Platelet-fibrin(ogen) interactions in flowing blood

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Cover illustration: Scanning electron microphotograph of a platelet thrombus adhered to a fibrin network formed under flow conditions.
(by Y.P. Wu and J.A. Remijn)
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Platelet-fibrin(ogen) interactions in flowing blood

Interacties van bloedplaatjes met fibrine en fibrinogeen bestudeerd onder stromingscondities

(met een samenvatting in het Nederlands)

Proefschrift

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"De wetenschap is een prachtig project tot ordening van het betrekkelijke met de betrekkelijke vermogens van de menselijke geest"

K.H. Miskotte (1894-1976)

Aan mijn vader en moeder

Voor Rosanne

Contents

CHAPTER 1 | General introduction

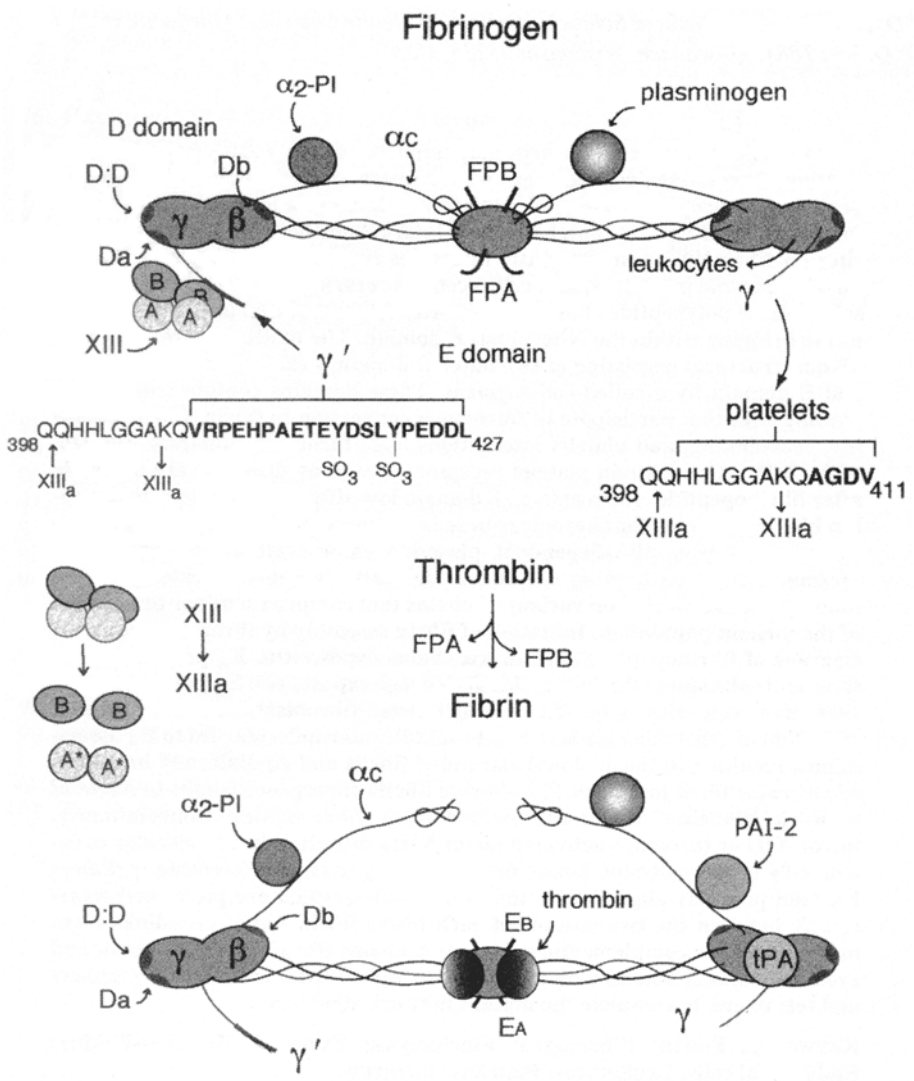
Introduction

Haemostasis, the arrest of haemorrhage at the site of vascular injury, is one of the most important host defense mechanisms. It preserves the integrity of the closed, high-pressure circulatory system by limiting blood loss. The main processes involved in the arrest of bleeding are platelet plug formation (platelet adhesion and aggregation) and fibrin formation (coagulation). Platelet aggregate formation starts when platelets adhere to subendothelial proteins, such as collagen fibrils, exposed to the blood stream upon vascular damage. The adhered platelets become activated and undergo a shape change accompanied by the secretion of granules. An important constituent of these granules is adenosine diphosphate (ADP), which enables newly arrived platelets to become activated via binding to specific ADP receptors on the platelet membrane. The final result of platelet activation is the exposure of the platelet integrin receptor $\alpha_{IIb}\beta_3$. Platelet aggregation is mainly mediated by fibrinogen, a dimeric molecule serving as bridge between platelets via binding to the exposed $\alpha_{IIb}\beta_3$. At the same time of platelet plug formation, the coagulation system is activated upon exposure of tissue factor to the blood stream. Activation of coagulation leads to thrombin formation, which is a strong platelet activator and which cleaves fibrinogen into fibrin monomers. These monomers polymerize to an insoluble fibrin network. The interaction of fibrin(ogen) with platelets is important for the stabilization of the haemostatic plug sealing the injured vessel.[1]

Fibrinogen

Fibrinogen is a large plasma glycoprotein of 340 kDa.[2] It is synthesized by hepatocytes and circulates in plasma at a concentration of 2-4 mg/ml with a half life of about 5 days. It is comprised of two sets of three polypeptide chains termed Aα (64 kDa), Bβ (56 kDa), and γ (47 kDa), which are joined together within their N-terminal domains by five symmetrical disulfide bridges.[3-7] The fibrinogen molecules are elongated 45 nm structures. It consists of

Figure 1. Schematic representation of fibrinogen and fibrin showing the major structural domains, the association sites that participate in fibrin polymerization, cross-linking, and other molecular and cellular binding interactions. (Reprinted with permission)[18]



three nodules: one central nodule, the E-domain, and two identical outer nodules, the D-domains, each connected by a coiled-coil segment to the central E-domain (Figure 1). The two D-domains represent the carboxyl-terminal regions of the Bβ- and γ-chains. The carboxyl

terminus of the Aα-chain is a more flexible appendix and is folded back onto or near the E-domain.[8,9-12] The three chains are encoded by different genes, clustered in a region of approximately 50 kilobases on chromosome 4 (q28-q31).[13] The Aα-chain consists of 610, the Bβ-chain 461, and the major form of the γ-chain 411 amino acids. A minor γ-chain variant termed γ', consists of 427 residues and amounts to about 8 % of the total γ-chain population. It arises through alternative processing of the primary mRNA transcript.[14-16] A similar alternative mRNA processing is responsible for an extension of the α-chain termed α_E, with 236 extra amino acids at the C-terminus and accounts 1-2 % of the total amount of circulating fibrinogen. Fibrinogen in which both α-chains have been replaced by α_E has been named fibrinogen-420 instead of the fibrinogen-340, using nomenclature based on its molecular weight.[17]

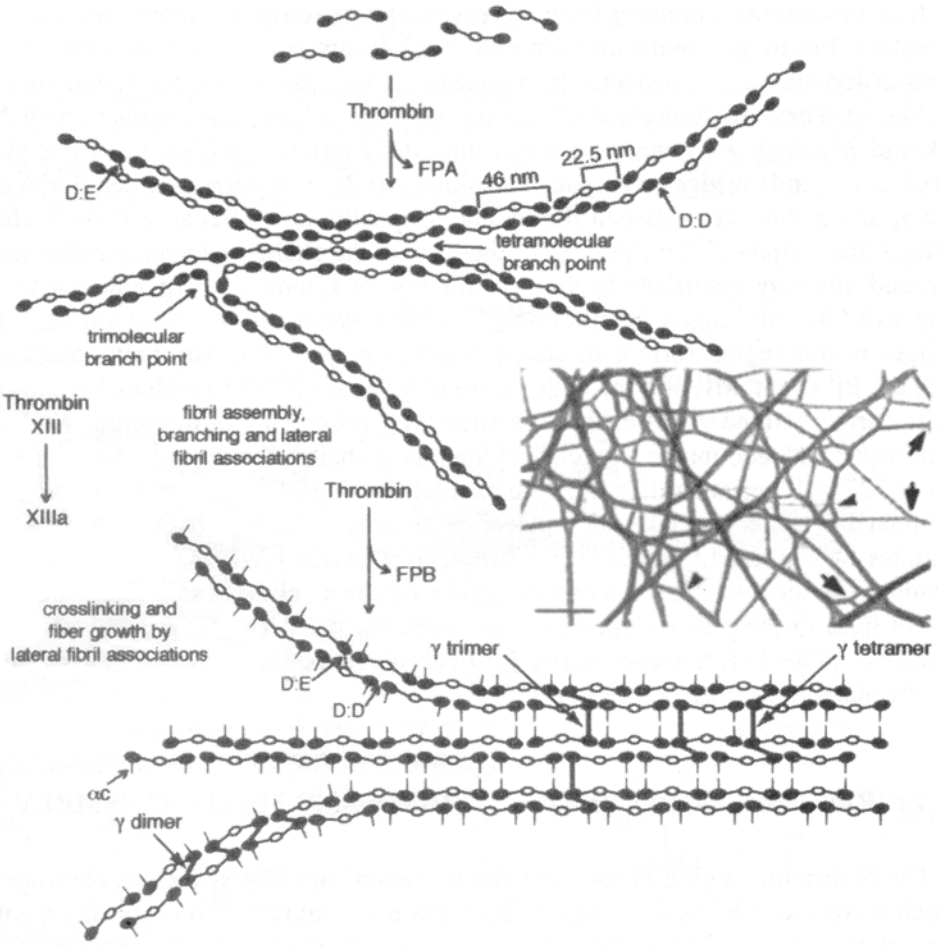
Fibrin formation

Fibrin network formation takes place in three major steps: conversion of fibrinogen to fibrin monomer by thrombin (1), association of half-molecule overlapping double-stranded fibrin protofibrils (2), and lateral association of protofibrils to form thick fibrin bundles and networks (3) (Figure 2) (for a review see Mosesson et al.).[18]

(1) Thrombin binds to recognition sites in the E-domain of fibrinogen[19], and then causes fibrin monomer formation by a specific proteolytic cleavage of the Arg16-Gly17 bond of the Aα-chain, releasing the 16 residues fibrinopeptide A (FPA). The newly exposed N-terminus is a polymerization site, E_A, of the fibrin α-chain comprising residues 17 to 20 (GPRV).[20] The E_A site subsequently combines with a complementary binding pocket (D_a) located in the D domain (γ337-379).[21-23] These initial E_A:D_a associations result in the formation of double-stranded twisting fibrils in which fibrin molecules become aligned in an end-to-middle staggered overlapping domain arrangement. Fibrils undergo lateral associations and form

branches (tetramolecular or bilateral and trimolecular or equilateral) that result in a complex fiber network (Figure 2).[24-28]

Figure 2. Schematic representation of fibrin assembly and cross-linking.



Assembly of fibrin begins with non-covalent interactions (D:E) between the E_A and D_a sites to form end-to-middle staggered overlapping double-stranded fibrils (upper). Fibrils also branch and undergo lateral associations to form wider fibrils and fibers. [Inset: fibril matrix containing equilateral (arrows) and bilateral (arrowheads) branch junctions; Bar = 100 nm]. After cleavage of FPB (lower), lateral fibril association and fibrin assembly occurs. Factor XIIIa introduces covalent C-terminal bindings (thick lines between D domains) to form γ dimers. (Reprinted with permission)[18]

More extensive lateral fibril associations result in thicker fiber bundles consisting of multiple fibrils and more condensed bilateral branch structures. This type of structure confers strength and rigidity to the network fibers. Equilateral branches probably form with greater frequency when fibrinopeptide cleavage is relatively low. The architecture of fibrin networks formed at low thrombin levels is more branched and therefore tighter (i.e. less porous) than networks formed at high levels of thrombin.[29] The type of network architecture that characterizes the tight fibrin network is entirely consistent with the higher degree of equilateral fibril branching that occurs at low thrombin levels. Such branches enhance clot elasticity.[30] Next to E:D association, also a self-association site between the two D domains participates in the fibrin assembly. These D:D sites are situated at the outer portion of each fibrin(ogen) D domain between residues 275 and 300 of the γ module. They are necessary for proper end-to-end alignment of fibrinogen and fibrin molecules in assembling polymer structures.[31]

(2) Following the action of thrombin in FPA generation, thrombin cleaves also the Arg14-Gly15 bond of the Bβ-chain releasing the 14 residues fibrinopeptide B (FPB) exposing the polymerization site, E_B (residues 15-18: GHRP).[20] FPB release occurs more slowly than the release of FPA.[32] E_B interacts with a complementary D_b site located in the C-terminal β-chain segment of the D domain.[23] The E_B:D_b interaction contributes to lateral fibril and fiber associations through cooperative interactions resulting from alignment of D domains in the assembling polymer.[33,34]

(3) In addition to these non-covalent intermolecular interactions in the fibrin assembly process, the C-terminal region participates in covalent cross-linking of fibrinogen and fibrin. Bound thrombin activates transglutaminase factor XIII. Factor XIIIa links fibrin molecules covalently by introducing covalent bonds between a lysine γ-chain (on position 406) and a glutamine acceptor on position 398 or 399 of another γ-chain.[35] This reaction increases the mechanical rigidity of the clot. The formation of a thrombin-fibrin-factor XIII ternary complex greatly enhances factor XIII activation.[36] Cross-linking also occurs, although more

slowly, between α- and γ-chains and among α-chains.[27,37] Furthermore, other plasma proteins such as fibronectin[38], vitronectin[39], thrombospondin[40], von Willebrand Factor[41] and α_2-plasmin inhibitor[38] are also either covalently or non-covalently incorporated into the fibrin mesh.

The fibrin clot acts as a reservoir for enzymatically active thrombin and represents a localized thrombogenic surface. Clot bound thrombin is resistant to inactivation by antithrombin, and it thereby regulates propagation of the clot.[42-44]

Fibrinolysis

Fibrinolysis, the dissolution of the fibrin clots, can be considered as an essential host defense mechanism against propagation of fibrin deposition within the vasculature. The physiological importance of the fibrinolytic system is demonstrated by the association between abnormal fibrinolysis and a tendency towards bleeding or thrombosis (i.e. the occlusion of a vessel by the haemostatic plug). The fibrinolytic system comprises an inactive proenzyme, plasminogen, that can be converted into the active enzyme, plasmin, by the action of specific enzymes collectively known as plasminogen activators. Other proteases, such as leukocytic elastase, also may participate in the breakdown of fibrin.[45,46] Plasmin is a serine protease and hydrolyzes susceptible lysine and arginine bonds. In this way, fibrin is degraded into soluble fibrin degradation products (FDP).[47] The fibrin-bound thrombin is thereby released.[48] Two immunologically distinct physiologic plasminogen activators (PA) have been identified: the tissue-type PA (t-PA) and the urokinase type PA (u-PA). t-PA-mediated plasminogen activation is mainly involved in the dissolution of fibrin in the circulation. The action of t-PA is accelerated in the presence of fibrin. Plasminogen activation occurs through t-PA binding to fibrin followed by the addition of plasminogen to form a ternary complex.[49] Two sites on fibrin are involved in the enhancement of plasminogen activation by t-PA: α148-160 and γ312-324.[50,51] These sites are cryptic in fibrinogen, but become exposed as a consequence of non-covalent D:E interactions in assembling fibrin fibrils. Inhibition of the fibrinolytic system

may occur either at the level of the plasminogen activators, by specific plasminogen activator inhibitors (PAI), or at the level of plasmin, mainly by α_2-antiplasmin.

Fibrinogen disorders

Diseases affecting fibrinogen may be either acquired or inherited. Acquired fibrinogen disorders include disseminated intravascular coagulation, primary fibrinolysis and liver disease. Inherited disorders of fibrinogen are rare and affect quantity (hypofibrinogenemia and afibrinogenemia) or quality of the circulating fibrinogen (dysfibrinogenemia).

Dysfibrinogenemia, is due to structural defects in the molecule.[52,53] 55 % of the reported dysfibrinogenemias are clinically asymptomatic; some present with a bleeding diathesis, others with thrombophilia, and occasionally with both, bleeding and thromboembolism.[52] They are associated with prolonged clotting times and low functional activity, but normal antigenic fibrinogen levels. Dysfibrinogenemia is usually caused by point mutations in the coding region of one of the three fibrinogen genes. Dysfibrinogenemia causes either impaired release of fibrinopeptides, defective fibrin polymerization, or abnormal cross-linking by factor XIIIa. Abnormal interactions with platelets, defective fibrinolysis, defective assembly of the fibrinolytic system, and abnormal calcium binding have also been described. In rare cases dysfibrinogenemias can be associated with hypofibrinogenemia.[52]

Hypofibrinogenemia, is characterized by low functional and antigenic fibrinogen concentrations (< 1.5 mg/ml) and can result from a variety of different mutations.[54] These can affect either, transcription, mRNA processing, translation, polypeptide chain processing and assembly, export from the hepatocyte, or the stability of the mature protein.

Congenital afibrinogenemia, is characterized by a complete absence or extremely low fibrinogen antigen and activity levels. Afibrinogenemia has been described in over 150

families. It is associated with bleeding complications that often start at birth with uncontrolled umbilical cord haemorrhages and has an estimated prevalence of 2 per million births.[55] Surprisingly however, the clinical symptoms of afibrinogenemia are less severe than those of haemophilia A or B. Spontaneous intracerebral haemorrhage and splenic rupture can occur throughout life and there is often significant bleeding after minor trauma. Patients mostly respond well to replacement therapy.[54,56]

Integrin binding sites on fibrinogen

Platelet integrin $\alpha_{IIb}\beta_3$ plays a central role in adhesion and aggregation of platelets. Resting platelets express their $\alpha_{IIb}\beta_3$ integrins in a low affinity state. After activation, mediated by other platelet receptors, $\alpha_{IIb}\beta_3$ shifts to an high affinity state and is able to bind its main ligand, fibrinogen.[57,58] In the past, three sites on fibrinogen have been postulated to bind to platelet integrin $\alpha_{IIb}\beta_3$: two Arg-Gly-Asp (RGD) sequences in the fibrinogen α-chain (both located in the α-chain at positions 95-97 and 572-574)[59] and the dodecapeptide sequence HHLGGAKQADVG present in the γ-chain (positions 400-411).[60,61] RGD is a consensus binding sequence for integrins and was identified using synthetic peptides.[62] The RGD sequence(s) mediate(s) fibrinogen binding to $\alpha_V\beta_3$ integrins on endothelial cells[63], melanoma cells[64], and fibroblasts[65]. Furthermore, there are other integrins on endothelial cells or fibroblasts that can bind to fibrin(ogen) RGD sites.[66,67] Experiments with antibodies and genetically engineered fibrinogen variants lacking the RGD sequences have demonstrated that the α-chain RGD sequences are not required in platelet-fibrin(ogen) interactions.[68-71] Instead, genetic variants of fibrinogen with either an extension or truncation of the γ-chain show that the carboxy-terminal sequence is an important determinant in platelet-fibrinogen binding.[72-74] These studies, however, do not exclude that other domains of fibrinogen may also participate in platelet-fibrin(ogen) interactions.[75] In fact, it has been reported that β15-42 peptides inhibit platelet aggregation[76] and support platelet spreading.[77]

In addition to its role in haemostasis, fibrinogen also participates in the inflammatory response via specific interactions with leukocyte cell surface adhesion integrins. The main high affinity fibrinogen receptor expressed on monocytes, macrophages and neutrophils is the integrin $\alpha_M\beta_2$ (CD11b/CD18, MAC-1).[78] The recognition site of MAC-1 on fibrinogen does not involve the RGD sites on the α-chain nor the dodecapeptide on the γ-chain.[79] The region γ190-202 has been identified as the mediating sequence of ligand binding to MAC-1.[80] Furthermore, it has been shown that the sequence γ377-395[81] and the alternatively spliced α_E domain also participate in the interaction with MAC-1.[82]

Platelet adhesion and thrombus formation

Blood platelets are small, disc-shaped, anuclear cells with a mean diameter of 2-3 μm and a mean volume of 8 fl. Platelets are produced and released from megakaryocytes present in bone marrow and they circulate for approximately 10 days in blood. Normal blood contains 150.000 to 400.000 platelets/μl.[83] Upon vascular and tissue trauma, platelets immediately respond by establishing adhesive interactions with exposed proteins present in the subendothelial extracellular matrix (ECM). They become activated through contact with thrombogenic surfaces or by locally released or generated chemical agonists. Once activated, platelets bind soluble adhesive molecules present in plasma and become the reactive surface for continuing platelet depositions. Initial platelet tethering to a surface and subsequent platelet-platelet cohesion are typically identified as two separate stages of thrombus formation, defined as adhesion and aggregation.[84] The ECM of the vessel wall is rich in collagens, which play an essential role in thrombus formation by providing a substrate for platelet adhesion and by activating platelets.[85] Adhesion to collagen requires besides the platelet glycoprotein (GP) Ib-IX-V complex, which interacts indirectly with collagen via von Willebrand Factor (vWF)[86], a number of collagen receptors, including integrin $\alpha_2\beta_1$ and GPVI. At high flow conditions, the first step of the overall platelet-surface interaction mechanism is the transient interaction of platelet GPIb with collagen-bound vWF, which

results in a translocation (rolling) of the platelets over the surface.[87] The platelets slow down and subsequently become firmly attached to the surface via $\alpha_2\beta_1$ and GPVI, which is responsible for platelet activation. This leads to the activation of $\alpha_{IIb}\beta_3$, content release of the secretion granules and subsequent platelet thrombus formation.[88] The important role of GPIb, $\alpha_2\beta_1$ and GPVI in haemostasis is illustrated respectively by the haemorrhagic conditions of patients with the Bernard-Soulier syndrome (impaired or absent interaction between vWF and GPIb)[89], von Willebrand's disease (dysfunctional or deficiency of vWF)[89,90], α_2 subunit deficiency (unresponsive to collagen induced aggregation and affected adhesion to the vessel wall)[91,92] and GPVI deficiency (reduced response to collagen).[93] However, concerning the involvement of $\alpha_2\beta_1$ and GPVI, it has been reported recently in β_1-null mice that GPVI plays a more central role in platelet-collagen interactions by activating different adhesive receptors, including $\alpha_2\beta_1$ integrin, suggesting that $\alpha_2\beta_1$ strengthens adhesion without being essential.[94]

Platelet integrin $\alpha_{IIb}\beta_3$

Platelet integrin $\alpha_{IIb}\beta_3$ is important for thrombus build up through platelet-platelet interactions mediated by its multivalent ligands like fibrin(ogen) and vWF. Integrin $\alpha_{IIb}\beta_3$ is the most abundant platelet-cell surface protein. A normal platelet contains approximately 50.000-100.000 $\alpha_{IIb}\beta_3$ receptors, randomly distributed on the platelet surface. Integrin $\alpha_{IIb}\beta_3$ consists of non-covalently associated α and β subunits and requires micromolar extracellular calcium concentration for its stability. Glanzmann's thrombasthenia is the inherited disorder of platelet function caused by a deficiency or abnormality of the integrin $\alpha_{IIb}\beta_3$ associated with bleeding.[95] Thrombasthenic platelets that lack $\alpha_{IIb}\beta_3$ do not aggregate in response to any agonist[96,97], nor do they bind to immobilized fibrinogen.[98] Integrin $\alpha_{IIb}\beta_3$ is also located on the surface-connected canalicular system and in the inner membrane of cytoplasmic α granules. This internal pool of $\alpha_{IIb}\beta_3$ becomes functionally active upon platelet activation. Activation of the platelet involves a change shape and fusion of the α granule membrane with the platelet

surface. Integrin $\alpha_{IIb}\beta_3$ is responsible for presence of fibrinogen within the α granules. Fibrinogen is endocytozed by megakaryocytes and platelets via $\alpha_{IIb}\beta_3$.[99,100] This process is mediated by the γ400-411 sequence, since fibrinogen with the extended form γ' is not observed within platelets and furthermore, fibrinogen of patients with mutations in the γ400-411 is absent in platelets.[101,102] In addition to fibrinogen, $\alpha_{IIb}\beta_3$ binds also to adhesive proteins like vWF, fibronectin, thrombospondin and vitronectin. This binding is mainly mediated by RGD sequences.[103,104]

ADP induced platelet activation

The receptor function of $\alpha_{IIb}\beta_3$ is dependent on platelet activation.[105] Activation occurs upon stimulation by agonists such as thrombin, collagen and epinephrine or by agonists secreted from platelets such as thromboxane A_2 (TxA_2) and ADP. The platelet release reaction enhances further platelet aggregation. TxA_2 is one of the final products of the prostaglandin synthesis and amplifies platelet aggregation via its binding to the TxA_2 receptors.[106,107] Aspirin is an inhibitor of this amplification mechanism via the irreversible acetylation of the platelet cyclo-oxygenase and it blocks thereby the formation of the prostaglandin metabolite TxA_2.[108] In addition to TxA_2, ADP is an important agonist of platelets.[109,110] ADP is present at near molar concentrations in platelet dense granules and reinforces platelet aggregation via additional activation of $\alpha_{IIb}\beta_3$. Inhibitors of ADP induced platelet aggregation are effective antithrombotic drugs.[111,112] ADP removing enzymes display antithrombotic properties in animal models, while patients with defects of ADP receptors or lacking ADP in their platelet granules suffer from a bleeding diathesis.[113,114] Adenine nucleotides interact with P2 receptors. These receptors are divided into two main groups: the G protein coupled or "metabotropic" superfamily termed P2Y and the ligand gated ion channel or "ionotropic" superfamily termed P2X.[115] Two P2 receptors contribute to platelet aggregation in response to ADP: the $P2Y_1$ through mobilization of calcium stores, whereas the more recently identified $P2Y_{12}$ receptor coupled to adenylyl cyclase inhibition is essential for a full aggregation via the

activation of $\alpha_{IIb}\beta_3$ en its participation in the potentiation of platelet secretion.[116] In addition to the G protein-coupled $P2Y_1$ and $P2Y_{12}$ receptors, platelets also express the $P2X_1$ receptor, which has been shown to be responsible for the fast calcium entry induced by ADP.[116] However, the exact functional activity of this receptor is still a matter of debate.[117,118]

Influence of flow on blood-vessel wall interactions

Blood flow determines the transport of platelets and clotting factors towards and away from the vessel wall surface.[119] Two fluid dynamic parameters are frequently used to describe the influence of blood flow on platelet adhesion: the shear rate (γ) and shear stress (τ).[120] The shear rate (s^{-1}) is a measure of how rapid fluid layers are flowing past each other (derivative of the velocity profile). The shear rate is zero in the center of the flow and maximal at the vessel wall. Shear stress is defined as the tangential force per unit area exerted in the direction of flow. In the case of a liquid obeying Newton's Second Law, shear stress (τ) is linearly related to the shear rate: $\tau = \eta \, . \, \gamma$, in which η is the viscosity of the blood. Shear rates and shear stresses can be calculated for various parts of the vasculature from the known vascular diameter and volume flow rates (see section Perfusion models). The shear rates are low in large vessels (large veins: diameter 0.5-10 cm: 200 s^{-1}; ascending aorta: diameter: 2.3-4.5 cm: 50-300 s^{-1}; small arteries: diameter 0.03 cm: 1500 s^{-1}), but increase up to 5.000 s^{-1} in capillaries (diameter: 0.0006 cm).[120] The shear rates in stenozed arteries can be much higher still (up to 40.000 s^{-1}). However, calculations of shear rate and stress are based on a simplified model of steady and non-pulsatile flow and they assume laminar flow, while branching and abnormal vascular curvature will cause local turbulence. Furthermore, blood is not a Newtonian fluid: the viscosity of blood is not constant, but decreases with increasing shear rates. The non-Newtonian flow properties of whole blood are due to the presence of red blood cells (occupying 40 % of the volume). Nevertheless, the use of shear rates and stress allows a description of the effect of blood flow on platelet adhesion.[121]

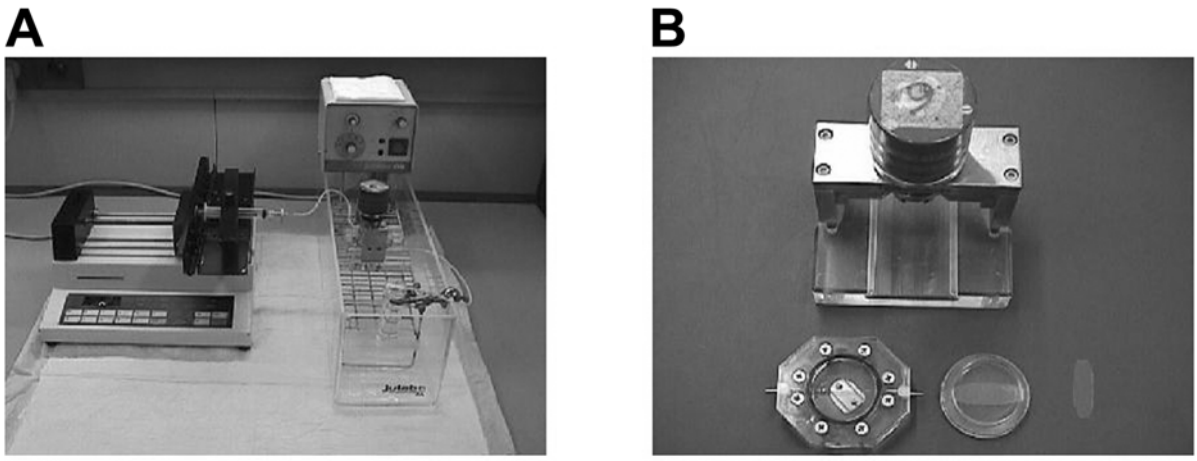
Platelet transport to the vessel wall is one of the main factors determining platelet adhesion. Platelet transport occurs by two processes: diffusion and convection. Diffusion is random and is the result of the Brownian motion of the platelets representing the movement of material relative to the average fluid motion. Convection is the movement of platelets with the fluid and is the result of flow. In the absence of flow, diffusion (which is a slow process) will be responsible for platelet transport, while in flowing blood convection forces will be dominant. In flowing blood, platelet transport is driven by the shear rate of the blood in combination with the presence of red cells. Platelet adhesion increases with increasing shear rate. The distribution of red cells in flowing blood is not homogeneous. Because of shear, red cells migrate to the middle of the flow stream. The smaller platelets are pushed aside towards the vessel surface due to collisions with red cells. The consequence is that the local concentration of platelets near the vessel wall increases upon increasing shear. The transport of platelets by red cells towards the vessel wall is dependent on the haematocrit, red cell rigidity and red cell size. Together with the plasma viscosity, the red cell rigidity determines the viscosity of the blood.[122] An increase in total viscosity will result in an increase in platelet deposition.[123]

Perfusion models

To study platelet-vessel wall interactions under more physiological conditions, an experimental perfusion system has been developed. A perfusion system consists of a perfusion chamber, a roller pump or infusion pump, containers, and silastic tubing to connect chamber and pump (Figure 3). In the past, Baumgartner developed an annular perfusion chamber which allowed to study platelet adhesion and thrombus formation on de-endothelialized rabbit or human arterial vessel wall segments. The segments were inverted on a central rod mounted in a cylinder and subsequently exposed to anticoagulated or non-anticoagulated blood.[124] The disadvantage of this type of chamber is that the evaluation through cross-sections of the vessel wall segment is very time consuming and laborious. The most commonly used flow chamber and also used in the present studies is the parallel plate

perfusion chamber.[125,126] It consists of a polycarbonate knob that can contain a coverslip (Figure 3). The thrombogenic surface is introduced on the coverslip, placed on the knob and exposed to flowing blood. The variation in shear rates is achieved by varying the flow rate and/or the dimensions of the chamber. These dimensions of the flow chamber (height, h; width, d) in combination with the volumetric flow rate (Q) and the viscosity (η) determines the shear stress (τ) in the chamber: $\tau = 6\ Q.\eta.h^{-2}.d^{-1}$. The role of vessel wall components can be studied by using purified proteins or extracellular matrices of cultured cells as thrombogenic surfaces.[127] After perfusion, the coverslip is removed and the platelets are fixed, stained and subsequently platelet deposition can be evaluated automatically with computer-assisted analysis.

Figure 3. Image of the experimental perfusion system.



Perfusions are performed using a small perfusion chamber (Panel B). Coverslips (coated with an adhesive protein or the extracellular matrix of cultured cells) placed on a circular knob in the perfusion chamber are exposed to whole blood under flow conditions using a syringe-pump. Perfusions are performed at 37 °C using a waterbath (Panel A).

Aim and outline of the present thesis

The objective of the studies described in this thesis is to investigate the interactions between platelets and fibrinogen/fibrin during the formation and degradation of a platelet-fibrin(ogen) plug under well-defined physiological flow conditions.

In *Chapter 2* the contribution of fibrinogen in the formation and packing of a platelet thrombus has been investigated under flow conditions. A perfusion model is developed to study thrombus growth and stability in real-time. For this study blood was used from a patient suffering from congenital afibrinogenemia. *Chapter 3* describes the genetic characterization of the fibrinogen disorder underlying the haemorrhagic condition of this patient. In *Chapter 4* the role of ADP was examined in platelet adhesion to fibrinogen and in thrombus formation on collagen. Fibrinogen contains a fibrin-specific region (γ312-322), which becomes exposed upon polymerization to fibrin. The participation of this epitope in platelet adhesion to immobilized fibrinogen has been investigated under conditions of flow in *Chapter 5*. Adhesion of platelets to a fibrin network during fibrinolysis is compared to neutrophil adhesion in *Chapter 6*. In *Chapter 7* a newly developed model is described to study the dynamics of fibrin formation and deposition in flow. Using this model the binding of fibrin fibers was studied to proteins present in the extracellular matrix. In *Chapter 8*, the findings of the studies described in *Chapters 2-7* are integrated and discussed in a broader context and future prospects are presented.

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CHAPTER 2

Absence of fibrinogen in afibrinogenemia results in large but loosely packed thrombi under flow conditions

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Summary

We studied the role of fibrinogen in platelet thrombus formation under flow on adhesive proteins using anticoagulated afibrinogenemic blood in a perfusion system. Perfusions with afibrinogenemic blood showed strongly increased surface coverage and thrombus volume that normalized upon addition of fibrinogen. Similar studies using citrate anticoagulated blood showed that this was due to fibrinogen and not fibrin. Morphological analysis showed that afibrinogenemic thrombi were loosely packed and consisted mainly of dendritic platelets that contacted one another through filopodia. However, in the presence of fibrinogen, platelets formed lamellipodia and spread out on top of one another. Studies with radiolabeled platelets showed similar numbers of platelets in both conditions demonstrating that the difference is one of packing and the larger size is due to absence of lamellipodia formation and spreading. The found increased thrombus size and loosely packed platelets might help to understand thrombotic complications sometimes seen in patients with afibrinogenemia.

