrombin.[22] Alternatively, the interaction of FXIIa with VWF may result in

an increase of the affinity of VWF for GPIbα, subsequently resulting in increased platelet deposition to VWF. In this respect, the effects of FXIIa mimic the effects of ristocetin, which may explain the observed inhibition of ristocetin-induced agglutination by FXIIa.

In conclusion, FXII interacts with VWF, which results in the generation of FXIIa Furthermore, FXIIa increases platelet deposition to VWF, which could have important implications in thrombus formation. Targeting the interaction between VWF and FXII would therefore be an interesting therapeutic implication without interfering with normal hemostasis.

Acknowledgements

REFERENCES

1. Renne T, Pozgajova M, Gruner S et al. Defective thrombus formation in mice lacking coagulation factor XII. J.Exp. Med. 2005;202:271-281.

2. Siedlecki CA, Lestini BJ, Kottke-Marchant KK et al. Shear-dependent changes in the three-dimensional structure of human von Willebrand factor. Blood 1996;88:2939-2950.

3. Schneider SW, Nuschele S, Wixforth A et al. Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. Proc.Natl.Acad.Sci.U.S.A 2007;104:7899-7903.

4. Maas C, Govers-Riemslag JW, Bouma B et al. Misfolded proteins activate factor XII in humans, leading to kallikrein formation without initiating coagulation. J.Clin.Invest 2008;118:3208-3218.

5. Sodetz JM, Pizzo SV, McKee PA. Relationship of sialic acid to function and in vivo survival of human factor VIII/ von Willebrand factor protein. J.Biol.Chem. 1977;252:5538-5546.

6. Lankhof H, Wu YP, Vink T et al. Role of the glycoprotein Ib-binding A1 repeat and the RGD sequence in platelet adhesion to human recombinant von Willebrand factor. Blood 1995;86:1035-1042.

7. Lenting PJ, Neels JG, van den Berg BM et al. The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. J.Biol.Chem. 1999;274:23734-23739.

8. Lisman T, Moschatsis S, Adelmeijer J, Nieuwenhuis HK, de Groot PG. 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. Blood 2003;101:1864-1870.

9. Born GV. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 1962;194:927-9.

10. Sixma JJ, de Groot PG, van ZH, IJsseldijk M. A new perfusion chamber to detect platelet adhesion using a small volume of blood. Thromb.Res. 1998;92:S43-S46.

11. Sakariassen KS, Aarts PA, de Groot PG, Houdijk WP, Sixma JJ. A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components. J.Lab Clin.Med. 1983;102:522-535.

12. Huizinga EG, Tsuji S, Romijn RA et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. Science 2002;297:1176-1179.

13. Kleinschnitz C, Stoll G, Bendszus M et al. Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. J.Exp.Med. 2006;203:513-518.

14. Doggen CJ, Rosendaal FR, Meijers JC. Levels of intrinsic coagulation factors and the risk of myocardial infarction among men: Opposite and synergistic effects of factors XI and XII. Blood 2006;108:4045-4051.

15. Colhoun HM, Zito F, Norman CN et al. Activated factor XII levels and factor XII 46C>T genotype in relation to coronary artery calcification in patients with type 1 diabetes and healthy subjects. Atherosclerosis 2002;163:363-369.

16. Coppola R, Cristilli P, Cugno M et al. Measurement of activated factor XII in health and disease. Blood Coagul.Fibrinolysis 1996;7:530-535.

17. Minnema MC, Peters RJ, de WR et al. Activation of clotting factors XI and IX in patients with acute myocardial infarction. Arterioscler.Thromb. Vasc.Biol. 2000;20:2489-2493.

18. Kohler HP, Carter AM, Stickland MH, Grant PJ. Levels of activated FXII in survivors of myocardial infarction-- association with circulating risk factors and extent of coronary artery disease. Thromb. Haemost. 1998;79:14-18.

19. Ulrichts H, Udvardy M, Lenting PJ et al. Shielding of the A1 domain by the D'D3 domains of von Willebrand factor modulates its interaction with platelet glycoprotein Ib-IX-V. J.Biol.Chem. 2006;281:4699-4707.

20. Martin C, Morales LD, Cruz MA. Purified A2 domain of von Willebrand factor binds to the active conformation of von Willebrand factor and blocks the interaction with platelet glycoprotein Ibalpha. J.Thromb. Haemost. 2007;5:1363-1370.

21. Bradford HN, Pixley RA, Colman RW. Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. J.Biol.Chem. 2000;275:22756-22763.

22. Mendolicchio GL, Celikel R, Varughese KI, Savage B, Ruggeri ZM. Modulation of von Willebrand Factor A1 Domain-Mediated Platelet Aggregation/ Agglutination by alpha-Thrombin. [abstract]. Blood 2004;104:182a

Chapter 7

Staphylococcal superantigen-like 5 activates platelets and supports platelet adhesion under flow conditions, which involves glycoprotein Ibα and αIIbβ3

Cees Weeterings [1,3] *, Carla JC de Haas [2] *, Mignon M Vughs [2], Philip G de Groot [1,3], Jos A van Strijp [2], Ton Lisman [1,4]

From [1] the Department of Clinical Chemistry and Haematology and [2] the Department of Medical Microbiology, University Medical Center Utrecht, The Netherlands; [3] the Institute of Biomembranes, Utrecht University, Utrecht, The Netherlands and [4] Surgical Research Laboratory, Department of Surgery, University Medical Center Groningen, University of Groningen, The Netherlands
* authors contributed equally to this work

Submitted

ABSTRACT

Objectives - Staphylococcal superantigen-like 5 (SSL5) is an exoprotein secreted by *Staphylococcus aureus* and has been shown to inhibit neutrophil rolling over activated endothelial cells via a direct interaction with P-selectin glycoprotein ligand 1 (PSGL-1).

Methods & Results - When purified recombinant SSL5 was added to washed platelets in an aggregometry set-up, complete and irreversible aggregation was observed. Proteolysis of the extracellular part of GPIbα or the addition of dRGDW abrogated platelet aggregation. When a mixture of isolated platelets and red cells was perfused over immobilized SSL5 at a shear rate of 300 s^{-1}, stable platelet aggregates were observed, and platelet deposition was substantially reduced after proteolysis of GPIb or after addition of dRGDW. SSL5 was shown to interact with glycocalicin, a soluble GPIbα fragment, and binding of SSL5 to platelets resulted in GPIb-mediated signal transduction as evidenced by translocation of 14-3-3ζ. In addition, SSL5 was shown to interact with endothelial cell matrix (ECM) and this interaction enhanced aggregation of platelets from whole blood to this ECM.

Conclusion – SSL5 activates and aggregates platelets in a GPIbα-dependent manner, which could be important in colonization of the vascular bed and evasion of the immune system by *S. aureus*.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a common human pathogen that induces both community-acquired as well as nosocomial infections. Infections with *S. aureus* are a growing concern, considering the increasing incidence of antibiotic resistant strains, such as methicillin resistant *S. aureus* (MRSA).[1] *S. aureus* causes infections that can be accompanied by infective endocarditis, sepsis and toxic shock syndrome.[2,3] Furthermore, infections with *S. aureus* may be accompanied by thrombocytopenia as a result of local platelet activation, and disseminated intravascular coagulation may occur when activation of hemostasis by the bacterium becomes systemic. In addition, infective endocarditis is regularly accompanied by embolic events such as stroke.[4] The interaction of *S. aureus* with platelets has been studied extensively and it has been shown that *S. aureus* can activate platelets, which is mediated by several surface-expressed proteins (reviewed by Fitzgerald et al.[5]), such as clumping factor A and fibronectin-binding protein A. Furthermore, *S. aureus* is able to bind to adhered platelets, which might be an important mechanism contributing to the colonization of the vascular bed or damaged heart valves.[6,7]

The invasiveness of *S. aureus* is dependent on a combination of surface-expressed virulence factors, such as clumping factor A and fibronectin-binding protein A, as well as excreted virulence factors, such as coagulase and alpha-toxin.[3,8] We and others have described several excreted virulence factors, such as staphylococcal complement inhibitor (SCIN) and chemotaxis inhibitory protein of *S. aureus* (CHIPS), to be important in inhibiting pathogen clearance by the immune system. SCIN was shown to interfere with the complement system, whereas we have shown that CHIPS inhibits neutrophil chemotaxis.[9,10] CHIPS is closely homologous to another family of excreted factors referred to as staphylococcal superantigen-like proteins (SSLs). The SSL-family of proteins is encoded on staphylococcal pathogenicity island 2 and each strain of *S. aureus* expresses at least seven to a maximum of eleven SSLs. Recently, it has been suggested that SSL members 5 and 7 play a role in staphylococcal evasion of the immune system.[11,12] We previously showed that SSL5 inhibits PSGL-1-mediated neutrophil rolling on activated endothelium, and we speculated that this mechanism is important for immune evasion.[11] Similar to PSGL-1, glycoprotein Ibα (GPIbα), one of the most abundant receptors on the platelet surface, is a tyrosine sulphated sialomucin. GPIbα is widely known as the receptor for von Willebrand factor (VWF),[13] but an increasing number of ligands, including coagulation factors IIa, VIIa, XII, XI(a), high-molecular weight kininogen, (activated) protein C, the leukocyte receptor MAC-1, and a dimeric form of beta2 glycoprotein I have been described in recent years.[14-21] Recently, GPIbα was shown to interact with P-selectin,[22] the main ligand of PSGL-1, and conversely, PSGL-1 was shown to interact with the main GPIbα ligand VWF.[23] These observations led us to the hypothesis that more ligands may be able to bind both GPIbα and PSGL-1. As platelets are known to be an important mediator in the colonization of *S. aureus*, we hypothesized that SSL5 could have an effect on platelets via GPIbα. Here, we show that SSL5 is capable of activation,

adhesion and aggregation of platelets, in which both GPIbα and αIIbβ3 play a role.

METHODS

Materials

Recombinant SSL5 was produced in E. coli and purified as described previously by Bestebroer et al.[11] The PAR1-agonist peptide SFLLRN was from Bachem (Bubendorf, Switzerland). The RGD-containing peptide D-arginyl-glycyl-L-aspartyl-L-tryptophane (dRGDW) was synthesized at the Department of Membrane Enzymology, Faculty of Chemistry (University of Utrecht, The Netherlands). O-sialoglycoprotein endopeptidase (OSE) was purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). The stable prostacyclin analog, iloprost, was a kind gift from Schering AG (Berlin, Germany). Neuraminidase (from Clostridium perfringens) was from Roche Applied Sciences (Indianapolis, IN). Glycocalicin (GC) was purified as described previously.[17] Fully sulphated wild-type recombinant GPIbα, comprising residues 1-290, was produced and purified as described previously.[24] Purified human αIIbβ3 was purchased from VWR International (Amsterdam, The Netherlands). Plasma-derived von Willebrand Factor (VWF) was purified from VWF/factor VIII concentrate (Haemate-P; Behringerwerke AG, Marburg, Germany).[25] Ristocetin was purchased from DiaMed (Cressier sur Morat, Switzerland). Calcein-AM was from Molecular Probes (Leiden, The Netherlands). Polyclonal antibody rabbit-anti-14-3-3ζ and goat-anti-rabbit-IRDye were obtained from Santa Cruz (Santa Cruz, CA) and Li-Cor Biosciences (Lincoln, NE) respectively. Monoclonal antibody against GPIbα, AK2, was purchased from AbD Serotec (Düsseldorf, Germany). ELK® milk powder was purchased from Campina (Eindhoven, the Netherlands). Vitronectin, fibronectin, fibrinogen, laminin, collagens type I to V and essentially fatty acid free bovine serum albumin (BSA) were all obtained from Sigma-Aldrich (St Louis, MO). All other chemicals used in the experiments were of analytical grade.

Platelet preparation

Blood from healthy volunteers, who claimed not to have used aspirin or other nonsteroidal anti-inflammatory drugs for the preceding 10 days, was drawn into one-tenth volume of 3.4% sodium citrate. This was done with the approval from the Institutional Review Board from the University Medical Center Utrecht (Utrecht, the Netherlands) and in accordance with the Declaration of Helsinki. Washed platelets were prepared as described previously.[26] The blood was centrifuged at 200g for 15 minutes at room temperature. The platelet-rich plasma (PRP) was removed and acidified by addition of one-tenth volume of ACD (2.5% trisodium citrate, 1.5% citric acid, and 2% D-glucose). Platelets were centrifuged (500g, 15 minutes) and the platelet pellet was resuspended in Hepes-Tyrode buffer at pH 6.5 (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgSO_4$, 5 mM D-glucose). Prostacyclin (PGI_2, 10 ng/ml) was added to prevent platelet activation during the subsequent washing step. Platelets were centrifuged (500g, 15 minutes) and resuspended in

a small volume of HEPES-Tyrode buffer. Platelet suspension was further diluted in HEPES-Tyrode buffer at pH 7.35 to a platelet count of 200 x 10⁹/L (200.000/µl). GPIbα-depleted platelets were prepared by treating the platelets for 30 minutes at 37°C with 30 µg/ml OSE. Proteolysis of GPIbα was monitored by assessing binding of a GPIbα-specific antibody (AN51, Dako, Glostrup, Denmark) to platelets by FACS analysis. OSE-treatment specifically reduced GPIbα expression on platelets to less than 5%.[17,27] Where indicated, platelets were first treated with 0.2 U/ml neuraminidase at 37°C for 45 minutes at pH 7.35.

Platelet aggregation

Platelet aggregation was measured using an optical aggregometer (Chrono-Log Corporation, Haverford, PA).[28] Aggregations were performed at 37°C with a sample stir speed of 900 rpm.

Perfusion studies

Perfusions were carried out in a single-pass perfusion chamber as described previously.[29] Coverslips were coated with SSL5 (1 µM) as described previously[30] and reconstituted blood was perfused over the coated coverslips for 5 minutes at a constant flow rate. In selected experiments, ECM-coated coverslips were prepared as described in the section "cell culture" and incubated with 1 µM SSL5 for 60 minutes at 37°C. Subsequently, citrated whole blood was perfused over the coated coverslips for 5 minutes at a constant flow rate.

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.[31] Next, the slides were examined using conventional light microscopy with a 40/1.00 PL APO oil immersion lens on a Leitz Diaplan (Carl Zeiss MicroImaging GmbH, Gottingen, Germany). Platelet adhesion was evaluated using computer-assisted analysis with OPTIMAS 6.2 software (Dutch Vision Systems [DVS], Breda, The Netherlands). The total surface area stained with May-Grünwald and Giemsa was calculated and expressed as the percentage of the surface covered with platelets. Aggregate coverage was investigated by measuring the most intense areas of staining, which include mostly dense aggregates and only some single non-spread platelets.

Real time perfusion studies

Real time perfusions were carried out in a single-pass perfusion chamber consisting of a silicon sheet gasket that maintained a flow path height of 0.125 mm and a width of 2 mm.[32,33] Platelet adhesion was continuously monitored using differential interference contrast microscopy with a Zeiss 100x/1.3 EC Plan-NeoFluar oil immersion lens on a Carl Zeiss AxioCam MRm and recorded using Carl Zeiss AxioVision imaging software (Carl Zeiss MicroImaging GmbH, Gottingen, Germany).

