Platelet procoagulant activity and thrombus formation

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Platelet procoagulant activity and thrombus formation

PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit Maastricht,

op gezag van de Rector Magnificus,

Prof. Mr. G.P.M.F. Mols,

volgens het besluit van het College van Decanen,

in het openbaar te verdedigen

op vrijdag 10 december 2004 om 12.00 uur

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Contents		Page
Chapter I	General Introduction	7
Chapter II	Integrin $\alpha_{IIb}\beta_3$ and shear-dependent action of glycoprotein Ib α stimulate platelet-dependent thrombin formation in stirred plasma. <i>J Lab Clin Med. 2003;141:350-8</i>	15
Chapter III	Von Willebrand factor C1C2 domain is involved in platelet adhesion to polymerized fibrin at high shear rate. <i>Blood. 2004;103:1741-6</i>	25
Chapter IV	Synergistic effect of thrombin on collagen-induced platelet procoagulant activity is mediated through PAR-1. <i>Submitted for publication</i>	33
Chapter V	Fibrinogen adsorption, platelet adhesion and thrombin generation at heparinized surfaces exposed to flowing blood. <i>Thromb Haemost. 2002;87:742-7</i>	45
Chapter VI	Thrombogenicity of polysaccharide-coated surfaces. <i>Biomaterials. 2003;24:1917-24</i>	53
Chapter VII	Covalently-bound heparin makes collagen thromboresistant. <i>Arterioscler Thromb Vasc Biol. 2004;24:613-7</i>	63
Chapter VIII	General Discussion	69
Nederlandse Samenvatting		75
Nawoord		79
Curriculum vitae		81
Publications		83

GENERAL INTRODUCTION

Preface

Arterial thrombosis is an acute complication that develops on chronic atherosclerotic lesions and causes heart attacks and stroke, at present the main causes of mortality in developed countries. The key components of thrombi that occlude arteries are platelets and fibrin.

Upon vascular injury platelets adhere to the damaged vascular wall and generate a procoagulant surface to promote thrombin production and consequently fibrin formation. Progress in understanding the mechanisms that cause platelet procoagulant activity might improve the ability to prevent occlusive thrombosis in diseased vessels. This chapter reviews current understandings and debates with respect to properties and generation of platelet procoagulant activity.

Thrombus formation

The core trigger of thrombus formation after vascular damage is the loss of the endothelial cell barrier between blood and extracellular matrix proteins. In response to this event a primary platelet plug is formed via platelet adhesion and aggregation processes. Under conditions of high shear stress, as found in arteries, the initial tethering of platelets to the subendothelium is dependent on the binding of glycoprotein (GP) Ib α to subendothelial bound von Willebrand factor (VWF).¹ This interaction is characterized by a rapid dissociation rate, resulting in platelet translocation. The deceleration in platelet velocity allows adhesive contacts through platelet receptors with slower intrinsic binding kinetics.²⁻⁴ Integrins are the major class of platelet receptors, mediating stable adhesion.⁵ Integrins are heterodimeric proteins consisting of α and β subunits and are usually present in a resting conformation, with low affinity for the natural ligand.⁶ A state of high affinity occurs after activation. A well defined example of this phenomenon is platelet adhesion to subendothelial collagen. After initial tethering of platelets via the GP Ib α -VWF axis, collagen can interact directly and indirectly with activated platelet integrin $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ (via VWF), respectively. Collagen first activates these integrins via GP VI, which is a member of the immunoglobulin (Ig) receptor superfamily

due to its Ig homology unit.⁷ GP VI signalling triggers platelets to extrude their storage granule content to the platelet's environment.⁸ The secondary agonist ADP, released from α -granules, further supports integrin activation via the G-protein coupled P2Y₁ and P2Y₁₂ receptors.⁹ A further mechanism important for the amplification of the GP VI response is the synthesis and release of thromboxane A₂ (TXA₂) via the cyclooxygenase-mediated arachidonic metabolic pathway.¹⁰ TXA₂ exerts its actions via heterotrimeric G protein-coupled thromboxane-prostanoid receptors (TP receptors).¹¹ In addition, activation of the integrin $\alpha_{IIb}\beta_3$ can be mediated by GP Ib α -VWF signaling or localized thrombin.¹²

Once adhered, adhesive proteins (VWF, fibrinogen, fibronectin), present in plasma or released from storage granules by activated platelets, bind to activated $\alpha_{IIb}\beta_3$. Like primary adhesion under arterial shear stress conditions, VWF is necessary for initial tethering of platelets. Stable platelet-platelet bridging is then mediated via activated integrin $\alpha_{IIb}\beta_3$ and the adhesive proteins.

