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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

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1. 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.

2. 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

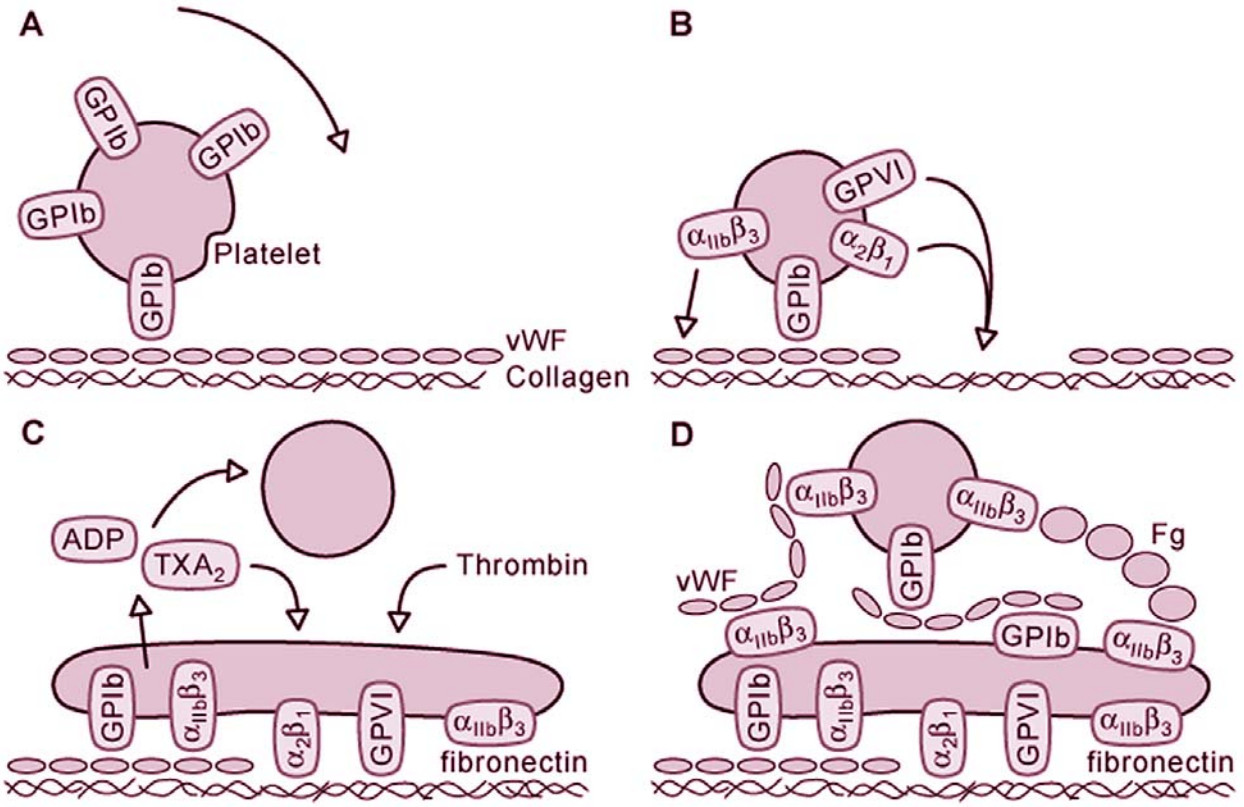

Figure 1. Schematic representation of platelet adhesion and aggregation under flow conditions. A) Rolling of platelets over collagen-bound vWF mediated by GPIb. B) Firm attachment mediated by alpha(2)beta(1) and glycoprotein VI (GP VI) binding to collagen, and by alpha(IIb)beta(3) binding to collagen-bound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

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), 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.