Cell culture

Wild-type Chinese Hamster Ovary (CHO) cells and CHO cells stably expressing the integrin αIIbβ3 (CHO-αIIbβ3, a generous gift of Dr. J. Ylänne (Helsinki, Finland)) were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium with 10% fetal calf serum and 400 µg/ml G418 in the presence of penicillin and streptomycin.

Human umbilical vein endothelial cells were isolated and grown to confluence in EBM-2 medium (Lonza, Walkersville), as described.[34] Thermanox coverslips (Nunc, Naperville, IL) were rinsed in 96% ethanol overnight, subsequently washed with phosphate buffered saline (PBS) and coated with 0.5% gelatin in phosphate buffered saline (PBS). Cells of the second passage were seeded on Thermanox coverslips (Nunc, Naperville, IL), placed in 96-wells tissue culture plates (Costar) and grown until confluence. The cells were stimulated for 6 to 8 hours with phorbol myristate acetate (PMA; Sigma; 20 ng/ml final concentration). After stimulation, the endothelial cell matrix (ECM) was isolated by removing the cells with 0.1 M NH_4OH for 5 minutes at room temperature, and subsequently the matrices were washed 3 times with PBS.

Binding studies

SSL5 was immobilized for 2 hours at 37ºC on a Costar 96-wells plate at the indicated concentrations. After blocking the wells with 4% ELK for at least 30 minutes, the wells were incubated with 2 µg/ml GC for 90 minutes at 22ºC. In some experiments, GC was pretreated with neuraminidase (from Clostridium perfringens, 0.2 U/ml, 45 minutes, 37ºC) or vehicle. Bound GC was detected with an in house rabbit polyclonal antibody against GPIbα, followed by a peroxidase-labeled swine-anti-rabbit antibody detected with TMB peroxidase substrate solution (Tebu-Bio, Heerhugowaard, the Netherlands). Results were obtained by measuring optical density at 450 nm on a SpectroMax Reader (Molecular Devices, Wokingham, United Kingdom).

For binding of SSL5 to HUVEC-derived ECM, wells were coated with ECM, as described in the cell culture section, or indicated ECM-proteins (10 µg/ml, 4°C overnight). After blocking with 4% ELK for 1 hour at 37°C, the wells were incubated with histidine-tagged SSL5 at the indicated concentrations for 1 hour at 37°C. Bound SSL5 was detected with anti-X-press-HRP (Invitrogen, Paisley, United Kingdom) followed by TMB peroxidase substrate solution. To determine binding of SSL5 to isolated platelets, SSL5 was labeled with FITC as described previously.[11] For binding of SSL5-FITC to platelets, isolated platelets were incubated with increasing concentrations of SSL5-FITC in Hepes-Tyrode buffer (pH 7.35) for 30 minutes. After washing, fluorescence was measured on a flow cytometer (FACSCalibur; Becton Dickinson, Franklin Lanes, NJ).

To investigate adhesion of CHO-αIIbβ3 to SSL5, CHO-αIIbβ3 were loaded with 4 µM calcein-AM in Hanks buffered salt solution (BioWhittaker) with 0.05% human serum albumin (HSA; Sanquin, Amsterdam, The Netherlands). A 96-wells plate (Greiner Bio-One, Frickenhausen, Germany) was coated with 400 nM SSL5 for 1 hour at 37°C. After washing with PBS, the plate was blocked with 4% ELK for 90

minutes at 37°C. The plate was then washed and 3 x 10^5 calcein-labeled CHO-αIIbβ3 were added to duplicate wells and allowed to adhere for 15 minutes at room temperature. After washing twice, adherent cells were quantified using a platereader fluorometer (FlexStation; Molecular Devices, Sunnyvale, CA).

14-3-3ζ translocation

Aliquots of 450 µl washed platelets (200.000/µl) were aggregated with SSL5 or ristocetin-activated VWF (10 µg/ml VWF and 1 mg/ml ristocetin) in the aggregometry set-up as described before. One minute after addition of the agonist, platelets were lysed by adding 10% (v/v) of a 10x concentrated Triton lysis buffer (TxLB; 10% Triton-100, 200 mM Tris, 50 mM EGTA supplemented with Complete Mini EDTA free proteinase inhibitor cocktail tablets (Roche) according to instructions of the manufacturer). Triton-100 insoluble fractions were spun down at 20.000g for 30 minutes and the supernatant was collected (cytosol fraction). Pellets were washed twice with 1x TxLB and resuspended in 60 µl reducing sample buffer (cytoskeletal fraction). Samples were separated by means of SDS-PAGE and transferred to PVDF membrane using western blotting. 14-3-3ζ was detected using rabbit anti-14-3-3ζ and goat-anti-rabbit-IRDye. Analysis was performed on a Li-Cor Odyssey scanner (Li-Cor Biosciences, Lincoln, Nebraska).

Surface plasmon resonance analysis

Binding studies were performed using a BIAcore2000 biosensor system (Biacore AB, Uppsala, Sweden) and surface plasmon resonance (SPR) analysis was done as described before.[35] αIIbβ3 was immobilized on a CM5-sensor chip, using the amine-coupling kit as prescribed by the supplier. A control channel was routinely activated and blocked in the absence of protein. Binding to coated channels was corrected for binding to non-coated channels. SPR analysis was performed in HEPES (25 mM NaCl, 125 mM HEPES, 3 mM $CaCl_2$, pH 7.4) buffer with a flow rate of 30 µl/min. Regeneration of the sensor chip surface was performed by incubating with 50 mM NaOH for 30 seconds at a flow rate of 30 µl/min.

Statistical analysis

Statistical analysis was performed using the GraphPad InStat (San Diego, CA) software package. Statistical differences in binding and adhesion were analyzed by Student's t-test or standard one-way analysis of variance (ANOVA). P values less than 0.05 were considered statistically significant.

RESULTS

Aggregation of washed platelets by SSL5 requires GPIb and αIIbβ3

When purified recombinant SSL5 was added to washed platelets in an aggregometry set-up, irreversible aggregation was observed as shown in figure 1A. Maximal aggregation was observed with 400 nM of SSL5. Aggregation occurred in the absence of exogenously added fibrinogen, and addition of fibrinogen did not
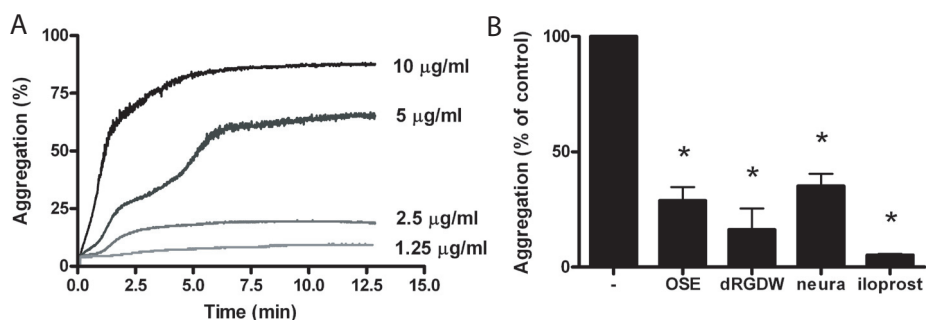
Figure 1 – SSL5 is able to aggregate washed platelets. (A) Washed platelets (200.000/µl) were activated with SSL5 at indicated concentrations. Aggregation was monitored using standard suspension aggregometry at 37°C at a stir speed of 900 rpm. Graph shows results of a single experiment. Data are representative for at least 3 independent experiments. (B) Platelets were pretreated with OSE, dRGDW, neuraminidase (neura) or iloprost and aggregation was initiated with 200 nM SSL5. Graph indicates platelet aggregation at 10 minutes. Platelet aggregation of untreated platelets with 200 nM SSL5 was set at 100% (-). Mean data of 3 independent experiments are shown. Error bars indicate SEM. * = p<0.01.

alter the aggregation profiles (data not shown). Aggregation was inhibited by dRGDW (200 µM), a peptide which blocks ligand binding to integrin αIIbβ3 (Figure 1B). Furthermore, treatment of platelets with O-sialoglycoprotein endopeptidase (OSE, 30 µg/ml), which cleaves the extracellular part of GPIbα, reduced platelet aggregation substantially (Figure 1B). Platelet aggregation was dependent on intracellular signaling, as treatment with a stable prostacyclin analog, iloprost (20 ng/ml), inhibited platelet aggregation induced by SSL5 (Figure 1B). As the interaction between SSL5 and PSGL-1 was shown to be critically dependent on sialic acid residues present on PSGL-1, we investigated the effect of neuraminidase treatment of platelets on aggregation induced by SSL5. As shown in Figure 1B, platelets treated with neuraminidase (0.2 U/ml, 45 minutes) showed reduced aggregation upon addition of SSL5.

Although SSL5 was able to fully aggregate platelets at 400 nM in a washed platelet suspension, SSL5 did not induce any platelet aggregation in platelet rich plasma (PRP), even after 60 minutes of incubation, with concentrations up to 4 µM of SSL5 (data not shown). However, subsequent experiments demonstrated that SSL5 was active towards platelets in a more physiological environment (see below).

SSL5 interacts with platelet GPIbα
Since OSE treatment reduced platelet aggregation by SSL5, we assessed a direct interaction between SSL5 and GPIbα. First, we immobilized SSL5 on a 96-wells plate and incubated with increasing concentrations of glycocalicin (GC), a plasma-purified extracellular part of GPIbα. As shown in Figure 2A, GC readily bound to SSL5. However, a recombinant truncated GPIbα (recGPIbα, residues 1-290) did not bind SSL5 (data not shown). Pretreatment of GC with neuraminidase resulted in a significant decrease of binding of GC to immobilized SSL5 (Figure 2A). These results suggests that the binding site on GC for SSL5 is located in the highly glycosylated stack of GPIbα.
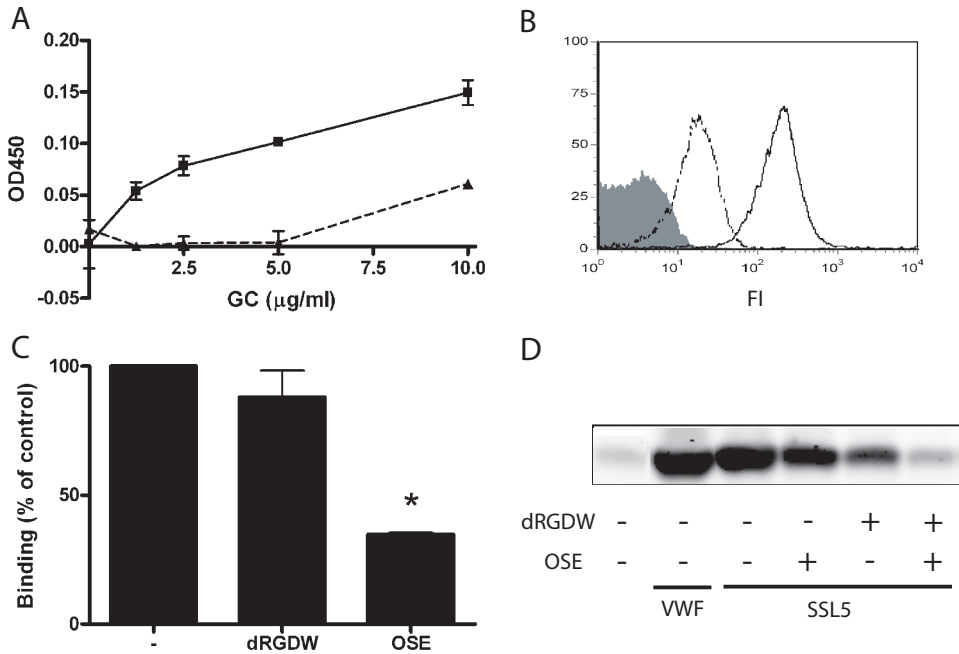
Figure 2 – SSL5 interacts with GPIbα. (A) SSL5 (400 nM) was immobilized on a microtiter plate. Subsequently, GC was pretreated with neuraminidase (dotted line) or vehicle (straight line) and wells were incubated with GC at concentrations indicated. Bound GC was detected by using a polyclonal antibody against GPIbα (2 μg/ml). Mean data of a single experiment is shown, error bars indicate SD. Data is representative for 3 independent experiments in which the same difference was observed. (B) Washed platelets (3000/μl) were incubated with FITC-labeled SSL5 for 30 minutes. Binding of SSL5-FITC (400 nM, straight line, 1 μg/ml dotted line) to washed platelets was compared to washed platelets alone (grey line) as expressed by fluorescence intensity (FI). (C) Washed platelets (3000/μl) were pretreated with dRGDW or OSE, after which binding of SSL5-FITC (400 nM) was investigated as mentioned under B. Binding of SSL5-FITC to untreated platelets was indicated as 100% binding. Mean data of 3 independent experiments are shown. Error bars indicate SEM. * = p<0.01. (D) Washed platelets were pretreated with OSE, dRGDW, or a combination of OSE and dRGDW as indicated. Subsequently, platelets were treated with SSL5 (400 nM) or ristocetin-activated VWF (VWF) in an aggregometer at a stir speed of 900 s^{-1}. After 1 minute, samples were lysed, and cytoskeletal fractions were isolated by centrifugation. Samples were subjected to SDS-PAGE followed by western blotting using a polyclonal antibody against 14-3-3ζ.

Subsequently, we investigated the binding of SSL5 to platelets using flow cytometry analysis. As shown in Figure 2B, FITC-labeled SSL5 (SSL5-FITC) was able to bind to washed platelets. Binding of SSL5-FITC to platelets was not affected by dRGDW, but pretreatment of platelets with OSE reduced the binding of SSL5-FITC by 65%, indicating a role for GPIbα in the initial interaction of SSL5 with platelets (Figure 2C).

SSL5 triggers translocation of the GPIbα-associated adapter protein 14-3-3ζ

To assess consequences of the interaction of SSL5 with GPIbα on platelets, we examined 14-3-3ζ translocation to the actin cytoskeleton, which was previously shown to indicate a GPIbα-dependent signaling event[36,37] As shown in figure 2D,

increased 14-3-3ζ association with the cytoskeleton was observed upon stimulation with SSL5. Removal of GPIbα by OSE partially prevented 14-3-3ζ translocation, whereas combined treatment with OSE and dRGDW fully abrogated SSL5 induced 14-3-3ζ translocation.