Introduction

Congenital afibrinogenemia is a rare autosomal recessive haemorrhagic disorder characterized by markedly reduced or undetectable levels of fibrinogen in the blood. It has an estimated incidence of 2 per million births.[1] Clinical manifestations range from minor to severe bleeding, often with long asymptomatic intervals. Bleeding may occur spontaneously or is related to trauma.[2] Afibrinogenemia patients not only have prolonged clotting times related to lack of or inadequate fibrin formation, but also have long bleeding times indicating a defect in platelet dependent haemostasis. Accordingly, in vitro aggregation studies show impaired aggregation in response to ADP and collagen that is corrected by fibrinogen.[1-4] Absence of fibrinogen as a bridging molecule between integrin $\alpha_{IIb}\beta_3$ (glycoprotein GPIIbIIIa) on activated platelets explains these findings. Although other adhesive proteins containing an RGD sequence like fibronectin, vitronectin and von Willebrand Factor (vWF) can also serve as ligand and even compete for $\alpha_{IIb}\beta_3$[5], static aggregation studies performed in an aggregometer are completely dependent on fibrinogen at plasma concentrations.[6] Such aggregation studies differ, however, from mural thrombus formation under physiological flow conditions because vWF is a major ligand for $\alpha_{IIb}\beta_3$ at high shear rates.[7-9]

In the present study we examined the role of fibrinogen in platelet thrombus formation on several in vivo thrombogenic surfaces under flow conditions, representing conditions encountered in veins, arteries and the microcirculation. We performed these studies with both afbrinogenemic (*Af*) and reconstituted blood (replacing plasma by human albumin solution). The results indicate that the absence of fibrinogen in afibrinogenemia results in large but loosely packed platelet aggregates. The increased thrombus size and more loosely packed platelets found in afibrinogenemia might help to understand the thrombotic complications that are sometimes seen in afibrinogenemic patients.[10-14]

Materials and methods

Patient profile

The male afibrinogenemic (*Af*) patient suffered from umbilical bleeding at birth. Later moderate epistaxis and easily bleeding gums after dental manipulation occurred and hemostasis of wounds was delayed. The patient did not experience thrombotic events and thrombotic or bleeding tendencies were absent in his family, which included his 2 daughters. In repeated laboratory measurements the simplate bleeding time was prolonged (> 30 min); platelet counts were normal (270 x 10^9/l), but platelet aggregation was disturbed. Additionally, the aPTT, prothrombin time, Russel Viper Venom and thrombin clotting times were unmeasurably long. Normal levels of coagulation factors II, V, VII, VIII, IX and X were present as well as normal levels of antithrombin III and plasminogen. The plasma fibrinogen antigen level was approximately 2 μg/ml, platelet fibrinogen concentration was 10 μg/10^9 platelets and vWF antigen was 18.7 μg/ml. All abnormal tests normalized with fibrinogen administration (to an equivalent of 1 g/l) to the patient or his plasma. The molecular basis of the congenital afibrinogenemia in this family has been described in Chapter 3.

Materials

Human placenta collagen type I and III and mepacrin were obtained from Sigma Chemical Co (St. Louis, MO, USA). Plasma-vWF was purified from cryoprecipitate by gel filtration on Biogel A15-m (Biorad, Richmond, CA) as described.[15] Human fibrinogen (plasminogen, vWF and fibronectin free) was obtained from Enzyme Research Labs (South Bend, IN, USA). Low Molecular weight heparin (LMWH: Fragmin®) was from Kabi Pharmacia (Uppsala, Sweden). Human albumin was obtained from Behring (Marburg, Germany). ^{111}Indium-oxine was obtained from Mallinckrodt (Petten, the Netherlands).

Coating of the coverslips

Thermanox® coverslips (Nunc, Inc., Naperville, IL, USA; surface area 1.2 cm^2) were soaked overnight in 80 % ethanol, rinsed thoroughly with distilled water and air-dried. Glass coverslips (Renes 24x60 mm, Zeist, the Netherlands) were cleaned overnight by a chromosulfuric acid (2 % chromium trioxide) solution, and rinsed with distilled water before spraying. Monomeric collagen type I or III was sprayed on coverslips and coverslips were blocked as described.[16] Human umbilical vein endothelial cells (HUVECs) were grown on coverslips in order to obtain the extracellular matrix (ECM). HUVECs were isolated from human umbilical cord veins according to the method of Jaffe et al. with minor modifications.[17,18] The cells were cultured and stimulated with phorbol myristate acetate (PMA; 20 ng/ml overnight) to obtain a tissue factor-rich ECM as described previously.[19]

Blood collection and reconstitution

Whole blood, obtained from the patient and healthy volunteer donors, who denied having taken aspirin or other platelet function inhibitors in the preceding week, was anticoagulated with 1/10 (v/v) of 200 U/ml LMWH in 0.15 M NaCl or with 1/10 (v/v) in 110 mmol/l trisodium citrate. In order to obtain reconstituted blood, the platelets and red cells were washed and reconstituted in a 4 % human albumin solution as described.[16]

Perfusion studies

Perfusions were performed with a single-pass perfusion chamber under non-pulsatile flow conditions using a modified small perfusion chamber with a slit height of 0.1 mm and a slit width of 2 mm.[20] Whole or reconstituted blood was prewarmed to 37 °C for 5 min and was drawn for 5 min through the perfusion chamber by a syringe placed in an Harvard infusion pump (Pump 22, model 2400-004; Natick, MA, USA) by which different wall shear rates were maintained. After a perfusion run, the coverslips were taken from the chamber, rinsed in Hepes buffered saline (10 mM Hepes, 150 mM NaCl, pH 7.4), fixed in 0.5 % glutaraldehyde in PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.4), dehydrated in methanol and

stained with May-Grünwald - Giemsa, as described previously.[21] Evaluation of platelet deposition and thrombus formation was performed using computer-assisted analysis or scanning electron microscopy as described.[16]

Real time perfusion studies

A perfusion chamber was developed for real time measurements with a confocal laser scanning microscope (CLSM) (Leica TCS 4D, Heidelberg, Germany). The chamber contained an inlet, outlet and a vacuum channel. On the top of the chamber a silicone rubber gasket was placed. A flow hole was cut in this silicon sheet to create a perfusion slit (slit width: 2 mm; length: 3 cm; height (= thickness of sheet): 0.125 mm) connecting the inlet with the outlet. On both sides, next to the flow area, a slit was cut in the silicon sheet to obtain two vacuum compartments. The coverslip was mounted on this silicone sheet by a vacuum force through the vacuum channel. Blood was incubated for 10 minutes with mepacrin (10 μM) for fluorescent labeling of the platelets.[22,23] Blood was aspirated through the chamber placed on an inverted CLSM. Confocal optical sections were obtained with an argon/krypton laser at a wavelength of 488 nm with longpass filter of 515 nm on an area of 25,186 μm^2 by selecting the highest thrombus (z = x) and subsequently scanning of 8 sections till z = 0. One confocal scanning section takes 1.5 seconds.

Radiolabeling perfusion studies

^{111}Indium-oxine solution (pH 6.1) was added to the washed platelet suspension, incubated for 10 minutes at 37 ºC and washed with Krebs-Ringer buffer (pH 6.0).[21] Blood was reconstituted and after perfusion the radioactivity of the coverslips and platelet suspension was measured with a Minaxi γ-counter (Packard, USA). Subsequently platelet deposition/cm^2 was calculated.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM) for data obtained from

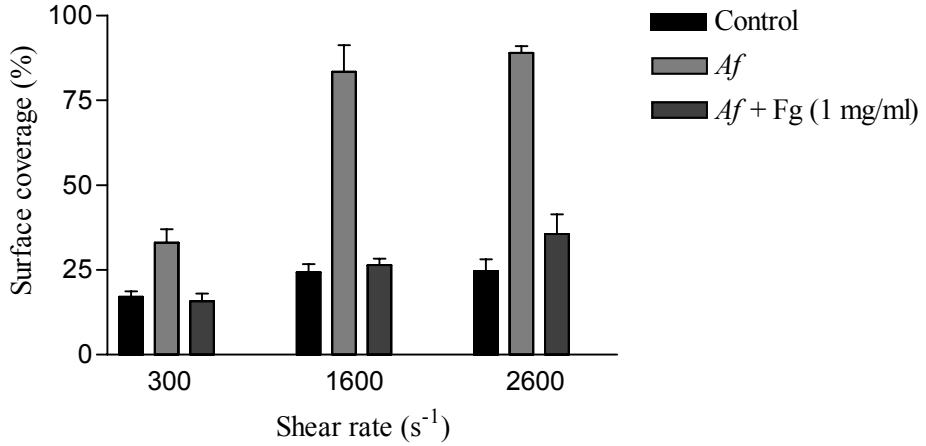
different experiments. The Student-*t*-test was used to test for significance of differences between groups. P-values < 0.05 were considered significant.

Results

Perfusion studies with afibrinogenemic (Af) blood on collagen type III

The role of fibrinogen in platelet thrombus formation on collagen type III was studied under flow conditions with blood from a patient with congenital afibrinogenemia at three different wall shear rates 300 s^{-1}, 1600 s^{-1} and 2600 s^{-1} simulating veins, large arteries and arterioles respectively. Platelet surface coverage is shown in Figure 1 as the percentage of the surface covered by platelets for control blood and patient blood with or without addition of fibrinogen.

Figure 1. Platelet surface coverage on collagen type III after perfusion of control blood, patient (afibrinogenemic, *Af)* blood and patient blood after addition of fibrinogen (1 mg/ml) at different shear rates for 5 minutes).



Platelet deposition is expressed as percentage of the surface covered by platelets. Data are the mean ± SEM of three separate experiments (300 s^{-1}; n =7) or at least two separate experiments (1600 s^{-1} and 2600 s^{-1}; 4 ≤ n ≤ 7) (n = number of evaluated coverslips; Af vs. Af + Fg: p < 0.0001).

At all shear rates we found an increased surface coverage with blood from the patient (300 s^{-1}: 33.0 $\pm$ 3.9 (SEM) %; 1600 s^{-1}: 83.4 $\pm$ 7.8 %; 2600 s^{-1}: 88.9 $\pm$ 2.0 %) compared with blood from normal donors (300 s^{-1}: 17.1 $\pm$ 1.6 %; 1600 s^{-1}: 24.3 $\pm$ 2.4 %; 2600 s^{-1}: 24.6 $\pm$ 3.6 %). Addition of 1 mg/ml of fibrinogen to patient blood resulted in decreased surface coverage (300 s^{-1}: 15.8 $\pm$ 2.3 %; 1600 s^{-1}: 26.4 $\pm$ 1.9 %; 2600 s^{-1}: 35.6 $\pm$ 5.8 %). Comparable results were found in perfusion studies performed with reconstituted blood using patient platelets and plasma of a healthy donor (with the same bloodgroup and comparable vWF concentration), which was mixed in different volume ratios with patient plasma resulting in a variation of different fibrinogen concentrations (data not shown).

The size of thrombi was quantified by aggregate analysis and results are shown in Table I for a shear rate of 1600 s^{-1} as percentage of total surface coverage present in a certain thrombus size category. Platelet thrombi of the patient were much larger (56 % of total surface coverage were thrombi > 400 μm^2) than thrombi formed with control blood (7 % > 400 μm^2).

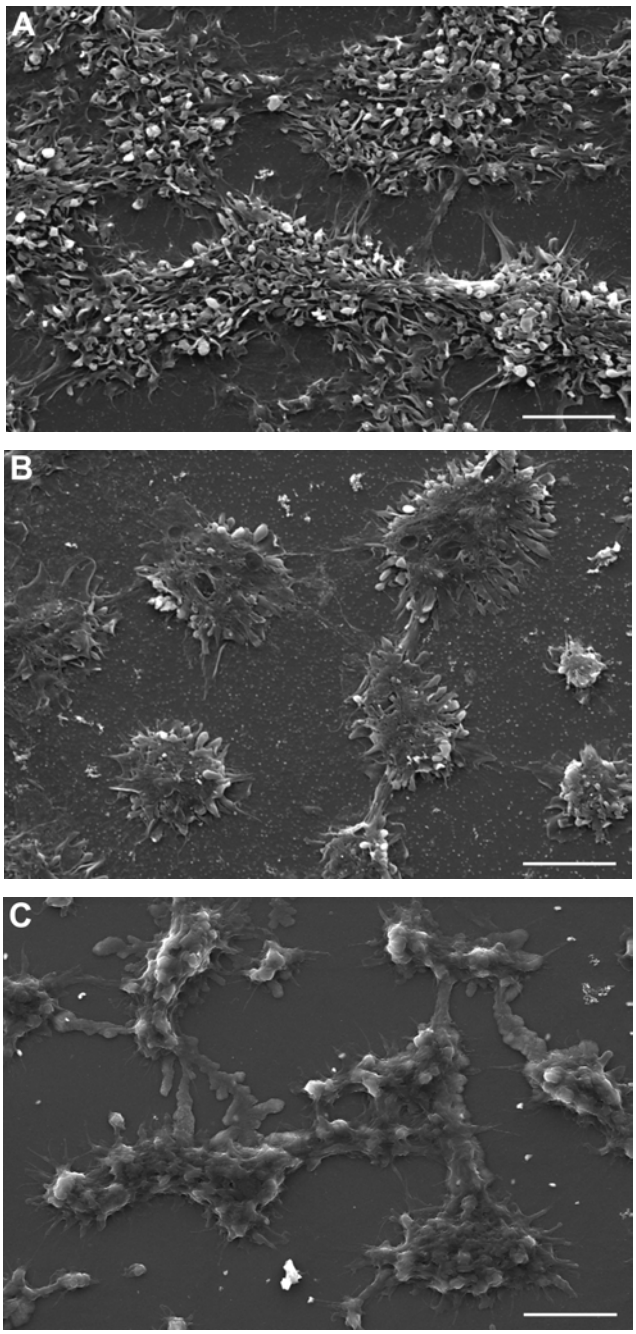
The morphology of the platelet thrombi on the collagen coverslips after perfusion (1600 s^{-1}) of patient blood (A), patient blood with addition of 1 mg/ml fibrinogen (B) and control blood (C) as shown by scanning electron microscopy, is presented in Figure 2.

Table I. Aggregate analysis of thrombi on collagen type III after perfusion of patient blood compared with control blood (1600 s^{-1}).

Size (μm^2)	< 8	8-40	40-400	400-1000	1000-3000	> 3000
Patient	4	7	34	20	12	24
Control	4	21	68	7	0	0

Thrombus size was evaluated using watershed provided by the OPTIMAS 6.0 software package.[16] Results are expressed as percentage of total surface coverage present in a certain thrombus size category. Platelet surface coverage of this perfusion experiment is given in Figure 1 for shear rate 1600 s^{-1}.

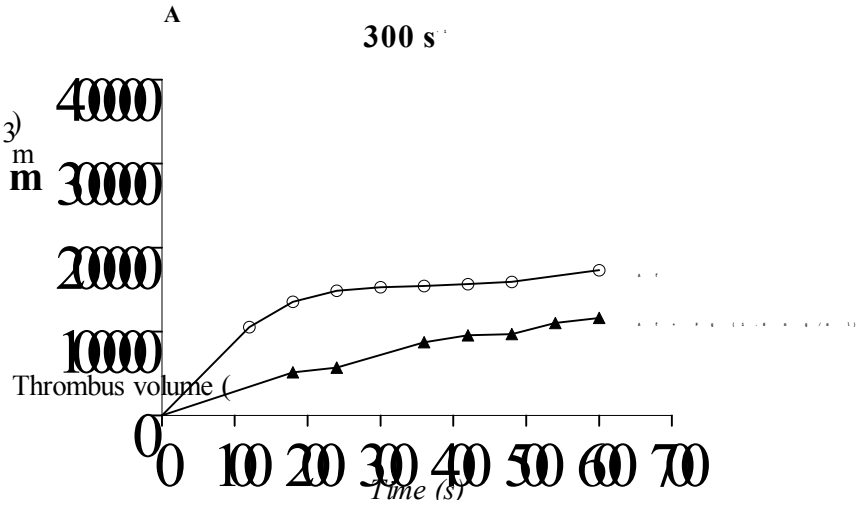
Figure 2. Morphology of platelet thrombi on collagen type III after perfusion of patient blood (A), patient blood after addition of 1 mg/ml fibrinogen (B) and control blood (C) as shown by scanning electron microscopy. Bar = 10 μm.

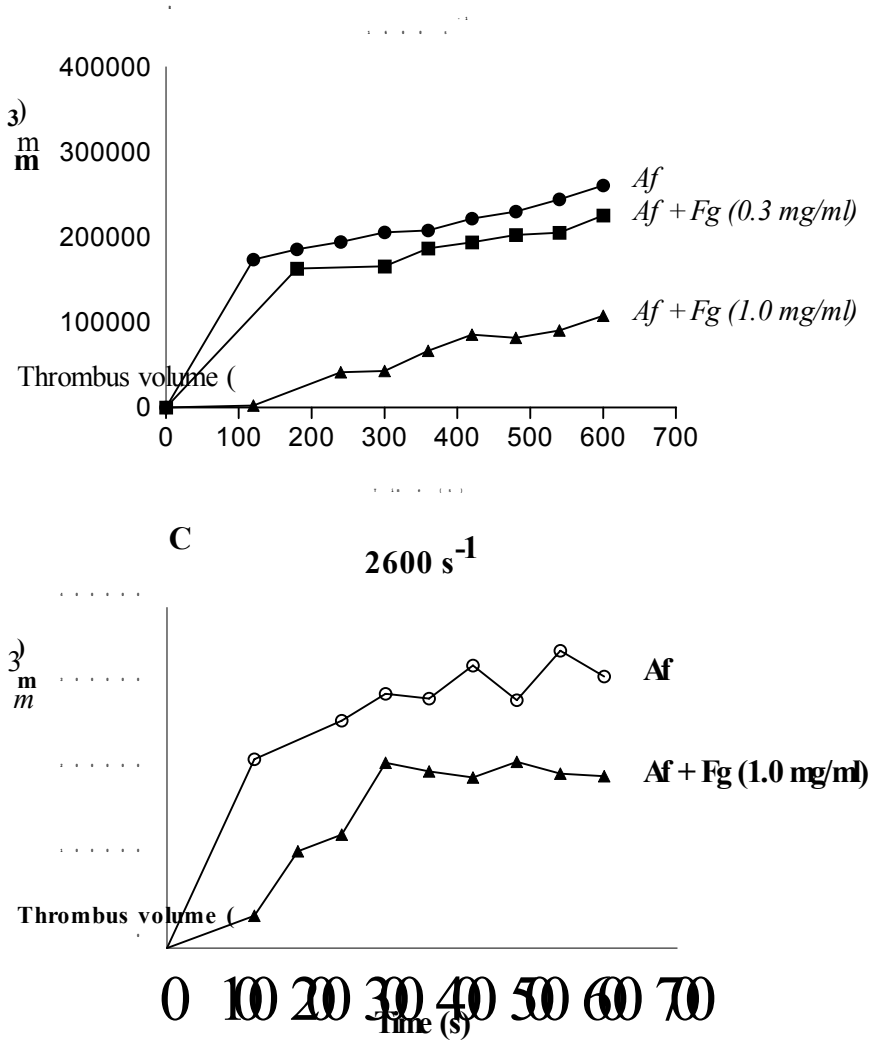


Thrombi formed from patient blood are larger than from control blood. In particular at high shear rates patient platelet deposition consisted not only of larger thrombi, but also of many single platelets adherent to the surface between the thrombi. Addition of fibrinogen to patient blood decreased thrombus heights as observed with scanning electron microscopy. Platelets were more loosely packed in patient thrombi. After addition of fibrinogen, spread platelets were seen that are so tightly packed that they are hardly distinguishable.

To study the stability and volume of thrombi, perfusions over collagen type III were performed in real time coupled to a confocal laser scanning microscopy (CLSM) for three-dimensional analysis. *Af* blood without addition of fibrinogen showed a large thrombus volume (261 x 10^3 µm^3; 10 minutes, 1600 s^{-1}), which decreased in a dose dependent manner upon presence of different fibrinogen concentrations (226 x 10^3 µm^3 at 0.3 mg/ml fibrinogen, 108 x 10^3 µm^3 at 1.0 mg/ml fibrinogen; 10 minutes, 1600 s^{-1}; Figure 3). Thrombi formed at low shear rates are smaller in volume (116 x 10^3 µm^3; *Af* blood with addition of 1 mg/ml fibrinogen; 300 s^{-1}) than thrombi formed at high shear rates (202 x 10^3 µm^3; *Af* blood with addition of 1 mg/ml fibrinogen, 2600 s^{-1}) as depicted in Figure 3.

Figure 3. Time course of thrombus formation on collagen type III at 300 s^{-1} (A), 1600 s^{-1} (B) and 2600 s^{-1} (C) with patient blood -/+ addition of fibrinogen.





Before perfusion, blood was pre-incubated for 10 minutes with mepacrin (10 μM) to label the platelets. Patient blood -/+ addition of fibrinogen (1 mg/ml and 0.3 mg/ml (1600 s^{-1})) was perfused for 10 minutes over collagen type III coated glass coverslips and thrombus volume was measured with confocal laser scanning microscopy.

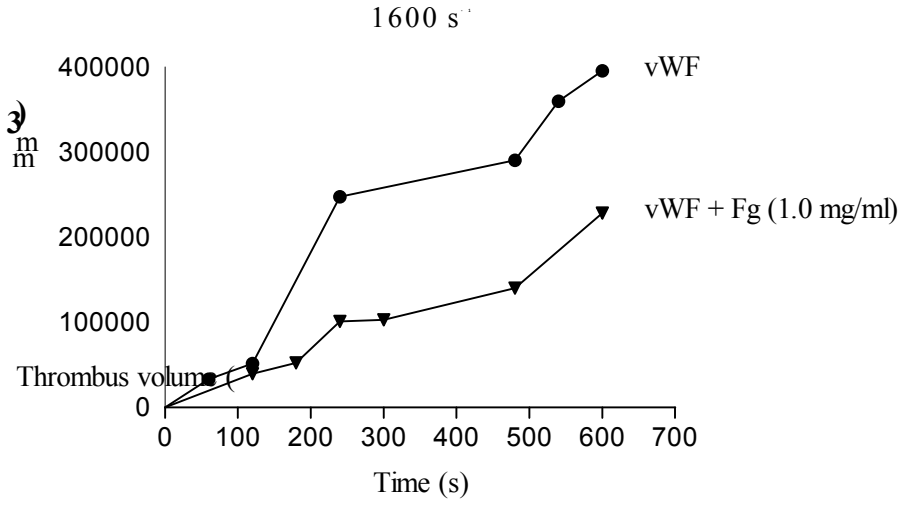
At all shear rates, fibrinogen inhibited thrombus volume growth on collagen type III (mean inhibition: 43 %). In our experimental design no signs of thrombus instability and embolization were observed. Thrombus volume stabilized at 2600 s^{-1} and continued to enlarge

after 10 minutes perfusion at 300 s^{-1} and 1600 s^{-1} for both conditions (with and without addition of fibrinogen).

Perfusion studies with reconstituted blood on collagen type III

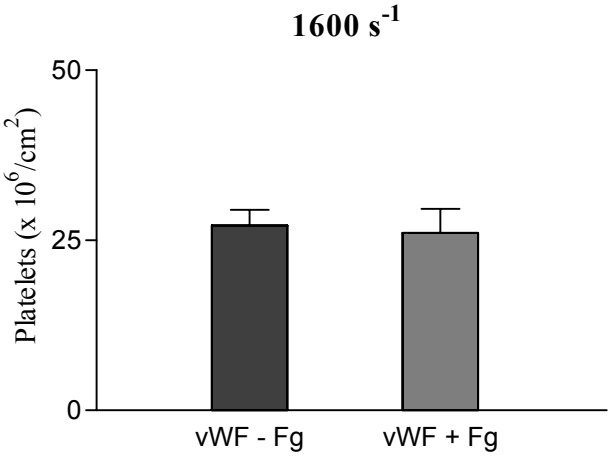
Perfusion experiments were repeated with reconstituted blood instead of native patient blood. The important difference is that platelet fibrinogen levels are normal in reconstituted blood. In reconstituted blood platelet surface coverage was also inhibited by addition of fibrinogen (41.0 $\pm$ 2.0 % (- Fg) vs. 14.8 $\pm$ 1.7 % (+ Fg); p:<0.0001). Consistent with the observations in *Af* blood, thrombus volume growth with reconstituted blood was also inhibited (42 %) under flow when fibrinogen was added to the blood, in three-dimensional real time analysis by CLSM (Figure 4). In order to study whether the inhibition was due to fibrinogen or fibrin we performed identical experiments using citrate anticoagulated blood (data not shown). Similar results were found, indicating that the inhibition was due to fibrinogen and not fibrin.

Figure 4. Time course of thrombus formation on collagen type III at a shear rate of 1600 s^{-1} with reconstituted blood -/+ addition of fibrinogen.



Reconst ituted blood (+ 10 μg/ml vWF) -/+ addition of fibrinogen (1 mg/ml) was perfused for 10 minutes over glass coverslips sprayed with collagen type III and thrombus volume was measured.

Figure 5. Platelet deposition on collagen type III after perfusion of reconstituted blood -/+ addition of fibrinogen as measured by radiolabeling.



The platelet rich suspension was incubated with ^{111}Indium-oxine. Reconstituted blood (+ 10 μg/ml vWF -/+ 1 mg/ml Fg) was perfused over a collagen surface at a wall shear rate of 1600 s^{-1} for 5 minutes. After fixation, radioactivity of the coverslips and the platelet rich suspension was measured with a Minaxi γ-counter and expressed as radioactivity/cm^2 stream area.

Radiolabeling perfusion studies with reconstituted blood on collagen type III

Because of the differences in aggregate morphology as shown by scanning electron microscopy (Figure 2), the packing of platelets was studied with radiolabeled platelets added to reconstituted blood. Although after 5 minutes thrombi were larger in the absence of fibrinogen (Table II and Figure 4), similar numbers of platelets were found in thrombi formed in the presence and absence of fibrinogen (Figure 5). This finding suggests that loose packing of platelets occurs in the absence of fibrinogen. Confirming results were found with fluorescent images of mepacrin labeled platelets in thrombi after perfusion. Single platelets in thrombi formed in the absence of fibrinogen were more easily distinguished than in thrombi formed in the presence of fibrinogen suggesting that these thrombi contained more densely packed platelets (data not shown).

Perfusion studies with Af blood on collagen type I and ECM

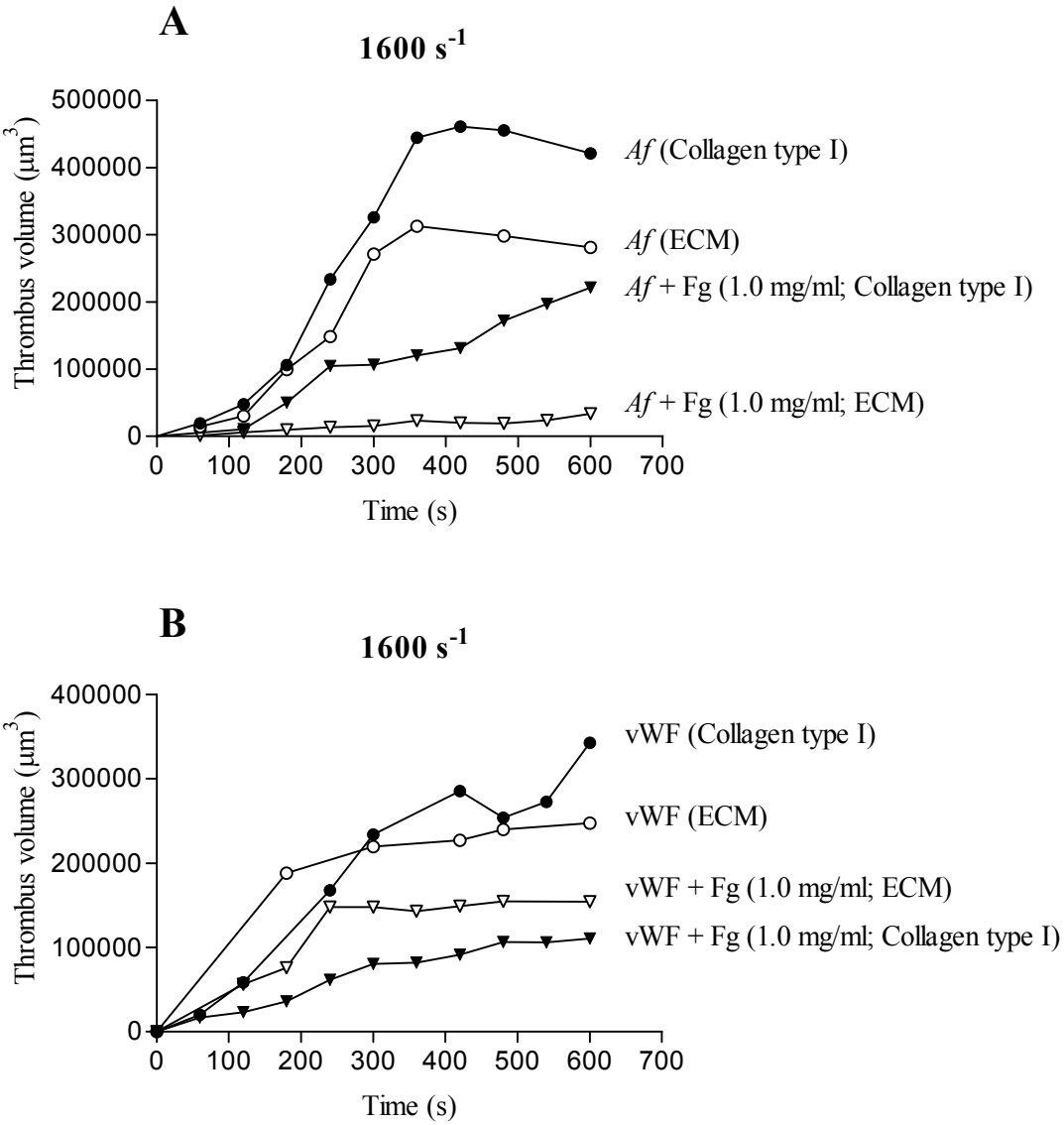
The role of fibrinogen in thrombus formation was further studied on other adhesive surfaces such as the extracellular matrix (ECM) of endothelial cells after PMA stimulation and on collagen type I. Consistent with observations on collagen type III, platelet surface coverage on collagen type I and ECM was strongly increased in *Af* blood and this increase was inhibited when fibrinogen was added to patient blood (Table II). Thrombi formed from *Af* blood were much larger than those from *Af* blood with added fibrinogen. PMA stimulated ECM disclosed similar results. At low shear rates (100 s^{-1}) fibrin was present between thrombi formed after perfusion of control blood. *Af* blood yielded extremely high platelet surface coverage, and no fibrin was detected (data not shown). Patient platelet surface coverage on both surfaces existed not only of larger thrombi, but also of many single platelets adhered between the thrombi.

Table II. Surface coverage to collagen type III, collagen type I and PMA stimulated ECM after perfusion (1600 s^{-1}) of reconstituted, control or patient blood -/+ addition of fibrinogen (1 mg/ml).

Perfusion	Collagen type III	Collagen type I	ECM
A. vWF – Fg	41.0 ± 2.0 (n=9)	36.5 ± 9.5 (n=9)	53.4 ± 9.5 (n=9)
B. vWF + Fg	14.8 ± 1.7 (n=9)***	6.7 ± 2.0 (n=8)*	13.1 ± 1.7 (n=8)*
C. Patient	83.4 ± 7.8 (n=5)	77.0 (n=2)	79.8 (n=2)
D. Patient + Fg	26.4 ± 1.9 (n=4)	23.0 (n=2)	30.7 (n=2)
E. Control	24.3 ± 2.4 (n=7)	20.0 (n=2)	28.9 (n=2)

Blood was perfused (5 min) over coverslips cultured with PMA stimulated ECM or sprayed with collagen type III or type I. Data are the mean percentage surface coverage ± SEM of three separate experiments (reconstituted blood) or the mean of one experiment performed in duplo (patient and control blood on collagen type I and ECM). See Figure 1 for statistics for patient and control blood on collagen type III. $^{*}p < 0.05$; $^{***}p < 0.001$*; B versus A; n = number of evaluated coverslips.*

Figure 6. Time course of thrombus formation on collagen type I and PMA stimulated ECM at different shear rates with patient (A) and reconstituted blood (B) -/+ addition of fibrinogen.



Thrombus development in real time was measured for collagen type I and ECM at a shear rate of 1600 s^{-1} (Figure 6A). Thrombus volume after 10 minutes perfusion on collagen type I was much higher than on collagen type III (421 x 10^3 μm^3 vs. 261 x 10^3 μm^3; 1600 s^{-1}). Thrombus volume on ECM (281 x 10^3 μm^3; 1600 s^{-1}) was comparable with collagen type III. Addition

of fibrinogen decreased thrombus volume on collagen type I by 47 % and on ECM by 88 %. Both surfaces showed after circa 7 minutes a slight decrease in thrombus volume without addition of fibrinogen.

Perfusion studies with reconstituted blood on collagen type I and ECM

The role of fibrinogen in thrombus formation on collagen type I and ECM, as studied with reconstituted blood is consistent with that of collagen type III: platelet surface coverage (Table II) and platelet thrombus volume (Figure 6B) are both strongly decreased in the presence of fibrinogen. Similar results were found with citrate anticoagulated blood (data not shown).

Discussion

We recently published data which show that vWF can compete with fibrinogen in thrombus formation under flow conditions.[16] In that article we performed perfusion studies with blood of a patient using severe von Willebrand's disease (vWD) and found increased platelet surface coverage and larger thrombi in vWD blood with addition of RGGS-vWF (which cannot interact with $\alpha_{IIb}\beta_3$) than with recombinant vWF (binds both GPIb and $\alpha_{IIb}\beta_3$). We postulated on the basis of these results that platelet-platelet interaction becomes less efficient via competition for the occupancy of $\alpha_{IIb}\beta_3$. In the present article we investigated whether absence of fibrinogen could give similar results compatible with the hypothesis that platelet-platelet interaction is more efficient when either vWF or fibrinogen is present alone as the main ligand.

We demonstrate that perfusion of *Af* blood over several adhesive surfaces such as collagen type I, collagen type III and tissue factor containing endothelial cell matrix showed a strong increase in platelet surface coverage as compared with control blood. Patient platelet thrombus volume was also increased at all studied shear rates (300 s^{-1}, 1600 s^{-1} and 2600 s^{-1}). Absence of fibrinogen was the cause for increased thrombus formation for all surfaces,

because thrombus formation was normalized upon addition of fibrinogen. Perfusion studies with reconstituted blood supported these findings using *Af* blood.