3. 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

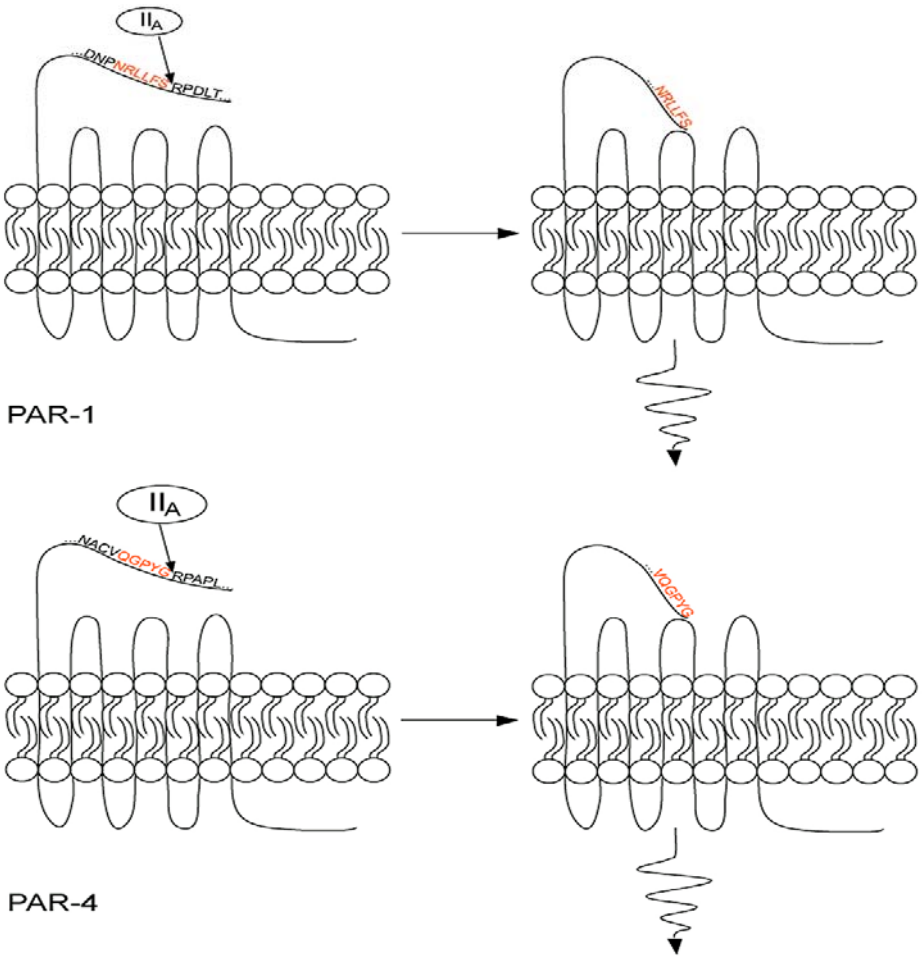

Figure 2. Thrombin-mediated activation of PARs in human platelets. Human platelets express both PAR-1 and –4. Thrombin cleaves at the N-terminal extracellular part of the receptor, thereby exposing a new N-terminus (SFLLRN and GYPGQV, respectively), which binds to the body of the receptor, leading to G-protein-coupled signal transduction.

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.

3.1. 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). 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.