SSL5 interacts with platelet integrin αIIbβ3

As dRGDW blocked both SSL5 induced aggregation and 14-3-3ζ translocation, we assessed a possible interaction between SSL5 and platelet integrin αIIbβ3, using CHO cells transfected with αIIbβ3 (CHO-αIIbβ3). As shown in figure 3A, CHO-αIIbβ3 adhered to immobilized SSL5, whereas untransfected CHO cells did not. Furthermore, direct interaction between SSL5 and αIIbβ3 was investigated using surface plasmon resonance. αIIbβ3 was coated to a CM5 sensor chip via amine-coupling with a maximum adsorption of 1500 RU and perfused with different concentrations of SSL5. Non-linear regression analysis resulted in an affinity constant (K_d) of 0.82 ± 0.13 μM (Mean ± SD, figure 3B).
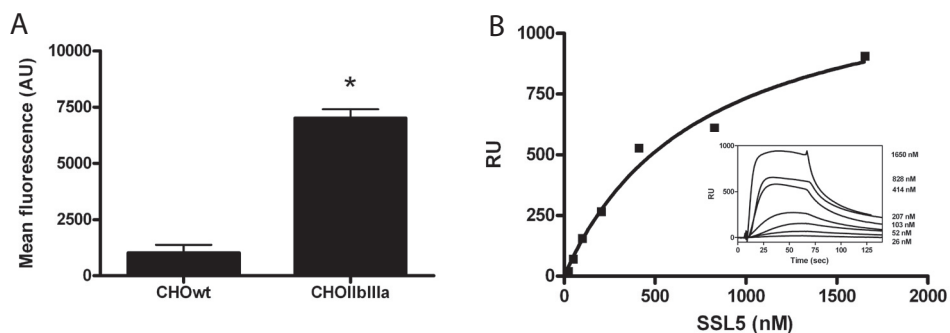

Figure 3 – SSL5 interacts with αIIbβ3. (A) SSL5 (10 μg/ml) was immobilized on a microtiter plate. Subsequently, calcein-labeled CHO cells transfected with αIIbβ3 (CHOIIbIIIa) or wild-type CHO cells (CHOwt) were allowed to adhere to SSL5 for 90 minutes at 37°C. Subsequently, wells were washed and bound cells were quantified by fluorescence readings. Mean data of 3 independent experiments, * = p<0.01, error bars indicate SEM. (B) αIIbβ3 (1500 RU) was immobilized on a CM5 sensor chip and binding of SSL5 was investigated by surface plasmon resonance. After adjusting for binding to a blanc channel, the response of SSL5 at equilibrium was determined and plotted against the concentration applied. Inset shows representative surface plasmon resonance traces of indicated concentrations of SSL5.

Platelet adhesion to immobilized SSL5 under flow conditions is dependent on GPIb and αIIbβ3

To examine whether platelets can interact with SSL5 under flow conditions, we perfused reconstituted blood (a mixture of washed platelets in HEPES-Tyrode buffer at pH 7.35 and isolated red cells, 40% hematocrit, 200.000 platelets/μl) over immobilized SSL5. Platelets readily adhered to immobilized SSL5 and were able to form large and stable aggregates (Figure 4A, supplemental video 1). Platelet adhesion and aggregate size was dependent on shear rate and adhesion was observed with shear rates up to 1600 s^{-1} (data not shown). Maximal surface coverage was observed at 300 s^{-1}, and therefore we continued investigation of platelet adhesion to SSL5 at 300 s^{-1}. Proteolysis of GPIbα by OSE reduced platelet

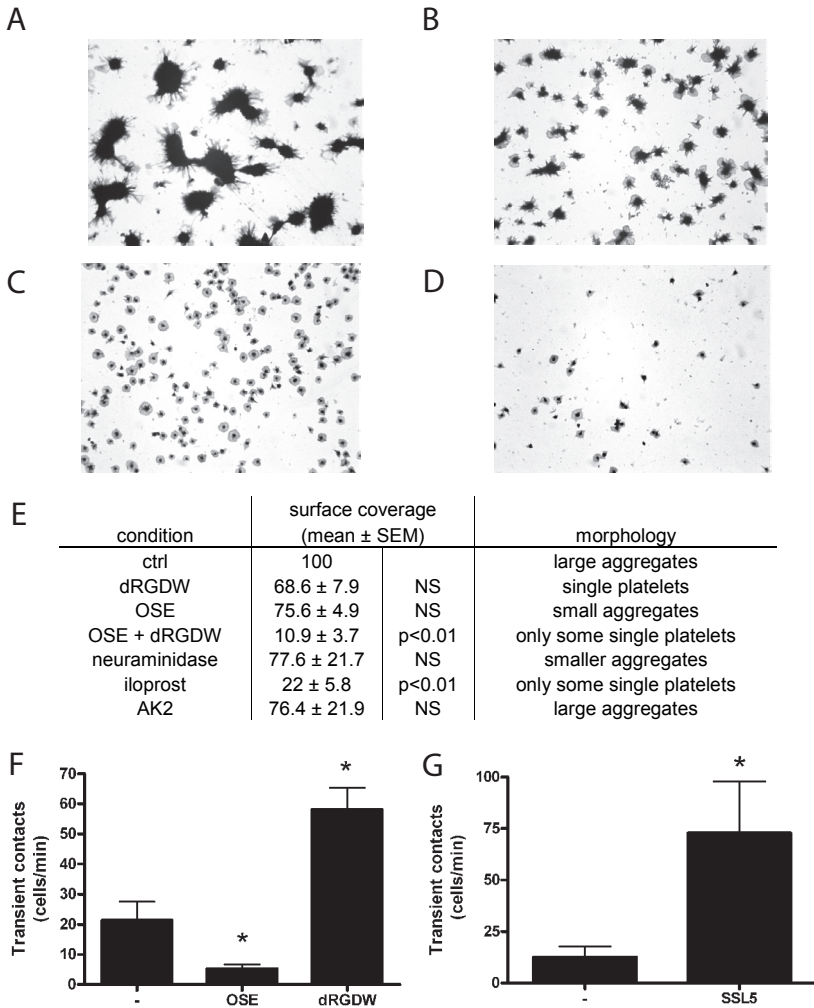| condition	surface coverage (mean ± SEM)		morphology
ctrl	100		large aggregates
dRGDW	68.6 ± 7.9	NS	single platelets
OSE	75.6 ± 4.9	NS	small aggregates
OSE + dRGDW	10.9 ± 3.7	p<0.01	only some single platelets
neuraminidase	77.6 ± 21.7	NS	smaller aggregates
iloprost	22 ± 5.8	p<0.01	only some single platelets
AK2	76.4 ± 21.9	NS	large aggregates

Figure 4 – Platelet adhesion to immobilized SSL5. Washed platelets were pretreated with (A) vehicle, (B) OSE, (C) dRGDW or (D) both OSE and dRGDW. Subsequently, reconstituted blood (washed platelets in HEPES-Tyrode buffer at pH 7.35 and isolated red cells, 40% hematocrit, 200.000 platelets/μl) was perfused over a coverslip coated with 400 nM SSL5 at a shear rate of 300 s^{-1} for 5 min at 37°C using a single-pass perfusion chamber. After perfusion, coverslips were fixed and stained with May-Grünwald/Giemsa and examined by light microscopy. Representative images of at least 3 independent experiments performed in triplicate are shown. (E) Washed platelets were pretreated with agents indicated. Subsequently, reconstituted blood was perfused over a coverslip coated with 400 nM SSL5 at a shear rate of 300 s^{-1} for 5 minutes at 37°C using a single-pass perfusion chamber. After perfusion, coverslips were fixed and stained with May-Grünwald/Giemsa and examined by light microscopy. Representative data of at least 2 independent experiments performed in triplicate is shown. (F) Washed platelets were pretreated with OSE or dRGDW and subsequently reconstituted blood was perfused over immobilized SSL5. Using real-time video analysis, transient contacts (defined as platelets adhering for less than 3 seconds) were scored. Mean data of 3 independent experiments are shown. Error bars indicate SEM. * = p<0.01. (G) Citrated whole blood was pre-activated with SFLLRN (15 μM) for 15 minutes at 37°C degrees and subsequently perfused over a coverslip coated with SSL5 or a coverslip coated with vehicle. Using real-time video analysis, transient contacts (defined as platelets adhering for less than 3 seconds) were scored. Mean data of 3 independent experiments. Error bars indicate SEM. * = p<0.05.

adhesion to SSL5, and resulted in formation of much smaller aggregates as compared to adhesion of platelets with functional GPIbα (Figure 4B, Figure 4E). Furthermore, addition of dRGDW, abrogated aggregate formation, and only single adhered platelets were observed (Figure 4C, Figure 4E). When platelets were pretreated with both OSE and dRGDW, platelet adhesion was almost completely abolished (Figure 4D, Figure 4E). Platelet activation was necessary for the interaction with immobilized SSL5, as iloprost (20 ng/ml) completely abolished platelet adhesion (Figure 4E). In contrast, an antibody against GPIbα (AK2, 10 µg/ml), interfering with the binding site for VWF on GPIbα, did not inhibit the interaction of platelets with SSL5 (Figure 4E).

These results suggest a role for GPIbα in initial adhesion to SSL5, after which αIIbβ3 is responsible for stable adhesion. To test this hypothesis, we performed real time analysis using differential interference contrast (DIC) microscopy and investigated the number of transient contacts as a measure for initial adhesion. As seen in Figure 4F, pretreatment of platelets with OSE resulted in a decrease in the number of transient contacts to SSL5 (supplemental video 2A). In contrast, dRGDW resulted in an increase in the number of transient contacts (Figure 4F, supplemental video 2B), although stable platelet adhesion was slightly reduced (Figure 4C, Figure 4E). These results support our hypothesis that GPIbα is necessary for initial adhesion and αIIbβ3 is necessary for stable adhesion of platelets to immobilized SSL5.

The results discussed so far were obtained using plasma-free, reconstituted blood. When citrated or low-molecular weight heparin-anticoagulated whole blood was perfused over immobilized SSL5, no adhesion was observed. To investigate whether platelets present in whole blood interacted at all with immobilized SSL5, we analyzed platelet adhesion by real-time video microscopy. We observed only minor interaction of platelets from whole blood with immobilized SSL5. However, when whole blood was pre-activated with the PAR-1 activating peptide SFLLRN (15 µM, 10 minutes), substantial platelet rolling and adhesion was observed, although these interactions did not lead to the formation of stable platelet aggregates (Figure 4G, Supplemental movie 3).

SSL5 increases platelet adhesion to endothelial cell matrix

To investigate the relevance of the SSL5-platelet interaction in a more physiologic context, we assessed platelet adhesion to endothelial cell matrix (ECM). First, we prepared ECM in a 96-wells plate and subsequently incubated the ECM with increasing concentrations of SSL5. As shown in Figure 5A, SSL5 readily bound to ECM with an apparent K_d of 0.8 ± 0.3 µM (Mean ± SD). In subsequent experiments we investigated SSL5 binding to purified components of the ECM and observed that SSL5 bound to vitronectin (K_d = 0.29 ± 0.08 µM, Mean ± SD), but did not interact with fibronectin, fibrinogen, laminin or collagens type I to V (data not shown).

To investigate the effect of SSL5 on platelet adhesion to ECM, we preincubated ECM-coated coverslips with SSL5 (1 µM, 60 minutes) before perfusing with whole blood for 5 minutes at 300 s^{-1}. As shown in Figure 5B, platelets primarily showed
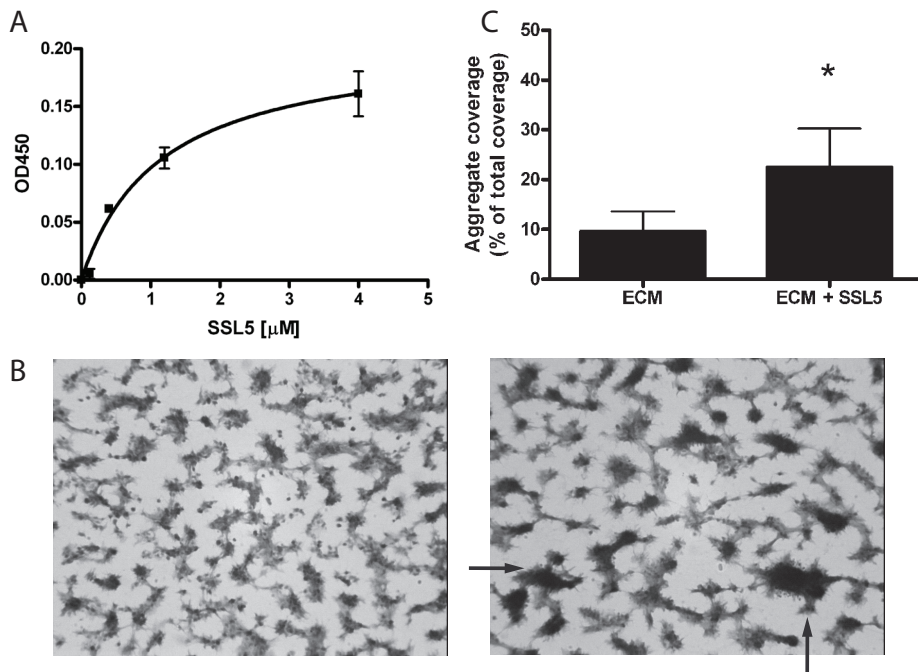
Figure 5 – SSL5 increases platelet aggregation to endothelial cell matrix. (A) ECM coated microtiter wells were incubated with increasing concentrations of SSL5 and bound SSL5 was detected by anti-X-press-HRP. Data of a single experiment representative for 3 independent experiments is shown (B) Whole blood was perfused over ECM coated coverslips incubated (right panel) with or (left panel) without SSL5 (1 µM). After perfusion, coverslips were fixed and stained with May-Grünwald/Giemsa and examined by light microscopy. Arrows indicate aggregates. Representative images of at least 3 independent experiments performed in triplicate are shown. (C) Platelet aggregates from experiment as described under B were quantified by calculating the percentage of the most intense areas compared to total surface coverage. Mean data of 6 independent experiments performed in triplicate is shown. Error bars indicate SEM. * = p<0.01.

spreading on ECM, whereas they additionally formed large aggregates when ECM was pretreated with SSL5. Interestingly, aggregates could only be observed on locations of initial platelet adhesion, indicating that the platelets did not show stable adhesion to SSL5 itself, but that SSL5 increases platelet aggregation rather than platelet adhesion. This was further demonstrated by calculating the amount of surface that was covered by the formed aggregates. Total surface coverage was not significantly different in the presence or absence of SSL5. However, when the surface coverage of areas with high density of staining was calculated as a measure for formed aggregates, preincubation of ECM with SSL5 showed a significant increase in the amount of aggregates formed as compared to ECM alone. Figure 5C shows the amount of aggregates as a percentage of total surface coverage, which was significantly increased upon preincubation with SSL5.

DISCUSSION

S. aureus excretes a variety of soluble proteins that are pivotal in host infection. Specifically, many of these proteins facilitate evasion of the host immune system. Virulence factors such as CHIPS and SCIN have been extensively characterized, and recently, we have identified SSL5 as an additional immune modulator excreted by *S. aureus*. SSL5 was shown to inhibit neutrophil rolling by interference with the PSGL-1-P-selectin interaction.[11] Here, we show that SSL5 interacts with GPIbα resulting in activation of platelets. In addition, SSL5 interacts with αIIbβ3 and the combined interaction of SSL5 with both adhesive proteins is sufficiently strong to support platelet adhesion under conditions of flow.

Platelets play an important role in the colonization of subendothelial tissue by *S. aureus*. Therefore, the enhancement of platelet aggregation to ECM by SSL5 might be an important mechanism to attract *S. aureus* to 'weak' spots in the endothelial layer. Subsequently, increased platelet adhesion will cause the attraction of additional neutrophils to the site of injury. However, SSL5 has already been shown to inhibit the attraction of neutrophils. In this way, SSL5 will first cause enhanced platelet aggregation at the site of injury, which is beneficial for the colonization of subendothelial tissue by *S. aureus*, while at the same time it anticipates on the counter effect of the immune system by interfering with the attraction of neutrophils (Figure 6).