At first sight, our present results appear to support this hypothesis of mutual competition when both vWF and fibrinogen are present as ligands for $\alpha_{IIb}\beta_3$. Closer inspection however, suggests otherwise. There was a marked difference in morphology between platelet thrombi formed in the absence or in the presence of fibrinogen. In its absence, thrombi were loosely packed and consisted mainly of dendritic platelets that contacted one another through filopodia. In the presence of fibrinogen, platelets formed lamellipodia and spread out on top of one another like "shingles on a roof". We therefore decided to obtain more information about the number of platelets that are involved in thrombus formation in both situations using radiolabeled platelets. These studies showed that exactly similar numbers of platelets were present in both conditions demonstrating that the difference is one of packing and that the larger size is simply due to the absence of lamellipodia formation and platelet spreading.

Our results confirm and extent those of Tsuji et al.[24] and Ruggeri et al.[25] who also found that fibrinogen was required for thrombus stability. Tsuji et al.[24] also found increased platelet surface coverage and no differences in mobility index (measure of stability) in perfusion studies with blood of two patients with afibrinogenemia on collagen type I at a shear rate of 1500 s^{-1}. Only at 4500 s^{-1} they could find a higher mobility index with *Af* blood, indicating that *Af* thrombi are less stable under extremely high shears. Ruggeri et al.[25] studied thrombus formation on collagen type I with PPACK anticoagulated reconstituted blood and found in the absence of fibrinogen unstable aggregates even at 1500 s^{-1}.

We extended these studies to other shear rates and other surfaces such as collagen type III and tissue factor containing endothelial cell matrix. We explored the reason of why the platelets were larger and less stable. Under our experimental conditions we did not observe embolization in the shear rate range studied, but the effect on platelet packing was observed under all conditions. Ruggeri's results suggested that fibrin formation might be a critical factor for thrombus stability. Our own results indicate that it is fibrinogen itself and not fibrin which is required, because similar results were obtained using citrated blood on collagen

under conditions which preclude the generation of thrombin. Although it is tempting to suggest that the many "bridges" that can form when fibrinogen is present, work as a "zipper" between platelets, the other possibility that activation of $\alpha_{IIb}\beta_3$ by occupancy with fibrinogen may lead to formation of lamellipodia and platelet spreading, should also be borne in mind.

The observed increase in thrombus size suggests that patients with afibrinogenemia could be at thrombotic risk. This is a rather paradoxical statement about a severe coagulation defect as afibrinogenemia, strongly associated with haemorrhagic manifestations. However, several cases are reported in the literature about an association between afibrinogenemia or hypofibrinogenemia and thrombosis. In three patients with hypofibrinogenemia (fibrinogen levels ranging from 0.2 to 0.7 g/l)[10-12] and in one patient with afibrinogenemia[13], thrombotic events were observed in the absence of any substitutive therapy. These patients suffered from arterial thrombosis with ischemic toe lesions in three cases and venous thrombosis in one patient.[12] Recently a study of 55 *Af* patients showed two young patients who developed spontaneous thrombotic episodes in cerebral sagittal sinus and popliteal artery respectively.[14] Different explanations have been offered for the observed thrombosis in afibrinogenemia. Marchall et al.[11] suggested that the disappearance of a protective film of fibrin that covers the thrombotic lesion might be responsible for thrombotic complications. This might be a possible explanation at low shear conditions, but at arterial shear rates fibrin formation is a minor factor in thrombogenesis.[26,27] Chafa et al.[28] suggested that thrombotic events are related to thrombin-induced platelet aggregation in vivo due to poor neutralization of this enzyme in turn due to lack of its adsorption on fibrin. An alternative explanation for these observed thrombotic events in afibrinogenemia patients might be that the absence of fibrinogen will cause larger thrombi which are more loosely packed than when it is present, but that at extremely high arterial shear rates instability of these high volume thrombi increase the risk for arterial thrombosis as observed in some afibrinogenemia patients. Similar suggestions were made according a recently published study on thrombus formation in fibrinogen- and vWF-deficient mice by Ni et al.[29]

Acknowledgments

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CHAPTER 3

Molecular basis of congenital afibrinogenaemia in a Dutch family

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Summary

Congenital afibrinogenemia is a rare autosomal recessive disorder characterized by complete absence or trace amounts of fibrinogen. Here we report the identification of the molecular defect underlying afibrinogenaemia in a Dutch patient. DNA sequence analysis of the fibrinogen Aα-, Bβ- and γ-genes revealed a homozygous deletion of two adenines between nucleotides 3120 and 3122 in exon 4 of the gene coding for the Aα-chain. This deletion results in a frameshift with a predicted premature end of translation at codon 140. This is the first report of a patient homozygous for this rare mutation associated with afibrinogenaemia.

Introduction

Fibrinogen is a 340 kDa glycoprotein synthesized by hepatocytes and secreted as a hexamer composed of three pairs of polypeptide chains (Aα, Bβ and γ). It circulates in plasma at a concentration of 2-4 mg/ml The Aα-, Bβ- and γ-chain are encoded by three different genes, respectively *FGA, FGB, FGG*, and clustered in a region of approximately 50 kilobases on chromosome 4 (q28-q31).[1] Inherited fibrinogen disorders are rare and affect either the quantity (afibrinogenemia and hypofibrinogenemia) or the quality of circulating fibrinogen (dysfibrinogenemia). Congenital afibrinogenaemia, originally described in 1920[2], is a rare haemorrhagic disorder inherited as an autosomal recessive trait with an estimated prevalence of two per million births.[3] Despite the central role of fibrinogen in haemostasis, the haemorrhagic diathesis ranges from mild to severe. This is probably partly due to the presence of functional von Willebrand Factor, which allows platelet adhesion/aggregation and formation of (loosely packed) thrombi even in the absence of fibrin(ogen).[4,5] Bleeding from the umbilical cord often reflects the first sign of the disorder. Furthermore, gum bleeding, epistaxis, menorrhagia, gastro-intestinal bleeding and haemarthrosis occur with varying intensity, and spontaneous intracerebral bleeding and splenic ruputure can occur throughout life.[6] Patients respond well to prophylactic fibrinogen administration therapy. The half-life of infused fibrinogen is essentially normal, therefore the genetic defect underlying afibrinogenaemia most likely resides at the level of fibrinogen synthesis.[3] Mutations that are associated with afibrinogenaemia have been identified in all three fibrinogen genes with a relative high frequency of a donor splice site mutation in intron 4 of *FGA*.[7]

Previously, we performed several studies on the role of fibrinogen in thrombosis and haemostasis using blood of a Dutch patient diagnozed with congenital afibrinogenaemia.[4,8] Here we report the molecular basis underlying the haemorrhagic state in this patient.

Patient, materials and methods

Case report

The patient is a 41-year old Dutch male, born of a consanguineous marriage (Figure 1: IX:1). Congenital afibrinogenaemia was diagnozed at birth after umbilical cord bleeding, prolonged bleeding (Simplate: > 30 min; Ivy: > 15 min) and clotting times (thrombin time: > 150 s; activated partial thromboplastin time: > 120 s; reptilase time: > 250 s). Coagulation factors were normal[8], except fibrinogen activity (< 0.1 mg/ml) and antigen (< 10 μg/ml).[4] Tests could be corrected by administration of fibrinogen to the patient or his plasma. His parents, brother, sister and two daughters were asymptomatic. Plasma fibrinogen antigen and activity of the father (VIII:1: activity 2.7 g/l; antigen 3.2 g/l) and the mother of the proband (VII:4: activity 2.3 g/l; antigen 2.2 g/l) were normal.

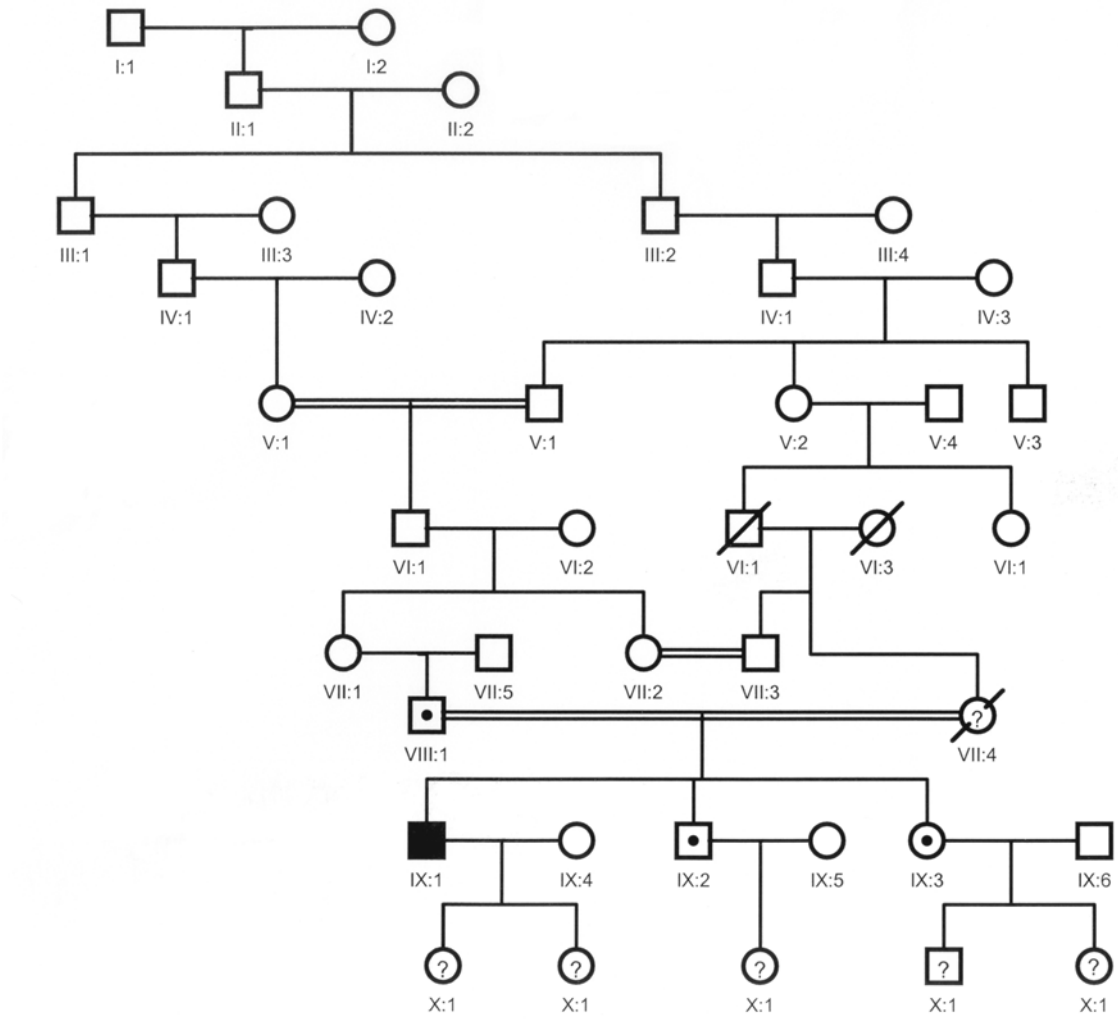
Preparation of plasma and isolation of DNA

Venous blood was collected into plastic tubes in 1/10 volume of 3.2 % sodium citrate. Platelet-poor plasma was obtained by centrifugation at 1200 g for 20 minutes at room temperature and aliquots were immediately stored at –80 °C. DNA was isolated from peripheral white blood cells using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Amplification of the Aα-, Bβ- and γ-genes

The individual exons of the fibrinogen Aα-, Bβ- and γ-genes were amplified by the polymerase chain reaction (PCR). Aα-gene (GenBank, accession no. M64982) primers were a generous gift from Dr. A. Czwalinna and Dr. M. von Depka (Hannover Medical School, Germany), while the exons of the γ-gene (GenBank, accession no. M10014) were amplified as described.[9] Bβ-gene (GenBank, accession no. M64983) primers are available on request.

Figure 1. Family tree of the proband with congenital afibrinogenaemia.



The filled symbol denotes the homozygous proband (IX:1). The open (IX:4, IX:5, IX:6), half-filled (IX:2, IX:3) and questionmarked (X:1, VII:4) symbols denote genotypically normal, heterozygous and not analyzed individuals respectively.

The PCR reactions were carried out with 50-100 ng DNA in 100 µl volumes containing 10 mmol/l Tris-HCL, pH 8.3, 50 mmol/l KCL, 1.5 mmol/l $MgCl_2$, 0.01% (wt/vol) gelatine, 0.2 mmol/l of each dNTP, 0.3 µmol/l of each primer and 2.5 U AmpliTaq Gold DNA polymerase. All reagents were obtained from Perkin Elmer (Roche Molecular Systems Inc., Branchburg, NJ, USA). The samples were subjected to 35 cycles of amplification with denaturation at 94 °C for 30 seconds (10 minutes at 95 °C prior to the first cycle), annealing for 30 seconds at 57 °C and extension at 72 °C for 60 seconds followed by an elongated extension time of 10 minutes after the last cycle.

DNA sequence analysis

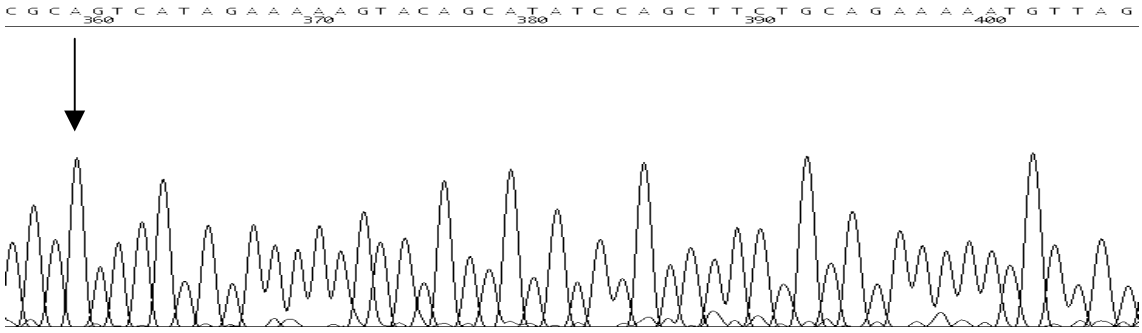
Automated DNA sequence analysis was performed with the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Warrington, England), according to the manufacturer's instructions. Sequencing reactions were all carried out in forward and reverse direction, and samples were analyzed on a Perkin Elmer Applied Biosystems ABI 310 Genetic Analyzer (Perkin Elmer Applied Biosystems, Foster City, CA). PCR products were purified prior to DNA sequence analysis with the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA, USA).

Results and discussion

In the present study we identified the molecular basis of congenital afibrinogenaemia of a patient (Figure 1: IX:1) born out of a consanguineous marriage (VIII:1-VII:4). This is the first report on the molecular characterization of congenital afibrinogenaemia in a Dutch family. DNA sequence analysis of *FGB* and *FGG* of the patient revealed no abnormalities. However, the fibrinogen Aα-gene of the patient showed a homozygous deletion in exon 4 where two adenines were deleted out of three adenines normally present at nucleotides 3120-3122. This mutation results in a shift of the reading frame and predicts a premature translational stop at codon 140 (Figure 2). Furthermore, the patient was (-/-) for the *Taq*I polymorphism in exon 6

of *FGA* previously described[10] (Appendix to Chapter 3). Subsequent testing of family members showed that the father (VIII:1), brother (IX:2) and sister (IX:3) of the patient were heterozygous for this mutation (Figure 1). DNA from the deceased mother was unavailable but with regard to the consanguinity, it is most likely that she was heterozygous for the same mutation. However, hemizygosity of the proband, for example due to a large deletion in the Aα-chain gene[11], can not be excluded. Neerman-Arbez et al. reported two compound heterozygous patients with congenital afibrinogenaemia with the same 2-base pair deletion as described in this study.[12] Here we report for the first time a patient with congenital afibrinogenaemia, who is homozygous for this mutation.

Figure 2. ***Top*****: DNA sequencing electropherogram showing the homozygous 2-base pair (AA) deletion (arrow) between nucleotides 3120 and 3122 in exon 4 of the fibrinogen Aα-chain gene as identified in the Dutch patient (IX:1; Figure 1) with congenital afibrinogenaemia.** ***Bottom*****: Predicted shift in the reading frame resulting in a premature translational stop at Aα-chain codon 140.**



Wild type Aα-chain:

```
CGC AAA GTC ATA GAA AAA GTA CAG CAT ATC CAG CTT CTG CAG AAA AAT GTT AGA
Arg Lys Val Ile Glu Lys Val Gln His Ile Gln Leu Leu Gln Lys Asn Val Arg
124
```

Mutant Aα-chain:

```
CGC AGT CAT AGA AAA AGT ACA GCA TAT CCA GCT TCT GCA GAA AAA TGT TAG AGC
Arg Ser His Arg Lys Ser Thr Ala Tyr Pro Ala Ser Ala Glu Lys Cys  *   *
124                                                             139
```

In general, the mutant mRNAs harbouring a premature termination codon will be subjected to nonsense-mediated mRNA decay (NMD)[13] that prevents the potentially deleterious effects of truncated proteins. However, recently Asselta et al. described five α-chain mutations giving rise to premature stop codons, localized within the first 4 exons of the Aα-chain gene. These mutations escaped from NMD and were thereby responsible for an impaired secretion of the hexameric fibrinogen molecule.[14] Moreover, Fellowes et al.[15] recently described a case of congenital afibrinogenaemia caused by a mutation that created a stop codon (Arg149Term) at a similar position as the one described here. They postulated the lack of truncated Aα-chain in patient's plasma to be caused by either a shortened intracellular half-life or lack of functionality in a region critical to stability i.e. the coiled coil (amino acids 45-165). Moreover, they suggested a minimal fibrinogen Aα-chain length necessary for supporting the assembly and secretion of stable fibrinogen.[15] Mutations coding for truncated proteins shorter than the minimal length might thus lead to afibrinogenaemia whereas truncation after these residues may lead to partial secretion of the α-chain, and consequent hypofibrinogenemia and/or dysfibrinogenemia.

In conclusion, we report a Dutch patient with a homozygous deletion of two adenines in exon 4 of the Aα-chain resulting in a frameshift with a predicted premature end of translation at codon 140. Although, the precise mechanism responsible for the afibrinogenaemia in our patient is unclear, we postulate that synthesis of the fibrinogen molecule will be abolished due to a defective folding and/or secretion of the hexameric molecule.

Acknowledgments

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APPENDIX
CHAPTER 3

Nature of the fibrinogen Aα-gene *Taq*I polymorphism

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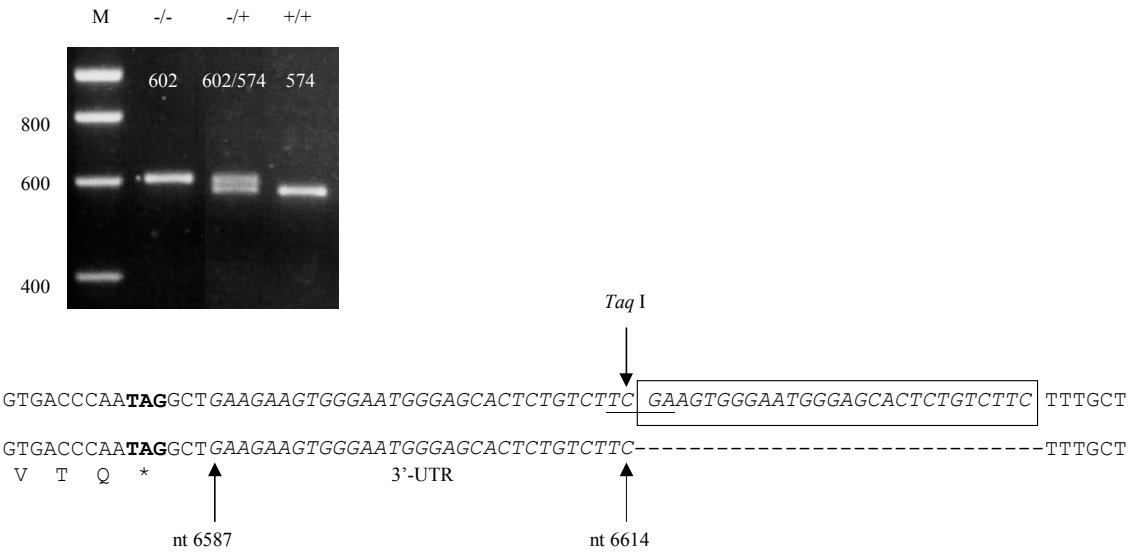
(Thrombosis and Haemostasis 2001: 86 (3): 935-936; Letter to the Editor)

The investigation of genotype to phenotype interactions is increasingly important in understanding the pathogenesis of coagulation disorders. Hereditary afibrinogenemia is a rare haemorrhagic disorder inherited in an autosomal recessive manner, with an estimated incidence of two per million births. Markedly reduced or undetectable levels of fibrinogen in the blood characterize the disease. The clinical phenotype is variable and ranges from minor to severe bleeding, often with long asymptomatic intervals.[1] In order to elucidate the mechanisms involved in this variance in phenotype we recently started to unravel the molecular basis of afibrinogenemia in a patient (Chapter 3) well characterized previously at the protein level in our laboratory.[2] To detect mutations in the fibrinogen Aα-, Bβ- and γ-genes, we used direct DNA sequencing of all coding sequences using primers located in introns to include splice sites.

During our investigations we detected a 28 base pair (bp) insertion located 3' to the termination codon in exon 6 of the fibrinogen Aα-gene in patients as well as in our control DNA. This insertion consisted of a duplication of nucleotides 6587-6614 (GenBank, accession no. M64982) and creates a *Taq*I recognition sequence (Figure 1). Since the presence of a polymorphic *Taq*I site in the fibrinogen Aα-gene is known, but considered to be associated with a single base change[3], we investigated if this duplication was the cause of the previously reported polymorphism. Using primers FGA-15-SN (sense, 5'-CTGGCTAGGCAATGACTAC-3') and FGA-15ASN (antisense, 5'GGTTGTAGAGAATC-TCAACTGC-3'), we amplified by PCR fragments of 574 bp and 602 bp depending on the absence or presence of the duplication, respectively. The PCR products were subsequently electrophorized on a 2.5 % agarose gel. We established the allele frequency in a normal control population (n=65) to be 0.29. The reported *Taq*I allelic frequency, as determined using restriction enzyme analysis, was 0.27.[4] We conclude the 28 bp duplication to be the nature of the *Taq*I polymorphism in the fibrinogen Aα-chain gene.

Figure 1. ***Top*****: Agarose gel electrophoresis of PCR products amplified from individuals without (-/-), heterozygous (-/+) or homozygous (+/+) for the 28 bp duplication in the 3' untranslated region (UTR) of the Aα–gene. M = marker.** ***Bottom*****: 3' Translation end of the α_E subunit. The lower sequence represents the wild-type sequence, the upper sequence shows the sequence with the 28 bp duplication (boxed). The duplication involves nucleotides 6587-6614 3' to the termination codon (bold) and creates, if present, a** ***Taq*****I recognition sequence (underlined).**



Exon 6 of the fibrinogen Aα-chain gene encodes 236 amino acids of an extended fibrinogen α subunit (α_E) that accounts for 1-2 % of the total amount of fibrinogen in the circulation.[5] The function of this fibrinogen α isoform is not known. Because the duplication is located downstream of the termination codon, in the 3'-untranslated region (3'-UTR), it does not result in an amino acid insertion in the protein. However, possible effects on mRNA stability or translational efficiency of the transcript that affect protein levels are feasible. Several studies reported no association of the *Taq*I polymorphism with fibrinogen levels[6-8] or with myocardial infarction.[9] However, in these studies total fibrinogen was measured and thus variations in fibrinogen α_E subunit concentrations would go by undetected due to low physiological levels of this fibrinogen subclass molecule. Recently, a study among obese

individuals reported an association of heterozygosity for a polymorphic pentanucleotide insertion in the 3'-UTR of the leptin receptor gene with lower serum insulin concentrations.[10] Therefore, to determine if the duplication had an effect on α_E levels, we performed Western blotting using a polyclonal rabbit antibody against human fibrinogen (Dako, Glostrup, Denmark). Plasma of individuals homozygous without duplication, homozygous with duplication and heterozygous individuals was used (n=28). No correlation between the α_E level and the polymorphism could be demonstrated with this antibody.

Because of the unusual type of polymorphism and because of its localization in the trailer of the transcript we suggest more elaborate studies on RNA and the possibility of an effect on the phenotype should be undertaken.

Acknowledgements

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CHAPTER 4

Role of ADP receptor $P2Y_{12}$ in platelet adhesion and thrombus formation in flowing blood

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(Arteriosclerosis, Thrombosis, and Vascular Biology 2002: 22 (4): 686-691)

Summary

ADP plays a central role in regulating platelet function. It induces platelet aggregation via the activation of two major ADP receptors, $P2Y_1$ and $P2Y_{12}$. We have investigated the role of $P2Y_{12}$ in platelet adhesion and thrombus formation under physiological flow using blood from a patient with a defect in the gene encoding $P2Y_{12}$. Anticoagulated blood from the patient and healthy volunteers was perfused over collagen-coated coverslips. The patient's thrombi were smaller and consisted of spread platelets overlayered with non-spread platelets, whereas control thrombi were large and densely packed. Identical platelet surface coverage, aggregate size and morphology were found when a $P2Y_{12}$ antagonist, N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (also known as AR-C69931MX), was added to control blood. Addition of a $P2Y_1$ antagonist (A3P5P) to control blood resulted in small, but normally structured, thrombi. Thus the ADP – $P2Y_{12}$ interaction is essential for normal thrombus buildup on collagen. Patient's blood also showed reduced platelet adhesion on fibrinogen, which was not due to changes in morphology. Comparable results were found by using control blood with AR-C69931MX and also with adenosine-3',5'-diphosphate (A3P5P). This suggested that $P2Y_{12}$ and $P2Y_1$ are both involved in platelet adhesion on immobilized fibrinogen, thereby revealing it as ADP-dependent. This was confirmed by complete inhibition upon addition of creatine phosphate (CP)/creatine phophokinase (CPK).

Introduction

Platelets play an important role in primary haemostasis via adhesion, aggregation and subsequent thrombus formation on collagen exposed at the site of vascular damage. Perturbations of this system may lead to pathological thrombus formation and vascular occlusion, resulting in stroke, myocardial or cerebral infarction. Under conditions of high shear, platelet thrombus formation is dependent on the interaction between von Willebrand Factor and platelet glycoprotein (GP) Ib. This interaction leads to platelet activation and conformational changes in the platelet integrin $\alpha_{IIb}\beta_3$[1], which is followed by binding of fibrinogen to the integrin and the formation of stable bridges between aggregating platelets.[2] The $\alpha_{IIb}\beta_3$ receptor has been the recent target for antithrombotic agents such as abciximab, eptifibatide and tirofiban as adjunctive therapy to decrease the ischemic complications of percutaneous coronary interventions and/or unstable angina.[3] During the past decade, ADP receptors on the platelet membrane have also become a target for antithrombotic strategies with compounds such as ticlopidine[4], clopidogrel[4,5] (Plavix®) and the AR-C compounds.[6,7]

ADP is an important agonist, released from damaged vessels and red cells, that induces platelet aggregation through activation of $\alpha_{IIb}\beta_3$. Another major source of ADP, as illustrated by the platelet functional defects in patients with storage pool deficiency[8], is secretion from platelet dense granules upon activation, providing a positive feedback through its binding to ADP receptors contributing to platelet aggregation with other agonists including collagen.

Human platelets possess two major ADP receptors: (1) the $P2Y_1$ receptor, which initiates platelet shape change and ADP-induced aggregation through mobilization of internal calcium stores; and (2) the receptor $P2Y_{12}$, that is coupled to adenylyl cyclase inhibition and which is essential for a full aggregation response to ADP and the stabilization of aggregates (see review).[9] A third receptor, $P2X_1$ that mediates ionic fluxes, has been demonstrated to have ATP as the most potent physiological nucleotide agonist, and ADP is clearly less potent.[10] Recently, Hollopeter et al.[11] described the cloning of the ADP receptor $P2Y_{12}$, which appeared to be the target of ticlopidine, clopidogrel and the AR-C analogues. They also

provided evidence that patient M.L. with a mild bleeding disorder has a defect in the gene coding for $P2Y_{12}$. The platelets of patient M.L. showed impaired ADP-dependent platelet aggregation, had a much reduced ADP binding capacity and lacked the ability to inhibit cAMP levels in response to ADP.[12]

Until now, studies on the role of ADP in platelet thrombus formation under physiological flow conditions have been performed either (1) in vivo, with the use of mice with a knock-out for $P2Y_{1,}$[13,14] and in animal models of arterial thrombosis using AR-C69931MX,[15] or (2) in perfusion models ex vivo, with the use of blood from healthy volunteers treated with clopidogrel[16] and, in a preliminary report, with the use of whole blood in the presence of the $P2Y_{12}$ antagonist: AR-C69931MX and the $P2Y_1$ antagonist: A3P5P.[17] However, no studies have been reported on platelet adhesion and thrombus formation in flow using human blood deficient for the ADP receptor $P2Y_{12}$. In the present study, we have investigated the role of the $P2Y_{12}$ receptor in platelet adhesion to fibrinogen and platelet thrombus formation on collagen by using blood from patient M.L. in comparison with control blood in the presence or absence of ADP receptor antagonists by using our ex vivo perfusion model.[18]

The data indicate for the first time that $P2Y_{12}$-dependent activation of platelets by ADP is involved in platelet adhesion to fibrinogen as well as in collagen induced formation of densely packed large thrombi under physiological flow conditions.

Patient, materials and methods

Patient

The patient (M.L.), with a familial bleeding disorder linked to a defective interaction between ADP and its receptor on platelets, was first described in detail by Nurden et al.[12] In brief, the patient is a 67-year-old man who had shown excessive bleeding mainly after surgery or trauma. Bleeding times were consistently prolonged. ADP induced aggregation always showed low maximal intensity and rapid reversibility of aggregation at all doses of ADP, whereas aggregation with medium to high doses of other agonists was normal. Furthermore, a

much decreased activation of $\alpha_{IIb}\beta_3$ complexes was found in response to ADP. ADP induced lowering of cAMP levels was absent for prostaglandin E_1 (PGE_1)-treated platelets from the patient. Binding of (^{3}H)2-methylthio-ADP to his platelets was severely decreased.

Materials

Human albumin was purchased from ICN Biomedicals B.V. (Zoetermeer, the Netherlands). Human fibrinogen (plasminogen, vWF and fibronectin free) was from Kordia Life Sciences (Leiden, the Netherlands). Pentasaccharide was a generous gift from Dr. D.G. Meuleman (Organon B.V., Oss, the Netherlands). PPACK was purchased from Bachem AG (Bubendorf, Switzerland). The ADP receptor $P2Y_{12}$ antagonist, the ATP analogue: N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (AR-C69931MX), was a kind gift from AstraZeneca (Loughborough, United Kingdom). The $P2Y_1$ inhibitor adenosine-3',5'-diphospate (A3P5P) and human placenta collagen type III were purchased from Sigma-Aldrich Chemicals B.V. (Zwijndrecht, the Netherlands). Creatine phosphate (CP) and creatine phosphokinase (CPK) were from Boehringer Mannheim (Germany). D-arginyl-glycyl-L-aspartyl-L-trypthophan (dRGDW) was provided by Dr. J. Bouchaudon (Rhône-Poulenc Rorer, France).

Blood collection

Whole blood, obtained from patient M.L. (platelet count: 180.000/μl) and healthy volunteers (platelet counts: 150.000 – 250.000/μl), who denied having taken aspirin or other platelet function inhibitors in the preceding week, anticoagulated with 1/10 (v/v) of 200 U/ml of a synthetic derivative of the pentasaccharide containing the minimal binding region of heparin to antithrombin III[19] in 0.15 M NaCl in combination with 50 μM PPACK, an inhibitor of α-thrombin. Thrombin generation was inhibited for at least 4 hours after blood collection. Perfusion studies using pentasaccharide/PPACK anticoagulated blood were performed within 4 hours of blood collection.

Perfusion studies

Perfusion experiments were performed over Thermanox® coverslips sprayed with collagen type III[2] or coated with fibrinogen (100 μg/ml) using a single-pass small perfusion chamber with a slit height of 0.1 mm and a slit width of 2 mm.[18] Blood was prewarmed to 37 ºC for 5 minutes, treated or not with ADP receptor antagonists, and subsequently drawn through the chamber for the indicated time period. After perfusion, the coverslips were fixed, dehydrated and stained as described.[20] Platelet deposition and thrombus formation were evaluated as platelet surface coverage and thrombus area using light microscopy (Leitz Diaplan, Leica, Rijswijk, the Netherlands) and computer-assisted analysis with OPTIMAS 6.0 software (Watershed module) (DVS, Breda, the Netherlands). Thrombus volume analysis was measured in real time using confocal laser scanning microscopy as described previously.[2]

Statistical analysis

Unless stated otherwise, results obtained with control blood (with or without antagonist) were expressed as mean ± standard error of the mean (SEM) for data obtained from three or more separate experiments each performed at least in triplicate. The collagen results for patient M.L. are data from at least three coverslips obtained from one experiment (expressed as mean ± standard deviation (SD)) and the data on fibrinogen surfaces are the results from two separate experiments each performed at least in triplicate (expressed as mean ± SD). One-way Analysis of Variance (ANOVA) was used to compare statistical significance between groups. P-values < 0.05 were considered significant. No p-values are reported for data from patient M.L., because it concerns only one patient.

Results

Role of $P2Y_{12}$ in thrombus formation on collagen in flowing blood

Role of ADP in thrombus formation

When whole blood from control donors was treated with the ADP scavenging system, CP/CPK (50 mM /40 μg/ml), and subsequently perfused over collagen type III at a wall shear rate of 1600 s^{-1}, platelet surface coverage was strongly reduced (Table I). This decrease in surface coverage at 5 minutes was due to reduced platelet attachment (adhesion) to the collagen, a reduced spreading and inhibited thrombus formation, as is shown in Figure 1.

Perfusion experiments with blood from patient deficient in $P2Y_{12}$

The contribution of the interaction of ADP with $P2Y_{12}$ to thrombus formation on collagen under conditions of flow was investigated using blood from patient M.L., who was deficient in the ADP receptor $P2Y_{12}$. Perfusions with the patient's blood showed a rapid coverage of the surface (3 minutes; Table I) with a small increase in surface covered with platelets after 5 minutes perfusion (Table I) compared to blood from control donors. This increase in surface coverage was not due to an increased thrombus size as shown by computer analysis. The bulk of thrombi (89.3 ± 11.4 %) formed with blood from the patient covered areas below 400 μm^2, whereas control thrombi were much larger (only 24.0 ± 2.0 % of the thrombi had thrombus areas lower than 400 μm^2). Control thrombi were large and tightly packed, in which individual platelets could hardly be distinguished (Figure 1). In contrast, the patient's thrombi consisted of a layer of spread platelets adhered as a patchwork on the collagen surface, while on top of this monolayer, non-spread (contact) platelets were attached (Figure 1).