3.1.1. 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

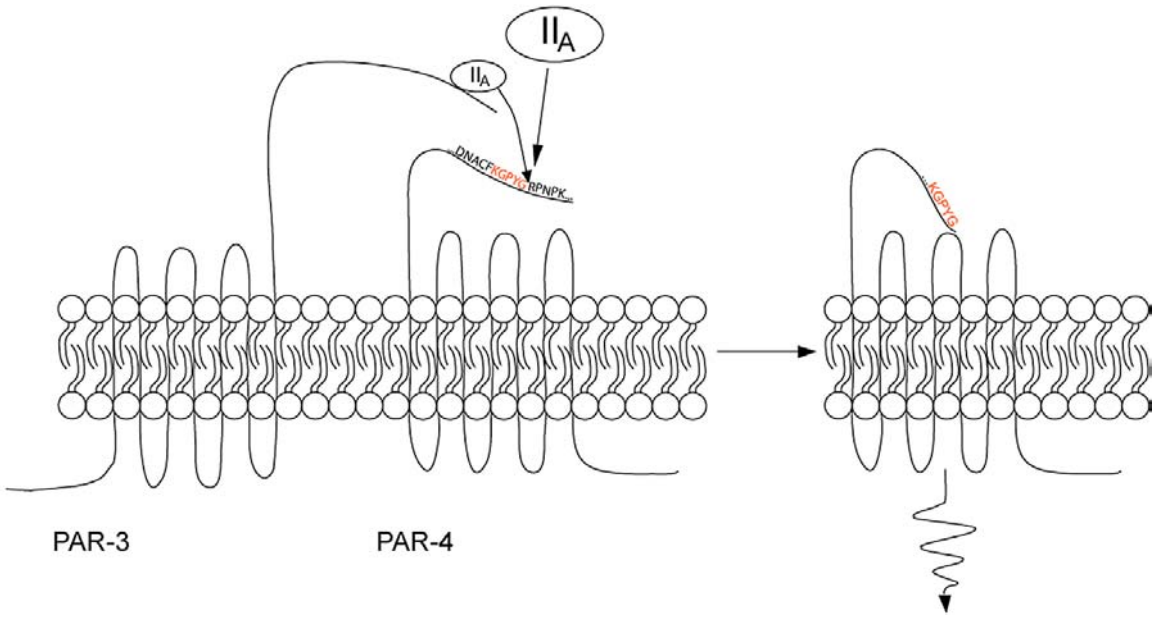

Figure 3. Thrombin-mediated activation of PARs in mouse platelets. Mouse platelets express PAR-3 and –4. PAR-3, however, is not cleaved by thrombin, but rather binds thrombin with high affinity and serves as a docking molecule to present thrombin to PAR-4, which is cleaved by thrombin. PAR-3 is necessary for PAR-4 activation at low concentrations of thrombin. At higher thrombin levels, PAR-4 can be activated without the requirement for PAR-3.

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 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).

3.1.2. 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.

3.1.3. 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).

3.2. 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).