We showed SSL5 to interact directly with GPIbα, which was anticipated from the notion that due to analogy in structure, multiple proteins that interact with GPIbα are also able to interact with PSGL-1. The interaction of SSL5 with GPIbα triggers signaling which is sufficiently strong to result in complete platelet aggregation even in the absence of exogenous fibrinogen, as evidenced by inhibition of aggregation by specific proteolysis of GPIbα by OSE, and translocation of 14-3-3ζ. In addition, αIIbβ3 was shown to interact with SSL5, and this interaction contributes to the adhesion of platelets to immobilized SSL5 under conditions of flow. SSL5 might interact with initially deposited platelets and thereby contribute to attraction of additional platelets, a mechanism we have previously postulated for GPIb-bound thrombin.[30]
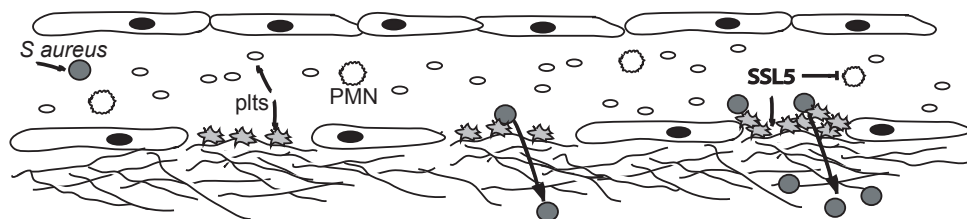
Figure 6 – Potential role for SSL5 in *S. aureus* colonization. Upon vascular damage, platelets adhere to exposed subendothelial proteins (left part). Subsequently, *S. aureus* interacts with platelets, which mediate colonization of the underlying vascular bed (middle part). SSL5 both inhibits the attraction of neutrophils and it increases platelet adhesion to the site of vascular damage, which accelerates the colonization of *S. aureus* (right part).

In our initial experiments, we studied platelet responses to SSL5 in plasma-free systems. Surprisingly, in platelet rich plasma or whole blood, the potent activatory effects of SSL5 initially could not be replicated. At present, the component(s) blocking SSL5 function in plasma are unknown but may involve albumin, or antibodies against SSL5 which are present in almost all healthy individuals. To investigate whether SSL5 may exert more subtle effects on platelets in plasma-containing environments, we performed experiments in a more physiological relevant context. First we showed that although platelets in a whole blood system did not interact with immobilized SSL5 under flow conditions, substantial interactions were observed when platelets were pre-activated by the PAR-1 activating peptide SFLLRN. In this model, no permanent platelet-SSL5 contacts could be demonstrated. In contrast, when we studied platelet adhesion to extracellular matrix generated from cultured primary endothelial cells, we observed SSL5 to significantly enhance platelet aggregate formation. Thus, in a plasma environment, SSL5 is not capable of directly activating platelets, but does facilitate platelet adhesion to a damaged vasculature. This implies that in vivo, platelets might need prior stimulation before bacterial proteins such as SSL5 can exert their activity, which prevents systemic platelet activation.

In conclusion, we have identified SSL5 as a novel ligand of GPIbα. In a purified system, the SSL5-GPIbα interaction is sufficiently potent to result in complete platelet aggregation. However, in a whole blood system, effects of SSL5 are more subtle, but still contribute significantly to a local increase in platelet adhesion and aggregation. This mechanism could stimulate the colonization of *S. aureus*, but may also contribute to some extent to *S. aureus*-induced thrombocytopenia. Inhibition of SSL5 could therefore be a potential target for treatment of *S. aureus* infection.

Acknowledgements

REFERENCES

1. Klevens RM, Morrison MA, Nadle J et al. Invasive methicillin-resistant Staphylococcus aureus infections in the United States. JAMA 2007;298:1763-1771.
2. Lowy FD. Staphylococcus aureus infections. N.Engl.J.Med. 1998;339:520-532.
3. Foster TJ. The Staphylococcus aureus "superbug". J.Clin.Invest 2004;114:1693-1696.
4. Hart RG, Foster JW, Luther MF, Kanter MC. Stroke in infective endocarditis. Stroke 1990;21:695-700.
5. Fitzgerald JR, Foster TJ, Cox D. The interaction of bacterial pathogens with platelets. Nat. Rev.Microbiol. 2006;4:445-457.
6. George NP, Konstantopoulos K, Ross JM. Differential kinetics and molecular recognition mechanisms involved in early versus late growth phase Staphylococcus aureus cell binding to platelet layers under physiological shear conditions. J.Infect.Dis. 2007;196:639-646.
7. Kerrigan SW, Clarke N, Loughman A et al. Molecular basis for Staphylococcus aureus-mediated platelet aggregate formation under arterial shear in vitro. Arterioscler.Thromb.Vasc.Biol. 2008;28:335-340.
8. Rooijakkers SH, van Kessel KP, van Strijp JA. Staphylococcal innate immune evasion. Trends Microbiol. 2005;13:596-601.
9. Rooijakkers SH, Ruyken M, Roos A et al. Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. Nat. Immunol. 2005;6:920-927.
10. de Haas CJ, Veldkamp KE, Peschel A et al. Chemotaxis inhibitory protein of Staphylococcus aureus, a bacterial antiinflammatory agent. J.Exp.Med. 2004;199:687-695.
11. Bestebroer J, Poppelier MJ, Ulfman LH et al. Staphylococcal superantigen-like 5 binds PSGL-1 and inhibits P-selectin-mediated neutrophil rolling. Blood 2007;109:2936-2943.
12. Langley R, Wines B, Willoughby N et al. The staphylococcal superantigen-like protein 7 binds IgA and complement C5 and inhibits IgA-Fc alpha RI binding and serum killing of bacteria. J.Immunol. 2005;174:2926-2933.
13. George JN, Nurden AT, Phillips DR. Molecular defects in interactions of platelets with the vessel wall. N.Engl.J.Med.

1984;311:1084-1098.
14. Harmon JT, Jamieson GA. The glycocalicin portion of platelet glycoprotein Ib expresses both high and moderate affinity receptor sites for thrombin. A soluble radioreceptor assay for the interaction of thrombin with platelets. J.Biol.Chem. 1986;261:13224-13229.
15. Weeterings C, de Groot PG, Adelmeijer J, Lisman T. The glycoprotein Ib-IX-V complex contributes to tissue factor-independent thrombin generation by recombinant factor VIIa on the activated platelet surface. Blood 2008;112:3227-3233.
16. Bradford HN, Pixley RA, Colman RW. Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. J.Biol.Chem. 2000;275:22756-22763.
17. Baglia FA, Shrimpton CN, Emsley J et al. Factor XI interacts with the leucine-rich repeats of glycoprotein Ibalpha on the activated platelet. J.Biol. Chem. 2004;279:49323-49329.
18. Joseph K, Nakazawa Y, Bahou WF, Ghebrehiwet B, Kaplan AP. Platelet glycoprotein Ib: a zinc-dependent binding protein for the heavy chain of high-molecular-weight kininogen. Mol.Med. 1999;5:555-563.
19. White TC, Berny MA, Tucker EI et al. Protein C supports platelet binding and activation under flow: role of glycoprotein Ib and apolipoprotein E receptor 2. J.Thromb.Haemost. 2008;6:995-1002.
20. Simon DI, Chen Z, Xu H et al. Platelet glycoprotein ibalpha is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). J.Exp.Med. 2000;192:193-204.
21. Pennings MT, Derksen RH, van LM et al. Platelet adhesion to dimeric beta-glycoprotein I under conditions of flow is mediated by at least two receptors: glycoprotein Ibalpha and apolipoprotein E receptor 2'. J.Thromb.Haemost. 2007;5:369-377.
22. Romo GM, Dong JF, Schade AJ et al. The glycoprotein Ib-IX-V complex is a platelet counterreceptor for P-selectin. J.Exp.Med. 1999;190:803-814.
23. Pendu R, Terraube V, Christophe OD et al. P-selectin glycoprotein ligand 1 and beta2-integrins cooperate in the adhesion of leukocytes to von Willebrand factor. Blood 2006;108:3746-3752.

24. Huizinga EG, Tsuji S, Romijn RA et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. Science 2002;297:1176-1179.
25. Sodetz JM, Pizzo SV, McKee PA. Relationship of sialic acid to function and in vivo survival of human factor VIII/von Willebrand factor protein. J.Biol.Chem. 1977;252:5538-5546.
26. Lisman T, Moschatsis S, Adelmeijer J, Nieuwenhuis HK, de Groot PG. 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. Blood 2003;101:1864-1870.
27. Kinlough-Rathbone RL, Perry DW, Rand ML, Packham MA. Responses to aggregating agents after cleavage of GPIb of human platelets by the O-sialoglycoprotein endoprotease from Pasteurella haemolytica- potential surrogates for Bernard-Soulier platelets? Thromb.Res. 2000;99:165-172.
28. Born GV. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 1962;194:927-929.
29. Sixma JJ, de Groot PG, van ZH, IJsseldijk M. A new perfusion chamber to detect platelet adhesion using a small volume of blood. Thromb.Res. 1998;92:S43-S46.
30. Weeterings C, Adelmeijer J, Myles T, de Groot PG, Lisman T. Glycoprotein Ibalpha-mediated platelet adhesion and aggregation to immobilized thrombin under conditions of flow. Arterioscler.Thromb.Vasc. Biol. 2006;26:670-675.
31. Sakariassen KS, Aarts PA, de Groot PG, Houdijk WP, Sixma JJ. A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components. J.Lab Clin.Med. 1983;102:522-535.
32. Remijn JA, Wu YP, Ijsseldijk MJ et al. Absence of fibrinogen in afibrinogenemia results in large but loosely packed thrombi under flow conditions. Thromb. Haemost. 2001;85:736-742.
33. Van Os E, Wu YP, Pouwels JG et al. Thrombopoietin increases platelet adhesion under flow and decreases rolling.

Br.J.Haematol. 2003;121:482-490.

34. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J.Clin.Invest 1973;52:2745-2756.

35. Lenting PJ, Neels JG, van den Berg BM et al. The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. J.Biol.Chem. 1999;274:23734-23739.

36. Munday AD, Berndt MC, Mitchell CA. Phosphoinositide 3-kinase forms a complex with platelet membrane glycoprotein Ib-IX-V complex and 14-3-3zeta. Blood 2000;96:577-584.

37. Urbanus RT, Pennings MT, Derksen RH, de Groot PG. Platelet activation by dimeric beta2-glycoprotein I requires signaling via both glycoprotein Ibalpha and apolipoprotein E receptor 2'. J.Thromb.Haemost. 2008;6:1405-1412.

Chapter 8

Discussion

Platelet activation and blood coagulation are two processes often studied separately, but which cannot be seen independently from each other. Platelets play a pivotal role in coagulation, not only by providing negatively charged phospholipids, but also in localizing the coagulation process from a diffuse plasma environment to an anionic surface, most likely via specific receptors on the platelets. On the other hand, the main product of coagulation, thrombin, is a potent platelet activator, thereby contributing to the exposure of negatively charged phospholipids on the activated platelet surface.

The main aim of this thesis was to investigate the interplay between platelets and coagulation in the haemostatic process. In this chapter, the contribution of our results to the current knowledge on the interplay between coagulation and platelets will be discussed in a broader perspective. First, the role of the platelet surface on the function of coagulation factors was investigated. Furthermore, the role coagulation factors could play in platelet deposition was examined. The central role of glycoprotein Ibα (GPIbα) in connecting coagulation and platelet function will be discussed and more light will be shed on how GPIbα can fulfill its various roles in haemostasis. Finally, the role of platelets in *S. aureus* infection will be discussed.

GPIbα is a multiligand receptor

After the discovery of GPIbα as the major platelet receptor for von Willebrand factor (VWF), multiple ligands for GPIbα have been described during the past 20 years, including coagulation factors IIa, XII, XI(a), high-molecular weight kininogen, (activated) protein C, the leukocyte receptor MAC-1, P-selectin and a dimeric form of beta2 glycoprotein I.[1-8] In this thesis, several additional ligands for GPIbα have been described.

First, we showed that rFVIIa was able to interact with platelets via GPIbα (chapter 3). We studied the interaction between the purified proteins in an ELISA setup, as well as with surface plasmon resonance. The presence of GPIbα resulted in acceleration of rFVIIa-mediated thrombin generation. Furthermore, we identified factor IX(a) as a novel ligand for GPIbα (chapter 4). Activated platelets were able to adhere to FIX(a), which was dependent on GPIbα. Proteolysis of GPIbα resulted in an increase in FIXa-mediated FXa-generation. Finally, the interaction of SSL5, a protein secreted by *S. aureus*, with GPIbα resulted in platelet activation and aggregation (chapter 7). Also, platelets were able to adhere to and form large stable aggregates on SSL5, which was dependent on GPIbα. Binding of SSL5 to endothelial cell matrix contributed to platelet deposition to endothelial cell matrix.

The role of GPIb in coagulation – mechanism of action of rFVIIa

rFVIIa has been developed for the treatment of haemophilia A and B patients, whose treatment has been complicated by the formation of inhibitors against FVIII or FIX, respectively. The mechanism of action was initially thought to proceed

exclusively by increased thrombin generation via a TF-dependent pathway. However, several groups speculated about a role for TF-independent thrombin generation as well.[9-11] This idea came from the notion that the optimal treatment for haemophilia patients requires much higher dosages of rFVIIa than would be expected from TF-dependent thrombin generation alone. The now widely used standard dose of rFVIIa (90 μg/kg) results in plasma levels that by far exceed the affinity constant (K_d) for TF binding, suggesting a TF-independent pathway would also account in part for the therapeutic efficacy of the drug.

It has been shown that rFVIIa can directly activate FX in the presence of calcium ions and negatively charged phospholipids, even in the absence of TF.[11-13] Furthermore, rFVIIa was shown to activate FX on monocytes or platelets independent of TF. However, the activation of FX by rFVIIa on anionic phospholipids in the absence of TF is an inefficient process and therefore we hypothesized the presence of a platelet receptor to catalyze this process. In chapter 3, we showed that rFVIIa interacts with GPIbα on the activated platelet surface and that this interaction contributes to rFVIIa-mediated thrombin generation. This observation is important in unraveling the exact mechanism of action of rFVIIa.

In chapter 3, we report an approximate K_d of about 80 nM of rFVIIa for GPIbα. The affinity for GPIbα resembles the predicted affinity of rFVIIa for the platelet surface, which was estimated to be about 90 nM.[9] Therefore, it seems plausible that GPIbα is the receptor responsible for platelet binding of rFVIIa. However, surface plasmon resonance studies show that although there is about 1000 RU of GPIbα immobilized on the sensor chip, only a small fraction can interact with rFVIIa, as evidenced by the relatively low response units after saturating binding of rFVIIa. A similar discrepancy has been observed for the binding of thrombin to GPIbα. Although there are about 25000 copies of GPIbα present on the platelet surface, only a small fraction (100 to 400) is involved in high-affinity binding of thrombin.[14,15] This difference might be ascribed to the localization of GPIbα in lipid rafts. Lipid rafts are specific patches on the membranes of many cells, enriched in cholesterol and glycosphingolipids and it was suggested that only GPIbα that is localized in lipid rafts might be able to bind thrombin.[16,17] In our study, we investigated the interaction between purified soluble GPIbα and rFVIIa, and therefore the partial localization of GPIbα in rafts could not explain the observed differences. An explanation could be that GPIbα immobilizes to the sensor chip preferentially with the same domain that is involved in the interaction with rFVIIa, although protein immobilization on a sensor chip is assumed to proceed randomly. Another interesting explanation could be that the plasma purified form of GPIbα is in a different conformation from GPIbα on the platelet surface. Obviously, the purified form is not in complex with the other subunits of the GPIb-complex, which makes it different from GPIbα present on the platelet surface, but also the conformation of the Ibα subunit may differ in the purified form. It would be of interest to investigate the number of binding sites for rFVIIa on the activated platelet surface in combination with the number of binding sites for rFVIIa on the activated platelet surface in which GPIbα is involved. In this way, it can be estimated how many GPIbα molecules are involved in the interaction with rFVIIa.