The decrease in thrombus size was confirmed with three-dimensional thrombus volume analysis measured after 1, 3 and 5 minutes perfusion using confocal laser scanning microscopy. The results showed impaired thrombus growth with the patient's blood compared to the control blood (at 1, 3 and 5 min, 10.9 ± 1.8, 14.4 ± 2.0, and 16.5 ± 1.3, respectively, for

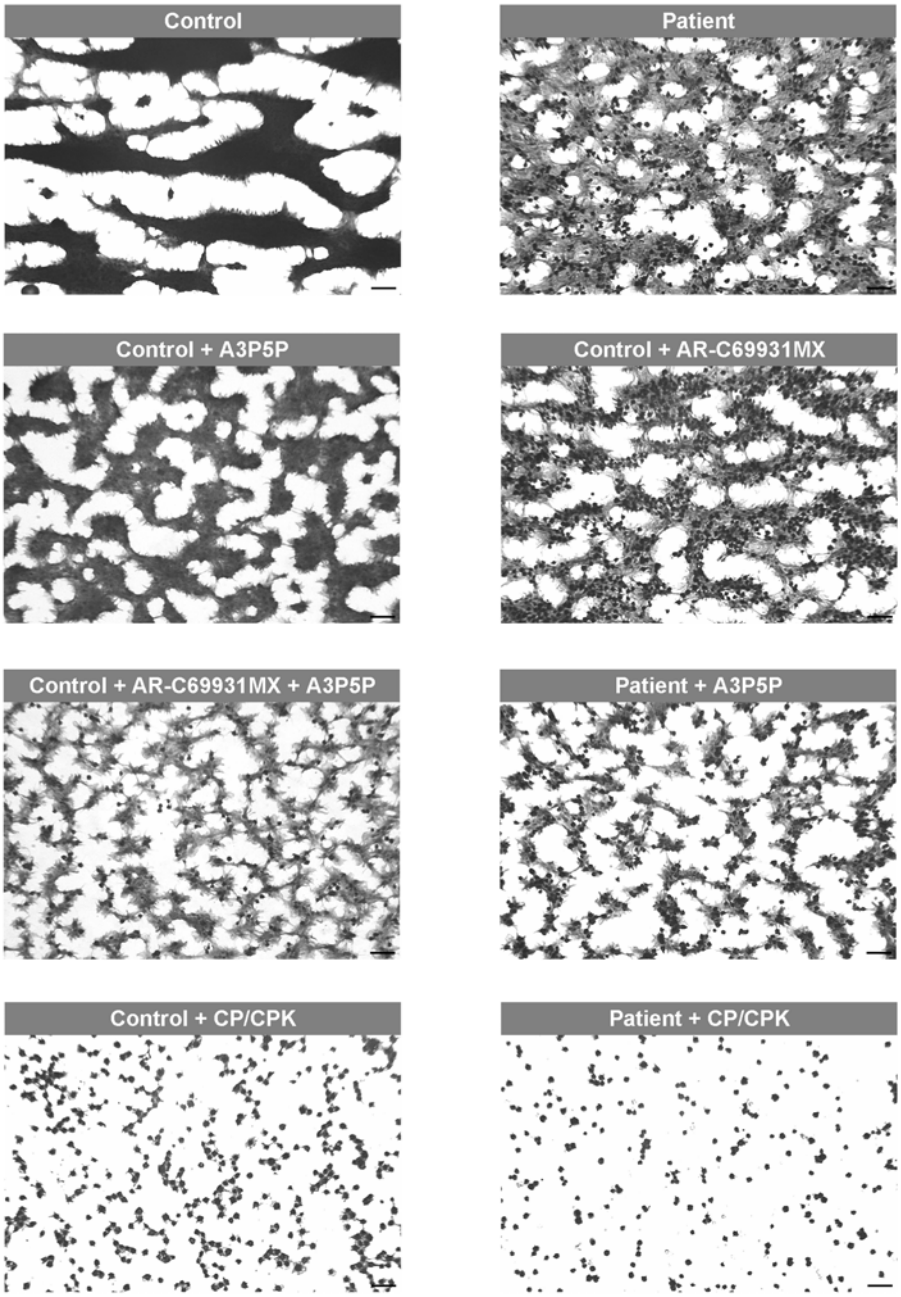
Control versus 7.7 ± 1.1, 9.6 ± 0.76, and 12.7 ± 0.6, respectively, for the Patient; data are the mean (x 10^4 μm^3) ± SD of a single experiment performed in duplicate). No destabilization was observed.

Table I. Effects of ADP on platelet surface coverage on collagen as shown after perfusion of blood from patient M.L. or a control donor in the presence or absence of ADP receptor antagonists at a wall shear rate of 1600 s^{-1}.

	1 min	3 min	5 min
Control	14.3 ± 1.3	40.3 ± 2.2	51.5 ± 1.5
+ AR-C69931MX	7.7 ± 1.3*	29.7 ± 3.4^{ns}	53.8 ± 2.6^{ns}
+ A3P5P	11.7 ± 1.7^{ns}	31.7 ± 5.1^{ns}	49.3 ± 3.6^{ns}
+ AR-C69931MX + A3P5P	5.0 ± 0.9**	21.1 ± 4.2**	33.4 ± 5.1**
+ CP/CPK	0.9 ± 0.6**	1.5 ± 0.5**	5.3 ± 1.6**
Patient	17.1 ± 1.9	53.9 ± 4.4	59.0 ± 5.3
+ AR-C69931MX	nd	nd	59.5 ± 2.6
+ A3P5P	5.1 ± 0.7	21.8 ± 4.0	41.6 ± 6.5
+ CP/CPK	nd	nd	8.7 ± 3.6

Anticoagulated blood was perfused for the given times over collagen type III coverslips. Before perfusion, blood was incubated with the respective ADP receptor antagonists: AR-C69931MX (1 μM), A3P5P (300 μM) and CP/CPK (50 mM /40 μg/ml). Platelet deposition is expressed as the percentage of the surface covered by platelets. Data are the mean ± SEM of at least three separate experiments each performed in triplicate (control blood experiments) or the mean ± SD of one experiment performed at least in triplicate (patient blood experiments). $^{ns}p > 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; *compared to control; nd = not determined.*

Figure 1. Representative light micrographs of thrombi formed on collagen after perfusion of blood from patient M.L. or a control donor in the presence (+) or absence of the ADP receptor antagonists (AR-C69931MX, A3P5P and CP/CPK). Surface coverage from these perfusion experiments is presented in Table I. Original magnification x 400; Bar = 100 μm.



Perfusion experiments with blood in the presence of AR-C69931MX

Perfusions over collagen with control blood in the presence of a selective $P2Y_{12}$ antagonist, the ATP analogue AR-C69931MX (1 μM), showed no significant differences in platelet surface coverage compared to the control without the antagonist after 3 and 5 minutes of perfusion (Table I). However, two-dimensional computer analysis confirmed that thrombus area was reduced and was similar to that observed with patient's blood (Control + AR-C69931MX: 82.5 ± 7.1 % versus Patient: 89.3 ± 11.4 % of thrombus areas were lower than 400 μm^2) and significantly reduced compared to control (Control: 24.0 ± 2.0 %; $p < 0.001$). Thrombi formed in the presence of AR-C69931MX had an identical morphology to those obtained with the patient's blood: an initial layer of spread platelets with single non-spread platelets on top of these (Figure 1). Increasing the concentration of AR-C69931MX up to 10 μM did not change the results. No significant additive effect of AR-C69931MX on platelet deposition and morphology was observed when the antagonist was added to the patient's blood. Although the thrombi were loosely packed in the presence of AR-C69931MX compared with the control, no emboli were observed with the results of three-dimensional thrombus growth analysis being 7.6 ± 2.3, 10.3 ± 2.8 and 9.7 ± 2.0 (x 10^4 μm^3 ± SD) after respectively 1, 3 and 5 minutes perfusion.

Perfusion experiments with blood in the presence of A3P5P

The contribution of both the ADP receptors $P2Y_{12}$ and $P2Y_1$ to thrombus formation was investigated with the patient's blood treated with a selective $P2Y_1$ antagonist: A3P5P (300 μM). These thrombi showed similar morphology in terms of platelet packing to those with obtained with the patient's blood alone (Figure 1), although the surface covered with platelets was decreased at each time point (Table I) and thrombus area was reduced (Patient + A3P5P: 99.5 ± 0.9 % versus Patient: 89.3 ± 11.4 % thrombus areas lower than 400 μm^2). When AR-C69931MX and A3P5P were added simultaneously to control blood similar results for thrombus area were obtained (Control + AR-C69931MX + A3P5P: 99.8 ± 0.3 % versus

Control + AR-C69931MX: 82.5 ± 7.1 % thrombus areas lower than 400 μm^2; $p > 0.05$) as well as for morphology and surface coverage (Table I and Figure 1).

Addition of A3P5P alone to control blood did not significantly affect platelet surface coverage (Table I), but decreased thrombus size (Control + A3P5P: 92.0 ± 5.2 % versus Control: 24.0 ± 2.0 % thrombus areas lower than 400 μm^2; $p < 0.001$). The small platelet aggregates were not as densely packed as with the control blood, but no single contact platelets were observed on top of the thrombi as was found when $P2Y_{12}$ was inhibited (Figure 1). Increasing the concentration of A3P5P up to 1 mM did not change the observed results from those at 300 μM.

Effect of AR-C69931MX and A3P5P on platelet adhesion to collagen

In order to study the effects of AR-C69931MX and A3P5P on the (primary) adhesion of platelets to collagen, we have performed platelet adhesion studies in the presence of dRGDW (50 μM). dRGDW completely inhibits aggregate formation without inhibiting platelet adhesion.[21] Presence of dRGDW in control blood resulted in the adhesion of single platelets to the collagen without the formation of aggregates (surface coverage: 39.8 ± 6.7 %). Addition of AR-C69931MX or A3P5P to control blood in the presence of dRGDW did not significantly alter platelet adhesion compared with control (AR-C69931MX: 32.1 ± 4.4 %; A3P5P: 34.7 ± 8.5 %; $p > 0.05$). Data are the mean ± SEM of three independent experiments each performed in triplicate.

Role of $P2Y_{12}$ in platelet adhesion to fibrinogen in flowing blood

Role of ADP in platelet adhesion

When whole blood is perfused at a wall shear rate of 300 s^{-1} over immobilized fibrinogen platelets adhere in a $\alpha_{IIb}\beta_3$ dependent manner without the formation of thrombi.[22] After perfusion, a single layer of adhered platelets is detectable on the fibrinogen surface. These consist of contact, dendritic and completely spread platelets. Upon addition of the ADP

scavenging system, CP/CPK, platelet adhesion was completely inhibited after 5 minutes perfusion (Table II), suggesting that platelet adhesion to fibrinogen requires ADP.

Table II. Effects of ADP on platelet adhesion to fibrinogen after perfusion of blood from patient M.L. or control blood in the presence or absence of ADP receptor antagonists at a wall shear rate of 300 s^{-1}.

	2 min	**5 min**
Control	31.4 ± 3.7	65.3 ± 4.5
+ AR-C69931MX	17.7 ± 3.3*	60.7 ± 8.6^{ns}
+ A3P5P	14.1 ± 1.2*	45.0 ± 6.2*
+ AR-C69931MX + A3P5P	4.9 ± 1.1**	15.4 ± 3.2**
+ CP/CPK	1.7 ± 0.4**	2.2 ± 0.4**
Patient	5.9 ± 1.3	30.2 ± 8.9
+ AR-C69931MX	7.3 ± 0.6	nd
+ A3P5P	8.2 ± 2.1	nd
+ CP/CPK	0.4 ± 0.5	nd

Anticoagulated blood was perfused for the given times over immobilized fibrinogen (100 μg/ml). Before perfusion, blood was preincubated with the respective ADP receptor antagonist: AR-C69931MX (1 μM), A3P5P (300 μM) and CP/CPK (50 mM / 40 μg/ml). Platelet deposition is expressed as a percentage of the surface covered by platelets. Data are the mean ± SEM of at least 3 separate experiments each performed in triplicate (control blood experiments) or the mean ± SD of one experiment at least performed in triplicate. Results for patient M.L. at 2 and 5 minutes are obtained from experiments performed at two separate moments. $^{ns}p > 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; *compared to control; nd = not determined.*

Perfusion experiments with blood from the patient

The specific role of the ADP receptor $P2Y_{12}$ in this process was studied by performing perfusion experiments over immobilized fibrinogen with blood from the patient. Table II shows reduced platelet adhesion after 2 as well as after 5 minutes perfusion. The density of adhered platelets was manually counted from 15 representative images of non-overlapping fields on each coverslip and showed that this reduction in surface coverage was not due to changes in morphology of the adhered platelets, but was due to a decreased number of platelets adhering to the immobilized fibrinogen (data not shown). The control platelets and the patient's platelets showed contact platelets as well as dendritic and completely spread platelets that had adhered to the immobilized fibrinogen.

Perfusion experiments with blood in the presence of AR-C69931MX and A3P5P

Addition of the $P2Y_{12}$ antagonist AR-C69931MX to control blood resulted in a delayed platelet deposition: after 2 minutes perfusion platelet deposition was significantly reduced, but this was normalized when blood was perfused for 5 minutes (Table II). Addition of AR-C69931MX to control blood did not influence the morphology of the adhered platelets. No additive effect was observed when AR-C69931MX was added to blood from the patient (Table II).

Inhibition of $P2Y_1$ with A3P5P in control blood decreased significantly the adhesion to fibrinogen after 2 and 5 minutes perfusion (Table II), but did not influence platelet morphology. Addition of A3P5P to the patient's blood did not further reduce platelet adhesion after 2 minutes perfusion compared to the control (Table II). Addition of A3P5P and AR-C69931MX together to control blood resulted in a significant reduction in platelet adhesion after 2 minutes as well as after 5 minutes perfusion (Table II).

Discussion

We report on the crucial role of ADP in platelet adhesion and thrombus formation in flowing blood. The presence of the ADP scavenging system, CP/CPK, resulted in a reduced platelet adhesion to collagen, whereas thrombus formation was completely inhibited. In agreement are early perfusion studies involving subendothelium and collagen that reported reduced platelet deposition with blood from patients deficient in the contents of dense granules (where ADP is stored in platelets)[23] and with control blood to which an ADP-utilizing enzyme system was added.[24,25] In addition, in vivo studies reported effects of infusing ADP-scavenging systems on the bleeding time from rat mesenteric arteries.[26] The contribution of the interaction of ADP with $P2Y_{12}$, the target of the ADP antagonists: ticlopidine, clopidogrel (Plavix®) and the AR-C compounds AR-C66096MX, AR-C67085MX and AR-C69931MX, was investigated using blood from control donors and from patient M.L. Until now, two unrelated patients have been fully characterized whose platelets, on exposure to ADP, have normal shape change, but show a reversible aggregation and do not exhibit the normal inhibition of PGE_1-stimulated adenylyl cyclase.[12,27] The clinical profile and platelet functions of these patients are comparable to that observed with humans or animals who have received thienopyridine drugs, suggesting that the patients have a $P2Y_{12}$ deficiency, which was confirmed by genetic analysis for patient M.L.[11] Northern blotting showed that platelets from this patient contain no mRNA for $P2Y_{12}$, and Western blotting has confirmed the absence of $P2Y_{12}$ protein (Dr. P.B. Conley, unpublished data, 2001). Therefore, the availability of this patient provides a unique way of investigating the role of $P2Y_{12}$ in thrombus formation.

We found a similarly impaired platelet aggregation under physiological flow conditions for blood from the patient and control blood incubated with the $P2Y_{12}$ antagonist: AR-C69931MX. Although primary platelet adhesion on collagen (as shown with dRGDW) was not affected, the thrombi were smaller and morphologically different, consisting of clusters of spread platelets on top of which were single non-spread platelets. This is compatible with a previous observation that the interaction of ADP with $P2Y_{12}$ is responsible for the full

activation of $\alpha_{IIb}\beta_3$[28] and, thus, supports the formation and growth of a densely packed thrombus under physiological flow conditions. In agreement are unpublished real-time observations of increased rolling of platelets over spread platelets in the presence of AR-C69931MX (J.A. Remijn, unpublished data, 2001). The reduced platelet consumption by the growing thrombus apparently resulted in a higher platelet concentration at the collagen surface, which enhances platelet-collagen adhesion and explains the increased surface coverage observed with blood from the patient at early perfusion times. ADP-induced platelet aggregates from patient M.L. formed in suspension and studied using electron microscopy showed similar loosely bound platelets with few contact points as reported here and showed similarities with aggregates of platelets from subjects receiving clopidogrel.[28] The formation of loosely packed thrombi was also observed with clopidogrel without decreased platelet adhesion to collagen under conditions of flow in an human ex vivo perfusion model using nonanticoagulated blood by Roald et al.[16,29] Our results obtained with blood from patient M.L. and control blood with AR-C69931MX are consistent with these flow studies on clopidogrel. One of the interesting aspects of $P2Y_{12}$ as a potential antithrombotic target is that thrombus growth is inhibited, whereas primary adhesion to collagen is not affected. The latter might explain why patient M.L. does not have major bleeding complications in the absence of trauma or surgery. The ADP-mediated downregulation of cAMP via $P2Y_{12}$ and/or other unidentified signalling pathways emanating from Giα- and β-, γ-chain dissociation, are evidently important for the activation of $\alpha_{IIb}\beta_3$ to support thrombus growth and not for the primary platelet-collagen interaction.

Furthermore, inhibition of $P2Y_1$ alone did also not affect primary platelet adhesion to collagen but resulted in reduced thrombus area, although these thrombi were still densely packed. Indeed platelet aggregation studies in $P2Y_1$-null mice showed impaired aggregation at low dose of collagen, a response that was restored at higher collagen concentrations.[13,14] This restoration of normal aggregation by increasing collagen concentrations in combination with the observed dense aggregate packing could be due explained by the participation of secreted ADP in the activation of $\alpha_{IIb}\beta_3$ via $P2Y_{12}$. The latter is inhibited when patient M.L. blood is

incubated with A3P5P or control blood is incubated with a combination of A3P5P and AR-C69931MX, resulting in reduced platelet surface coverage and further reduced thrombus size, which consisted of loosely bound, non-activated platelets. These results show that both ADP receptors participate in collagen induced platelet thrombus formation under conditions of flow. However, the inhibition of the separate receptors results in different morphology. A differential participation of $P2Y_1$ and $P2Y_{12}$ in the activation of $\alpha_2\beta_1$[30] may also need to be considered. The quantitative differences in adhesion between the presence of CP/CPK and the residual adhesion when both ADP receptors $P2Y_1$ and $P2Y_{12}$ are inhibited might be due to an incomplete inhibition of the receptors with recycling of internal pools or due to a role of the largely uncharacterized $P2X_1$ receptor for ATP and ADP.

The involvement of ADP interaction with $P2Y_{12}$ for the activation of $\alpha_{IIb}\beta_3$ was confirmed by the observed reduced platelet adhesion at both 2 and 5 minutes perfusion of the patient's blood over immobilized fibrinogen, an interaction which is totally $\alpha_{IIb}\beta_3$ dependent at wall shear rates of 300 s^{-1}.[22] Similar results to the patient were obtained upon addition of AR-C66931MX to control blood after 2 minutes perfusion. However, with this antagonist, platelet adhesion was normal after 5 minutes perfusion, indicating that platelet adhesion was delayed rather than prevented. The differences in inhibition between the patient's platelets and control platelets in the presence of AR-C69931MX could be due to the total absence of $P2Y_{12}$ on the patient's platelets; in control blood, AR-C69931MX probably inhibits the surface expressed receptors only, whereas any internal receptor pool may not be inhibited as it would be inaccessible to the antagonist. Inhibition of $P2Y_1$ with A3P5P also resulted in decreased platelet adhesion, probably explained by the fact that the ADP – $P2Y_1$ interaction plays a role in the initial rate of activation of $\alpha_{IIb}\beta_3$. Addition of the combination of AR-C69931MX and A3P5P did markedly reduce platelet adhesion after 5 minutes perfusion, but did not abrogate it. Furthermore, the morphology of the adhered platelets was not affected. When the total released ADP was scavenged by CP/CPK, platelet adhesion was totally inhibited, confirming the role of ADP in adhesion to immobilized fibrinogen. Our results regarding ADP dependence in platelet adhesion to immobilized fibrinogen suggest that platelets need to be

preactivated to adhere. This is in contrast with previous studies under static or flow conditions, which suggested that (resting) platelets can adhere to surface-immobilized fibrinogen, presumably via unactivated $\alpha_{IIb}\beta_3$.[31-33] These studies were based on observations that platelets treated with PGE_1 still adhered to immobilized fibrinogen. Our results performed in whole blood under physiological flow conditions are more consistent with a recent study on coaggregation of unactivated or ADP-activated platelets with fibrinogen-coated beads in flowing suspensions showing that only preactivated platelets can adhere to the fibrinogen coated surface.[34] In the present study, we show that the interaction of ADP with $P2Y_{12}$ and $P2Y_1$ are both necessary to obtain platelet adhesion on immobilized fibrinogen under conditions of flow. Under shear conditions using whole blood, ADP could be secreted from red blood cells or from preactivated platelets.

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CHAPTER 5

Reduced platelet adhesion in flowing blood to fibrinogen by alterations in segment γ316-322, part of the fibrin-specific region

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Summary

The interaction of platelets with fibrinogen is a key event in the maintenance of an haemostatic response. It has been shown that the twelve-carboxyterminal residues of the γ-chain of fibrinogen mediate platelet adhesion to immobilized fibrinogen. These studies, however, do not exclude the possibility that other domains of fibrinogen are involved in interactions with platelets. In order to obtain more insight into the involvement of other domains of fibrinogen in platelet adhesion, we studied platelet adhesion in flowing blood to patient dysfibrinogen Vlissingen/Frankfurt IV (V/F IV), to several variant recombinant fibrinogens with abnormalities in γ-chain segment γ318-320 and segment γ408-411. Perfusion studies at physiological shear rates showed that platelet adhesion was absent to γΔ408-411, slightly reduced to the heterozygous patient dysfibrinogen V/F IV and strongly reduced to the homozygous recombinant fibrinogens: γΔ319-320, γ318Asp→Ala and γ320Asp→Ala. Furthermore, antibodies raised against the sequences γ308-322 and γ316-333 inhibited platelet adhesion under conditions of shear. These experiments indicated that the overlapping segment γ316-322 contains amino acids, which could be involved in platelet adhesion to immobilized fibrinogen under flow conditions. In soluble fibrinogen this sequence is buried inside the fibrinogen molecule and becomes exposed after polymerization. In addition, we show that this fibrin-specific sequence also becomes exposed when fibrinogen is immobilized on a surface.

Introduction

Fibrinogen plays a major role in haemostatic plug formation not only as the precursor of fibrin and as a mediator in platelet thrombus formation, but also as an adhesive molecule for platelets.[1] Resting, unstimulated platelets do not interact with fibrinogen in the fluid phase. Following activation of platelets, glycoprotein (GP) IIbIIIa (integrin $\alpha_{IIb}\beta_3$) undergoes a conformational change and provides a binding site for fibrinogen.[2,3] The integrin receptor $\alpha_{IIb}\beta_3$, plays an important role in mediating (multivalent) interactions of platelets with both (immobilized) fibrinogen and fibrin.[4,5] Fibrinogen exists in plasma predominantly as a homodimer of α-, β-, and γ-polypeptide chains.[6] Three sites on fibrinogen have been postulated to bind to $\alpha_{IIb}\beta_3$ during the interaction with platelets: two Arg-Gly-Asp (RGD) sites and the carboxyterminal dodecapeptide of the γ-chain. RGD is a consensus binding sequence for integrins and was identified using synthetic peptides.[7] The two RGD-sequences are both located in the α-chain at positions 95-97 and 572-574[8] and the third binding site is specific for fibrinogen and present in the carboxy-terminal segment of the γ-chain at positions 400-411 with the sequence HHLGGAKQAGDV.[9,10] Experiments with antibodies and genetically engineered fibrinogen variants lacking the RGD sequences in the α-chain have demonstrated that the α-chain RGD sequences are not required for $\alpha_{IIb}\beta_3$ binding to soluble and surface bound fibrinogen or for platelet aggregation.[11-15] Instead, genetic variants of fibrinogen with either an extension of the γ-chain or truncation of the γ-chain show that the C-terminal sequence of the γ-chain is an important determinant on fibrinogen for platelet binding and aggregation.[16,17] These studies, however, do not exclude that other domains of fibrinogen[5,18] may also be playing a role in the interaction with platelets, assuming that the γ-chain dodecapeptide sequence is necessary but not sufficient to mediate irreversible platelet attachment. Fibrinogen isolated from patients with dysfibrinogenemia offers the possibility to correlate structural abnormalities of the fibrinogen molecule with functional alterations. Congenital dysfibrinogenemia is characterized by the biosynthesis of a structurally abnormal

fibrinogen molecule that exhibits altered functional properties.[19] Studies on dysfibrinogen Vlissingen/Frankfurt IV (V/FIV), in which the residues Asn319 and Asp320 on the γ-chain are absent, showed defective calcium binding and impaired fibrin polymerization.[20] Furthermore, aggregation studies showed impaired platelet aggregation with patient fibrinogen V/IV[21] as well as with the similar dysfibrinogen made with recombinant techniques.[22] In the present study, we investigated the capacity of dysfibrinogen V/IV to support platelet adhesion in flowing blood. Furthermore we studied several recombinant fibrinogens with known abnormalities in the γ-chain segments γ318-320 and γ408-411.
We report a reduced platelet adhesion by alterations in the fibrinogen γ-chain sequence 316-322, indicating that besides the last 4 amino acids of the γ-chain, an additional site could be involved in platelet-fibrinogen interactions in flowing blood.

Materials and methods

Fibrinogens

Human fibrinogen was purchased from American Diagnostica (Greenwich, CT, USA) and further purified by size exclusion chromatography on Sepharose 4B in 0.05 M ammonium sulphate, pH 7.4, containing 0.01 mg/ml bovine pancreas trypsin inhibitor (Sigma Chemical Co., St Louis, MO, USA). Peak fractions were pooled, lyophilized and stored in aliquots at – 70 ^{o}C for up to 6 months. Lyophilized fibrinogen was reconstituted in phosphate buffered saline (PBS, 10 mM phosphate buffer, 150 mM NaCl, pH 7.4) prior to coating coverslips. The protein concentration was determined by absorbance measurements using an extinction coefficient at 280 nm of 1.6 ml mg^{-1} cm^{-1}.[23]

Fibrinogen Vlissingen/Frankfurt IV (V/F IV) was purified from patient plasma as described.[24] The defect was elucidated by protein sequence analysis and by DNA sequencing of the corresponding exon, and is in detail described elsewhere.[20,25]

Recombinant human fibrinogen and *fibrinogen with γ-chain variants* γ318Asp→Ala, γ320Asp→Ala[26], deletion of γ319-320 in γΔ319, 320[22] and deletion of γ408-411 in γΔ408-411[27] were prepared, expressed in CHO-cells and isolated as described. The purity and quality of the studied dysfibrinogens was tested by SDS-PAGE showing that the α, β and γ-chain were intact without visible contamination or degradation as reported for V/F IV[25], γ318Asp→Ala and γ320Asp→Ala[26], γΔ319, 320[22] and γΔ408-411[27].

Antibodies

The monoclonal antibody (mAb) 4A5 (courtesy of Dr. Gary R. Matsueda) used in enzyme-linked immunosorbent assays (ELISA) binds to the carboxyl-terminus of the fibrinogen γ-chain (γ402-411)[28] and prevents platelet adhesion.[29] The monoclonal antibody directed against β_3 of $\alpha_{IIb}\beta_3$ (C17), was a gift from Dr. A.E.G.Kr. von dem Borne (CLB, Amsterdam, the Netherlands).The polyclonal rabbit antibody against human fibrinogen, the peroxidase-conjugated swine antibody against rabbit IgG (SWARPO) and the peroxidase-conjugated rabbit antibody against mouse IgG (RAMPO) were from Dako (Glostrup, Denmark).

Synthetic peptides

Two peptides representing residues 308-322 (NGMQFSTWDNDNDKF) and residues 316-333 (DNDNDKFEGNCAEQDGSG) of the γ-chain of fibrinogen were synthesized by Pepscan systems (Lelystad, the Netherlands). Both peptides were also synthesized with an extra C-terminal cysteine. The presence of the cysteine allows the coupling of the peptides to Imject 7 Maleimide activated Keyhole Limpet Hemocyanin (KLH, Pierce Chemical Co. Oud-Beijerland, the Netherlands). Each peptide was purified by high performance liquid chromatography using an Aquapore C-18 reverse phase column with a gradient applied over 30 min from 10 % acetonitrile/water with 0.05 % trifluoroacetic acid to 70 % acetonitrile/water with 0.05 % trifluoroacetic acid. Each peptide was shown to have the expected amino acid composition and the correct sequence determined by sequence analysis. Aliquots of each peptide were dissolved in 0.1 mol/l acetic acid and freeze dried.

Preparation of polyclonal antibodies

Each peptide was used to make polyclonal antibodies in two New Zealand White rabbits. 1.5 mg of each peptide was coupled to 1 mg/ml KLH as a carrier protein, using 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDAC) HCL as the coupling agent. KLH (0.5 ml) and peptide (10 mg/ml in H_2O) were mixed and EDAC (110 mg/ml in PBS, Bio-Rad Laboratories, Richmond, CA, USA) was added. Coupling was allowed to proceed for two hours at room temperature under rotation. After exhaustive dialysis against PBS at 4^{o}C overnight, the solution was diluted 1:1 with (In)Complete Freunds Adjuvant. The rabbits were injected with protein with Complete Freunds Adjuvant at day 1 and boostered every 4th week with protein and Incomplete Freunds Adjuvant. The antibodies were measured by screening plasma samples of the rabbits with an ELISA in which the peptides were coated on an ELISA-tray. The rabbits were boostered 8 times before the antibody titre was high enough for functional experiments.

Preparation of F(ab)$_2$ fragments

IgG was purified from rabbit serum with protein-G-Sepharose column chromatography (Pharmacia-LKB, Piscataway, NY, USA). After extensive washing with PBS, the bound material was eluted with 0.1 M glycine pH 2.7. Each fraction was adjusted to pH 7.4 prior to dialysis against PBS at 4 ^{o}C overnight. F(ab)$_2$ fragments were prepared from each IgG by digesting the IgG-preparation with pepsin (80:1) at a pH of 3.5 at 38 ^{o}C for 1 hour according to the instructions of the supplier (Pierce Chemical Co., Rockford, IL, USA). The digestion was stopped with 2 M Tris/HCl. The digests were isolated on Protein A-agarose using a Pierce ImmunoPure F(ab)$_2$ Preparation Kit, according the instruction of the suppliers. The concentration of the F(ab)$_2$ fragments was determined with absorbance at 280 nm using an extinction coefficient of 1.4 ml mg^{-1} cm^{-1}.[30] SDS-PAGE analysis confirmed the purity of the F(ab)$_2$ samples.

ELISA

An ELISA was used to measure affinity of mAb 4A5 for the fibrinogen γ-chain variants. Furthermore the affinity of the IgG's, raised against the peptides γ308-322 and γ316-333, was studied for fibrinogen and the peptides against they were raised. All incubations were carried out for 2 hour at 37 ^{o}C, and were followed by extensive washing with distilled water. Fibrinogen or peptide (10 μg/ml) was coated in PBS, subsequently each well was blocked with 3 % bovine serum albumin (BSA) and 0.1 % Tween. The first antibody consisted of mAb 4A5 (1:15,000 dilution from ascites) or the indicated antibodies, anti γ308-322 or anti γ316-333, (concentrations ranging from 0.78 μg/ml to 100 μg /ml). Detection involved RAMPO (for mAb 4A5) or SWARPO for the IgG's with ortho-phenylenediamine used as a substrate. Absorbance was measured at 490 nm in a Vmax Microtiter Plate Reader (Molecular Devices, Palo Alto, CA, USA).

Coverslip preparation

Thermanox® coverslips (Nunc, Inc., Naperville, IL, USA; surface area 1.2 cm^2) were soaked overnight in 80 % ethanol, rinsed thoroughly with distilled water and dried before use. Coverslips were coated with 100 μg/ml (variant) fibrinogen in PBS for 1 hour at room temperature. Where indicated, coated coverslips were incubated with 100 μg/ml IgG of $F(ab)_2$ fragments. As a control, coverslips coated with fibrinogen were incubated with $F(ab)_2$ directed against ED-1 fibronectin (Cappel Organon Technica Corporation, West Chester, PA, USA).

Perfusion experiments

Perfusion studies were performed with a single-pass perfusion chamber as described.[31] Whole blood obtained by venipuncture from healthy volunteer donors was anticoagulated with 1/10 volume 110 mM trisodium citrate and prewarmed at 37 ^{o}C for 10 minutes. In some experiments peptides were added to the perfusate 10 minutes before perfusion. Perfusions were performed by drawing blood for 5 minutes directly through the perfusion chamber using

a syringe placed in a Harvard infusion pump (Pump 22, model 2400-004; Natick, MA, USA) by which different wall shear rates were maintained. No fibrin was formed during perfusions of citrate anticoagulated blood over immobilized fibrinogen as concluded by the lack of FPA generation. Platelet adhesion to immobilized fibrinogen under conditions of flow was already optimal at calcium concentrations > 50 μM as shown by comparable platelet adhesion to immobilized fibrinogen after perfusion of citrate- versus low molecular weight heparin anticoagulated blood (1) and platelets with red cells resuspended in 4 % human albumin solution in the presence or absence of calcium (2). After perfusion with citrated blood, the coverslips were removed, rinsed with 10 mM Hepes buffer containing 150 mM NaCl pH 7.4, fixed with 0.5 % glutaraldehyde, dehydrated in methanol and stained with May-Grünwald - Giemsa as described previously.[32] The percentage of the surface covered with platelets was measured using light microscopy (Leitz Diaplan, Leica, Rijswijk, the Netherlands) and computer-assisted analysis with OPTIMAS 6.0 software (DVS, Breda, the Netherlands). Platelet coverage, expressed as percentage of the surface covered, is the average of 15 fields/coverslip.