3.2.1 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

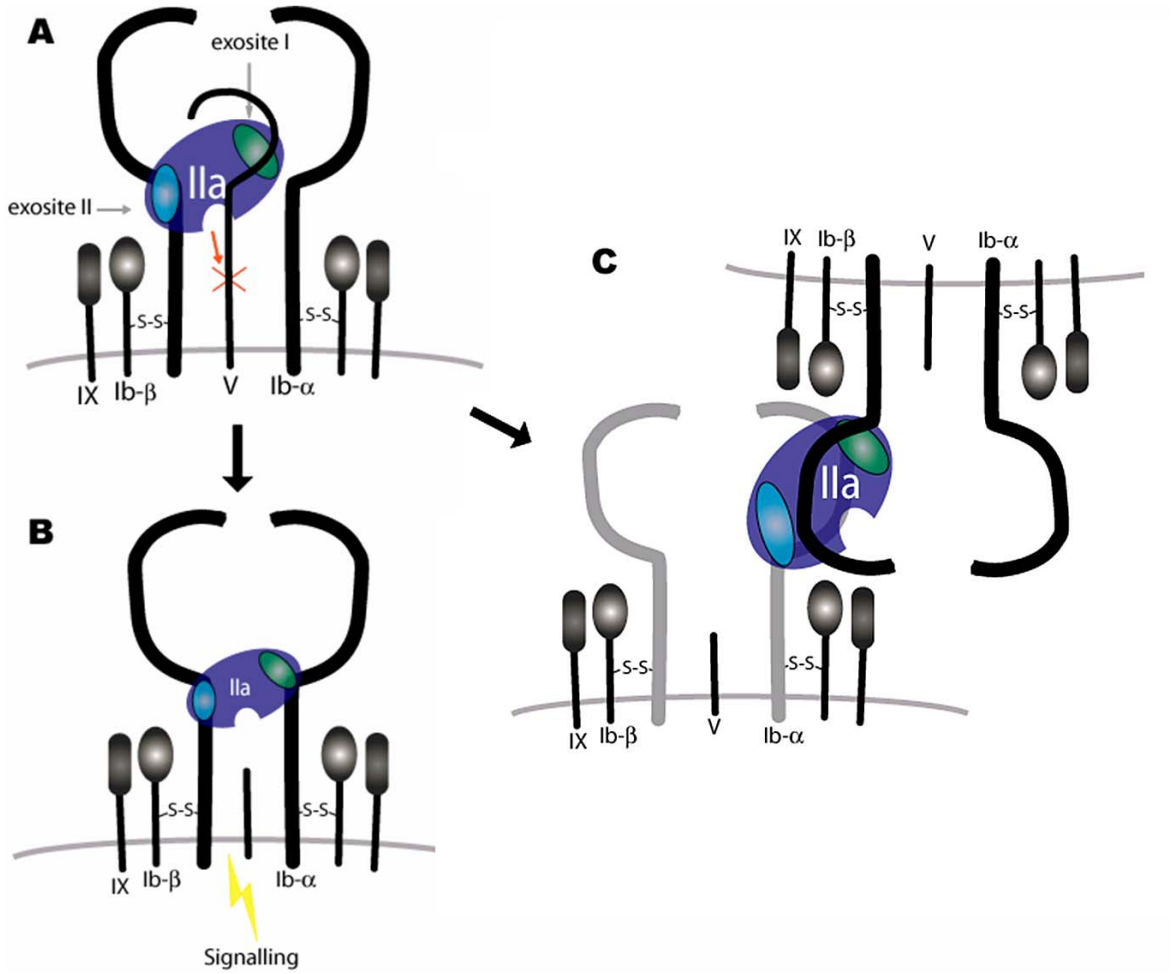

Figure 4. Schematic representation of thrombin binding to GPIb(alpha). (A) Thrombin binds to the tyrosine-sulphated region of GPIb(alpha) via its exosite II. After binding to GPIb(alpha) thrombin is able to cleave GPV, resulting in the ability to bind via exosite I to another GPIbα molecule. The cleavage 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.

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 (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.

3.2.2. 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).

3.2.3. 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 section 3.2.2) 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.

3.3 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 (see section 3.2). 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.

4. 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).

4.1. 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,

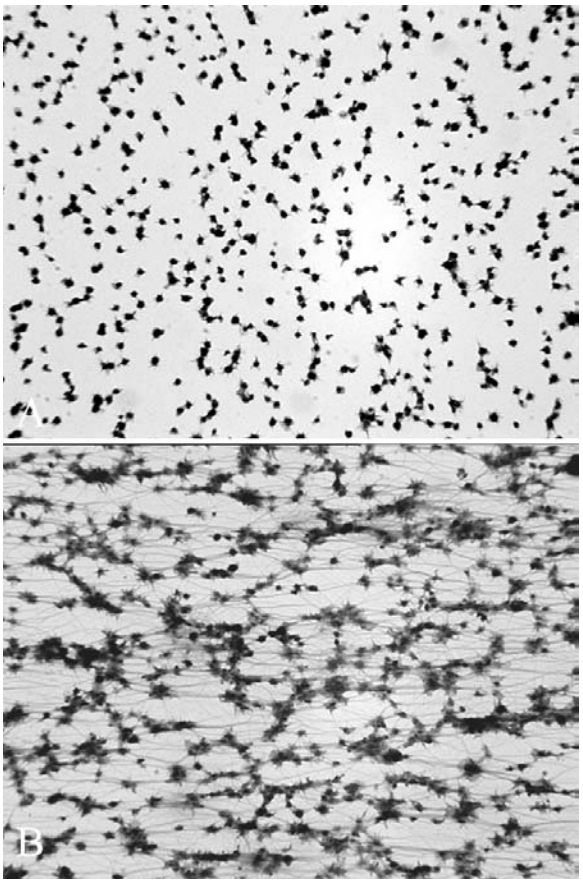

Figure 5. Platelet aggregation independent of alpha(IIb)beta(3) via polymerizing fibrin. In this experiment, the adhesion of platelets treated with an alpha(IIb)beta(3) inhibitor to collagen was studied under flow conditions in absence (panel A) or presence (B) of rFVIIa. In absence of thrombin generation 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

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).

4.2. 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 αIIbβ3-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.

5. 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.

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Send correspondence to: Dr Ton Lisman, Thrombosis and Haemostasis Laboratory, Department of Haematology G.03.647, University Medical Centre, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands, Tel: 31-30-2506513, Fax: 31-30-2511893, E-mail: j.a.lisman@azu.nl

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