This would provide more insight into whether any specific lipid environment or conformational change in GPIbα is required for the interaction with rFVIIa.

It has been speculated by Munnix et al[18] that only a small fraction of the platelet population contributes to procoagulant activity after stimulation, whereas another fraction of the platelets is involved in platelet aggregation. Furthemore, Kjalke et al showed that FITC-labeled rFVIIa preferentially bound to a subgroup of platelets after activation with a combination of thrombin and convulxin.[19] Studies by our group did not particularly point towards a preferential binding of rFVIIa to a specific subpopulation of platelets, although we cannot exclude this to occur (chapter 3 and [20]). A potential explanation for this discrepancy could be that the experiments by Munnix et al were mostly performed under conditions of flow, whereas studies by our group were mostly done under static conditions, although experiments under conditions of flow also did not suggest a specific localization of rFVIIa to specific platelets.[20] Shear stress could select platelets to perform a specific role in thrombus formation, e.g. participate in platelet aggregation or participate in fibrin generation. Thus, it would be interesting to investigate the distribution of rFVIIa in a thrombus formed under shear flow, using high-resolution microscopy methods that are currently becoming available.

The interaction of rFVIIa with GPIbα offers an interesting opportunity for the improvement of its therapeutic efficacy. Recently, Persson et al reported a variant of rFVIIa with similar TF-dependent, but improved TF-independent thrombin generation capacity compared to wild-type rFVIIa.[21] Whether the efficacy of this variant is dependent on GPIbα remains to be established, but based upon our observations, constructing a variant of rFVIIa with an improved binding capacity for activated platelets could benefit its therapeutic efficacy. However, the exact residues within rFVIIa and GPIbα that are required for the interaction are not yet known and therefore need to be identified.

Besides its use in haemophilia patients, rFVIIa also showed indications for use in several platelet disorders, such as Glanzmann's thrombasthenia and thrombocytopenia.[20,22-24] Patients with the rare platelet disorder Bernard-Soulier syndrome (BSS) are characterized with a bleeding tendency, caused by the lack of (functional) GPIbα on their platelet surface. The involvement of GPIbα in the mechanism of action of rFVIIa would imply that BSS-patients do not benefit or benefit to a lesser extent from treatment with rFVIIa, as patients with other platelet disorders. However, several reports have described the successful treatment of BSS-patients with rFVIIa.[25-27] This most likely is due to thrombin generation independent of the activated platelet surface, which seems plausible considering the low platelet counts observed in BSS-patients. Furthermore, platelets from BSS patients showed an increase in PS exposure on the platelet surface, which results in a higher coagulation potential and could compensate to some extent for the lack in platelet numbers.[28] However, it is not clear yet whether BSS patients require higher or multiple doses of rFVIIa to treat bleeding compared to haemophilia patients for example. Therefore, it is difficult to compare the efficacy of rFVIIa in BSS patients with its use in other patient groups and more research is required to study the exact efficacy of rFVIIa in these patients.

The role of GPIbα in coagulation – consequences for other coagulation factors

While the presence of GPIbα increases thrombin generation mediated by rFVIIa, it inhibits FXa-generation (and subsequent thrombin generation) by FIXa (chapter 4). Thus the role of GPIbα in coagulation is not to only accelerate coagulation, but it seems to carefully modulate thrombin generation mediated by different coagulation pathways. The reason for this differential regulation remains unclear and the function of GPIbα in coagulation should be studied in more complex systems. The discrepancy between FXa-generation by rFVIIa and FIXa could be explained by a model depicted in Figure 1, which shows the potential role for GPIbα in different stages of coagulation. rFVIIa is important in the initiation phase of coagulation, where platelets are only slightly activated and the GPIb-complex is present on the platelet surface. GPIbα accelerates rFVIIa-mediated thrombin generation and the formed thrombin then further activates platelets and activates the feedback loop of coagulation via activation of FXI. Following platelet activation, surface expression of GPIbα is reduced, which is most likely due to shedding from the surface by proteases such as ADAM17 and internalization to intracellular compartments.[29-31] The absence of GPIbα then results in increased FXa-generation by FIXa, thereby accelerating the propagation phase of coagulation, as depicted in Figure 1. However, whether proteolysis of GPIbα precedes the propagation phase of coagulation is currently not known. Future studies will have to elucidate the exact role GPIbα plays in the sequence of events that ultimately lead to thrombin generation.

It has been shown that factor XI interacts with GPIbα. The binding site for FXI is located within the leucine-rich repeats of GPIbα and the interaction of FXI with GPIbα could be inhibited with the A1-domain of VWF.[3] It was demonstrated that the interaction of both thrombin and FXI with one GPIb-molecule or two adjacent GPIb-molecules could accelerate the rate of FXI activation by thrombin, but those results were recently challenged.[32] Furthermore, GPIbα has been identified as the high-affinity receptor on platelets for thrombin.[1] Recently, two groups independently reported the crystal structure of thrombin with GPIbα, suggesting thrombin can bind to one molecule of GPIbα on two separate locations.[33,34] However, the consequences of thrombin binding are not completely understood, but might have implications for the function of GPIbα. The interaction between thrombin and GPIbα does accelerate proteolysis of protease-activated receptor 1 (PAR1) by thrombin.[35] In this way, low concentrations of thrombin are able to activate platelets, which contributes to the exposure of negatively charged phospholipids on the platelet surface. So, multiple coagulation factors interact with GPIbα and it seems that in this way GPIbα can modulate coagulation on the activated platelet surface, although the exact mechanisms remain to be identified.

Thrombin contributes to platelet adhesion via interaction with GPIbα

Thrombin has a central role in hemostasis. It activates platelets, cleaves fibrinogen into fibrin, and activates factor XIII. Furthermore, thrombin enhances coagulation
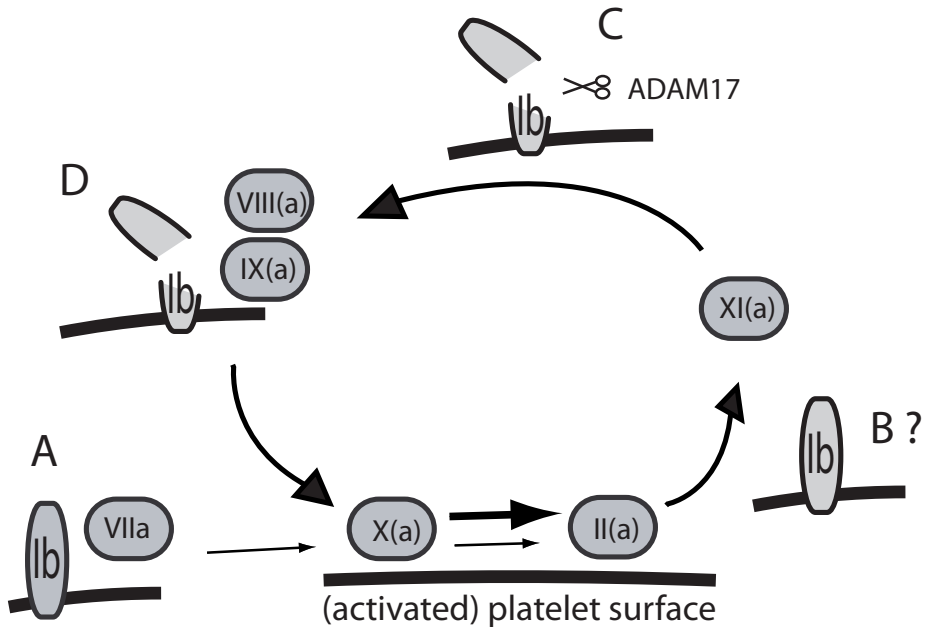
Figure 1 – A general model for the role of GPIbα in coagulation. (A) rFVIIa-mediated generation of FXa and subsequent thrombin is accelerated in the presence of GPIbα (Ib). (B) Thrombin in its turn activates FXI in which the role for GPIbα remains elusive. Furthermore it activates platelets via a combination of GPIbα and the protease-activated receptors 1 and 4. (C) Upon platelet activation, sheddases such as ADAM17 can cleave GPIbα from the platelet surface. (D) FIXa-mediated FXa generation is accelerated upon proteolysis of GPIbα.

by activating factors V, VIII, and XI, but it also inhibits coagulation by activating protein C, and attenuates fibrinolysis by activating thrombin activatable fibrinolysis inhibitor (TAFI). In chapter 5, we have shown that the interaction between thrombin and GPIbα can result in stable platelet adhesion and the formation of large aggregates. When thrombin is bound to fibrin, it is able to contribute to platelet deposition to fibrin as well as to directly capture platelets in a GPIbα-dependent manner. This identifies yet another role for thrombin in haemostasis.

In a review by Lane et al, the sequence of events leading to stable platelet plug formation is described and the role thrombin plays in this process is highlighted.[36] Crucial functions for thrombin in this process are the generation of fibrin and the activation of platelets. How thrombin that is bound to the fibrin clot, is able to activate platelets is not explained and most likely requires the transfer from the fibrin clot to the platelet surface. The findings in chapter 5 demonstrate that thrombin is able to interact at the same time with fibrin via exosite I and with GPIbα on the platelet surface via exosite II. Our results provide a possible explanation for how thrombin is able to transfer from the fibrin clot to the platelet surface, which is crucial for thrombin to perform its diverse functions. Thrombin is initially involved in the conversion from fibrinogen to fibrin and will be bound to fibrin upon formation of the fibrin clot. Platelets can bind to fibrin via αIIbβ3 and to fibrin-

bound thrombin via GPIbα, as demonstrated in chapter 5. In this way, thrombin could be able to transfer from fibrin to the platelet surface via the interaction with GPIbα, ultimately leading to activation of the platelet via PAR1.

The role of FXII in thrombus formation

The prominent role of FXII in coagulation in vitro combined with the lack of a bleeding diathesis in FXII-deficient patients raises an interesting question about the relevance of FXII in thrombosis and haemostasis in vivo. In addition, epidemiological studies showed that low FXII levels in plasma resulted in an increased risk for thrombosis,[37] indicating a protective role for FXII that is most likely not related to its coagulant properties. Besides its potential to propagate coagulation in vitro, FXII has also been shown to be able to activate the kallikrein-kinin system by activation of prekallikrein. Activation of the kallikrein-kinin system by FXIIa leads to release of bradykinin from high-molecular-weight kininogen and regulates inflammation, blood pressure control, and pain. Therefore, Maas et al suggested that the potential of FXII to activate the kallikrein-kinin system is responsible for the protective effect of high FXII levels on arterial thrombosis.[38]

In chapter 6, we observed that FXII can interact with immobilized VWF and this suggests a potential third role for FXII in haemostasis. Studies using flow models showed that FXIIa resulted in increased platelet deposition to VWF. Platelet adhesion was studied in a plasma-free system using reconstituted blood, which rules out a potential effect of coagulation or the kallikrein-kinin system. Studies by Renné and coworkers demonstrated that FXII-deficient mice, which as similar to their human counterparts do not suffer from a bleeding disorder, showed reduced thrombus formation upon vascular injury, and this could be restored by infusion of FXII.[39] Although experiments by Johne et al suggest that FXII-dependent coagulation is responsible for the role of FXII in thrombus formation,[40] we suggest that it could be the interaction of FXII with VWF and its contribution to platelet deposition to VWF that might be responsible for the effect of FXII in thrombus formation. The epidemiological studies investigating the role of FXII in thrombosis mainly focused on its function in coagulation, but it could be more interesting to focus on the relation between FXII and (activated) VWF levels and the risk for thrombosis.

The effect of coagulation factor binding on the function of GPIbα

The studies in this thesis focus mainly on the role of GPIbα in modulating coagulation. The effect of the interaction of GPIbα with clotting factors on the function of GPIbα remains to be established. It has been speculated that the interaction of thrombin with GPIbα increases its affinity for VWF.[33,41]

An interesting study regarding the role of thrombin in platelet adhesion to VWF was performed by the group of McCarty.[42] They have used a thrombin mutant, which selectively activates protein C, but has a diminished capacity to cleave fibrinogen and hydrolyse PAR1. This thrombin mutant did not support platelet adhesion to the same extent as wild-type thrombin, but platelets could roll and tether under conditions of flow to this thrombin mutant in a GPIbα-dependent

manner, which confirmed our results that stable platelet adhesion to immobilized thrombin was dependent on both PAR1 and GPIbα. Interestingly, although still able to bind GPIbα, the mutant thrombin inhibited platelet deposition to collagen and VWF. The exact consequences of thrombin binding to GPIbα therefore remain to be established.

FXIIa has been shown to interact with GPIbα and this interaction inhibited platelet aggregation induced by low concentrations of thrombin.[2] In chapter 6, we describe that FXIIa increases platelet deposition to VWF. Combining these two observations could suggest that FXIIa can interact with both GPIbα on the platelet and immobilized VWF at the same time, thereby increasing the affinity of GPIbα for VWF. Whether the interaction between GPIbα and FXIIa is responsible for the increased platelet deposition to VWF remains to be investigated.

"Active" GPIbα

The interaction between rFVIIa and FIX(a) with GPIbα on the platelet surface described in this thesis, seems to predominantly occur after stimulation of platelets. Also, the interaction of FXI with GPIbα on platelets benefits from stimulation with SFLLRN or thrombin.[3] Why platelet activation is necessary for optimal binding has not yet been elucidated. A possible explanation is that GPIbα requires a conformational change or a change in lipid environment in order to become able to bind coagulation factors.[43]

It has already been described that, upon platelet stimulation, GPV is rapidly shed by caspase-dependent proteolysis.[29,44] This might have several implications for the GPIb-complex. It has been speculated that proteolysis of GPV is necessary for potential clustering of GPIbα. This could occur via interaction of two GPIbα molecules of the same complex, leading to intracellular signaling, or via interaction of two GPIbα molecules from different complexes on the same platelet or even on two opposing platelets (chapter 1A and [45]). Whether potential GPIbα clustering is only induced by ligand binding is not yet clear. The possibility remains that proteolysis of GPV by itself is already sufficient for the clustering of GPIbα. Studies from GPV-deficient mice show that GPV might play a role in the platelet response to thrombin and proteolysis of GPV could expose a new receptor for thrombin.[46] A similar mechanism could be involved in the interaction of rFVIIa or FIXa with the GPIb-complex as well and proteolysis of GPV might be required to expose new binding sites on the platelet surface.