Real-time perfusions were performed using a previously described perfusion chamber.[33] Before perfusion, whole blood was preincubated with mepacrin (10 μM; Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands) for fluorescent labeling of the platelets. The perfusion chamber was mounted on a fluorescent microscope, which was connected to a camera (Leitz Wetzlar, Leica, Rijswijk, the Netherlands) and a video cassette recorder (JVC, Germany).

Static adhesion assay

Platelet rich plasma was obtained from whole citrated blood by centrifugation at 160 g for 10 min. Coverslips, one per well, in a 6-well plate (Costar, Cambridge, MA, USA), were incubated with PRP for 1 hour at 37 ^{o}C. Platelets were stained and platelet coverage was analyzed as described for the perfusion experiments.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM) for data obtained from different experiments or ± standard deviation (SD) for data obtained within one experiment. The Student *t*-test was used to test for significance of differences groups. P-values < 0.05 were considered significant.

Results

Platelet adhesion to dysfibrinogen Vlissingen/Frankfurt IV (V/F IV) and recombinant fibrinogen γΔ319, 320

Perfusion experiments demonstrated a reduced platelet adhesion to plasma fibrinogen V/F IV and its recombinant counterpart γΔ319, 320 in flowing blood at physiological shear rates of 300 s^{-1} and 1600 s^{-1}. These shear rates represent the conditions within veins and arteries, respectively. Table I shows the platelet adhesion of fibrinogen V/F IV and γΔ319, 320 compared to control fibrinogen (plasma fibrinogen for fibrinogen V/F IV and recombinant fibrinogen for fibrinogen γΔ319, 320) after 5 minutes perfusion. Platelet adhesion to γΔ319, 320 was significantly reduced (300 s^{-1}: 35 % decrease ($p < 0.001$); 1600 s^{-1}: 79 % decrease ($p < 0.01$) and was much more inhibited than to the patient fibrinogen (300 s^{-1}: 16 % decrease ($p > 0.05$); 1600 s^{-1}: 29 % decrease ($p > 0.05$)). Similarly, static platelet adhesion studies showed a reduced platelet adhesion for both the patient fibrinogen and the recombinant fibrinogen compared to control. To exclude the possibility that the reduced platelet adhesion was due to improper coating of the variant fibrinogens, the amount of immobilized fibrinogen on the surface was measured by ELISA. Comparable amounts of fibrinogen were present with all fibrinogen preparations (data not shown).

Table I. Platelet adhesion to patient dysfibrinogen Vlissingen/Frankfurt IV (Fg V/F IV) and recombinant fibrinogen γ-chain variants under flow and static conditions.

Surface	300 s^{-1}	1600 s^{-1}	Static
Fg (plasma)	58 ± 6 %	38 ± 6 %	24 ± 11 %
Fg V/F IV	49 ± 3 %^{ns}	27 ± 4 %^{ns}	10 ± 3 %^{ns}
Fg (recombinant)	51 ± 2 %	29 ± 8 %	27 ± 10 %
Fg γΔ319, 320	33 ± 2 %***	6 ± 1 %**	4 ± 2 %*
Fg γΔ318D→ A	24 ± 3 %***	9 ± 1 %*	5 ± 3 %*
Fg γΔ320D→ A	30 ± 3 %***	8 ± 3 %*	5 ± 2 %*
Fg γΔ408-411	1 ± 0 %***	0 ± 0 %***	nd

Flow results were obtained from perfusion experiments using coverslips coated with the fibrinogens (Fg) (100 μg/ml), blocked with an excess of human albumin, and subsequently exposed to flowing whole blood for 5 min at wall shear rates of 300 s^{-1} and 1600 s^{-1}. Static results were obtained from incubation of the coverslips with PRP for 30 minutes at 37 °C. Platelet adhesion is expressed as percentage of the surface covered with platelets. Data are the mean ± SEM of minimal three separate experiments each performed in duplo or triplo. $^{ns}p > 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; *nd = not determined.*

Platelet adhesion to recombinant fibrinogen γ-chain variants

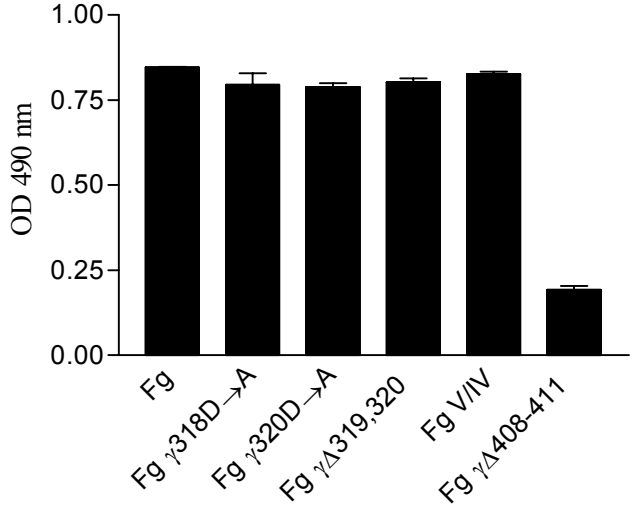
Results of perfusion experiments using recombinant fibrinogens with substitutions: γ318Asp→Ala (Fg γΔ318D→A) and γ320Asp→Ala (Fg γΔ320D→A) showed significant reduced platelet adhesion under flow conditions at shear rates of 300 s^{-1} and 1600 s^{-1} after 5 minutes perfusion (Table I). Increasing the perfusion times (tested for Fg γΔ320D→A) resulted in similar reduction in adhesion (up to 10 minutes perfusion: Control (87.8 ± 4.8 (SD) versus Fg γΔ320D→A 44.1 ± 10.6 (SD)). Real-time observations showed that the reduction in adhesion to immobilized dysfibrinogen was due to rolling and detachment of adhered platelets (data not shown). No significant differences in adhesion were found

between γ318Asp→Ala, γ320Asp→Ala and γΔ319, 320. Static platelet adhesion to the recombinant human fibrinogen γ-variants showed also a significant reduction in platelet adhesion compared to control. Platelet adhesion to Fg γΔ408-411 was completely absent at shear rates of 300 s^{-1} and 1600 s^{-1}.

Binding of mAb 4A5 to dysfibrinogen Vlissingen/Frankfurt IV (V/IV) and the recombinant fibrinogen γ-chain variants

The monoclonal antibody 4A5 binds to the carboxyl-terminus of human fibrinogen γ-chain[28] and thereby inhibits the adhesion of platelets to immobilized fibrinogen[29] by recognizing the sequence γ402-411.

Figure 1. Binding of mAb 4A5 (anti γ402-411) to dysfibrinogen Vlissingen/Frankfurt IV (V/IV) and the variant recombinant fibrinogens γ318D→A, γ320D→A, γΔ319, 320 and γΔ408-411.



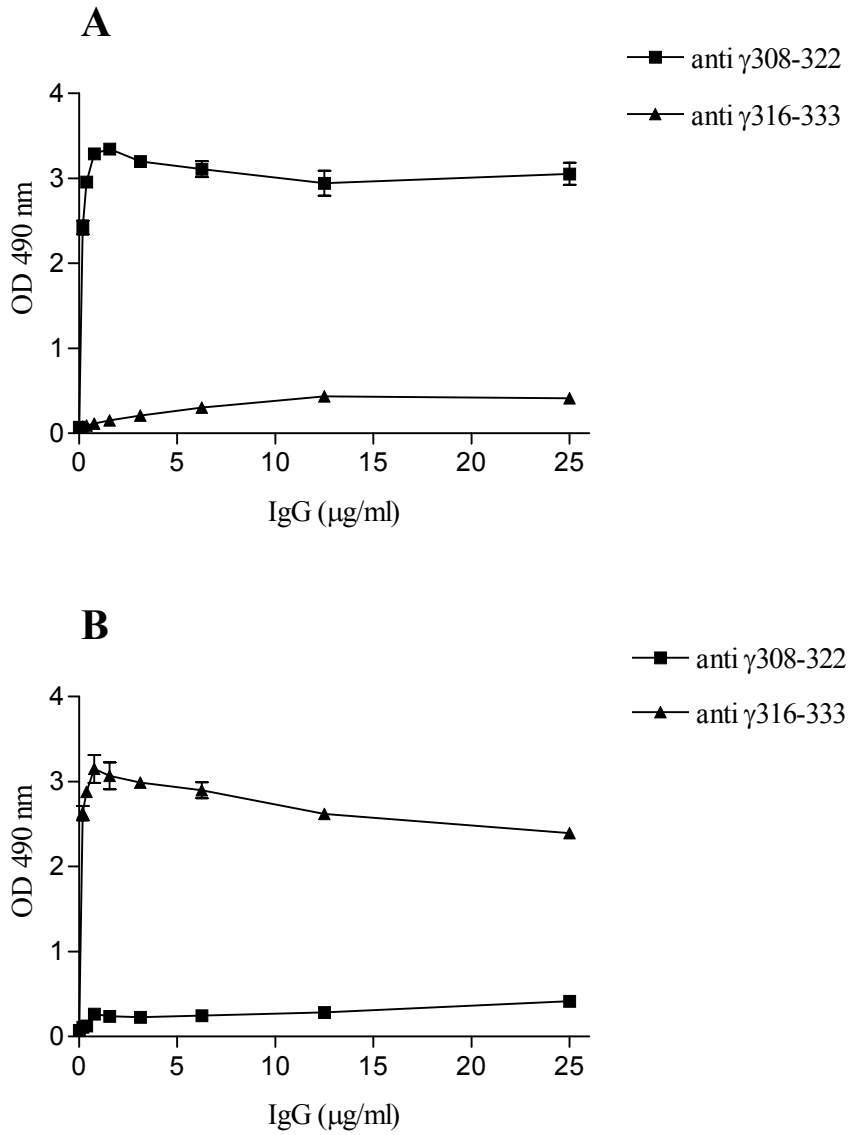
Fibrinogen was coated for 2 hours in an ELISA-tray and after a blocking procedure with albumin incubated for 2 hours with the monoclonal antibody (mAb) 4A5. Detection involved a rabbit antibody against mouse IgG conjugated to horseradish peroxidase with ortho-phenylenediamine used as a substrate. Absorbance was measured at 490 nm. Data represents the mean ± SEM of three experiments each performed in duplo.

ELISA binding studies using mAb 4A5 were performed to study whether the reduced platelet adhesion to the fibrinogen γ-chain variants was due to alterations in conformation affecting the primary platelet adhesion site γ400-411. No differences were observed in binding of mAb 4A5 to dysfibrinogen V/IV and the recombinant fibrinogens Δγ319-320, γΔ318D→A and γ320Asp→Ala, while no binding was found to γΔ408-411 (Figure 1). As the antibody completely blocks adhesion to the dysfibrinogens, this suggested that the primary platelet adhesion site γ400-411 is still surface exposed in the fibrinogen γ-chain variants, except γΔ408-411.

Characterization of antibodies against peptides γ308-322 and γ316-333

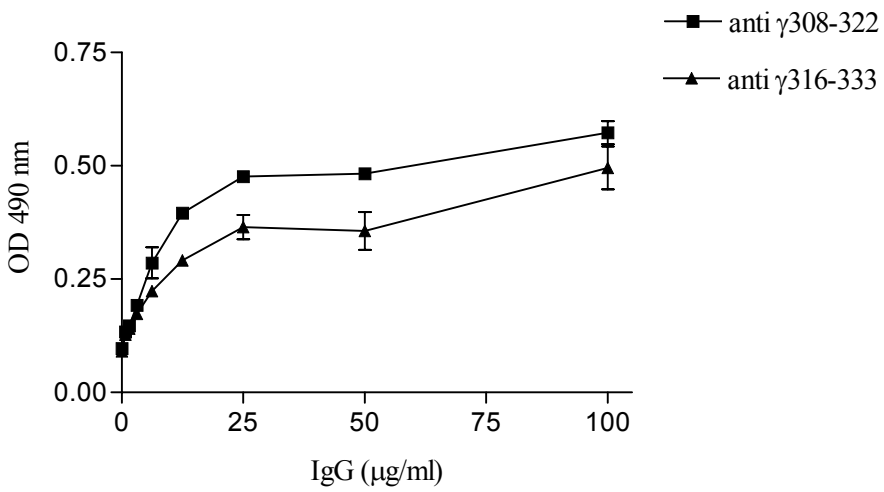
The inhibition of platelet adhesion to the fibrinogen γ-chain variants and availability of γ400-411 suggested a possible role of the sequence γ318-320 in the adhesion of platelets. Therefore peptides were made representing the amino acid sequence γ308-322 and γ316-333. Addition of both peptides in concentrations up to 1 mM to whole blood did not influence platelet adhesion to native fibrinogen. To obtain a peptide sequence in a proper conformation, we tested also circular peptides, but similar results were obtained as with the linear peptides (data not shown). Polyclonal antibodies were raised against both peptides coupled to KLH. These polyclonal antibodies recognized the peptide sequence to which they were raised, but they did not recognize the other peptide (Figure 2A and B). Both antibodies recognized native fibrinogen, although to a lesser extent than the peptides to which they were raised (Figure 3). The antibodies recognized preformed coated fibrin in a similar manner as coated fibrinogen (data not shown). The binding of antibodies against γ308-322 and γ316-333 was specific for the fibrinogen sequences against which they were raised, because peptide concentrations up to 250 μg/ml inhibited the binding of the respective antibody to fibrinogen completely.

Figure 2. Binding of rabbit IgG raised against peptides representing the fibrinogen sequences γ308-322 and γ316-333 to peptide γ308-322 (A) and peptide γ316-333 (B).



The peptide (10 μg/ml) was coated for 2 hours in an ELISA-tray and after a blocking procedure with albumin incubated for 2 hours with the antibody. Detection involved a swine antibody against rabbit IgG conjugated to horseradish peroxidase with ortho-phenylenediamine used as a substrate. Absorbance was measured at 490 nm. Data represents the mean ± SD of one experiment performed in duplo.

Figure 3. Binding of rabbit IgG raised against peptides representing the fibrinogen sequences γ308-322 and γ316-333 to fibrinogen.



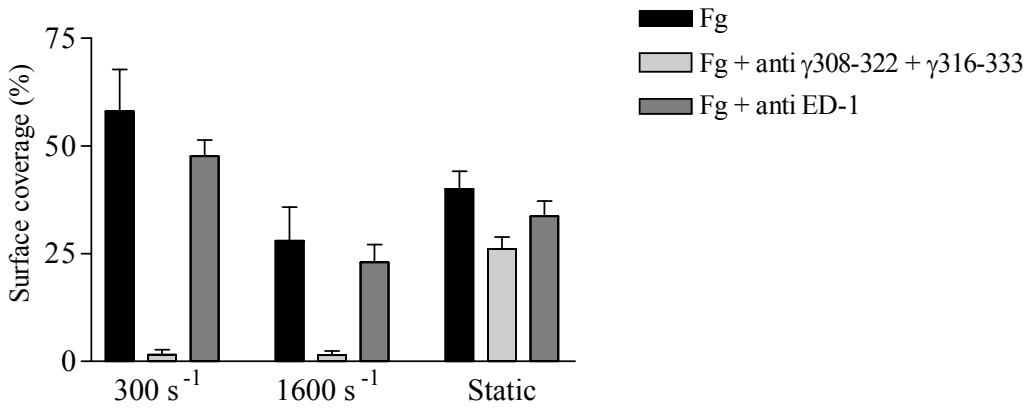
Fibrinogen (10 μg/ml) was coated for 2 hours in an ELISA-tray and after a blocking procedure with albumin incubated for 2 hours with the antibody. Detection involved a swine antibody against rabbit IgG conjugated to horseradish peroxidase with ortho-phenylenediamine used as a substrate. Absorbance was measured at 490 nm. Data represents the mean ± SD of one experiment performed in duplo.

To investigate whether the antibodies would also recognize plasma fibrinogen in solution, the binding of the antibodies to coated fibrinogen was studied in the presence of increasing amounts of fibrinogen in solution. Soluble fibrinogen (100 μg/ml) did not significantly inhibit the binding of either the antibody against γ308-322 (10 μg/ml) nor the antibody against γ308-322 to immobilized fibrinogen (coating concentration: 10 μg/ml). When a polyclonal antibody directed against fibrinogen was used, addition of 100 μg/ml soluble fibrinogen inhibited the binding of this commercial antibody to coated fibrinogen by 52 ± 0.7 % ($p < 0.001$; data based on three separate ELISA experiments each performed in triplo).

Effect of anti-peptide antibodies on platelet adhesion

To further examine the participation of the γ308-333 region of the γ-chain of fibrinogen in platelet adhesion, $F(ab)_2$ fragments of the antibodies directed against γ308-322 and γ316-333 peptides were tested both under flow conditions and in a static adhesion assay. The coverslips coated with fibrinogen were preincubated with $F(ab)_2$ fragments, because when the coverslips were incubated with IgG, large platelet aggregates were found. Preincubation of fibrinogen with either the $F(ab)_2$ fragments directed against γ308-322 or γ316-333 separately did not significantly affect platelet adhesion. However, when the coverslips coated with fibrinogen were incubated together with both antibodies, platelet adhesion was inhibited by more than 90% at both shear rates 300 s^{-1} and 1600 s^{-1} (Figure 4).

Figure 4. Effect of $F(ab)_2$ fragments against γ308-322 and γ316-333 on platelet adhesion to fibrinogen under flow and static conditions.



Coverslips were coated with fibrinogen (10 μg/ml) and incubated with a mixture of $F(ab)_2$ fragments (100 μg/ml). $F(ab)_2$ against ED-1 fibronectin was used as control. Perfusions were performed by flowing whole blood for 5 min at wall shear rates of 300 s^{-1} and 1600 s^{-1}. Results are expressed as % of the surface covered with platelets. Data are in the mean ± SEM of results obtained in three separate perfusion experiments each performed in duplo. ($p < 0.001$ for fibrinogen preincubated with the anti-peptide $F(ab)_2$ fragments versus fibrinogen preincubated with control $F(ab)_2$; $p > 0.05$ for static platelet adhesion experiments)

Incubation with a control $F(ab)_2$ fragment directed against fibronectin did not influence platelet adhesion. Combination of the $F(ab)_2$ fragments in a static platelet adhesion assay showed a small, but not significant, inhibition in platelet adhesion (Figure 4). ELISA bindingstudies with mAb 4A5 on fibrinogen preincubated with the $F(ab)_2$ fragments did not decrease the recognition of the sequence γ400-411 (data not shown, based on three separate experiments each performed in duplo). No difference in binding of $F(ab)_2$-fragments was found to immobilized fibrinogen under static conditions and immobilized fibrinogen exposed to shear.

Discussion

Platelet adhesion to fibrinogen depends on the interaction between $\alpha_{IIb}\beta_3$ on the platelet with the carboxyterminal dodecapeptide of the γ-chain. Using genetically modified fibrinogen and fibrinogen isolated from a patient with dysfibrinogenemia we found that an other domain in fibrinogen could also be involved in platelet adhesion under conditions of flow.

Deletion of the last four amino acids of the γ-chain (γΔ408-411) results in total loss of platelet adhesion. These observations are in agreement with studies by Farrell et al.[14] and Zaidi et al.[16], who showed that fibrinogen variants with an extension of the γ-chain have lost their ability to support platelet adhesion under physiological flow conditions. These results are also consistent with results of Holmbäck et al.[15], who showed that mice homozygous for a γ-chain with a deletion of the last 5 amino acids showed impaired platelet aggregation and with Rooney et al.[27], who demonstrated that recombinant human fibrinogen with a deletion of the last four amino acids showed absence of human platelet aggregation in suspension. Here, we show that these four amino acids are also essential for platelet adhesion to fibrinogen under physiological flow conditions.

In addition to the γ400-γ411 sequence, we show that the sequence around γ318-320 could also be involved in platelet interaction with immobilized fibrinogen. The use of recombinant

fibrinogen γΔ319, 320 resulted in a strongly reduced platelet adhesion. These results were confirmed by the use of dysfibrinogen Vlissingen/Frankfurt IV (V/F IV), which is characterized by a deletion of these two amino acids in the γ-chain. The reduction in platelet adhesion for the patient dysfibrinogen V/F IV is smaller than found with the recombinant fibrinogen γΔ319, 320. Patient dysfibrinogen V/F IV is a heterozygous form of dysfibrinogenemia with half of the γ-chains mutated. Whether the circulating forms of these dysfibrinogens are heterodimers, normal homodimers and abnormal homodimers has not been determined, but probably the normal homodimers and/or the heterodimers[25] support platelet adhesion. This explains the extent in reduction in platelet adhesion to the recombinant dysfibrinogen (only abnormal homodimers) compared with the heterozygous patient dysfibrinogen.

Substitution of amino acids 318 and 320 by alanines in the γ-chain of recombinant fibrinogens caused a similar strong decrease in platelet adhesion as found with γΔ319, 320 under physiological flow conditions. These amino acids participate in the calcium binding region as shown in the crystal structure of the 30 kDa C-terminal fragment of the γ-chain of human fibrinogen.[34,35] Binding studies with 4A5, a monoclonal antibody directed against the dodecapeptide, which completely inhibits platelet adhesion, revealed no differences in affinity to the fibrinogen γ-chain variants. This suggests that the primary platelet adhesion site γ400-411, which is not closely located to the region γ318-320 as deduced from the crystal structure, is optimally available and that the decrease in adhesion is not the result of a conformational change. Furthermore, when the calcium binding region of fibrinogen was affected by (pre)incubation the immobilized fibrinogen with EDTA no influence on platelet adhesion was found, indicating that the fibrinogen-bound calcium was not involved in the interaction with platelets. Addition of EDTA to whole blood completely inhibits platelet adhesion via the influence on $\alpha_{IIb}\beta_3$ dimerization.

Platelet adhesion to fibrinogen at low shear rates is completely dependent on the platelet receptor $\alpha_{IIb}\beta_3$ and no other platelet receptors have been found involved.[36,37] Therefore we

assume that $\alpha_{IIb}\beta_3$ is the receptor for the sequence around γ318-320, although it is difficult to prove, because antibodies against $\alpha_{IIb}\beta_3$ will always result in complete inhibition due to inhibition of $\alpha_{IIb}\beta_3$ - γ400-411 interaction.

To further exclude that the decrease in adhesion found with the dysfibrinogens resulted from a perturbation in the structure of fibrinogen, antibodies were raised against peptides covering the sequence γ318-320. Perfusion studies showed that platelet adhesion was completely absent, when both antibodies were added together. This indicates that the loss of adhesiveness was due to loss of an additional binding site for platelets, while γ400-411 is still exposed as shown by the fact that mAb 4A5 still binds normally. However, we can not exclude completely that the accessibility of the dodecapeptide is hindered by the presence of the antibodies directed against the peptides γ308-322 and γ316-333, although mAb 4A5 optimally recognizes fibrinogen in the presence of both antibodies. This discrepancy might be due to size differences between an antibody (4A5) and a integrin ($\alpha_{IIb}\beta_3$) on a platelet membrane. The differences between static and flow conditions might be explained by the fact that under static conditions a weaker interaction between platelets and fibrinogen is enough, while under conditions of flow multiple interactions are necessary to withstand the shear forces.

Interestingly, the peptide-antibodies did not inhibit adhesion when incubated separately with fibrinogen. The antibodies were raised against the sequence γ308-322 and γ316-333, respectively. Both antibodies did not recognize the joint sequence present in both peptides, indicating that the antibodies did not recognize the overlapping sequence of the peptides. Thus, the first antibody recognized the sequence γ308-316, while the second antibody recognized the sequence γ322-333. Clearly, these two sequences by themselves are not involved in the recognition by platelets and therefore we suppose that the overlapping region γ316-322 (which includes γ318-320) is the crucial domain, containing amino acids which are responsible in platelet-fibrinogen binding during adhesion. Only the combination of antibodies results in sufficient steric hindrance of the overlapping region to prevent platelet adhesion. This segment is part of a fibrin-specific epitope γ312-324, which is buried inside the

fibrinogen molecule in soluble fibrinogen and becomes exposed after fibrin formation.[38] The competition experiments between soluble and bound fibrinogen with the polyclonal IgG's directed against the peptides γ308-322 and γ316-333 indicated that these antibodies indeed recognize only bound fibrinogen. Furthermore, the antibodies did not inhibit platelet aggregation studies (data not shown). These results suggest that next to the conversion into fibrin the sequence γ308-333 also becomes exposed when fibrinogen is immobilized on a surface and not upon binding to $\alpha_{IIb}\beta_3$.

Clot retraction, another haemostatic process dependent on the interaction between $\alpha_{IIb}\beta_3$ and fibrin(ogen), is not affected by either a deletion of the last four amino acids of the γ-chain or by mutations in the RGD sites of the α-chain.[12,15] These observations indicate that additional binding sites on fibrin and/or fibrinogen for $\alpha_{IIb}\beta_3$ are present and critical for platelet mediated clot retraction. The γ-chain segment: 316-322, part of the fibrin-specific region, described here might also be a possible candidate for this interaction. Further studies to investigate this are in progress.[33]

Platelet adhesion to immobilized fibrinogen depends at low shear rates completely on $\alpha_{IIb}\beta_3$, which promotes immediate arrest onto immobilized fibrinogen without translocation over the surface.[37] The carboxy-terminal segment of the γ-chain at position 400-411 is the essential interaction site for $\alpha_{IIb}\beta_3$. In addition, our data indicate that the exposure of amino acids present in the sequence γ316-322 could co-operate with the primary platelet adhesion site γ400-411 to cause high-affinity interactions with platelets. Real-time observations showed that the reduced adhesion to the dysfibrinogens was due to increased rolling and detachment of platelets. From this we hypothesize that the region γ400-411 enables the interaction of fibrinogen with $\alpha_{IIb}\beta_3$ and subsequently γ316-322 becomes important to further stabilize the adhered platelet when stronger forces such as shear are exposed to the platelets. One of the conformational changes that occur in fibrinogen upon surface mobilization that is involved in the interaction of platelet integrin $\alpha_{IIb}\beta_3$ with fibrinogen is elucidated here.

Acknowledgments

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CHAPTER 6

Impaired platelet adhesion to lysed fibrin, whereas neutrophil adhesion remains intact under conditions of flow

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Summary

After vessel wall injury an haemostatic plug is formed. Restoration of vascular integrity should involve a stop to further platelet deposition and a subsequent removal of these thrombi by the fibrinolytic system and by proteases delivered by infiltrating inflammatory cells. Here we studied a possible functional and time spatial relation between platelet and inflammatory cell (PMN) adhesion to fibrin during fibrinolysis. Fibrin surfaces were exposed to fibrinolytic agents and platelet and PMN adhesion was studied under conditions of flow. Fibrinolysis of pre-formed fibrin surfaces reduced platelet adhesion rapidly, whereas PMN adhesion to fibrin was only slightly reduced even after 180 minutes exposure to plasmin. Binding studies using a polyclonal antibody showed that fibrinogen antigen remained present during the whole period of plasmin treatment. The impaired platelet adhesion under plasmin-induced fibrinolytic conditions coincided with a loss of the primary platelet adhesion site γ400-411. The PMN β_2-integrin interaction sites on fibrin remained present upon fibrinolysis as indicated by intact adhesion. Based on this in-vitro model, we hypothesize that fibrinolysis reduces the thrombogenicity of the haemostatic plug, whereas the inflammatory response is preserved and may participate in the long term removal and restructuration of the plug.

Introduction

The haemostatic plug is formed at the site of vascular injury to prevent excessive blood loss from the circulation. Blood platelets adhere to and aggregate by proteins exposed to the circulation after vascular damage.[1] Furthermore, tissue factor activates the coagulation cascade resulting in the formation of thrombin, which converts fibrinogen into the insoluble fibrin network.[2,3] Fibrin, in itself adhesive for platelets and platelet themselves are deposited in layers[4], both forming a tight seal until the haemostatic plug is disassembled by the fibrinolytic system.[5] Moreover, the haemostatic plug is adhesive for inflammatory cells, which role is not only essential for inflammatory defense but also for the dissolution and breakdown of the clot.[6] Polymorphonuclear leukocytes (PMN's) are known to be present in in-vivo clots, 4 to 13 hours old, in numbers up to 13 times greater than their number in flowing blood.[7] It has been suggested that neutrophil elastases, which produce fibrin degradation products, are also involved in the fibrinolytic process.[8,9]

The processes of clot build up and degradation are balanced to avoid pathological disorders such as bleeding and thrombosis. In case of formation of an haemostatic plug, restoration of vascular integrity requires a stop to further platelet and fibrin deposition and subsequent removal of these thrombi by the fibrinolytic system and proteases delivered by infiltrating PMN's. Treatment with fibrinolytic agents in this respect have shown to be useful in removing thrombi particularly in coronary arteries. If, however, adhesion of circulating platelets to fibrin in the clot would continue during thrombolysis, rapid restoration of blood flow would be antagonized. Our laboratory and others showed that fibrinolysis diminishes platelet adhesion to the remaining fibrin network under flow[10,11] and static conditions.[12] Here we show that the rapid removal of the platelet binding epitope by the fibrinolytic process coincides with the loss of platelet adhesion and we found that PMN adhesion is still intact indicating that the β_2 binding sites are still present in the degraded fibrin.

Materials and methods

Materials

Human fibrinogen was purchased from Enzyme Research Laboratories (Kordia, the Netherlands). Plasmin was purchased from Roche Diagnostics (Almere, the Netherlands). Human plasminogen and tissue type plasminogen activator (t-PA) were from Nodia/Chromogenix (Amsterdam, the Netherlands). Trasylol® (aprotinin) was obtained from Bayer B.V. (Mijdrecht, the Netherlands). Human α thrombin was purchased from Sigma Aldrich Chemie B.V. (Zwijndrecht, the Netherlands). Human albumin was purchased from ICN Biochemicals (Zoetermeer, the Netherlands).

Antibodies

The monoclonal antibody (mAb) 4A5 (generously provided by Dr. G.R. Matsueda) used in enzyme-linked immunosorbent assays (ELISA) binds to the carboxyl-terminus of the fibrinogen γ-chain (γ402-411) and prevents platelet adhesion.[13] The polyclonal rabbit antibody against human fibrinogen (peroxidase-conjugated), the peroxidase-conjugated swine antibody against rabbit IgG (SWARPO) and the peroxidase-conjugated rabbit antibody against mouse IgG (RAMPO) were from Dako (Glostrup, Denmark). The monoclonal antibody IB4 (anti-CD18), used in a concentration of 10 μg/ml, was from the American Type Culture Collection hybridoma cell line (Rockville, MD, USA).

Preparation of fibrin coated coverslips

Thermanox® (Nunc, Inc., Naperville, IL, USA) or glass coverslips (RENES, Zeist, the Netherlands) were soaked overnight in 80 % ethanol, rinsed thoroughly with distilled water and dried before use. Fibrinogen was diluted to 1 mg/ml in phosphate-buffered saline (PBS, 10 mM phosphate buffer, 150 mM NaCl, pH 7.4) and thrombin was added to the fibrinogen solution at a final concentration of 0.5 U/ml, just before spraying. Subsequently, the mixture was sprayed onto the coverslips using a Badger Model 100 air-brush (Franklin Park, IL,

USA). The final protein concentration on the coverslip was 3 μg/cm^2, and this was the minimal concentration needed for platelet adhesion at which the % surface covered by adherent platelets did not change with increasing density.[14] The fibrin coated coverslips were blocked in 1 % human albumin solution in PBS for 30 minutes and kept in PBS until treatment with plasmin.

Fibrinolysis of fibrin coated coverslips

Fibrin coated coverslips were exposed to an active fibrinolytic system to induce (partial) fibrin(ogen) degradation. The coverslips were exposed to either plasmin (0.25 U/ml or variable when stated) or to a mixture of plasminogen (60 nM) and t-PA (0.4 nM) (to obtain a so-called *limited* fibrinolysis as described by Hantgan et al.[10]) for up to 180 minutes (or else when stated) at 37 °C. Digestion was stopped by washing the coverslips with PBS and possible residual plasmin activity was inhibited by exposure to aprotinin (5000 U/ml). Subsequently, the lysed fibrin surfaces were blocked with human albumin and used for perfusion or ELISA studies.

Blood collection

Whole blood obtained by venipuncture from healthy volunteer donors who denied having taken aspirin or other platelet function inhibitors in the preceding week, was anticoagulated with 1/10 volume 110 mM trisodiumcitrate. The citrate anticoagulated blood was used directly in platelet adhesion studies or used to isolate PMN's for adhesion studies.

PMN isolation

Neutrophils were purified from the buffy coat of citrate anticoagulated blood as described previously.[15] In short, mononuclear cells were removed by centrifugation over Ficoll-Paque (density, 1.077/μl). The remaining red blood cells were lysed by incubation in isotonic 115 mM ammonium chloride solution (pH 7.4) at 4 °C for 20 minutes. Cells were washed twice, diluted in RPMI-Hepes buffer (20 mM Hepes, 132 mM NaCl, 6 mm KCl, 1.2 mM KH_2PO_4, 1

mM $MgSO_4.7H_2O$), 5mM glucose, 0.5 % (w/v) human albumin and 1 mM $CaCl_2$, pH7.4) (2 x 10^6 cells/ml), and kept at room temperature until start of perfusion. Neutrophil purity was greater than 95 %, and viability measured with Trypan blue exclusion was greater than 98 %; morphology was checked by light microscopy. For blocking experiments, neutrophils were preincubated with the indicated antibody for 30 minutes at room temperature.

Perfusion experiments

Perfusion studies were performed in a single-pass perfusion chamber as described.[16] Blood or neutrophils in suspension were prewarmed for 10 minutes at 37 ^{o} C. Perfusions were performed by drawing the perfusate for 5 minutes directly through the perfusion chamber using a syringe placed in a Harvard infusion pump (Pump 22, model 2400-004; Natick, MA, USA). In this way, the flow rate (Q) and thereby the shear rate through the chamber was precisely controlled. Perfusion experiments were performed at a shear rate of 300 s^{-1}; the wall shear rate (γ) can be calculated from the equation, $\gamma = (6.Q)/(d.h^2)$; w is the slit width, and h the slit height.[17]

Evaluation of platelet adhesion

After perfusion the coverslips were removed, rinsed with 10 mM Hepes buffer (containing 150 mM NaCl pH 7.4), fixed with 0.5 % glutaraldehyde, dehydrated in methanol and stained with May-Grünwald - Giemsa as described.[18] The percentage of the surface covered with platelets was measured with an image analyzer interfaced to a camera on a light microscope (400 x magnification) using OPTIMAS 6.0 software (DVS, Breda, the Netherlands). Platelet coverage, expressed as percentage of the surface covered, is the average of 15 fields/coverslip.