Another interesting thought is that GPIbα needs a specific lipid microenvironment in order to function properly, for example lipid rafts. Multiple cytoskeletal and signaling proteins have been shown to be associated with lipid rafts and therefore they are thought to play an important role in intracellular signaling. It has already been shown that a fraction of the GPIb-IX-V complex resides within lipid rafts, and that this localization is important for platelet adhesion to VWF and subsequent signaling.[16] Furthermore, lipid rafts are about two to three-fold enriched in negatively charged phospholipids.[47,48] Moreover, procoagulant microparticles were shown to be highly enriched in negatively charged phospholipids and appear to originate from lipid rafts (reviewed by Lopez et al[49]). Taken together, the presence

of GPIbα in lipid rafts, enriched in negatively charged phospholipids, would be an ideal environment for coagulation factors such as FVIIa. The interaction of their Gla domain with negatively charged phospholipids and another domain with GPIbα would specifically localize them to similar regions, thereby enhancing the potential of thrombin formation.

GPIbα as an intermediate station
The list of ligands for GPIbα seems to be an ever growing one and here we add several additional ligands to this list. It seems unthinkable that all these proteins bind to the same molecule of GPIbα at the same time, because of steric hindrance issues or potential overlap in binding sites between the several ligands. It could be that every protein has its specific set of GPIbα-receptors, but the question then arises how these different sets of receptors can distinguish between the different ligands. Another explanation could be that GPIbα acts as a docking site from which, depending on the ligand, it directs its ligand towards another receptor. This receptor then finally influences the function of the ligand or results in intracellular platelet signaling.

This hypothesis comes from observations that several receptors have been shown to colocalize with GPIbα on the platelet surface, mostly upon platelet activation. The binding of beta2-glycoprotein I has been demonstrated to be dependent on both GPIbα and the LDL-receptor family member apoER2',[8,50] which have been found to colocalize in electron microscopy studies. Also, both apoER2' and GPIbα are involved in the binding of (activated) protein C and FXI to the platelet surface.[5,51] Furthermore, GPIbα has been shown to be associated with the Fc receptor FcγRIIa, FcRγ-chain and GPVI.[52-54] In this thesis we describe that rFVIIa and FIX(a) interact with GPIbα. However, the role of other platelet receptors can not be excluded. Furthermore, platelet adhesion to thrombin was shown to be dependent on GPIbα as well as PAR1 and the interaction between thrombin and GPIbα accelerates PAR1 cleavage,[35] suggesting that GPIbα and PAR1 are in close vicinity of each other. In addition, we have shown that SSL5 activates and aggregates platelets via a combination of GPIbα and αIIbβ3, again indicating that GPIbα is most likely not the only receptor responsible for the actual effects of the ligand. So, as probably more ligands for GPIbα will be discovered in the future, one might suggest that these studies should not focus on GPIbα alone, but should also focus on the association of GPIbα with a second receptor to better understand subsequent events such as intracellular signaling.

GPIbα as a signaling receptor?
There are many different signaling pathways operating upon platelet activation. At present, not much is know about GPIbα as a signaling receptor. Only some signaling molecules have been shown to be exclusively linked to GPIbα. It has been shown that the adapter protein 14-3-3ζ is connected to GPIbα, as well as other signaling molecules such as Dab1 and PI3-kinase, and GPIbα is connected to the cytoskeleton via these proteins. It has been speculated that ligands binding to GPIbα can cause signaling, but the evidence for direct signaling via GPIbα

remains scarce and most signaling via GPIbα might involve other receptors as well. For example, several studies have shown that the interaction between porcine VWF and GPIbα results in tyrosine phosphorylation of FcRγ-chain,[53,55] which is responsible for most of the intracellular signaling. Furthermore, one of the most important consequences of GPIbα signaling might involve the activation of αIIbβ3. An interesting study by Kasirer-Friede et al[56] showed that after inducing clustering of GPIbα by using small molecule dimerizer technology, the signaling molecule Syk is tyrosine phosphorylated, resulting in the activation of αIIbβ3. Although Syk is a general signaling molecule in the activated platelet, this study shows that GPIbα is able to induce intracellular signaling, regardless whether this is via a direct or indirect mechanism. Another study by the same group showed that GPIbα is capable of signaling by itself after binding of VWF, as they showed that in a FcRγ(-/-) mouse, signaling was impaired but not completely abolished.[57]

In this thesis we showed that SSL5, a protein secreted by *S. aureus*, can induce signaling via GPIbα. Question remains whether other receptors are involved in this signaling pathway. Although we show 14-3-3ζ translocation to the cytoskeleton, a process thought to be a specific GPIbα signaling event, we cannot exclude the role of other receptors. SSL5 is such a potent platelet activator, and therefore it seems unlikely that it exerts its effect exclusively via GPIbα, because up to now no other GPIbα ligands have been found that are able to induce such strong effects on platelets via GPIbα alone. We already suggested that (activated) αIIbβ3 is involved in outside-in signaling by SSL5 and this might account for most of the signaling events that occur in SSL5-activated platelets. To a certain extent, the binding of SSL5 to platelets involves the same receptors as the interaction between platelets and VWF, and therefore a role of the FcRγ-chain, known to be involved in platelet signaling upon VWF binding, would be interesting to investigate.

S. aureus infection and hemostasis

S. aureus infection often is accompanied with thrombotic complications. Several surface-expressed factors as well as secreted virulence factors aid to the disease progress observed in *S. aureus* infection. In chapter 7, we identified the secreted virulence factor SSL5 as a strong platelet agonist in vitro, via interacting with GPIbα. In addition, SSL5 interacts with αIIbβ3 and the combined interaction of SSL5 with both adhesive proteins is sufficiently strong to support platelet adhesion under conditions of flow.

Platelets play an important role in the colonization of subendothelial tissue by *S. aureus*. Therefore, the enhancement of platelet aggregation to ECM by SSL5 might be an important mechanism to attract *S. aureus* to 'weak' spots in the endothelial layer. Platelets will attract neutrophils to the site of injury and therefore an increase in platelet adhesion will result in the attraction of additional neutrophils. However, SSL5 has already been shown to inhibit the attraction of neutrophils by interfering with the interaction between P-selectin glycoprotein ligand 1 (PGSL-1) and P-selectin,[58] thereby anticipating on the counter effect of the immune system. It remains to be established what the net effect of these two mechanisms will

be and it would be interesting to study both processes of platelet adhesion and neutrophil attraction together in an in vivo model.

The results in chapter 7 indicate that the interaction between SSL5 and GPIbα is involved in *S. aureus* infection. Furthermore, it has been shown that protein A expressed on the surface of *S. aureus* can interact via VWF with GPIbα as well (reviewed by Fitzgerald et al[59]). This suggests that GPIbα plays an important role in *S. aureus* infection. Therefore, targeting of GPIbα could be an interesting application to control the thrombotic complications that are observed in infections with *S. aureus*.

Concluding remarks

Since the discovery of GPIbα, multiple proteins have been found to interact with GPIbα, among which a substantial amount of proteins of the coagulation system. This led to our hypothesis that GPIbα might play a central role in modulating coagulation. Whether there are several subpopulations of GPIbα that are involved in the interaction with its variety of ligands and whether the clustering of GPIbα is important for its function, requires more exploration and offers interesting opportunities for future research. Most likely, more proteins will be identified to interact with GPIbα and future studies will have to deal with the interaction of multiple proteins with GPIbα in a more complex system and will have to study the consequences on the function of GPIbα.

REFERENCES

1. Harmon JT, Jamieson GA. The glycocalicin portion of platelet glycoprotein Ib expresses both high and moderate affinity receptor sites for thrombin. A soluble radioreceptor assay for the interaction of thrombin with platelets. J.Biol.Chem. 1986;261:13224-13229.

2. Bradford HN, Pixley RA, Colman RW. Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. J.Biol.Chem. 2000;275:22756-22763.

3. Baglia FA, Shrimpton CN, Emsley J et al. Factor XI interacts with the leucine-rich repeats of glycoprotein Ibalpha on the activated platelet. J.Biol.Chem. 2004;279:49323-49329.

4. Joseph K, Nakazawa Y, Bahou WF, Ghebrehiwet B, Kaplan AP. Platelet glycoprotein Ib: a zinc-dependent binding protein for the heavy chain of high-molecular-weight kininogen. Mol.Med. 1999;5:555-563.

5. White TC, Berny MA, Tucker EI et al. Protein C supports platelet binding and activation under flow: role of glycoprotein Ib and apolipoprotein E receptor 2. J.Thromb.Haemost. 2008;6:995-1002.

6. Simon DI, Chen Z, Xu H et al. Platelet glycoprotein ibalpha is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). J.Exp.Med. 2000;192:193-204.

7. Romo GM, Dong JF, Schade AJ et al. The glycoprotein Ib-IX-V complex is a platelet counterreceptor for P-selectin. J.Exp.Med. 1999;190:803-814.

8. Pennings MT, Derksen RH, van Lummel M et al. Platelet adhesion to dimeric beta-glycoprotein I under conditions of flow is mediated by at least two receptors: glycoprotein Ibalpha and apolipoprotein E receptor 2'. J.Thromb.Haemost. 2007;5:369-377.

9. Monroe DM, Hoffman M, Oliver JA, Roberts HR. Platelet activity of high-dose factor VIIa is independent of tissue factor. Br.J.Haematol. 1997;99:542-547.

10. Lisman T, de Groot PG. Mechanism of action of recombinant factor VIIa. J.Thromb.Haemost. 2003;1:1138-1139.

11. Telgt DS, Macik BG, McCord DM, Monroe DM, Roberts HR. Mechanism by which recombinant factor VIIa shortens the aPTT: activation of factor X in the absence of tissue factor. Thromb.Res. 1989;56:603-609.

12. Bom VJ, Bertina RM. The contributions of Ca2+, phospholipids and tissue-factor apoprotein to the activation of human blood-coagulation factor X by activated factor VII. Biochem.J. 1990;265:327-336.

13. Miletich JP, Jackson CM, Majerus PW. Interaction of coagulation factor Xa with human platelets. Proc.Natl. Acad.Sci.U.S.A 1977;74:4033-4036.

14. De Marco L, Mazzucato M, Fabris F et al. Variant Bernard-Soulier syndrome type bolzano. A congenital bleeding disorder due to a structural and functional abnormality of the platelet glycoprotein Ib-IX complex. J.Clin.Invest 1990;86:25-31.

15. De Marco L, Mazzucato M, Masotti A, Fenton JW, Ruggeri ZM. Function of glycoprotein Ib alpha in platelet activation induced by alpha-thrombin. J.Biol.Chem. 1991;266:23776-23783.

16. Shrimpton CN, Borthakur G, Larrucea S et al. Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation. J.Exp.Med. 2002;196:1057-1066.

17. VanHoorelbeke K, Ulrichts H, Romijn RA, Huizinga EG, Deckmyn H. The GPIbalpha-thrombin interaction: far from crystal clear. Trends Mol.Med. 2004;10:33-39.

18. Munnix IC, Kuijpers MJ, Auger J et al. Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation: regulation by transient integrin activation. Arterioscler.Thromb.Vasc.Biol. 2007;27:2484-2490.

19. Kjalke M, Kjellev S, Rojkjaer R. Preferential localization of recombinant factor VIIa to platelets activated with a combination of thrombin and a glycoprotein VI receptor agonist. J.Thromb.Haemost. 2007;5:774-780.

20. Lisman T, Moschatsis S, Adelmeijer J, Nieuwenhuis HK, de Groot PG. 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. Blood 2003;101:1864-1870.

21. Persson E, Kjalke M, Olsen OH. Rational design of coagulation factor VIIa variants with substantially increased intrinsic activity. Proc.Natl.Acad. Sci.U.S.A 2001;98:13583-13588.

22. Poon MC, d'Oiron R, Hann I et al. Use of recombinant factor VIIa (NovoSeven) in patients with Glanzmann thrombasthenia. Semin. Hematol. 2001;38:21-25.

23. Kristensen J, Killander A, Hippe E et al. Clinical experience with recombinant factor VIIa in patients with thrombocytopenia. Haemostasis 1996;26 Suppl 1:159-164.

24. Lisman T, Adelmeijer J, Cauwenberghs S et al. Recombinant factor VIIa enhances platelet adhesion and activation under flow conditions at normal and reduced platelet count. J.Thromb.Haemost. 2005;3:742-751.

25. Peters M, Heijboer H. Treatment of a patient with Bernard-Soulier syndrome and recurrent nosebleeds with recombinant factor VIIa. Thromb.Haemost. 1998;80:352.

26. Ozelo MC, Svirin P, Larina L. Use of recombinant factor VIIa in the management of severe bleeding episodes in patients with Bernard-Soulier syndrome. Ann.Hematol. 2005;84:816-822.

27. Tefre KL, Ingerslev J, Sorensen B. Clinical benefit of recombinant factor VIIa in management of bleeds and surgery in two brothers suffering from the Bernard-Soulier syndrome. Haemophilia. 2009;15:281-284.

28. Bevers EM, Comfurius P, Nieuwenhuis HK et al. Platelet prothrombin converting activity in hereditary disorders of platelet function. Br.J.Haematol. 1986;63:335-345.

29. Gardiner EE, Karunakaran D, Shen Y et al. Controlled shedding of platelet glycoprotein (GP)VI and GPIb-IX-V by ADAM family metalloproteinases. J.Thromb. Haemost. 2007;5:1530-1537.

30. Bergmeier W, Piffath CL, Cheng G et al. Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates GPIbalpha shedding from platelets in vitro and in vivo. Circ.Res. 2004;95:677-683.

31. van Zanten GH, Heijnen HF, Wu Y et al. A fifty percent reduction of platelet surface glycoprotein Ib does not affect platelet adhesion under flow conditions.

Blood 1998;91:2353-2359.

32. Baglia FA, Badellino KO, Li CQ, Lopez JA, Walsh PN. Factor XI binding to the platelet glycoprotein Ib-IX-V complex promotes factor XI activation by thrombin. J.Biol.Chem. 2002;277:1662-1668.

33. Celikel R, McClintock RA, Roberts JR et al. Modulation of alpha-thrombin function by distinct interactions with platelet glycoprotein Ibalpha. Science 2003;301:218-221.

34. Dumas JJ, Kumar R, Seehra J, Somers WS, Mosyak L. Crystal structure of the GpIbalpha-thrombin complex essential for platelet aggregation. Science 2003;301:222-226.

35. De Candia E, Hall SW, Rutella S et al. Binding of thrombin to glycoprotein Ib accelerates the hydrolysis of Par-1 on intact platelets. J.Biol.Chem. 2001;276:4692-4698.

36. Lane DA, Philippou H, Huntington JA. Directing thrombin. Blood 2005;106:2605-2612.

37. Doggen CJ, Rosendaal FR, Meijers JC. Levels of intrinsic coagulation factors and the risk of myocardial infarction among men: Opposite and synergistic effects of factors XI and XII. Blood 2006;108:4045-4051.

38. Maas C, Govers-Riemslag JW, Bouma B et al. Misfolded proteins activate factor XII in humans, leading to kallikrein formation without initiating coagulation. J.Clin.Invest 2008;118:3208-3218.

39. Renne T, Pozgajova M, Gruner S et al. Defective thrombus formation in mice lacking coagulation factor XII. J.Exp. Med. 2005;202:271-281.

40. Johne J, Blume C, Benz PM et al. Platelets promote coagulation factor XII-mediated proteolytic cascade systems in plasma. Biol.Chem. 2006;387:173-178.

41. Mendolicchio GL, Celikel R, Varughese KI, Savage B, Ruggeri ZM. Modulation of von Willebrand Factor A1 Domain-Mediated Platelet Aggregation/Agglutination by alpha-Thrombin. [abstract]. Blood 2004;104:182a.

42. Berny MA, White TC, Tucker EI et al. Thrombin mutant W215A/E217A acts as a platelet GPIb antagonist. Arterioscler.Thromb. Vasc.Biol. 2008;28:329-334.

43. Weeterings C, Lisman T, de Groot PG. Tissue factor-independent effects of recombinant factor VIIa on hemostasis. Semin.Hematol. 2008;45:S12-S15.

44. Rabie T, Strehl A, Ludwig A, Nieswandt B. Evidence for a role of ADAM17 (TACE) in the regulation of platelet glycoprotein V. J.Biol.Chem. 2005;280:14462-14468.

45. Lisman T, Weeterings C, de Groot PG. Platelet aggregation: involvement of thrombin and fibrin(ogen). Front Biosci. 2005;10:2504-2517.

46. Ramakrishnan V, DeGuzman F, Bao M et al. A thrombin receptor function for platelet glycoprotein Ib-IX unmasked by cleavage of glycoprotein V. Proc.Natl.Acad.Sci.U.S.A 2001;98:1823-1828.

47. Pike LJ. Lipid rafts: bringing order to chaos. J.Lipid Res. 2003;44:655-667.

48. Pike LJ, Han X, Gross RW. Epidermal growth factor receptors are localized to lipid rafts that contain a balance of inner and outer leaflet lipids: a shotgun lipidomics study. J.Biol. Chem. 2005;280:26796-26804.

49. Lopez JA, del Conde I, Shrimpton CN. Receptors, rafts, and microvesicles in thrombosis and inflammation. J.Thromb. Haemost. 2005;3:1737-1744.

50. Urbanus RT, Pennings MT, Derksen RH, de Groot PG. Platelet activation by dimeric beta2-glycoprotein I requires signaling via both glycoprotein Ibalpha and apolipoprotein E receptor 2'. J.Thromb.Haemost. 2008;6:1405-1412.

51. White TC, Berny MA, Tucker EI et al. Identification of a Role for Apolipoprotein E Receptor 2 as a Platelet Receptor for Factor XI [abstract]. Blood 2008;112:1334.

52. Sullam PM, Hyun WC, Szollosi J et al. Physical proximity and functional interplay of the glycoprotein Ib-IX-V complex and the Fc receptor FcgammaRIIA on the platelet plasma membrane. J.Biol. Chem. 1998;273:5331-5336.

53. Falati S, Edmead CE, Poole AW. Glycoprotein Ib-V-IX, a receptor for von Willebrand factor, couples physically and functionally to the Fc receptor gamma-chain, Fyn, and Lyn to activate human platelets. Blood 1999;94:1648-1656.

54. Arthur JF, Gardiner EE, Matzaris M et al. Glycoprotein VI is associated with GPIb-V-IX on the membrane of resting and activated platelets. Thromb. Haemost. 2005;93:716-723.

55. Wu Y, Suzuki-Inoue K, Satoh K et al. Role of Fc receptor gamma-chain in platelet glycoprotein Ib-mediated signaling. Blood 2001;97:3836-3845.

56. Kasirer-Friede A, Ware J, Leng L et al. Lateral clustering of platelet GP Ib-IX complexes leads to up-regulation of the adhesive function of integrin alpha IIbbeta 3. J.Biol.Chem. 2002;277:11949-11956.

57. Kasirer-Friede A, Cozzi MR, Mazzucato M et al. Signaling through GP Ib-IX-V activates alpha IIb beta 3 independently of other receptors. Blood 2004;103:3403-3411.

58. Bestebroer J, Poppelier MJ, Ulfman LH et al. Staphylococcal superantigen-like 5 binds PSGL-1 and inhibits P-selectin-mediated neutrophil rolling. Blood 2007;109:2936-2943.

59. Fitzgerald JR, Foster TJ, Cox D. The interaction of bacterial pathogens with platelets. Nat. Rev.Microbiol. 2006;4:445-457.

Chapter 9

Nederlandse samenvatting

Bloedplaatjes als katalysator van de bloedstolling

Bij het repareren van schade aan een bloedvat spelen de bloedstolling en bloedplaatjes een belangrijke rol. De bloedplaatjes vormen de bakstenen om het ontstane gat in de vaatwand op te vullen en de bloedstolling zorgt, door middel van de vorming van fibrine, voor het cement tussen de bakstenen. In dit onderzoek is gekeken naar de specifieke interactie tussen de componenten van de bloedstolling (stolfactoren) en de bloedplaatjes.

Dit onderzoek laat zien dat het eiwit glycoproteine Ib op bloedplaatjes belangrijk is voor de binding van stolfactoren aan de bloedplaatjes en dat deze interactie van invloed is op de activiteit van specifieke stolfactoren en de bloedstolling in het algemeen. Daarnaast beschrijft dit onderzoek dat stolfactoren ook de mogelijkheid hebben om de activiteit van bloedplaatjes te reguleren door als oppervlak te fungeren waaraan bloedplaatjes kunnen hechten.

De bevindingen in dit proefschrift bieden nieuwe inzichten in het samenspel tussen bloedstolling en de bloedplaatjes. In de toekomst kan dit onderzoek onder andere bijdragen aan de behandeling van mensen met haemofilie, waarbij een van de stolfactoren ontbreekt, en de betrokkenheid van glycoproteine Ib kan nieuwe mogelijkheden bieden tot verbeterde therapie.

Introductie

Bij het repareren van schade aan een bloedvat spelen de bloedstolling en bloedplaatjes een belangrijke rol. De bloedplaatjes vormen de bakstenen om het ontstane gat in de vaatwand op te vullen en de bloedstolling zorgt, door middel van de vorming van fibrine, voor het cement tussen de bakstenen. Bij schade aan de vaatwand komt bindweefsel vrij wat onder de cellen van de vaatwand ligt. Bloedplaatjes kunnen hechten aan de eiwitten in dit bindweefsel, zoals collageen en vitronectine, maar de hoge stroomsnelheid van het bloed zorgt ervoor, dat bloedplaatjes te hard gaan om stabiel te binden aan het vrijgekomen collageen. Von Willebrand factor uit het bloed bindt daarom aan collageen en zorgt er via de interactie met glycoproteine Ib (GPIb) op het bloedplaatje voor, dat het bloedplaatje wordt afgeremd. Hierna kunnen bloedplaatjes beter binden aan collageen Verder veranderen bloedplaatjes van vorm zodat ze het gat in de vaatwand perfect afdichten.
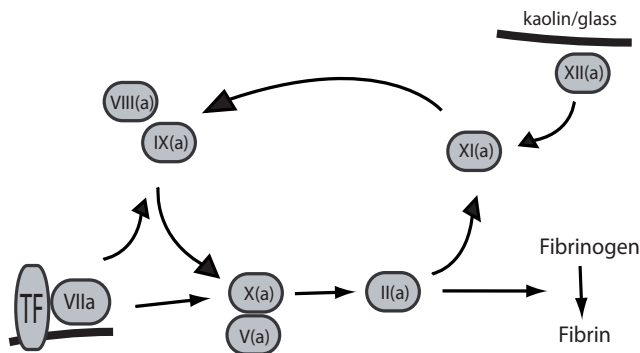
Figuur 1 - Versimpeld weergave van de bloedstolling. TF: weefselfactor.

De bloedstolling begint bij de blootstelling van weefselfactor (tissue factor; TF) aan stromend bloed. Weefselfactor komt vrij bij schade aan de vaatwand en activeert stolfactor VII. Het complex van weefselfactor en geactiveerd factor VII zorgt voor de activatie van een hele serie andere stolfactoren, die elkaar activeren als een rij dominostenen, en is schematisch weergegeven in Figuur 1. Het complex van weefselfactor en geactiveerd factor VII activeert factor X, wat op zijn beurt samen met factor V weer protrombine (factor II) activeert tot actief trombine. Trombine knipt een aantal stukjes van fibrinogeen af, waardoor er draden van fibrine gevormd kunnen worden, die de prop van bloedplaatjes verstevigen. Naast het knippen van fibrinogeen kan trombine ook factor XI activeren, wat op zijn beurt weer factor IX actief maakt. Factor IX kan dan in samenwerking met factor VIII wederom factor X activeren, wat uiteindelijk resulteert in nog meer trombine. Naast deze traditionele route van bloedstolling is er een alternatieve route, die begint bij de kunstmatige activatie van factor XII in een reageerbuis door glas of kaoline, een component uit Chinese klei. De activatie van factor XII resulteert in actief factor XI en haakt weer in op de reguliere route van bloedstolling. Als een van deze stolfactoren ontbreekt, zoals onder andere bij haemofilie het geval is waar factor VIII of IX ontbreekt, dan verloopt de bloedstolling stroef. Het cement

voor de bakstenen kan niet goed uitharden en de schade aan de vaatwand kan slecht gerepareerd worden.

Voor een optimale bloedstolling zijn ook de bloedplaatjes belangrijk. Als bloedplaatjes geactiveerd worden, vormen ze het ideale oppervlak om bloedstolling te laten plaatsvinden. Veel stolfactoren binden goed aan negatief geladen lipiden, die na de activatie van bloedplaatjes aan het oppervlak te vinden zijn. Dit proces is altijd gedacht redelijk willekeurig plaats te vinden, maar er zijn onderzoeksgroepen die speculeren dat er specifieke receptoren op de bloedplaatjes aanwezig zijn, die de stolfactoren bij elkaar brengen. Dit proefschrift beschrijft de rol die GPIb op het bloedplaatje speelt in de bloedstolling.

GPIb reguleert de bloedstolling op bloedplaatjes
Mensen met haemofilie worden behandeld met stolfactor VIII (of IX), maar ongeveer 25% van de mensen met haemofilie ontwikkelt antistoffen tegen deze medicijnen, waardoor deze niet meer goed werken. Geactiveerd factor VII (rFVIIa) wordt momenteel kunstmatig geproduceerd en dient als goed werkend alternatief medicijn voor mensen met haemofilie, die last hebben van antistoffen tegen factor VIII. De resultaten in hoofdstuk 3 laten zien dat actief factor VII aan de bloedplaatjes receptor GPIb kan binden en dat deze interactie van invloed is op de activiteit van de specifieke stolfactoren en de bloedstolling in het algemeen. Aan de ene kant zorgt de binding van geactiveerd factor VII aan GPIb voor een versnelde bloedstolling en door de ontwikkeling van een gemodificeerd factor VII, wat nog beter aan GPIb bindt, zouden haemofiliepatienten beter geholpen kunnen worden. Aan de andere kant remt de aanwezigheid van GPIb de bloedstolling die via factor IX verloopt, zoals beschreven in hoofdstuk 4. Op deze manier lijkt GPIb dus de bloedstolling te reguleren door aan verschillende componenten van de bloedstolling te binden.

Stolfactoren hebben invloed op de functie van bloedplaatjes
De interactie tussen stolfactoren en GPIb wordt op een andere manier belicht in hoofdstuk 5 en 6. Zoals in de introductie al beschreven, vervult GPIb een belangrijke functie in het hechten van bloedplaatjes aan de beschadigde vaatwand. In hoofdstuk 5 wordt beschreven dat bloedplaatjes niet alleen aan eiwitten zoals collageen kunnen binden, maar ook aan de stolfactor trombine wat op een stukje glas gezet is. Trombine bevindt zich normaal gesproken niet op een oppervlak, maar is wel betrokken bij het reparen van de vaatwand, doordat het in complex met fibrine aanwezig is. De combinatie van trombine en fibrine zorgt voor aanwas van extra bloedplaatjes naar het gebied van vaatschade. Daarnaast is trombine een sterke activator van bloedplaatjes en de combinatie van trombine en fibrine brengt de trombine dichter bij de bloedplaatjes, waardoor de activatie beter verloopt.

Waar de afwezigheid van alle stolfactoren leidt tot een bloedingsneiging, is dit bij factor XII als een van de weinige stolfactoren niet het geval. Daarom is van factor XII niet zo goed bekend, wat de exacte functie in het lichaam is. De

resultaten in hoofdstuk 6 laten zien dat ook factor XII een bijdrage levert aan de hechting van bloedplaatjes aan een beschadigde vaatwand. Van zowel factor XII als von Willebrand factor is bekend dat ze via GPIb aan bloedplaatjes kunnen binden, In hoofdstuk 6 wordt beschreven dat factor XII en von Willebrand factor ook aan elkaar kunnen binden en deze interactie verhoogt het aantrekken van bloedplaatjes. Deze resultaten beschrijven een nieuwe functie voor factor XII in het lichaam en zouden in de toekomst wellicht kunnen leiden tot een nieuwe behandelingsstrategie voor trombose.

Het bacteriele eiwit SSL5 activeert bloedplaatjes

In hoofdstuk 7 wordt tenslotte beschreven hoe het bacteriele eiwit SSL5 bloedplaatjes kan activeren. Infecties met bacteriën, zoals de bekende ziekenhuisbacterie Staphylococcus aureus (S. aureus), gaan vaak gepaard met de vorming van trombose in de kleinere vaten. De celwand van de bacterie bestaat uit verschillende eiwitten waar bloedplaatjes gevoelig voor zijn. Tevens stoot de bacterie een reeks van eiwitten uit, die de kans op overleving voor de bacterie verhogen. SSL5 is een van deze eiwitten en in een eerder onderzoek is aangetoond dat SSL5 de afweerreactie van het lichaam tegen S. aureus verzwakt. In dit proefschrift wordt beschreven dat SSL5 bloedplaatjes kan activeren en kan laten samenklonteren. Dit proces gaat onder andere via GPIb op het bloedplaatje. Het activeren van bloedplaatjes kan belangrijk zijn voor de overleving van S. aureus in het lichaam. Bloedplaatjes hebben de natuurlijke neiging om zich aan zwakke plekken in de vaatwand te gaan hechten, en hiervan kan S. aureus gebruik maken door via de uitstoot van SSL5 meer plaatjes te laten hechten. Vervolgens kan S. aureus zich voortplanten door te infiltreren in het onderliggende weefsel. Door uit te zoeken welke aspecten belangrijk zijn voor de infectie met S. aureus kan er een betere therapie gevonden worden.

Conclusie

GPIb vervult een centrale rol in de functie van bloedplaatjes. Het verzorgt niet alleen de primaire aanhechting van bloedplaatjes aan de beschadigde vaatwand, maar reguleert ook de bloedstolling die plaatsvindt op geactiveerde bloedplaatjes. Verder dragen stolfactoren weer bij aan de aanhechting van bloedplaatjes aan de beschadigde vaatwand, en versterken bloedstolling en bloedplaatjes zo elkaars functie. Tenslotte maakt S. aureus gebruik van GPIb door onder andere via deze receptor bloedplaatjes te activeren en zijn eigen overlevingskansen te verhogen.