Evaluation of PMN adhesion

Neutrophil perfusions were performed and evaluated as described.[19] During the perfusion, the flow chamber was mounted on a microscope stage (DM RXE; Leica, Weitzlar, Germany)

equipped with a B/W CCD video camera (Sanyo, Osaka, Japan) coupled to a VHS video recorder. Perfusion experiments were recorded on video tape. Video images were evaluated for the number of adhered cells with a Quantimet 570C image-analysis system (Leica, Cambridge, UK). The number of surface-adhered neutrophils was measured after 5 minutes of perfusion at a minimum of 30 randomized fields (total surface > 3 mm^2) and expressed as the number adhered cells/mm^2 surface.

ELISA on fibrin coated coverslips

ELISA's were used to study the presence of fibrinogen antigen on the fibrin coated coversips during fibrinolysis. After exposure to plasmin, washing and treatment with aprotinin, the coverslips were blocked with 3 % albumin. Subsequently, the fibrin(ogen) surfaces were incubated with the indicated antibody. All incubations were carried out for 2 hour at 37 ^{o} C, and were followed by extensive washing with PBS. The peroxidase-conjugated polyclonal antibody against fibrinogen (Dako, Denmark) was diluted 1:1000 and mAb 4A5: 1:15,000. Detection involved RAMPO (for mAb 4A5) with ortho-phenylenediamine used as a substrate. Absorbance was measured at 490 nm in a Vmax Microtiter Plate Reader (Molecular Devices, Palo Alto, CA, USA).

Statistical analysis

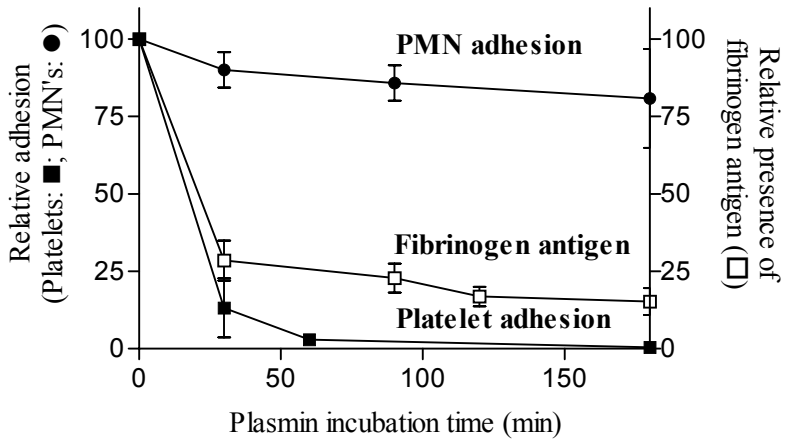
Results were expressed as mean ± standard error of the mean (SEM) for data obtained from at least three separate experiments each performed at least in duplicate. Results obtained from one experiment performed at least in duplicate are expressed as mean ± standard deviation (SD). The Student *t*-test was used to test for significance between groups. P-values < 0.05 were considered significant.

Results

Platelet and PMN adhesion to fibrin surfaces exposed to fibrinolysis

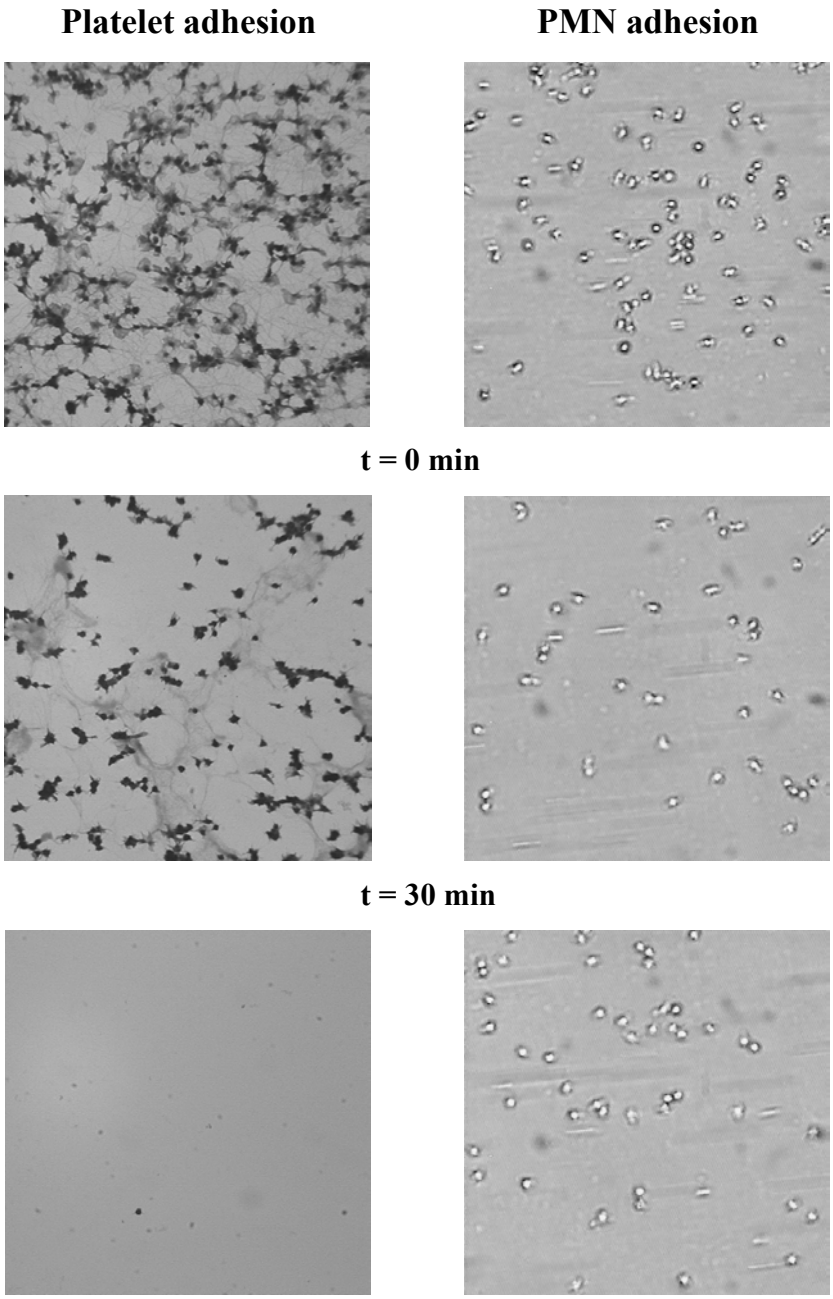
The capacity of fibrin coated surfaces to support platelet adhesion under conditions of flow during ongoing fibrinolysis was investigated using a perfusion chamber. Citrate anticoagulated blood was drawn at a wall shear rate of 300 s^{-1} over the fibrin surfaces, which had been exposed to plasmin (0.25 U/ml) for various times. Platelet adhesion was strongly impaired to fibrin treated with plasmin (0.25 U/ml) as illustrated in Figure 1 and 2.

Figure 1. Effects of fibrinolysis on platelet (▣) and PMN (●) adhesion to fibrin under flow conditions (left ordinate) in relation to the presence of fibrinogen antigen (☒) on the surface (right ordinate).



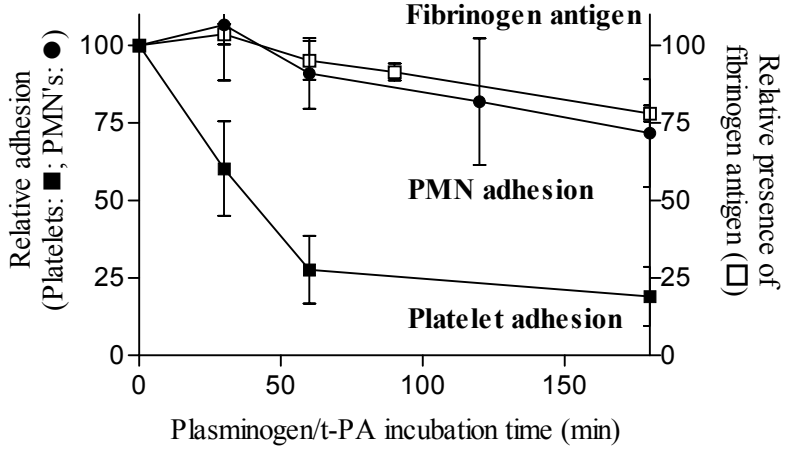
Coverslips were sprayed with fibrin (1 mg/ml) and exposed to plasmin (0.25 U/ml) for the time denoted at 37 °C. Fibrinolysis was stopped with aprotinin and perfusions were performed at a wall shear rate of 300 s^{-1} (5 min). Adhesion is expressed relatively as % of the surface covered with platelets or number of PMN's adhered to non-treated fibrin at t= 0 (absolute platelet adhesion: 48.2 ± 6.9 % surface coverage (= 100 %) at t = 0; absolute PMN adhesion: 614.1 ± 122.6 cells/mm^2 (= 100 %) at t = 0). Fibrinogen antigen on the fibrin coverslips during fibrinolysis was measured with ELISA using a peroxidase conjugated polyclonal antibody against fibrinogen. The relative absorbance is expressed as % of the absorbance of non-treated fibrin coated coverslips. All data represent the mean ± SEM of three separate experiments each performed at least in duplicate.

Figure 2. Effect of fibrinolysis on platelet and PMN adhesion to fibrin under flow conditions. Pre-formed fibrin coverslips were exposed to plasmin (0.25 U/ml) for the time denoted. The experimental conditions are given in the legend to Figure 1.



After 60 minutes of incubation platelet adhesion was reduced by > 95 %. The (residual) amount of fibrinogen antigen presence on the coverslips during fibrinolysis was measured using an ELISA-type setup with a polyclonal antibody raised against fibrinogen. Exposure of the fibrin coverslips to plasmin reduced the amount of antigen present on the surface with 80 % upon 180 minutes of incubation (Figure 1). Notwithstanding, the fact that fibrinogen antigen was reduced after 180 minutes of plasmin incubation, the digested fibrin surfaces remained adhesive for polymorphonuclear leukocytes (PMN's) as observed with perfusions of PMN's at 300 s^{-1} (Figure 1 and 2). Fibrin, after 180 min of plasmin incubation still showed 80 % of the cells that adhered to untreated fibrin. Adhesion of PMN's to fibrin with or without incubation of plasmin was completely inhibited upon addition of the monoclonal antibody IB4, which is directed against β_2 integrins (data not shown).

Figure 3. Effects of limited-fibrinolysis on platelet (▣) and PMN (●) adhesion to fibrin under flow conditions (left ordinate) in relation to the presence of fibrinogen antigen (▨) on the surface (right ordinate).



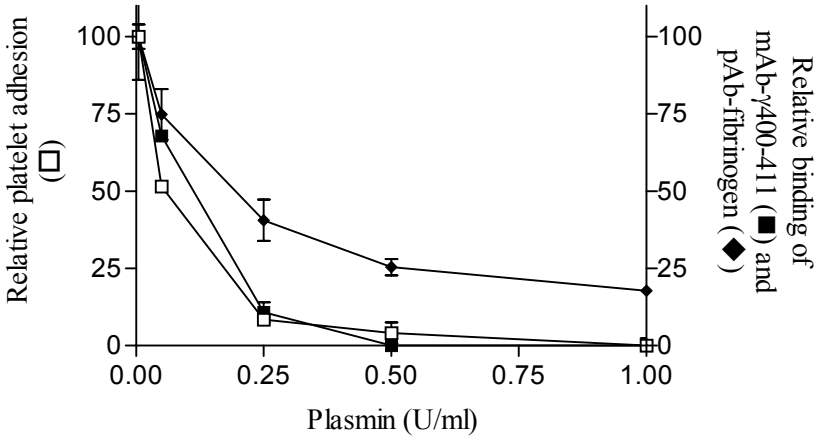
Coverslips were sprayed with fibrin (1 mg/ml) and exposed to a mixture of t-PA (0.4 nM) and plasminogen (60 nM) for the time denoted at 37 °C. The experimental conditions are given in the legend to Figure 1. Absolute platelet adhesion: 34.6 ± 3.9 % surface coverage (= 100 %) at t = 0; abolute PMN adhesion: 1266.7 ± 215.8 cells/mm^2 (= 100 %) at t = 0. All data represent the mean ± SEM of three or more separate experiments each performed at least in duplicate.

When the fibrin surfaces were exposed to *limited* fibrinolysis by plasminogen (60 nM) and t-PA (0.4 nM) treatment approximately 75 % fibrinogen antigen remained present after 180 min incubation. Still platelet adhesion decreased to 25 %, while PMN adhesion remained for 75 % (Figure 3).

Presence of the fibrinogen sequence γ400-411 during fibrinolysis

Exposure of the fibrin surfaces to plasmin resulted in a decrease in platelet adhesion upon increasing incubation time. The reduction of platelet adhesion could be explained by a specific proteolytic cleavage of the carboxyl-terminal part of the γ chain: the dodecapeptide γ400-411, known as the primary platelet adhesion site. Therefore the presence of the epitope was analyzed during fibrinolysis using a monoclonal antibody (mAb 4A5), which inhibits platelet adhesion to fibrinogen and is directed against the sequence γ402-411. Figure 4 shows a decreased platelet adhesion upon increasing plasmin concentrations (all incubated at 30 minutes). Platelet adhesion was completely absent when the fibrin surfaces were incubated with 1 U/ml plasmin. Under these conditions the sequence γ400-411 was completely absent, as shown by the loss of recognition by the monoclonal antibody 4A5, and 18 % of the fibrinogen antigen remained present on the surface as recognized by a polyclonal antibody (Figure 4). These binding studies of mAb 4A5 to similarly treated fibrin coverslips suggest a relation between the observed reduction in platelet adhesion and decreased binding of mAb 4A5 upon increasing plasmin concentration. A similar relation between mAb 4A5 binding and platelet adhesion was observed when the fibrin surfaces were exposed to plasmin (0.25 U/ml) with increasing incubation times (data not shown).

Figure 4. Effect of fibrinolysis on platelet adhesion (☒; left ordinate) in relation to the presence of the primary platelet adhesion site γ400-411 (▣; right ordinate) as measured with a monoclonal antibody (mAb) and the presence of fibrinogen antigen (▬; right ordinate) as measured with a polyclonal antibody (pAb).



The fibrin surfaces were incubated for 30 minutes with variable plasmin concentrations (U/ml). The experimental conditions are given in the legend to Figure 1. For the ELISA a monoclonal antibody (mAb 4A5) raised against the fibrinogen sequence γ400-411 was used. Detection involved a rabbit antibody against mouse IgG conjugated to horseradish peroxidase and ortho-phenylenediamine as substrate and absorbance was measured at 490 nm. Data represents the mean ± SD of one experiment performed in triplo.

Discussion

Thrombolytic therapies with plasminogen activators have been shown to be effective and of benefit for patients with acute myocardial infarction.[20-22] A major prerequisite for successful reperfusion, however, is the fact that during thrombolytic treatment, adhesion of circulating platelets to the degraded fibrin clot will be halted. Additional platelet adhesion would otherwise contribute to reocclusion of the vessel. Therefore, combinations of anti-fibrinolytic and platelet adhesion/aggregation inhibiting therapy are often used in clinical practice. The

ability of fibrin to support platelet adhesion after the action of fibrinolytic agents has been studied.[10-12] Our laboratory reported reduced platelet adhesion under flow conditions to fibrin exposed to so-called *limited* fibrinolytic conditions using t-PA (60 nM) and plasminogen (0.4 nM) leaving the alpha, beta and gamma chain intact as monitored by SDS-PAGE.[10] Hamaguchi et al. reported under static conditions similar decreased platelet adhesion to more extensively degraded fibrin using plasmin (0.5 U/ml).[12] Platelet adhesion to fibrinogen and fibrin is completely dependent on platelet integrin $\alpha_{IIb}\beta_3$ at low shear rates (300 s^{-1})[23], and while it has been shown that the fibrinogen sequence γ400-411 is the primary platelet adhesion site for $\alpha_{IIb}\beta_3$ under these conditions of flow[24], in the present study we have investigated the influence of fibrinolytic conditions on the presence or absence of the known platelet binding epitope γ400-411 on the remaining fibrin together with the actual adhesion of blood platelets and PMN's under conditions of flow.

Digestion of the fibrin surfaces resulted in a rapid loss of platelet adhesion under flow (Figures 1-3) and coincides with the loss of the primary platelet adhesion site γ400-411 as shown by the impaired binding of the monoclonal antibody 4A5 in ELISA studies (Figure 4). This confirms earlier suggestions reported by Hamaguchi et al. for static conditions.[12]

Sites of vascular damage not only contain fibrin and platelets but also inflammatory cells and in vitro experiments showed that PMN's adhere to thrombotic substrates as activated platelets, fibrinogen and fibrin.[25] We show clearly that neutrophil adhesion to fibrin remains present during fibrinolysis, whereas platelet adhesion is rapidly inhibited even at limited fibrinolytic conditions (Figure 1).[10] Adhesion of PMN's to fibrin is completely dependent on CD11b/CD18 (MAC-1; $\alpha_M\beta_2$) under conditions of flow.[26] Therefore the capacity of the fibrin network during fibrinolysis to maintain adhesion of neutrophils suggests that the site(s) on fibrin(ogen) involved in the interaction with the neutrophil receptor MAC-1 are still present and functional. In this respect, it has been shown that binding of fibrinogen to MAC-1 does not involve the two RGD sequences (α95-97 and α572-574) on the alpha chain nor the dodecapeptide γ400-411 on the gamma chain of fibrinogen.[27] Instead the region γ190-202 in the fibrinogen gamma chain has been identified as the mediating sequence of ligand binding

to MAC-1.[28] Furthermore, it has been shown that the sequence γ377-395 is also involved in the recognition of MAC-1 with the gamma chain of fibrinogen[29] and recently it has been reported that the extended alpha chains (α_E), present in a subclass of fibrinogen known as fibrinogen-420, can also serve as a ligand for MAC-1.[30] From the present adhesion results, we conclude that either one or both of these sequences are present and functional during extensive fibrinolytic conditions to support adhesion under flow conditions. Future experiments will be directed to study fibrinolytic influence on fibrin formed under flow conditions[31] and on mixed thrombi.

Based on this in-vitro model, we hypothesize that the difference between adhesion of platelet and neutrophils to the fibrin clot during fibrinolysis might illustrate a physiological mechanism that reduces the thrombogenicity of the haemostatic plug. Whereas the inflammatory response is preserved, this process might participate in the long term removal and restructuration of the plug.

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CHAPTER 7

Dynamics of fibrin deposition on matrices under conditions of flow

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(Preliminary report)

Summary

We have studied the influence of flow on the formation and deposition of fibrin. When anticoagulated whole blood was perfused over tissue factor rich extracellular matrices (ECM), a dense fibrin network was formed on the coverslip with fibrin fibers oriented in flow direction. Increasing shear rates resulted in a decreased thrombin and fibrin generation as reflected by F1+2- and FPA-levels in the perfusate, respectively. As expected fibrin deposition was also decreased. Real-time videomicroscopy and platelet poor plasma was used to study the influence of flow on the deposition of fibrin. Locally formed fibrin fibers remained bound to the surface, but some of the fibers detached and were transported by flow. Part of the flowing fibrin fibers adhered again downstream to the ECM. Binding of these circulating fibrin fibers to ECM proteins was studied on downstream placed coverslips coated with an adhesive protein. The circulating fibrin fibers bound to downstream placed fibrinogen coated coverslips. Vitronectin and fibronectin also bound fibrin fibers, although to a lesser extent than fibrinogen. Von Willebrand Factor (vWF) and albumin coated coverslips bound only traces or no fibrin, respectively. We conclude that the effect of shear on fibrin formation is due to both a decrease in fibrin generation and increased removal of fibrin exerted by shear forces. Furthermore, detached fibrin fibers can bind downstream to extracellular matrix proteins such as vitronectin, fibronectin and fibrinogen.

Introduction

When a vessel is injured, a platelet plug is rapidly formed to minimize blood loss. The formation of this plug depends on a complex set of regulated events called haemostasis. The two basic processes in haemostasis are (1) adhesion and aggregation of blood platelets onto collagens present in the perivascular connective tissue and (2) the conversion of soluble fibrinogen into an insoluble fibrin network.[1] Thrombosis and bleeding are the pathological counterparts of normal haemostasis, manifesting as an obstruction of the circulation by thrombi in veins or arteries and a delayed formation of an haemostatic plug, respectively. Fibrinogen and von Willebrand Factor (vWF) are two principal ligands known to mediate platelet adhesion and aggregation via the binding to the platelet receptors $\alpha_{IIb}\beta_3$ (fibrinogen and vWF) and GPIb (vWF). Fibrinogen stabilizes the platelet thrombus[2-4] via the formation of tightly packed platelet aggregate to resist shear forces.[5] In veins (shear rates of 300 s^{-1} or less), fibrin accounts for most of the mass of the thrombus.[6] Thrombi formed at the vessel wall of epicardial coronary vessels (300 - 800 s^{-1}) and arterioles (2000 s^{-1} or more) are mainly composed of aggregated platelets with relatively little fibrin.[7-9] Several studies have reported the shear rate dependence of fibrin formation using either in- or ex-vivo thrombosis models using (non)-anticoagulated blood and fixed end points.[10-13]

Here, we report that the shear rate dependence of fibrin formation is due to both a decreased generation of fibrin and an increased removal of fibrin due to shear forces. We have used a perfusion chamber and videomicroscopy to study real-time the formation of a fibrin network. We found that when fibrin was formed under flow, fibers were seen to detach, to circulate, and to bind downstream. This binding depends on the nature of the surface with good binding to fibrinogen, vitronectin and fibronectin coated surfaces.

Materials and methods

Materials

Low molecular weight heparin (LMWH; Fragmin®: anti-Xa) was obtained from Pharmacia (Woerden, the Netherlands). Thermanox® coverslips were from Nunc Inc. (Naperville, IL, USA). Human albumin was from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands). Innovin® was purchased from Baxter Diagnostics Inc. (Unterschleissheim, Germany). Human fibrinogen (plasminogen, vWF and fibronectin free) was from Enzyme Research Labs (South Bend, IN, USA). Human fibronectin was purified as described previously[14] via affinity chromatography from citrated plasma over a gelatin-Sepharose (Pharmacia, Uppsala, Sweden) column. Collagen type III was obtained from Sigma (St. Louis, MO, USA). Human vWF was purified from plasma cryoprecipitate by gel filtration on Biogel A15 (Biorad, Richmond, CA, USA) as described.[15] Human vitronectin was purified from plasma according the method as described.[16] Diazobicyclo-octane (DABCO) and 4β-phorbol, 12-myristate, 13-acetate (PMA) were purchased from Sigma (St. Louis, MO, USA). MOWIOL 40-88 was from Aldrich-Chemie (Steinheim, Germany) and plasmin was purchased from Boehringer Mannheim (Germany). Asserachrom® D-dimer ELISA was from Diagnostica Stago (Boehringer Mannheim, Germany). The dRGDW peptide (D-arginyl-glycyl-L-aspartyl-L-trypthophan) was generously provided by Dr. J. Bouchaudon (Rhône-Poulenc-Rorer, France).

Blood collection

Whole blood was obtained by venapuncture from healthy volunteer donors, who denied having taken aspirin or other platelet function inhibitors in the preceding week, and collected into 1/10 (v/v) of low molecular weight heparin (LMWH: 200 anti Xa-units/ml saline). Platelet poor plasma (PPP) was obtained after centrifugation (10 min at 2500 g, 20 °C) and filtration. Detection limit for platelet count was < 1.000/μl. Reconstituted blood was prepared with various platelet numbers as follows: platelet rich plasma (PRP) was obtained from citrate (1/10 volume 110 mM trisodium citrate) anticoagulated whole blood by centrifugation (10

min at 200 g, 20 °C). PRP and PPP were mixed to obtain the chosen platelet concentration. Red cells were washed three times with 154 mM NaCl and 5 mM D-glucose by centrifugation at 2000 g at 20 °C for 5 min and subsequently packed by centrifugation for 12 min at 2000 g. Packed red cells were added to the PPP/PRP mixture to obtain perfusate with a hematocrit of 40 %.

Cell culture to obtain tissue factor rich extracellular matrix (ECM)

Fibroblasts derived from human fetal lung tissue or human skin, were cultured on glass coverslips or on Thermanox® coverslips as described.[17] Human umbilical vein endothelial cells were cultured as described.[18] After confluence of the endothelial cells, the cells were stimulated with phorbol myristate acetate (PMA: 20 ng/ml overnight) to obtain a tissue-factor-rich extracellular matrix (ECM). The ECM was obtained upon removal of the endothelial cells or fibroblasts with 0.1 M NH_4OH at room temperature for 15 min and gently shaking.[17] The isolated tissue-factor rich matrixes of fibroblasts and of stimulated endothelial cells were washed three times with phosphate buffered saline (PBS, 10 mM phosphate buffer, 150 mM NaCl, pH 7.4) and stored with PBS at –20 °C before use.

Tissue factor coated coverslips

One vial of Innovin® was dissolved in 10 ml distilled water and diluted 1/10 (v/v) in coating buffer (0.1 M Na_2CO_3, pH 9.5). Thermanox® coverslips were stored in 70 % ethanol, rinsed with distilled water and air-dried. The coverslips were incubated with the Innovin® solution overnight at 4 °C as described.[19] After incubation, the coverslips were rinsed and stored in PBS until perfusions were performed.

Perfusion studies

Blood or plasma was used in perfusion experiments. Before perfusion, plasma or blood was prewarmed for 10 min at 37 ºC and perfused at 37 ºC. Perfusions were performed using a single-pass modified small perfusion chamber with a slit height of 0.1 mm and a slit width of

2 mm under non-pulsatile flow conditions.[20] Blood or plasma was drawn for the time indicated through the perfusion chamber by a syringe placed in an Harvard infusion pump (Pump 22, model 2400-004; Natick, MA, USA) by which different wall shear rates were maintained. After perfusion the coverslips were rinsed in Hepes-buffered saline (10 mM Hepes, 0.15 M NaCl, pH 7.4), fixed in 0.5 % glutaraldehyde in PBS, dehydrated in methanol and stained with May-Grünwald - Giemsa, as described.[21]

Thrombin and fibrin formation as reflected by F1+2- and FPA-levels was measured in the perfusate after perfusion over tissue factor rich surfaces. Directly after perfusion, the perfusate was collected in a syringe containing 3.8 % citrate to inhibit further coagulation. Subsequently F1+2- and FPA-levels were measured in platelet poor plasma using an ELISA for F1+2 (Enzygnost® F1+2 micro, Behring Diagnostics, Germany) and FPA (Sheep anti-human Fibrinopeptide A (SAFPA-AP), Kordia, the Netherlands; Human fibrinopeptide A, Bachem, Switzerland), respectively.

Real-time perfusion studies

As previously described[5], a vacuum perfusion chamber was assembled on a microscope (Leitz Diaplan, Leica Rijswijk, the Netherlands) (100x magnification) equipped with a camera (JAI-CCD, Copenhagen, Denmark) connected with monitor (RCA, Lancaster, USA) and videocassette recorder (VC-MH69, Sharp Electronics, United Kingdom). Platelet poor plasma was perfused over glass coverslips covered with the ECM of human fibroblasts or PMA stimulated endothelial cells for 10 min. Perfusion was recorded real-time with videotape. A frame grabber was used to digitize the video (Matrox Meteor Driver, Matrox Electronic Systems Ltd. Quebec, Canada) for OPTIMAS 6.0 software (DVS, Breda, the Netherlands) and frames were visualized and analyzed using the “Emboss” filter (Adobe Photoshop v5.02; Adobe Systems Inc., USA).

Quantification of fibrin deposition on coverslips

Surface coverage evaluation of fibrin deposition was performed on stained coverslips using computer-assisted analysis as described.[22] Quantification of fibrin deposition was also performed immunochemically. To do so, the coverlips were rinsed directly after perfusion with Buffer C (130 mM NaCl, 2 mM KCl, 12 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 0.9 mM $MgCl_2$; pH 7.4). Fibrin on the coverslips was digested with plasmin[23]: the coverslip was immersed in a plasmin solution (0.05 U in 500 μl) and incubated at 37 °C for 60 min with gently shaking. The samples were collected and snap frozen at – 20 °C until assayed for D-dimer levels as a measure for fibrin. D-dimer levels were measured with Asserachrom® D-Dimer ELISA.

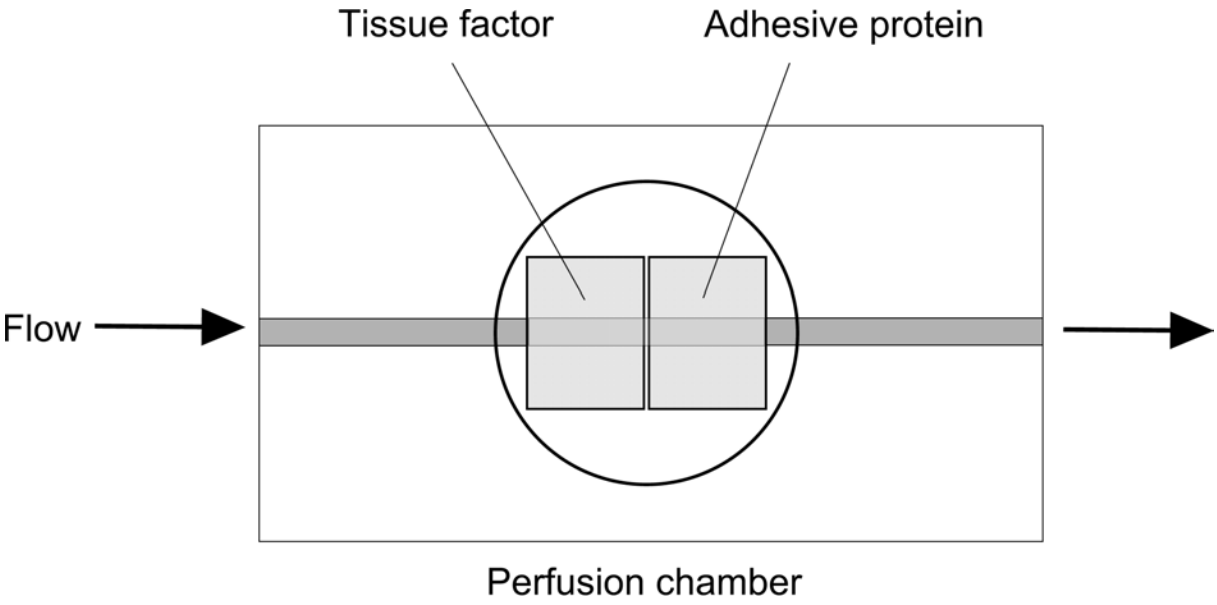
FITC-labeled fibrin network

FITC (Fluorescent isothiocyanaat)-labeled fibrinogen (generous gift of Roy Harris, Andaris Limited, Nottingham, United Kingdom) was added to the perfusate (100 μg/ml). Perfusion experiments were performed over glass coverslips covered with the ECM of human fibroblasts. After perfusion, the coverslips were removed, rinsed with Hepes-buffered saline and fixed in a 3 % paraformaldehyde solution with 0.025 % glutaraldehyde. After rinsing with PBS the coverslips were blocked with 1 % bovine albumin solution and mounted in MOWIOL 40-88 and 2.5 % DABCO (diazobicyclo-octane). The coverslips were analyzed with fluorescence confocal laser scanning microscopy.

Fibrin binding to different adhesive proteins under flow conditions

Plasma perfusions (20 s^{-1}) were performed using an one-way perfusion chamber with two coverslips in series (Figure 1). The upstream placed coverslip (A) was coated with tissue factor and the second (downstream) coverslip (B) was coated with the adhesive protein to be studied (100 μg/ml in PBS). The proteins were coated for 90 min at room temperature and blocked for 30 min with 1 % bovine serum albumin solution in PBS.

Figure 1. Model a perfusion chamber with two coverslips in series to study fibrin fiber binding to adhesive proteins.



The first coverslip (upstream: A) was coated with tissue factor and the second coverslip (downstream: B) was coated with the studied adhesive protein (fibrinogen, vWF, fibronectin, vitronectin or albumin).

Fibrin binding to pre-adhered platelets

Coverslips with pre-adhered platelets were obtained by perfusing whole blood over vWF coated coverslips for 5 min. The obtained coverslip with pre-adhered platelets were washed and mounted in a perfusion chamber immediately adjacent to a tissue factor coated coverslip (A). Subsequently, both coverslips were perfused with plasma.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM) for data obtained from three or more separate experiments each performed at least in duplicate. Experiments from one or two separate experiments each performed at least in duplicate are expressed as mean ± standard deviation (SD).

Results

Fibrin formation on tissue factor rich matrices in whole blood under flow conditions

Perfusions of anticoagulated whole blood over tissue factor rich extracellular matrices (ECM) resulted in fibrin deposition and thrombus formation. Fibrin deposition was visible with light microscopy only at wall shear rates below 300 s^{-1} (Figure 2A). The fibrin fibers were connected to each other to form a network bound to the ECM with fibers aligned in the direction of flow. The decreased fibrin deposition (as % of the surface coverage) upon increasing shear rate corresponded with thrombin and fibrin generation as reflected by respectively F1+2- and FPA-levels in the perfusate (Table 1). Perfusion of whole blood with normal platelet numbers (Table 1: > 75.000/µl) over tissue factor rich ECM at a shear rate of 300 s^{-1} generated 25.5 ± 6.7 ng/ml FPA and 3.2 ± 1.0 nM F1+2, whereas at shear rates of 1600 s^{-1} FPA and F1+2 decreased to 6.6 ± 7 and 0.8 ± 0.9 respectively.

Table 1. Effects of platelet number and shear rate on the surface covered with fibrin (% Surface Coverage: S.C.) in relation to the generation of fibrinopeptide A (FPA) and F1+2 in the perfusate.

	300 s^{-1}			1600 s^{-1}		
	FPA	**F1+2**	**% S.C.**	**FPA**	**F1+2**	**% S.C.**
0 - 25.000	18.8 ± 14	1.3 ± 0.6	50.2 ± 22	8.3 ± 12	0.5 ± 0.6	0
25.000-75.000	28.2 ± 0.1	3.2 ± 0.1	60.3 ± 7	7.0 ± 10	0.7 ± 0.8	0
> 75.000	25.5 ± 6.7	3.2 ± 1.0	55.4 ± 10	6.6 ± 7	0.8 ± 0.9	0

Perfusions were performed with several platelet numbers over tissue factor rich extracellular matrices (ECM) at 300 s^{-1} and 1600 s^{-1}. Surface coverage was measured with computer asissted analyis using OPTIMAS 6.0 Software. F1+2- and FPA-levels were measured in the perfusate with ELISA. Data represent the mean ± SD of two independent experiments each performed in triplicate.