Dankwoord

DANKWOORD

Het dankwoord schijnt het belangrijkste gedeelte te zijn van een proefschrift. Bijna iedereen zal een proefschrift in ontvangst nemen, de maker feliciteren en het boekwerk openslaan bij het dankwoord. Sta ik er in? Wat is er over me geschreven? Er is blijkbaar behoefte aan, dus wie ben ik om daar niet aan te voldoen... Nee, een woord van dank naar de mensen die het allemaal mogelijk hebben gemaakt, is absoluut op zijn plaats. In redelijk willekeurige volgorde:

Ton, ik heb grote bewondering voor je. Je bent buitengewoon getalenteerd en ik wilde daarom ook heel graag specifiek bij jou AIO worden. Verder ben je niet alleen in de wetenschap geïnteresseerd, maar heb je me geïntroduceerd in Herman Brusselmans, Kopenhagen en de blues. Naast `sweet home alabama´ heb ik toch maar al te graag ´toet-toet-toeter op mijn waterscooter' mee lopen blèren. Het was me een eer en genoegen om voor je te werken, en ik hoop dat we elkaar nog regelmatig tegenkomen in de toekomst.

Flip, ik heb veel van je geleerd. Ondanks dat je elke week de resultaten van de hele afdeling onder ogen krijgt, wist je altijd heel snel de vinger op de zere plek te leggen wat betreft mijn onderzoek. Een analyticus pur sang en daarmee van onschatbare waarde voor een AIO. Dank je wel.

De mensen van de Lisman groep. Regelmatig zijn we uitgenodigd bij Ton voor heerlijke diners, en moest ik 's avonds weer op zoek naar mijn schoenen. Jelle, Jelk Ademier. "Houthalen" is nog maar net voorbij, of je staat alweer te popelen voor het volgende jaar. Ik vraag me wel eens af: als ik je niet bevrijd had uit je benarde positie in de Basket, was je dan gewoon in Utrecht gebleven? (Jelk had zichzelf opgesloten op het toilet in de Basket, dacht ie). Verder heb ik die vloertegel ingelijst (en bij het grof vuil gezet :P).
Suzanne, de kletskous. Ik had het zelf niet beter kunnen bedenken. Als er wat te ouwehoeren was, dan was Suzanne in de buurt. Het was erg gezellig om met je samen te werken. Succes in het zuiden van het land!
Mirjam, rustig op een terras genietend van een koud biertje met de Lisman groep, en toen kwam daar een oude bekende langs. Het verleden van Mirjam werd geopenbaard, net zoals haar oude bijnaam: Jammie! Daar kom je niet meer vanaf! :P
Verder Dafna, 'oeps, het sleuteltje van mijn slot is afgebroken'. Sultana, een voorspoedige toekomst in het mooie Griekenland gewenst!

Coen, de ene dag een net overhemd, de andere dag een trainingsbroek. Je bent net zo wisselvallig als je kleding, maar dat is het kenmerk van een creatieve geest. Je bent een uniek persoon en het was een geweldige ervaring om je binnen en buiten het lab mee te maken! Vele engelse pints in de Jan Primus hebben we gedronken en tegelijkertijd de afdeling geanalyseerd. Ik hoop dat er nog vele halve liters zullen volgen, om te beginnen: New York city here we come!

Rolf, de toekomstige leider van het LKCH. Blijkbaar nogal ontoegankelijk, vanwege zijn boze blik. Maar achter die "evil eye" zit een vent met een hart van goud, die altijd behulpzaam is (ook al kun je af en toe lekker zeiken op mensen). Verder ben je een van de weinigen die mijn Theo Maassen quotes herkent. Hulde!

Silvie, je zal de Rabradio popquiz zonder mij moeten doen nu. En misschien komt er ooit nog eens een house-warming… Martin/Tinus, ouwe brombeer, geniet van je pensioen! Evelyn, succes met de laatste loodjes. Ya-Ping, maker van prachtige plaatjes. Annet, assertief als altijd en met een lach als een brandweer. Lab1 is weer een stuk gezelliger! :-) Valentina, tell me something funny! You definitely brought some new 'life in the brewery'. Anja, de stress voor het boekje slaat al wat toe. In San Francisco heb ik daar gelukkig niks van gemerkt. En ik viel ook best wel mee als roommate toch? Gwen, Cetin en Esther, succes met jullie promotie. Vivian, good luck for the future. Claudia, Thijs, Eelo, jullie zijn de nieuwe generatie. Maak vooral een leuke periode van je AIO-schap. Onthou mijn motto: je werkt om te leven en je leeft niet om te werken. ;-)

Mensen van lab III. Jan-Willem, interessant hoe je over één dia een half uur kan praten. Het was leuk in San Francisco speciaalbier met je te delen. Arnold, Koekman! Een vriendelijke vent met een aanstekelijke lach. Je straalt altijd rust uit en lijkt nooit gestressed te zijn, en dat is voor een toeziend analist wat mij betreft een voortreffelijke eigenschap. De lekkernijen uit de slagerij vallen zeer in de smaak, maar toch vind ik die van Lotgering beter :P Sandra, mijn promotie is in juni. Dus dat lijkt me de hoogste tijd om die nieuwe Birkenstocks (dat heb ik op moeten zoeken overigens) eens aan te trekken. Het was altijd weer leuk om een glimlach op je gezicht te toveren, als je je weer eens aan het ergeren was op lab III (ras-pessimist), ondanks die bovenlip :P Dianne, hoe jij opmerkingen verkeerd of anders kan opvatten is een gave. Dus zo zal ook deze opmerking wel weer in het verkeerde keelgat schieten. He, nee, zo bedoelde ik het niet! Wat is je nieuwste hobby trouwens? Probeer schilderen van Ravensburger eens, een echte aanrader! Gelukkig hoef ik je niet zo vaak in de zeik te zetten, want dat doe je zelf wel. Ik ben benieuwd naar jouw promotiefilmpje. :P Erik, met wie moet ik nu ouwehoeren over voetbal en de animatiequiz op vi.nl doen?

Mark, grote mond, klein hartje. Een ééntje graag, te vroeg teruggetrokken, anders krijg je er maar narigheid van. Arjan, organisator van het wekelijkse speelkwartier (ook wel voetballen genoemd). Niet mokken, lekker rocken! Maarten E, kweken is meestal saai, maar niet als jij schuine moppen aan het tappen bent. Robbert, altijd enthousiast en altijd in voor een praatje. Tuna, wat kun jij lekker zeveren over de kleinste dingen. Tineke, nee ik heet geen Niels. Kelly, Evelien, Michael, Berris. Teun, koning van de slidings in een lange broek. Martine. Wouter, de grote roerganger van het LKCH. Martijn, schaamteloze zelfpromotie. Rob, altijd enthousiast. Harry, ik kan je EM-foto's wel dromen inmiddels. Joukje, jouw werk maakt het leven van een AIO wat gemakkelijker, dank daarvoor. Mevrouw Rosmina, dank voor de afwas. Arno, ouwe hooligan. Daag me nog eens uit voor een oefenpotje!

Mensen van het rode cel lab: Jerney, Brigit, Marcel, Richard. Alle mensen van de speciële, waar ik zo vaak plaatjes kwam meten. Oud-collega's: Eszter, you stupid woman! When do you come watch television at my place? Ronan, crazy French guy. Maarten P, bakkie?! Suzanne Korporaal, heeeeyyy……Suus…... Remo, doldwaze Italiaan. Caro, Miranda, Vivianne, Menno, Sultana, Bettina, Mieke, Wessie, Sarah, Carina, Janine, Chantal. Alle studenten die ik heb zien komen en gaan. Ik ben gewoon te lui om iedereen bij naam te noemen. Jullie zorgen voor sfeer op de afdeling! Verder iedereen die ik verder vergeten ben te noemen: sorry, ik heb het niet expres gedaan. Ook jij bedankt voor al die jaren!

Carla en Mignon van het EWI. Een gezellige samenwerking. Regelmatig om een uurtje of drie (of was het vijf over drie?) afgesproken om de laatste resultaten door te spreken. Die resultaten staan in hoofdstuk 7 en hopelijk is dat binnenkort een gepubliceerd verhaal. Dank voor het meer dan interessante zijspoor!
Verder wil ik iedereen bedanken, die op een of andere manier aan een hoofdstuk heeft meegeholpen, door het sturen van cruciale materialen of het geven van advies. Especially, I would like to thank the people from Novo Nordisk in Malov for their warm welcome in their lab. You were really helpful and I have only good memories from my stay in Denmark.

Theo Maassen, bedankt voor de onuitputtelijke bron van quotes die ik met zeer grote regelmaat heb gebruikt. Bij elke situatie past wel een geniale quote en die heb ik dan ook te pas en te onpas misbruikt.

Mark, Jessica, Bob, Vanessa, Ad, Pieter, Frits, Ingmar, Bram, en nog vele anderen (ik vergeet ongetwijfeld, onbedoeld, mensen), mijn tijd op de Warande is voorbij gevlogen met jullie.
Dunnen, jammer maar ook wel gaaf dat je op dezelfde dag gaat promoveren. Sorry, dat ik er niet bij kan zijn, ik heb al wat anders ;-) EricD, ik heb er al één meer op dan jij (oh nee, dat was TT). Ik val met 10 legertjes tssstss, die!, aan ;-) Mulder, ja dat dacht ik al. Dé ontwerper van het etiket voor de Pater Cornelis en vele andere karikaturen. Heren, het wordt weer eens tijd voor een weekendje in een of ander hutje op de hei en biertjes drinken.
Jos, ook wel Josef, al jaren mijn buurman en tevens drinkebroeder. Fijn dat je aan mij dacht om dat lauwe Duitse pils mee te delen. Ok, over die Yeti's mag ik niet klagen. Hopelijk delen we nog vele bieren uit alle streken van de wereld.
Anke en Matthijs, mijn paranimfen. Wanneer is het weer decadente zondag? In Den Bosch, Amsterdam, of zelfs London of Parijs, waar is het de volgende keer? Mooi om jullie in zo'n net kloffie te zien, dan ben ik niet de enige die voor joker staat.

Mijn familie, ik ben niet iemand die elke week langs komt voor een bakkie koffie, maar jullie zijn me zeer dierbaar, niemand uitgezonderd: ik ben net zo trots op jullie als jullie op mij!

List of publications
&
Curriculum vitae

LIST OF PUBLICATIONS

1. <u>Weeterings C</u>, Lisman T, de Groot PG.
    Tissue factor-independent effects of recombinant factor VIIa on hemostasis.
    Semin Hematol 2008; 45(suppl 1):S12-S15.

2. <u>Weeterings C</u>, Adelmeijer J, de Groot PG, Lisman T.
    Glycoprotein Ib[alpha] contributes to tissue factor-independent thrombin
    generation by recombinant factor VIIa on the activated platelet surface.
    Blood 2008, 112:3227-33.

3. Heyligers JMM, Lisman T, Verhagen HJM, <u>Weeterings C</u>, de Groot PG, Moll FL.
    A heparinbonded vascular graft generates no systemic effect on markers of
    haemostasis activation or detectable HIT-inducing antibodies in humans.
    J Vasc Surg 2008, 47: 324-329.

4. Heyligers J, Verhagen HJM, Rotmans JI, <u>Weeterings C,</u> de Groot PG, Moll FL,
    Lisman T.
    Heparin immobilization reduces thrombogenicity of small-caliber ePTFE
    grafts.
    J Vasc Surg, 2006;43: 587-591.

5. <u>Weeterings C</u>, Adelmeijer J, Myles T, de Groot PG, Lisman T.
    Glycoprotein Ib(alpha)-mediated platelet adhesion and aggregation to
    immobilized thrombin under conditions of flow.
    Arterioscler Thromb Vasc Biol. 2006;26:670-675.

6. Lisman T, <u>Weeterings C</u>, de Groot PG.
    Platelet aggregation: involvement of thrombin and fibrin(ogen).
    Front Biosci. 2005;10:2504-17

7. Litjens PE, Van Willigen G, <u>Weeterings C</u>, Ijsseldijk MJ, Van Lier M, Koivunen
    E, Gahmberg CG, Akkerman JW.
    A tripeptide mimetic of von Willebrand factor residues 981-983 enhances
    platelet adhesion to fibrinogen by signaling through integrin alpha(IIb)beta3.
    J Thromb Haemost. 2005 Jun;3(6):1274-83.

OTHER

1. <u>Weeterings C</u>, de Groot PG, Lisman T.
   Glycoprotein Ib-alpha modulates factor IXa-mediated factor Xa-generation on the activated platelet surface.
   J Thromb Haemost 2009; Suppl: PP-MO-126

2. Maas C, Meijers JCM, <u>Weeterings C</u>, de Groot PG, Urbanus RT.
   Factor V is a cofactor for activation of factor XI by thrombin.
   J Thromb Haemost 2009; Suppl; AS-TU-017

3. <u>Weeterings C</u>, de Haas CJC, Vughs MM, de Groot PG, van Strijp JA, Lisman T.
   Staphylococcal superantigen-like 5 activates platelets, and supports platelet adhesion under flow conditions, which is mediated by GPIb-alpha and alpha-IIb-beta-3. [manuscript submitted]

4. <u>Weeterings C</u>, de Haas CJC, Vughs MM, de Groot PG, van Strijp JA, Lisman T.
   Staphylococcal superantigen-like 5 activates platelets, and supports platelet adhesion under flow conditions, which is mediated by GPIb-alpha and alpha-IIb-beta-3.
   Blood 2008; 112(11):1336. [abstract]

5. Pendu R, <u>Weeterings C</u>, Urbanus RT, Pennings MTT, Denis CV, de Groot PG, Lenting PJ.
   PSGL1 and LDL-receptors mediate adhesion of leukocytes to beta2-glycoprotein-I.
   J Thromb Haemost 2007; 5 Supplement 2: P-M-463. [abstract]

6. <u>Weeterings C</u>, Adelmeijer J, de Groot PG, Lisman T.
   Glycoprotein Ib[alpha] contributes to tissue factor-independent thrombin generation by recombinant factor VIIa on the activated platelet surface.
   J Thromb Haemost 2007; 5 Supplement 2: O-S-042. [abstract]

7. <u>Weeterings C</u>, Adelmeijer J, Myles T, de Groot PG, Lisman T.
   Glycoprotein Ib(alpha)-mediated platelet adhesion and aggregation to immobilized thrombin under conditions of flow.
   J Thromb Haemost 2005; 3 Suppl 1: P0336. [abstract]

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 21 januari 1981 te Leerbroek. Na het afronden van het VWO aan het Gymnasium Camphusianum te Gorinchem, is hij in 1998 begonnen met de studie Medische Biologie aan de Universiteit Utrecht. Als onderdeel van deze studie heeft hij stages gelopen bij de afdeling Haematologie van het Universitair Medisch Centrum Utrecht onder begeleiding van dr. G. van Willigen en prof. dr. J.W.N. Akkerman, en de afdeling Medische Microbiologie van het Universitair Medisch Centrum Utrecht onder begeleiding van dr. H.S. Nottet. Na het afronden van deze studie in 2003, heeft hij een jaar gewerkt als research-analist bij de afdeling Haematologie van het Universitair Medisch Centrum Utrecht, alvorens in 2005 te beginnen met zijn promotieonderzoek onder begeleiding van dr. T. Lisman en prof. dr. Ph.G. de Groot bij dezelfde afdeling. De resultaten van dit onderzoek zijn beschreven in dit proefschrift.