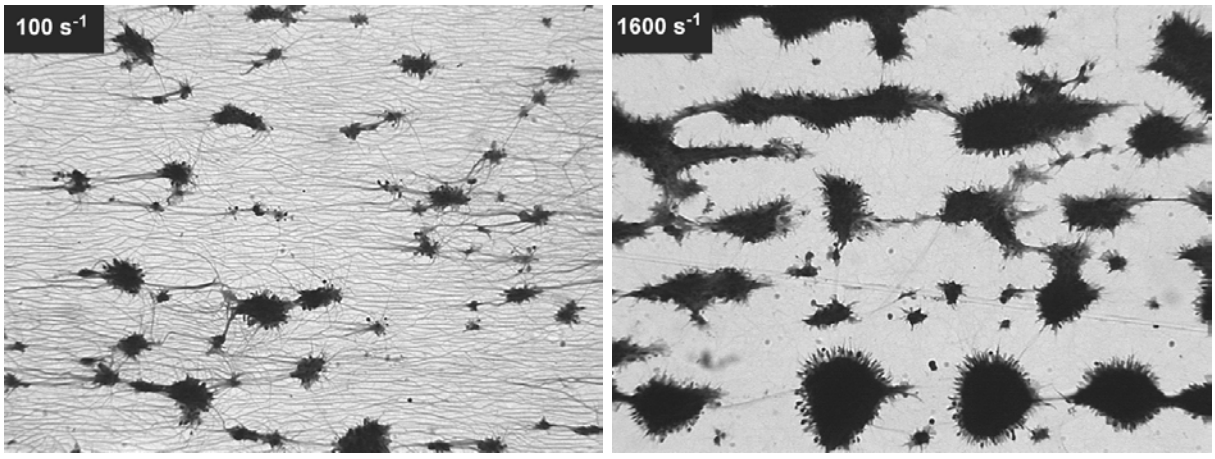
The contribution of platelets in fibrin formation was studied using reconstituted blood with decreasing platelet numbers. Perfusions at 300 s^{-1} with reconstituted blood with low platelet number (< 25.000/μl) resulted in a decreased F1+2 generation, but FPA generation was not significantly affected (Table 1). At higher shear rates (1600 s^{-1}) no effect of decreasing platelet number was observed on FPA and F1+2 generation.

Fibrin formation on tissue factor rich matrices in plasma under flow conditions

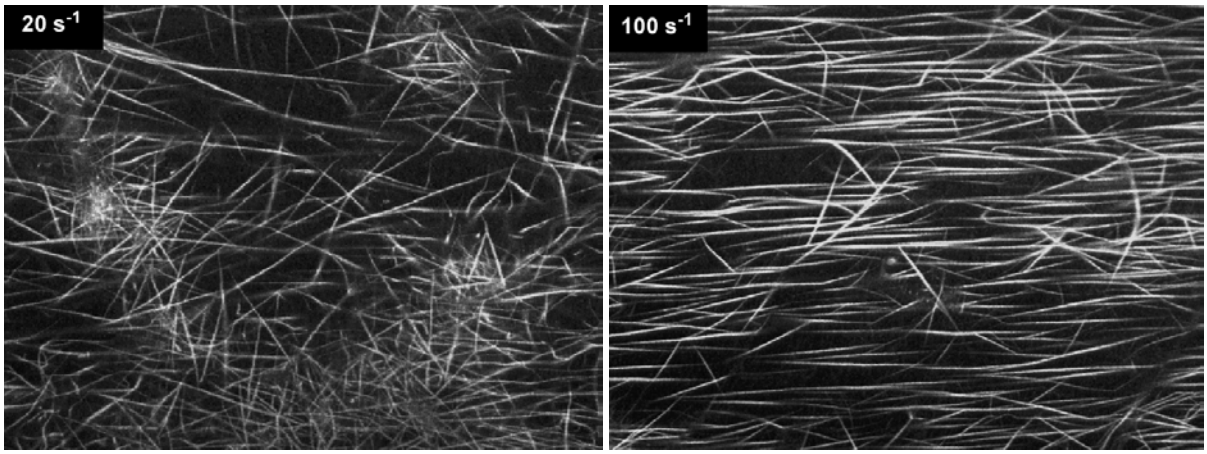
Fibrin formation under flow conditions was better visualized in real-time when red blood cells and platelets were absent (next section). Since fibrin was formed even at low platelet numbers (Table 1), we used platelet poor plasma (detection limit: < 1.000/μl). We first studied fibrin formation on tissue factor rich matrices using platelet poor plasma and fixed end points. FITC-labeled fibrinogen was added to platelet poor plasma, which was perfused at different shear rates (Figure 2B).

Figure 2. Fibrin deposition on tissue factor rich extracellular matrix (ECM) at several wall shear rates after whole blood perfusions measured by light microscopy (A) and after perfusion of platelet poor plasma to which FITC-labeled fibrinogen was added, measured by fluorescence confocal laser scanning microscopy (B).

A



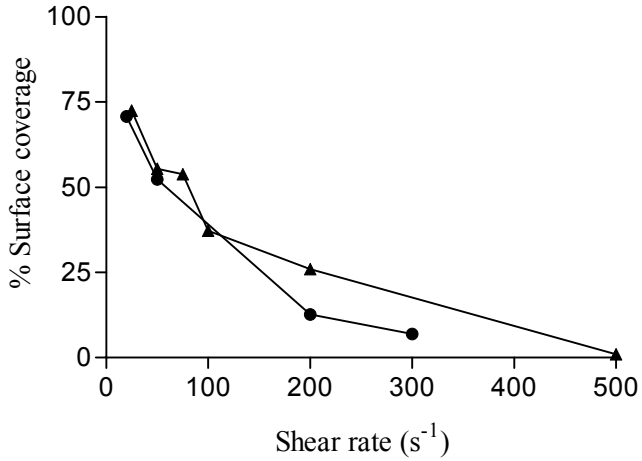
B



The fibrin network formed in the absence of platelets, was visualized with fluorescence microscopy (Figure 2B). At very low shear rates (20 s^{-1}), fibrin fibers formed a random network, while at 100 s^{-1} fibrin fibers were orientated in the flow direction (Figure 2B).

The shear rate dependence of fibrin deposition in plasma was quantified on both tissue factor rich ECM and tissue factor coated coverslips by measuring the surface covered with fibrin using computer-assisted analysis (Figure 3). Fibrin deposition was almost completely absent at shear rates higher than 300 s^{-1}. This shear rate dependence of fibrin deposition on tissue factor coated coverslips was confirmed immunochemically by D-dimer measurements after plasmin digestion of the deposited fibrin (20 s^{-1}: 53.7 ± 14; 300 s^{-1}: 0.2 ± 0.1; data expressed in fibrinogen equivalents: ng/mm^2 coverslip ± SD of four coverslips from two independent experiments).

Figure 3. Shear rate dependence of fibrin deposition.



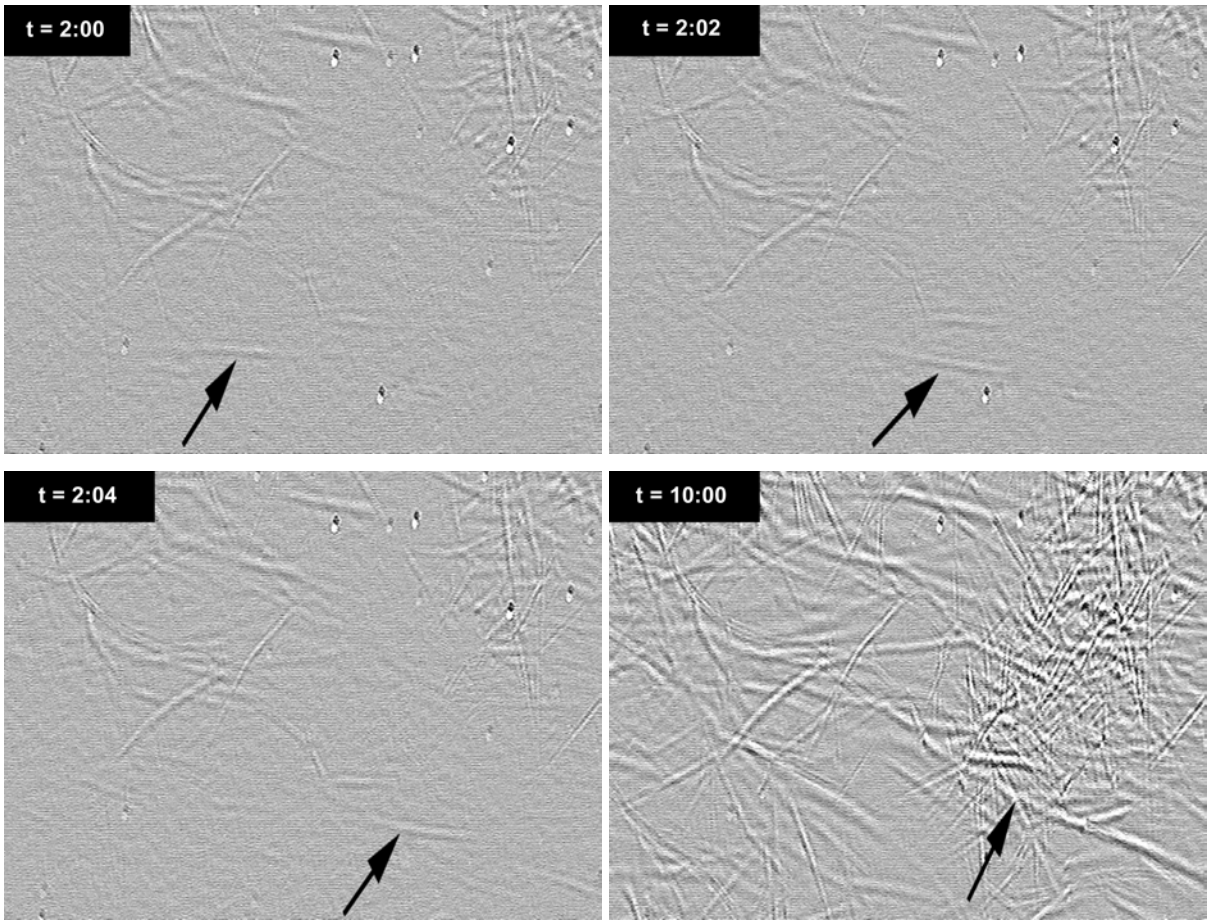
Fibrin on the coverslips was quantified by measuring surface coverage of fibrin deposited on coverslips coated with tissue factor (Innovin®) (▲) or tissue factor rich ECM (✯) using computer-assisted analysis. Data represent the mean from one perfusion experiment performed in duplicate.

Real-time fibrin network formation

The deposition of a fibrin network under conditions of flow was studied in real-time using a perfusion chamber connected to a light microscope on which a camera and videocassette recorder was mounted. Plasma perfusions were performed over tissue factor rich ECM at different shear rates. Fibrin fibers were observed after approximately 2 min perfusion. These fibers oriented in the direction of flow were not only attached to the surface, but also transported by plasma. At a shear rate of 20 s^{-1}, fibrin fibers moved over the surface, aligning in flow direction and became fixed to the surface. A part of the formed fibrin fibers were released from the surface and washed away. Some of these flowing fibrin fibers became incorporated into a fibrin network already formed downstream. Real-time observations of fibrin network formation at shear rates 20 s^{-1} and 100 s^{-1} are depicted in Figure 4A and 4B, respectively.

Figure 4. Real-time observations of fibrin network formation at 20 s^{-1} and 100 s^{-1}.

A



A perfusion chamber was assembled on a microscope equipped with camera, monitor and videocassette recorder. Plasma perfusions were performed over tissue factor rich ECM. During perfusion fibrin formation was recorded in real-time and after perfusion video images were frame grabbed with OPTIMAS 6.0 software and visualized with "Emboss" filter. Real-time observations of fibrin network formation showed that a small of the fibrin fibers were transported downstream by flow and were captured into a fibrin network downstream. ***Figure 4A*** *shows fibrin formation at 20 s^{-1} after 2 min perfusion (t = 2:00 min). Frames were taken in 2 s intervals (t = 2:02 and 2:04 min) and the end situation (t = 10 min). At t = 2:00 min a fibrin fiber (arrow) is transported by flow (t = 2:02 min, t = 2:02 min), captured into a fibrin network downstream (t = 2:04 s) and is part of the fibrin network after 10 min perfusion.*

B

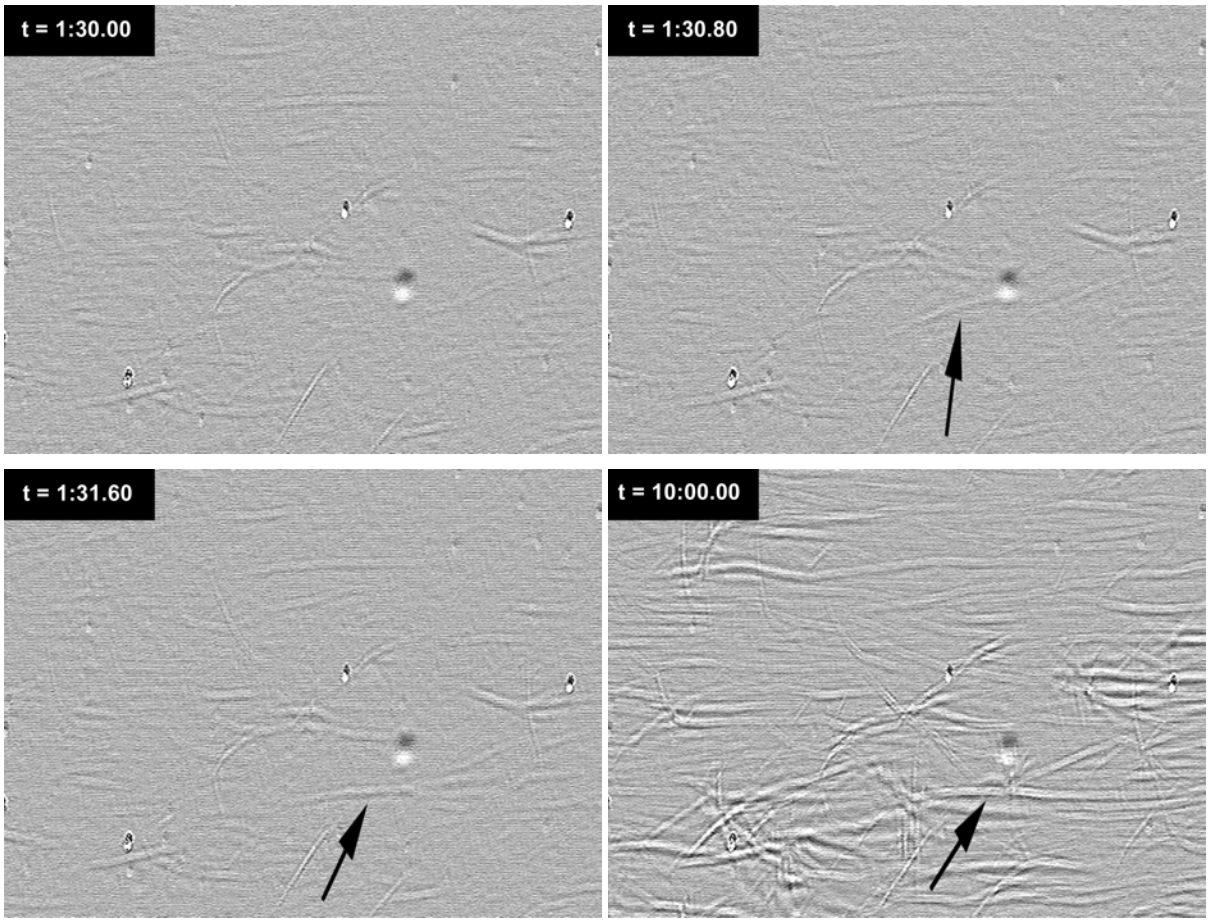


Figure 4B (t = 1:30 min): A fast moving fibrin fiber is captured to the surface (t = 1:30.80 min), bowed in flow direction (t = 1.31.60 min) and is part of the fibrin network after 10 min perfusion.

Figure 4A represents a perfusion after 2 min (t = 2:00 min). Frames were taken in 2 s intervals (t = 2:02 and 2:04 min) and the end situation (t = 10 min). At t = 2 min a fibrin fiber (arrow) is transported by flow (t = 2:00, 2:02), captured into a fibrin network downstream (t = 2:04) and has become a part of the fibrin network after 10 min perfusion. In Figure 4B a moving fibrin fiber (t = 1:30.00 min) is captured onto the surface (1:30.80 min), bowed in flow direction (1:31.60) and has become a part of the fibrin network after 10 min perfusion. Shear

rates of 300 s^{-1} or higher showed some fibrin fibers making contact with the surface, but most of the fibers were washed away by flow (data not shown).

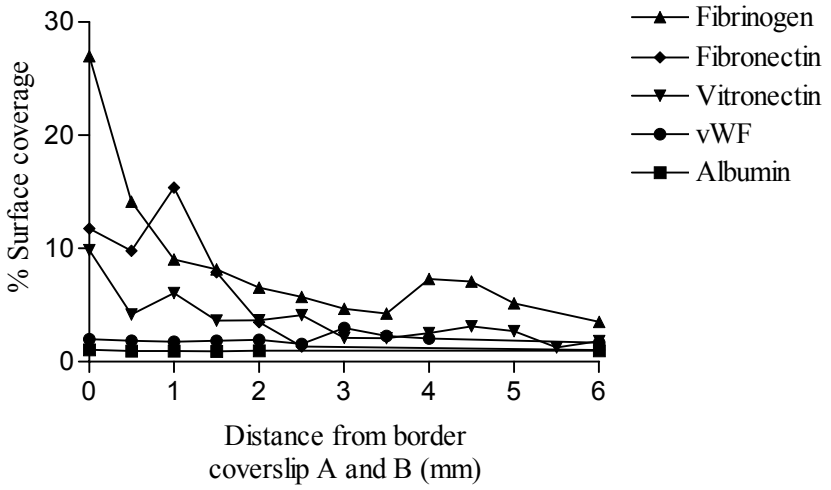
Fibrin binding to proteins present in the ECM

The fibrin network, formed upon perfusions of platelet poor plasma, was bound to the ECM without mediation by platelets. To exclude a possible role of traces of platelets possibly present in the filtered plasma (detection limit: < 1.000/μL) dRGDW peptide was added (up to 1 mM) to the plasma in order to inhibit platelet-fibrin(ogen) interactions. The presence of dRGDW did not affect fibrin deposition on the matrix (data not shown). This suggested that the ECM itself is able to bind fibrin fibers under low shear conditions.

The binding of flowing fibrin fibers to adhesive proteins present in the ECM was studied using a model, which consist of a perfusion chamber with two coverslips mounted in series (Figure 1). During perfusion, the plasma perfusate flows over the first coverslip (A), which is coated with tissue factor to initiate fibrin formation. The downstream placed coverslip (B) is coated with an adhesive protein to which fibrin fibers can bind. This binding was analyzed using computer-assisted surface coverage evaluation. When coverslip (B) was coated with albumin, no binding of fibrin fibers was observed. Therefore albumin was used as blocking protein to avoid non-specific binding to the coverslips. Perfusions of plasma over fibrinogen coated coverslips (B) revealed fibrin fiber deposition in flow direction in a gradient with high amounts in the beginning (27 % surface coverage at the entrance of the coverslip) and small amounts downstream on the coverslip (Figure 5). To exclude that this was due to fibrin formation of the immobilized fibrinogen, we tested whether fibrin was formed on immobilized fibrinogen after perfusion of a thrombin solution (up to 1 U/ml). No fibrin fibers were found on the fibrinogen coated coverslip (data not shown). Trace amounts of fibrin were found when vWF was coated on coverslip (B) (1 - 2 % surface coverage; Figure 5). Vitronectin and fibronectin pre-coated coverslips (B) contained an intermediate amount of fibrin fibers (10 and 12 % respectively at the entrance of the coverslip; Figure 5). To control whether the fibrin fibers were indeed related to the first coverslip coated with tissue factor: the

upstream coverslip (A) was coated with fibrinogen and the downstream coverslip (B) was coated with tissue factor. No fibrin fibers were observed on the fibrinogen coated coverslip. The results on albumin, fibrinogen, vitronectin and fibronectin were confirmed immunochemically with D-dimer measurements after digestion of the adhered fibrin with plasmin (albumin: 0.0 ± 0.0, fibrinogen: 1.5 ± 0.9, vitronectin: 0.8 ± 0.4, fibronectin: 0.3 ± 0.2; data expressed in fibrinogen equivalents: ng/mm^2 coverslip ± SEM of three independent experiments each performed at least in duplicate).

Figure 5. Binding of fibrin fibers to several adhesive proteins preadsorbed on a coverslip.

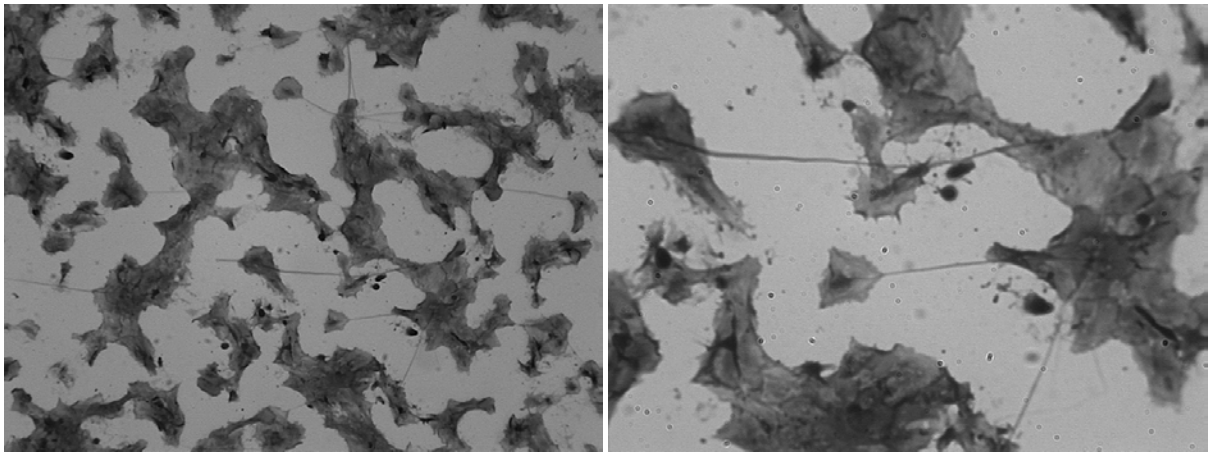


Perfusions were performed using the perfusion chamber depicted in Figure 4 with two coverslips in series. The first coverslip (coverslip A) was coated with tissue factor, to initiate fibrin formation, and the second coverslip (coverslip B) was coated with an adhesive protein. Plasma perfusions at 20 s^{-1} were performed for 10 min at 37 °C. After perfusion fibrin deposition on the adhesive protein (coverslip B) was measured using computer-assisted analysis starting at the borderline between coverslip A and B (=0 mm) towards downstream the coverslip B (increasing mm). Results are the mean of two independent perfusion experiments each performed in duplicate.

Binding of fibrin fibers to platelets pre-adhered to vWF

Platelets can participate in fibrin formation by serving as a pro-coagulant surface for thrombin generation. Here we investigated whether adhered platelets were also able to bind flowing fibrin fibers. Whole blood perfusions were performed over vWF coated coverslips to obtain a surface with a single layer of adhered platelets. Subsequently, this coverslip was placed downstream (B) to a tissue factor coated coverslip (A). Coverslips coated with vWF showed hardly any fibrin (as shown in Figure 5). However, on the single layer of platelets pre-adhered to vWF, fibrin fibers were observed. The fibers were bound to the pre-adhered platelets (Figure 6). The amount of fibrin fibers attached decreased when the distance to the entrance of the coverslip increased. The amount of fibrin fibers observed on pre-adhered platelets was lower than observed for vitronectin in Figure 5.

Figure 6. Binding of fibrin fibers to platelets pre-adhered to pre-coated vWF.



Coverslip B was coated with vWF and first perfused with whole blood to obtain pre-adhered platelets. Subsequently this coverslip was placed in series with a tissue factor coated coverslip (A) and perfused with platelet poor plasma. Original magnification: left panel 400 x; right panel: 1000 x).

Discussion

The shear rate dependence of fibrin formation in flowing blood has been extensively studied in the past. Animal studies showed that fibrin deposition at the subendothelial surface appeared to require venous blood flow, whereas platelet adhesion and aggregation play a dominant role at arterial shear rates.[10] Additionally, in vitro perfusion studies with (non)-anticoagulated blood reported decreased fibrin deposition upon increasing shear rates (50 – 2600 s^{-1}), whereas platelet deposition increased.[11-13] At low shear rates the generation of thrombin is dependent on the presence of platelets (Table 1) as platelets provide a procoagulant surface.[24] This dependence was not observed at higher shear rates, because at higher shear rates more platelets are transported to the surface and thus platelet number is no longer rate limiting.

Until now, studies on shear rate dependence of fibrin formation were performed at fixed-end points. Here we confirmed this shear rate dependence of fibrin deposition when we studied fibrin formation in real-time. Furthermore, we studied the ability of fibrin fibers to bind to ECM proteins under conditions of flow.

Videomicroscopy revealed that locally formed fibrin fibers were attached to the surface, but also detached and transported in the direction of flow. Part of these flowing fibrin fibers adhered downstream to the surface again. Detachment and transport increased upon increasing shear. Thus flow plays a distinct role on the formation and the removal of fibrin fibers. Fibrin fibers were able to bind to the extracellular matrix (ECM) under conditions of flow, without mediation by platelets: when plasma was filtered to remove the residual platelets and platelet-fibrin(ogen) interactions were inhibited with dRGDW, fibrin fibers were still found bound to the matrix. This suggests that proteins present in the ECM support fibrin binding under conditions of flow. From the studied proteins, fibrinogen revealed to be the best binding protein in the extracellular matrix that support binding of the flowing fibrin fibers. It has been shown before that fibronectin[25] and vitronectin[26] are able to bind fibrin. We show that this indeed takes place under conditions of flow. Von Willebrand Factor (vWF) did not

support detectable fibrin deposition under flow conditions. These results are consistent with earlier observations that no binding of vWF could be detected when plasma was perfused over a fibrin surface or when coated fibrinogen was incubated with plasma in ELISA.[27] Platelet pre-adhered to vWF changed fibrin binding, showing that platelets can act as intermediate for binding of fibrin to the vessel wall.

In conclusion, we describe an in vitro real-time model through which we were able to study the dynamics of fibrin formation under flow conditions. We found that flow not only affects fibrin generation, but also fibrin deposition via the transport of fibrin fibers downstream. These flowing fibrin fibers are able to bind to the ECM via binding to fibrinogen, fibronectin, vitronectin or via platelets. Although our results are based on in vitro experiments with anticoagulated plasma/blood, in vivo the presence of circulating fibrin monomers in patients with venous thrombosis or coronary artery disease may be a clinical counterpart of this.[28-31] Furthermore, the presented real-time model could be used to study the dynamic aspects of fibrin degradation via the fibrinolytic system.

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CHAPTER 8

Summary, discussion and future prospects

Introduction

The haemostatic response starts immediately upon damage of the vessel wall in order to prevent blood loss. Exposure of subendothelial proteins to the circulation results in platelet adhesion. After activation the platelets secrete their granule content and form a platelet aggregate, in which fibrinogen participates as an important ligand in bridging the platelets. The platelet plug seals the injury and is subsequently strengthened by the formation of a fibrin network. After the arrest of bleeding, the fibrinolytic system is responsible for the removal of the clot. Thrombosis and a bleeding tendency are the pathological counterparts of a normal haemostasis, manifesting as an obstruction of the circulation by thrombi in veins or arteries and a delayed formation of an haemostatic plug, respectively.

Formation and binding of fibrin to the vessel wall under flow

The composition of a thrombus is among others determined by the local flow conditions. At low shear conditions (< 100 s^{-1}), present in veins, fibrin deposition predominates.[1] Venous thrombosis may be the consequence of an increased coagulation or impaired fibrinolysis. In arteries (> 1600 s^{-1}), thrombi mainly consist of aggregated platelets usually induced by a (ruptured) atherosclerotic plaque.[2-4] Several studies have reported the inverse relation between increasing shear rate and decreasing fibrin formation using either in- or ex-vivo thrombosis models using (non)-anticoagulated blood and fixed end points.[5-8] Flow influences not only the generation of fibrin, but also the transport and deposition of already formed fibrin fibers as observed with real-time videomicroscopy (Chapter 6). These fibrin fibers transported by flow bind downstream to a locally formed fibrin network. This in-vitro observation may implicate that flow transports fibrin fibers, formed at and liberated from the vascular lesion, through the circulation. The presence of circulating fibrin monomers in patients may be a clinical counterpart of this in vitro observation.[9] It has been shown that thrombin bound to fibrin

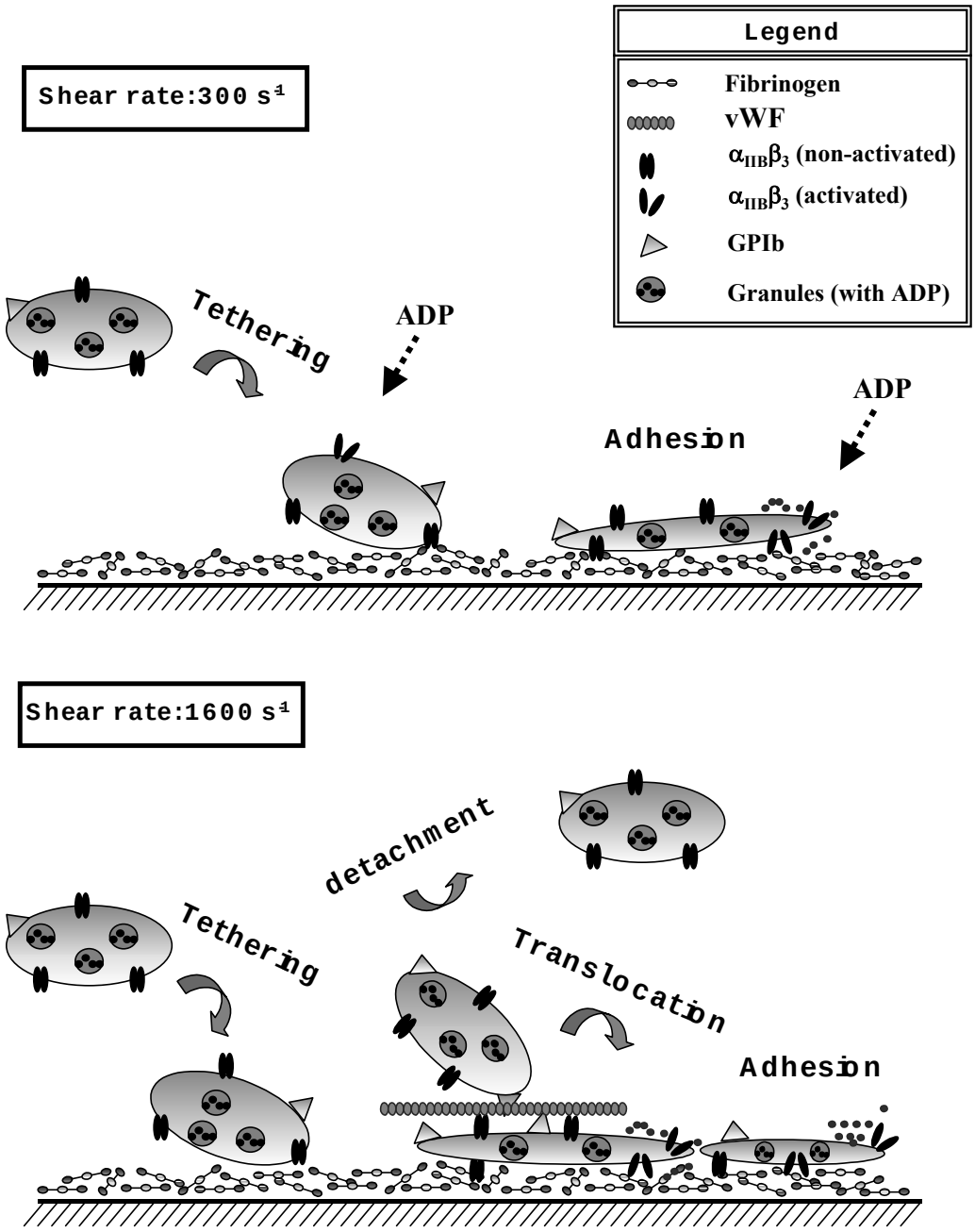
fibers is resistant to inactivation by antithrombin.[10-12] These fibers may thereby act as a thrombogenic risk factor in the circulation.

Pre-adhered platelets are capable to bind these flowing fibers (Chapter 6). In addition, the subendothelium itself also supports binding of the fibrin network. Several proteins (fibrinogen, vitronectin and fibronectin) present in the subendothelium were able to bind fibrin fibers, transported by flow (Chapter 6). The exact nature of this binding was not investigated, but anchorage of fibrin to proteins present in the subendothelium may occur non-covalently or covalently (via transglutaminase-mediated binding of fibrin) (Chapter 1).

Platelet adhesion to fibrinogen under flow

As described in the previous chapters, fibrinogen is one of the proteins important for platelet-platelet interactions. Resting, unstimulated platelets do not interact with fibrinogen in the fluid phase. However, when fibrinogen is immobilized on a surface platelets, adhere and spread on it without the formation of platelet aggregates. Platelet adhesion to fibrinogen has received much attention because it is has been shown that platelet adhesion to biomaterials is caused by fibrinogen adsorption followed by platelet adhesion to this adsorbed fibrinogen.[13] Platelet adhesion to fibrinogen at low shear rates (< 300 s^{-1}) is completely dependent on the platelet integrin $\alpha_{IIb}\beta_3$ and no other platelet receptors have been found involved.[14,15] At low shear rates the receptor $\alpha_{IIb}\beta_3$ promotes immediate arrest onto immobilized fibrinogen without translocation over the surface (Figure 1).[15] Platelet adhesion to fibrinogen at low shear conditions consists of a random distribution of spread and dendritic platelets, whereas at high shear rates platelets adhered in bands, aligned in the direction of flow.[16] Adhesion to fibrin(ogen) at shear rates above 1300 s^{-1} requires the presence of vWF.[17] Endenburg et al. showed that in blood from a patient with severe von Willebrand's disease (vWD) the typical morphology at high shear rates changed into a random distribution of adhered platelets usually observed for shear rates at 300 s^{-1}.[16]

Figure 1. Schematic representation of the mechanisms involved in platelet adhesion to fibrin(ogen) at shear rates of 300 s^{-1} and 1600 s^{-1}.



At 300 s^{-1} platelets are transported from the blood stream and make initial contact with the immobilized fibrinogen, adhere and spread via the platelet receptor $\alpha_{IIb}\beta_3$. At 1600 s^{-1} platelets are transported to the surface, make contact and detach again or translocate via the interaction of GPIb with vWF (from plasma

or released from platelets and bound to the membrane surface via GPIb and/or $\alpha_{IIb}\beta_3$) already adhered platelets and adheres next to the adhered platelet via $\alpha_{IIb}\beta_3$ forming a band of adhered platelet aligned in flow direction. ADP pre-activates platelets either before adhesion and/or after adhesion resulting in activation and stabilization.

Furthermore, they reported that GPIb – vWF interaction is necessary for the interaction of platelets with fibrinogen at high shear rates (1600 s^{-1}) (Figure 1). The exact mechanism was not resolved, but unpublished real-time observations of platelet adhesion to fibrinogen at these shear rates show translocation of new platelets over already adhered platelets. This might point to an interaction of GPIb with vWF bound to the surface of an already adhered platelet. The resulting slow down of the platelet allows a firm attachment to fibrinogen via $\alpha_{IIb}\beta_3$ (Figure 1).

Platelet-fibrinogen interaction is dependent on the carboxy-terminal segment of the γ-chain under low as well as high shear conditions. Perfusions over the recombinant fibrinogen in which γ408-411 was deleted resulted in a complete absence of platelet adhesion at 300 s^{-1} as well as 1600 s^{-1} (Chapter 5). This is further supported by studies on impaired platelet aggregation in suspension using genetic variants of fibrinogen with either an extension or truncation of the γ-chain[18,19] and perfusion studies in which a synthetic peptide covering the C-terminal sequence of the γ-chain inhibited platelet adhesion.[20] Interestingly, platelet adhesion to fibrinogen is completely inhibited in the presence of peptides containing the Arg-Gly-Asp (RGD) sequence.[20] This suggests an involvement of the two RGD sequences in the fibrinogen α-chain. However, experiments with antibodies and genetically engineered fibrinogen lacking the RGD sequences in the α-chain have demonstrated that the α-chain RGD sequences are not required for $\alpha_{IIb}\beta_3$ binding to soluble and surface bound fibrinogen or for platelet aggregation.[21-25] Until now, no flow experiments have been performed with recombinant fibrinogen lacking either one or two of the RGD sequences. This in contrast to other RGD containing proteins that can interact with $\alpha_{IIb}\beta_3$; studies performed with these

proteins showed that platelet adhesion to recombinant fibronectin[26] or to recombinant vWF[27] required the RGD sites for optimal adhesion and platelet spreading under flow conditions.

The absolute requirement of the sequence γ400-411, does not exclude that other domains of fibrinogen may also participate in the interaction with platelets, assuming that the C-terminal γ-chain sequence is necessary but not sufficient to mediate irreversible platelet attachment. In fact, several studies suggested that other domains should be involved in platelet-fibrinogen interactions.[28,29] Chapter 5 reports the involvement of the region γ316-322 in platelet adhesion to fibrinogen under flow conditions. Platelet adhesion was reduced to patient dysfibrinogen γΔ319-320, recombinant fibrinogens: γΔ319-320, γ318Asp→Ala and γ320Asp→Ala. Furthermore combined antibodies against the sequences γ308-322 and γ316-333, also inhibited platelet adhesion. We conclude that the overlapping region γ316-322 contains amino acids which are responsible for platelet adhesion to fibrinogen, especially under conditions of flow. Exposure of γ316-322 could co-operate with the primary platelet adhesion site γ400-411 to cause high-affinity interactions with platelets. Real-time observations show that the reduced adhesion to the dysfibrinogens is due to increased rolling and detachment of platelets. Increasing shear rates show increased inhibition of platelet adhesion to these dysfibrinogens. From this we conclude that the region γ400-411 enables the interaction of fibrinogen with $\alpha_{IIb}\beta_3$ and subsequently γ316-322 becomes important to further stabilize the adhered platelet when stronger forces such as shear are exposed to the platelets. The segment γ316-322 is part of a fibrin-specific epitope γ312-324, which is buried inside the fibrinogen molecule in soluble fibrinogen and becomes exposed after fibrin formation.[30] In Chapter 5 we show that this sequence also becomes exposed when fibrinogen is immobilized on a surface. Since platelet adhesion to fibrinogen is completely $\alpha_{IIb}\beta_3$ dependent, we assume that $\alpha_{IIb}\beta_3$ is the receptor for this sequence.

Role of ADP in platelet adhesion to fibrinogen under flow

Platelet aggregation is mediated by soluble fibrinogen and requires the activation of platelets; resting (non-activated) platelets do not interact with fibrinogen.[31] In contrast to platelet aggregation in suspension, it has been suggested that platelet adhesion to surface immobilized fibrinogen does not require pre-activation of platelets. Resting platelets in whole blood evaluated at shear rates up to 1600 s^{-1} have been reported to adhere to immobilized fibrinogen.[15,18] These studies were based on observations that platelets treated with PGE_1 still adhered to immobilized fibrinogen.[32] However, Hantgan et al. reported a reduced platelet adhesion to fibrin(ogen) in the presence of prostacyclin.[17] In Chapter 4 we describe that platelet adhesion to fibrinogen is dependent on ADP. Perfusions of blood from a patient deficient for the ADP receptor $P2Y_{12}$ showed reduced platelet adhesion to immobilized fibrinogen. Similar results were obtained with control blood in which the $P2Y_{12}$ receptor was antagonized, although this effect normalized upon increasing perfusion time. Inhibition of the $P2Y_1$ receptor resulted also in a reduced platelet adhesion, and when both receptors were inhibited platelet adhesion was strongly reduced. Addition of the ADP scavenging system inhibited platelet adhesion almost completely. These results suggest that platelet adhesion to surface bound fibrinogen involves (pre-)activated platelets (Figure 1). Unpublished real-time observations in which the $P2Y_{12}$ receptor was antagonized, showed an increased detachment of platelets, which suggest that ADP might particularly be important in the stabilization of the adhered platelet on the surface (Figure 1). Recently, Bonnefoy et al. reported a study on co-aggregation of non-activated or ADP-activated platelets with fibrinogen-coated beads in flowing suspensions, in which they suggested that platelets need to be pre-activated to adhere to immobilized fibrinogen.[33,34] Under shear conditions using whole blood, ADP could be secreted from red blood cells or from pre-activated platelets.

Fibrinogen versus fibrin

Platelet adhesion to fibrin is in many respects similar to platelet adhesion to fibrinogen. Adhesion occurs linearly with time, but the adhesion rate is faster than to fibrinogen and lower concentrations of fibrin are required to obtain optimal adhesion.[20] Furthermore larger concentrations of RGD peptides and dodecapeptide are required to inhibit platelet adhesion to fibrin.[20] This indicates that platelet binding epitopes (for example the fibrin-specific region, Chapter 5) are better accessible in fibrin compared with fibrinogen or that the density of epitopes on the surface is larger.

In vitro, aggregated platelets retract due to the presence of polymerized fibrin. Clot retraction differentiates between fibrinogen and fibrin, although the difference may be due to structural differences between fibrin and fibrinogen and not by different interaction sites with platelets. This platelet integrin $\alpha_{IIb}\beta_3$-dependent process is not affected by either a deletion of the last four amino acids of the γ-chain or by mutations in the RGD sites of the α-chain.[22,25,35] These observations indicate that additional binding sites on fibrin(ogen) for $\alpha_{IIb}\beta_3$ are present and critical for clot retraction. The γ-chain segment: 316-322, part of the fibrin-specific region, described in the present thesis (Chapter 5) may be a possible candidate for this interaction. In fact, preliminary results show that the antibodies raised against the sequences γ308-322 and γ316-333 inhibited platelet-mediated clot retraction.[36]

Effects of fibrinolysis on adhesion to fibrin under flow

The absolute requirement for the carboxy-terminal part of the γ-chain in platelet adhesion to pre-formed fibrin was also observed during fibrinolysis. Limited fibrinolytic conditions resulting in the removal of the dodecapeptide, as demonstrated with a specific monoclonal antibody, caused decreased platelet adhesion (Chapter 7). In vivo, this means that once fibrinolysis is started, platelet adhesion to a platelet-fibrin clot is impaired at an early stage.

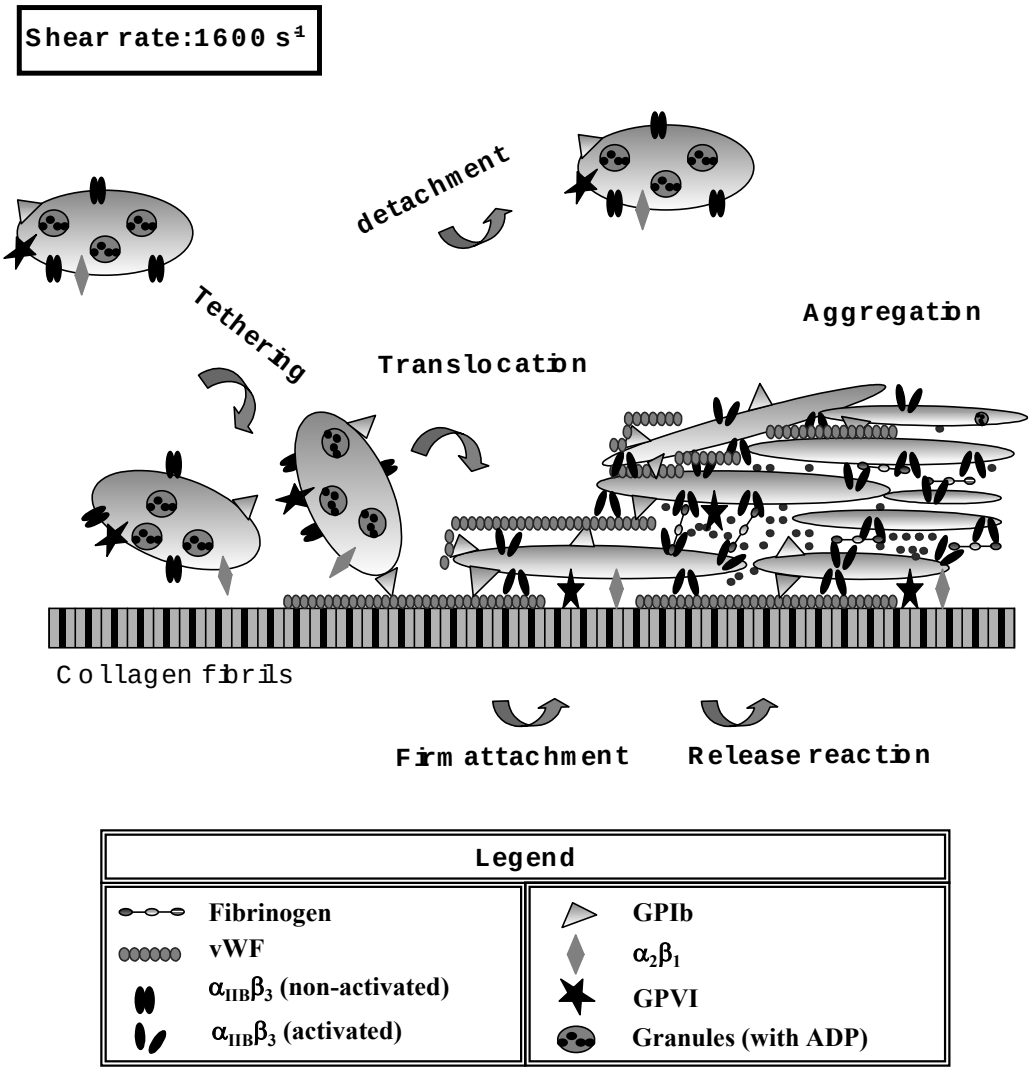
Fibrinolysis inhibits thereby the propagation of the clot and avoids possible pathological complications. A platelet-fibrin clot is an adhesive surface for PMN's under conditions of flow.[37] In Chapter 7 the adhesion of PMN's was studied under flow conditions to a pre-formed fibrin network, which had been subjected to fibrinolysis. At fibrinolytic conditions in which platelet adhesion was reduced, PMN adhesion was still preserved. Based on this in-vitro model, we hypothesize that under physiological conditions fibrinolysis reduces the thrombogenicity of the platelet-fibrin clot, whereas the inflammatory response is preserved and is able to participate in the long term removal and restructuration of the plug (Chapter 7).

Platelet thrombus formation under flow

At arterial shear rates, a rupture of the vessel wall will result in the formation of a thrombus consisting mainly of aggregated platelets. Collagen, a major determinant of the subendothelium, is one of the most important adhesive proteins responsible for primary adhesion to the vascular lesion. Figure 2 shows the multi-step process of platelet aggregation in flowing blood at high shear conditions (1600 s^{-1}). At arterial flow, binding of plasma vWF to collagen and subsequent GPIb binding to immobilized vWF is required for initial platelet-surface contact. This transient interaction causes translocation (rolling) of the platelet over the surface resulting in loss of velocity of the platelets.[15] After this initial adhesion step, different receptor-ligand interactions synergistically promote stable platelet adhesion.[38] Platelet integrin $\alpha_2\beta_1$ is involved in the subsequent firm attachment of the platelet through its interaction with collagen[39,40] and the collagen receptor GPVI leads to platelet activation, subsequent spreading and activation of $\alpha_{IIb}\beta_3$.[41-44] A recent publication reported that glycoprotein VI and not $\alpha_2\beta_1$ is essential for platelet interaction with collagen.[45] These findings with β_1-null platelets are in contrast to 1) observations in patients who suffered from bleeding and whose platelets showed low or no expression of $\alpha_2\beta_1$ and failed to respond to collagen[39,40]; 2) platelet adhesion studies using human blood under flow conditions, showing

an involvement of $\alpha_2\beta_1$[46-48]; 3) the finding that platelets with increased density of $\alpha_2\beta_1$ on the platelet membrane adhere significantly better to collagen.[47]

Figure 2. Schematic representation of the mechanisms involved in thrombus formation on collagen at arterial shear rates of 1600 s^{-1}.



At high shear rates only GPIb interaction with immobilized vWF multimers can initate the tethering of circulating platelets to collagen in the vessel wall and to already adhered platelets. This GPIb dependent interaction is a transient interaction, resulting in the translocation of the platelet over the surface (vWF

immobilized to the collagen and/or immobilized to an already adhered platelet). Binding of the platelet to the collagen via the collagen receptors ($\alpha_2\beta_1$ and GPVI) results in firm attachment and activation, which results in the exposure of activated $\alpha_{IIb}\beta_3$. Fibrinogen (and vWF) serves as stabilizing bridging ligand for $\alpha_{IIb}\beta_3$ connecting platelets together, and thereby the formation of a platelet aggregate. Aggregation is reinforced by the release of the granule content upon activation. ADP an important constituent of the dense granules reinforces platelet aggregation and thrombus growth through its binding to ADP receptors ($P2Y_{12}$ and $P2Y_1$) resulting in the full activation of $\alpha_{IIb}\beta_3$.

Thrombus formation or platelet aggregation starts from the first layer of adhered and activated platelets and is mediated via several ligands, such as fibrinogen, vWF, fibronectin or vitronectin. The concentration of these ligands at the platelet thrombus surface might increase locally due to the release from the α-granules, upon activation. The first studies on the role of vWF binding to $\alpha_{IIb}\beta_3$ of pre-activated platelets showed strong competition by fibrinogen.[49] Aggregation studies performed in an aggregometer are completely dependent on fibrinogen.[50] Mural thrombus formation under physiological flow conditions differs from these aggregation studies, because vWF has shown to be a major ligand for $\alpha_{IIb}\beta_3$.[51-53] Perfusion studies with vWD blood to which recombinant vWF (RGGS-vWF) was added (which cannot interact with $\alpha_{IIb}\beta_3$) showed increased thrombus formation compared to vWD blood to which non-mutated recombinant vWF (with normal interaction with GPIb and $\alpha_{IIb}\beta_3$) was added.[54] Based on these results, a model was proposed in which platelet-platelet interaction is more efficient when either vWF or fibrinogen is present alone, whereas the presence of both ligands yield an incomplete bridge formation.[54] In Chapter 2 we tested this hypothesis by examining the role of fibrinogen in platelet thrombus formation. For this study we used blood from a patient diagnozed with congenital afibrinogenemia. The molecular basis underlying the absence of fibrinogen in this patient is characterized in Chapter 3. An homozygous 2 base pair deletion in exon 4 of the fibrinogen alpha chain was found. This deletion results in a frameshift which leads to a premature stop codon. Perfusion studies with blood of the patient or reconstituted

blood in which fibrinogen was absent showed increased thrombus formation (surface coverage, thrombus volume). This observation appeared at first sight to support the hypothesis of incomplete bridge formation due to mutual competition when both vWF and fibrinogen are present as ligands. However, closer inspection of the morphology and platelet number, revealed that increased thrombus volume was due to a looser packing of the platelet thrombus, and not due to increased platelet deposition. In the presence of fibrinogen, platelets form lamellipodia and spread out on top of one another like "shingles on a roof". In the absence of fibrinogen, however, thrombi consisted mainly of dendritic platelets contacting one another through filopodia (Chapter 2). The loosely packing of the platelet aggregate in the absence of fibrinogen might implicate thrombus instability, although we did not observe thrombus instability at shear rates up to 2600 s^{-1}. Tsuji et al. reported an increased instability at shear rates of 4500 s^{-1} with afibrinogenemic blood[55], while Ruggeri et al. reported thrombus instability with reconstituted blood at shear rates of 1500 s^{-1}.[56] A study using intra-vital microscopy in fibrinogen deficient mice reported formation of abundant thrombi at the vascular lesion, but these thrombi were unstable and embolized, causing downstream occlusions.[57] As discussed in Chapter 2, this embolization might be a plausible explanation for thrombotic complications sometimes observed in afibrinogenemic patients. Mice deficient in both vWF and fibrinogen still formed thrombi succesfully, but these thrombi were highly unstable. Platelets of these mice specifically accumulated fibronectin in their α-granules, suggesting a possible mechanism for the participation of fibronectin in supporting platelet aggregation.[57]

Our results obtained using anticoagulated blood precludes thrombin generation, indicating that fibrinogen itself (and not fibrin) is sufficient to obtain a densely packed thrombus. The loose packing of the aggregates in the absence of fibrinogen suggests that either the many bridges that can form when fibrinogen is present, can act as a zipper between platelets, or activation of $\alpha_{IIb}\beta_3$ by occupancy of fibrinogen may lead to the formation of lamellipodia, platelet spreading and granule secretion.[58] We have measured the extent of secretion by measuring the presence of ^{14}C-labeled serotonin in platelet thrombi after perfusion of

reconstituted blood in the presence or absence of fibrinogen using platelets with ^{14}C-labeled serotonin. We found increased levels of serotonin in thrombi formed in the absence of fibrinogen, suggesting less secretion in the absence of fibrinogen (unpublished results). This indicates that absence of fibrinogen could result in a decreased outside-in signalling via $\alpha_{IIb}\beta_3$, and a reduced activation and granule secretion, leading to a reduced amplification of aggregation. In agreement with this hypothesis are morphologic studies on thrombi formed in the absence of ADP (secreted during the release of granule content and responsible for the amplification of aggregation) showing similar loose packing of platelet thrombi as observed in the absence of fibrinogen.

The importance of the release of ADP in the activation of newly arrived platelets in flowing blood has been shown in Chapter 4. In vivo, the importance of the release reaction is illustrated by the platelet functional defects in patients with storage pool deficiency.[59] Secretion of dense granules results in the release of ADP. ADP is an important agonist that induces platelet aggregation through a positive feedback via its binding to the ADP receptors, $P2Y_{12}$ and $P2Y_{1,}$ resulting in the activation of $\alpha_{IIb}\beta_3$.[60] Perfusion experiments described in Chapter 4 show that the presence of the ADP scavenging system results in complete inhibition of thrombus formation in flowing blood. The contribution of $P2Y_{12}$ in platelet thrombus formation was investigated with blood from a patient, deficient for the $P2Y_{12}$ receptor.[61,62] Patient blood or control blood in which $P2Y_{12}$ was antagonized showed impaired thrombus buildup. Thrombi were smaller and loosely packed consisting of spread platelets on top of which were single non-spread platelets. Our data suggests that interaction of ADP with $P2Y_{12}$ is responsible for the full activation of $\alpha_{IIb}\beta_3$ consistently with previous observations with subjects who had received the $P2Y_{12}$ antagonist: clopidogrel (Plavix®).[63-65] One of the interesting aspects of $P2Y_{12}$ as a potential antithrombotic target is that thrombus growth is inhibited, while primary adhesion to collagen is not affected.

Inhibition of the $P2Y_1$ receptor alone also affected thrombus formation, although these thrombi were still densely packed. This densely packing is explained by the remaining interaction of ADP with $P2Y_{12}$, responsible for the “normal” thrombus buildup. Consistent are

platelet aggregations studies in suspension $P2Y_1$-null mice showing a restoration of the initial impaired aggregation upon increasing collagen concentrations.[66,67] When both ADP receptors were antagonized in the patient or control, thrombus formation was further inhibited and consisted of loosely-bound non-activated platelets. These results show that both ADP receptors are required in platelet thrombus formation under conditions of flow. Recently, confirming results were reported using blood from normal donors in the presence of ADP receptor antagonists.[68]

Future prospects

The studies described in the present thesis contribute to a further knowledge of the mechanisms involved in the interaction of blood platelets with fibrinogen and fibrin under physiological flow conditions. Presence of fibrinogen resulted in the formation of a densely packed platelet thrombus resistant to shear forces, whereas absence of fibrinogen resulted in a loosely packed platelet aggregate. Similar morphology in thrombus packing was observed when the ADP receptor $P2Y_{12}$ was antagonized (in the presence of fibrinogen). As discussed, these observations may be related and future experiments have to be focused on the elucidation of the precise activation mechanisms involved. In this respect, the first layer of platelets adhered to collagen will be activated via tyrosine kinase-mediated pathway, and activation of the platelets in the following layers of the growing thrombus may then be dependent on G-proteins and outside-in signaling via $\alpha_{IIb}\beta_3$. In addition, it would be worthwhile to study the contribution of other possible ligands such as fibronectin, vitronectin and thrombospondin in platelet thrombus formation.

The important contribution of ADP in the formation of a platelet aggregate has been studied in Chapter 4, however the other platelet amplifying activation mechanism via TxA_2 was not investigated. The role of TxA_2 in platelet thrombus formation has been studied in the past and showed to be relevant only at very high shear rates.[69,70] Recently, it has been shown that the combination of aspirin and clopidogrel is beneficial for patients in reducing cardiovascular

events.[71] Therefore, it is interesting to study the contributions of both pathways in platelet activation and aggregate packing under conditions of flow. Thrombus packing and the effects of shear on stability and embolization can be studied efficiently using intra-vital microscopy in (knock-out) mice. Notwithstanding disadvantages like species-specific differences, intra-vital microscopy in mice precludes the use of anticoagulation. Although perfusions over tissue factor rich extracellular matrices with anticoagulated blood show local fibrin formation, the use of anticoagulant will influence the results.

In Chapter 7 an experimental perfusion model was developed to form a fibrin network under flow and to study this in real-time. In vivo a fibrin network is also formed under conditions of flow, but also in the presence of factor XIII that can cross-link the fibrin fibrils and other adhesive proteins that are often incorporated in the clot. The role of these proteins should be studied to extend the study described in Chapter 6, in which pre-formed fibrin surfaces were used, in order to understand the influence of fibrinolysis on the adhesion of platelets and PMN's to fibrin networks.

Furthermore, the interaction of platelets with fibrin during clot retraction is a process, which remains to be elucidated. The fibrin-specific region γ316-322 may participate in this unresolved process (Chapter 5).

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CHAPTER 9 | Nederlandse samenvatting

Inleiding

In een volwassen mens circuleert 5 à 6 liter bloed door de bloedvaten. Het bloed transporteert onder andere bloedcellen (rode bloedcellen, witte bloedcellen en bloedplaatjes), zuurstof, voedingsstoffen, afvalstoffen en hormonen. Deze stoffen dragen bij aan het functioneren van de organen. Het is van levensbelang dat de bloedcirculatie niet verstoord wordt. Het lichaam bezit daarvoor een systeem dat strikt gereguleerd is om enerzijds bloedverlies na beschadiging van de vaatwand tegen te gaan en anderzijds om verstopping van bloedvaten te voorkomen, een proces trombose genaamd. Dit nauw gereguleerde proces wordt ook wel haemostase genoemd. Bloedplaatjes (of trombocyten) spelen een belangrijke rol in de haemostase. Ze circuleren door de bloedvaten met zo'n 300 miljard bloedplaatjes per liter bloed. Wanneer een vaatwand beschadigd is, komen de bloedplaatjes in contact met bestanddelen van onderliggende lagen in het weefsel (bijvoorbeeld collageen). De bloedplaatjes hechten (adhereren) aan deze bestanddelen en worden hierdoor geactiveerd. Deze activatie van bloedplaatjes zorgt ervoor dat bloedplaatjes "samenklonteren" (aggregeren). Voor deze aggregatie dient het eiwit fibrinogeen als een brug tussen bloedplaatjes. De geaggregeerde bloedplaatjes vormen zodanig een bloedplaatjesprop (plug) die de vaatwandschade afdicht. Dit is de eerste fase van de bloedstelping. Wanneer een bloedplaatjesprop een bloedvat verstopt, wordt er gesproken over een trombus. Tegelijkertijd aan de vorming van een bloedplaatjesplug wordt de bloedstolling geactiveerd. Het eindprodukt van de bloedstollingscascade is het eiwit fibrine. Het polymeer fibrine is een netwerk van aaneengeschakelde fibrinogeen bouwstenen. Dit fibrinenetwerk is verantwoordelijk is voor een stevige afsluiting van de wond. Het fysiologische mechanisme dat verantwoordelijk is voor het opruimen van het fibrinestolsel na het herstellen van de vaatwand, wordt fibrinolyse genoemd.

Doel van het onderzoek

Inzicht in deze processen van haemostase en trombose is van belang voor de ontwikkeling van geneesmiddelen die vaatziekten bestrijden. In dit proefschrift zijn de interacties van bloedplaatjes met fibrine en fibrinogeen bestudeerd tijdens de vorming en afbraak van het bloedplaatjes-fibrinestolsel. In het lichaam vindt de vorming van een stolsel plaats in stromend bloed. Studies naar de hechting van bloedplaatjes aan een beschadigde vaatwand hebben laten zien dat dit proces, sterk afhankelijk is van de stromingscondities van het bloed. Daarom zijn de studies, beschreven in dit proefschrift, uitgevoerd onder stromingscondities met behulp van het perfusiemodel.

Onderzoeksmethode

Het perfusiemodel is een systeem waarmee de stroming van bloed over verschillende oppervlakken bij verschillende snelheden, representief voor slagaders (arteriën) en aders (venen), bestudeerd kan worden. De ontwikkeling van dit perfusiemodel heeft in grote mate bijgedragen aan de kennis van de factoren die een rol spelen in de adhesie van bloedplaatjes aan de vaatwand. Het perfusiemodel bestaat uit een perfusiekamer. Deze perfusiekamer is te vergelijken met een plastic bloedvat (Hoofdstuk 1, Figuur 3). Hierin worden dekglaasjes aangebracht die bedekt zijn met een eiwit (bijvoorbeeld collageen, fibrinogeen, fibrine, etc.), waaraan de interactie van bloedplaatjes bestudeerd kan worden. Met behulp van een pomp stroomt bloed over het dekglaasje. Na afloop van de perfusie worden de bloedplaatjes, die geadhereerd zijn aan het eiwit op het dekglaasje, gekleurd en met behulp van een microscoop geanalyseerd. Om het dynamische proces van bloedplaatjesadhesie niet alleen na afloop maar ook tijdens de perfusie te visualiseren, is een perfusiekamer ontwikkeld waarmee het mogelijk is om de interacties van het bloedplaatje continue (real-time) te bestuderen (zie Hoofdstuk 2). In Hoofdstuk 7 is tevens een model beschreven om de vorming van fibrine in real-time te bestuderen.

Resultaten

Fibrinogeen in de opbouw van een trombus

De rol van fibrinogeen in de opbouw en pakking van een bloedplaatjestrombus onder zowel veneuze als arteriële stromingscondities is onderzocht in Hoofdstuk 2. Hiervoor is onder andere gebruik gemaakt van bloed van een patiënt met congenitale afibrinogenemie. Dit is een uiterst zeldzame erfelijke aandoening gekarakteriseerd door volledige afwezigheid van fibrinogeen in het bloed. Patiënten met congenitale afibrinogenemie hebben een bloedingsneiging. Het moleculaire defect op DNA niveau is beschreven in Hoofdstuk 3. De resultaten van het onderzoek laten zien dat afwezigheid van fibrinogeen leidt tot grote, losgepakte (losgestapelde) bloedplaatjesaggregaten, terwijl de aanwezigheid van fibrinogeen leidt tot kleine, dichtgepakte trombi. Fibrinogeen speelt dus blijkbaar een belangrijke rol in de pakking van een trombus en is daarmee essentieel voor de stabiliteit van de trombus onder fysiologische stromingscondities.

ADP in bloedplaatjesadhesie en trombus vorming

Wanneer bloedplaatjes geactiveerd worden, komt onder andere adenosinedifosfaat (ADP) vrij. ADP kan een bloedplaatje (verder) activeren. Deze activatie is nodig opdat bloedplaatjes met elkaar kunnen aggregeren. Hoofdstuk 4 beschrijft de rol van ADP in de interactie van bloedplaatjes met fibrinogeen. Bloedplaatjes adhereren aan fibrinogeen. Fibrinogeen zelf is echter niet in staat om bloedplaatjes zodanig te activaren dat ze aggregeren, zoals bijvoorbeeld het geval is op collageen. In Hoofdstuk 4 wordt met behulp van remmers van de twee belangrijke ADP receptoren op het bloedplaatje ($P2Y_1$ en $P2Y_{12}$) bestudeerd in hoeverre de interactie van bloedplaatjes met fibrinogeen ADP afhankelijk is onder fysiologische stromingscondities. De resultaten laten zien dat ADP zeer belangrijk is voor de adhesie aan fibrinogeen.

De bijdrage van ADP in de opbouw van een trombus op collageen is tevens bestudeerd onder stromingscondities. Hiervoor is gebruik gemaakt van bloed van een patiënt met een zeer

zeldzaam voorkomende erfelijke $P2Y_{12}$ deficiëntie. Uit de resultaten blijkt het belang van ADP in de trombus opbouw onder stromingscondities. Met name $P2Y_{12}$ is van belang is voor de opbouw van een trombus. Afwezigheid van deze receptor vertoonde dezelfde losgepakte trombi vergelijkbaar met de afwezigheid van fibrinogeen (Hoofdstuk 2). $P2Y_{12}$ is verantwoordelijk voor de activatie van de receptor voor fibrinogeen op bloedplaatjes en is daarom een belangrijke doel voor de ontwikkeling van geneesmiddelen tegen trombose.

Adhesie van bloedplaatjes aan fibrinogeen

De interactie tussen een bloedplaatje en fibrinogeen is mogelijk doordat de fibrinogeen receptor op het bloedplaatje bindt aan een specifiek gebied op het fibrinogeen molecuul. Het specifieke gebied dat hiervoor verantwoordelijk is, wordt het dodecapeptide (γ400-411) genoemd. Een fibrinogeen molecuul heeft twee van deze dodecapeptiden en is zodanig in staat om als een brug te dienen tussen twee bloedplaatjes. De adhesie van bloedplaatjes aan fibrinogeen onder stromingscondities wordt hoofdzakelijk gemedieerd door het dodecapeptide (Hoofdstuk 5). Echter, in Hoofdstuk 5 is de rol van een ander domein (γ316-322) op het fibrinogeen molecuul onderzocht in bloedplaatjesadhesie aan fibrinogeen. Dit domein wordt ook wel het fibrine specifieke domein genoemd, omdat het pas geëxposeerd wordt na polymerisatie tot fibrine. De resultaten laten zien dat het fibrine specifieke domein ook een rol speelt in de bloedplaatjes-fibrinogeen interactie met name onder stromingscondities.

Invloed van fibrinolyse op bloedplaatjesadhesie

Hoofdstuk 6 beschrijft het effect van de fibrinolyse op de adhesie van bloedplaatjes aan fibrine. Uit deze studie blijkt dat al reeds in een vroeg stadium van fibrinolyse het dodecapeptide van fibrine afgeknipt wordt. Dit duidt op een (extra) anti-trombotische werking van de fibrinolyse, want tijdens fibrinolyse kunnen dus nieuw gearriveerde bloedplaatjes niet adhereren. In tegenstelling tot bloedplaatjes konden neutrofielen (witte bloedcellen), zelfs na intensieve fibrinolyse, nog gewoon adhereren onder stromingscondities. Dit zou in vivo van

betekenis kunnen zijn met betrekking tot de rol van neutrofielen in het opruimen (fagocyteren) van de stolselrestanten.

Fibrine vorming onder stromingscondities

In Hoofdstuk 7 is de invloed van flow op de vorming en depositie van fibrine onderzocht. Met behulp van een real-time model wordt aangetoond dat flow een belangrijke invloed heeft op het vervoer van fibrinedraden. De binding van deze door flow getransporteerde fibrinedraden aan vaatwandeiwitten is eveneens bestudeerd. Fibrinogeen en vitronectine bleken fibrinedraden te kunnen binden onder stromingscondities. Geadhereerde bloedplaatjes waren ook in staat om fibrinedraden in flow te binden.

Conclusies

Het onderzoek beschreven in dit proefschrift draagt bij aan de kennis van de mechanismen die betrokken zijn bij de interacties van bloedplaatjes met fibrine en fibrinogeen onder fysiologische stromingscondities. Fibrinogeen is verantwoordelijk voor de pakking van een trombus. Afwezigheid van fibrinogeen resulteerde in een losgepakt bloedplaatjesaggregaat. Een vergelijkend patroon (morfologie) in de pakking van een trombus is gevonden wanneer de ADP receptor $P2Y_{12}$ afwezig was. In Hoofdstuk 8 zijn de bevindingen in een model geplaatst en zijn aanbevelingen voor vervolgonderzoek gegeven. De resultaten uit dit proefschrift dragen bij aan een beter inzicht in de ontwikkeling van een in stromend bloed gevormde trombus, hetgeen van groot belang is voor de ontwikkeling van geneesmiddelen ter bestrijding van vaatziekten.

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Curriculum vitae

J.A. (Jasper) Remijn was born on May 8, 1974 in Veenendaal (the Netherlands). He finished secondary school (VWO, Van Lodensteijncollege) in 1992. In the same year, he started his study Chemistry at the Utrecht University. In 1997, he obtained a masters degree in Chemistry. From November 1997 until May 1998, he was employed as a software engineer at Baan Development BV. From May 1998 until May 2002, he joined the Department of Haematology as a research fellow (PhD student) at the Faculty of Medicine from the University Medical Center Utrecht. During this period, he performed research for the present thesis under supervision of Prof. Dr. Ph.G. de Groot and Prof. Dr. J.J. Sixma.