The previously described processes of platelet adhesion and aggregation are not sufficient for stable thrombus formation. To obtain a stable thrombus the end product of blood coagulation, fibrin, is required. Upon vascular injury, blood coagulation is initiated by exposure of tissue factor (TF) at the surfaces of smooth muscle cells and fibroblast in the vessel wall or inflammatory cells at the site of injury. Circulating factor VIIa or VII binds to TF and factor VII is consequently activated. The resulting membrane-bound TF/FVIIa enzymatic complex converts factors IX and X into their active forms. At this point procoagulant cell membranes, predominantly those of platelets, catalyse the key reactions of the coagulation cascade, namely, the formation of the tenase and prothrombinase complexes, which respectively convert the zymogens FX and prothrombin (FII) into their active forms Xa and thrombin (IIa), respectively (figure 1).¹³ Thrombin subsequently converts soluble fibrinogen into fibrin.

In vivo, procoagulant cell surfaces can be provided by platelets present in the growing thrombus. Yet, circulating platelets do not provide this catalytic surface and therefore the key question addressed in this introduction is: what induces platelet procoagulant activity and how?

Figure 1: Tissue factor induced thrombin generation. The membrane-bound TF/VIIa enzymatic complex activates factor IX and X. Factors IXa and Xa assemble into the tenase and prothrombinase complex, respectively. The catalytic phospholipid surface catalyses Xa and IIa formation. Black heads represent phosphatidylserine.

Membrane phospholipid asymmetry

Cell membranes of platelets, and other eukaryotic cells, are composed of a bilayer of phospholipids containing proteins. The plasma membrane of resting platelets is in a state of phospholipid asymmetry. That is, the outer leaflet of the plasma membrane is composed predominantly of the choline-phospholipids sphingomyelin (Spm) and phosphatidylcholine (PtdCho), whereas the inner leaflet mostly contains amino-phospholipids phosphatidylethanolamine (PtdEth) and phosphatidylserine (PtdSer). At least two transporter proteins are involved in maintaining lipid asymmetry, namely, 1) the ATP dependent aminophospholipid translocase, which transports PtdSer and PtdEth from outer to inner leaflet against the concentration gradient, and 2) the ATP dependent floppase, which transports all phospholipids outward with half times about 10 times longer than translocase. At physiological intracellular calcium concentrations (± 50 nM), the translocase and floppase are active and they maintain lipid asymmetry. Once platelets are activated, intracellular calcium levels rise and inhibit translocase and floppase activity. Additionally, the rise in cytoplasmic calcium activates a non-selective phospholipid transporter protein, called scramblase, which allows lipids to move randomly between inner and outer leaflet. It has been proposed that the calcium signal must be sustained at a high level during several minutes to significantly stimulate scramblase activity.¹⁴ In this situation, the anionic phospholipids PtdSer and PtdEth are exposed at the outer leaflet of the plasma membrane and this provides the catalytic surface for the

assembly of tenase and prothrombinase complexes (figure 2). Moreover, this process has been shown to be accompanied by shedding of procoagulant microparticles from the platelet plasma membrane (reviewed in¹⁵).

There is increasing evidence that besides negatively charged phospholipids, other components of the platelet membrane, like surface glycoproteins, are important for procoagulant activity. This is supported by a study of Ramström et al, demonstrating that PtdSer is necessary, but not sufficient for coagulation amplification.¹⁶ The clotting times were prolonged for blood stimulated with the non-physiologic calcium ionophore A23187, exposing the same amounts of PtdSer at the outer leaflet of the platelet plasma membranes when compared with blood treated with physiologic platelet activators like collagen and thrombin. This suggests that in response to physiologic stimuli platelets might exhibit additional features than PtdSer important for the assembly of tenase and prothrombinase complexes. Accordingly, recent studies indicate that platelets express specific binding sites for coagulation proteins (reviewed in¹⁷).

Platelet procoagulant activity induced by collagen

More than twenty years ago, Bevers et al published the effects of thrombin and collagen on the membrane phospholipid distribution of platelets.¹⁸ They demonstrated that thrombin and collagen only moderately affect the platelet procoagulant response. On the other hand, a dual

Figure 2: Regulation of membrane phospholipid asymmetry. The ATP dependent aminophospholipid translocase is responsible for inward movement of PtdSer and PtdEth, whereas the ATP dependent floppase transports all phospholipids outward. A rise in intracellular calcium concentration activates the non-specific scramblase which induces phospholipid randomisation. This randomisation causes exposure of PtdSer (Black heads) at the outer plasmamembrane which in turn promotes blood coagulation.

stimulation with the two agonists appeared to be the most potent accelerator of prothrombinase activity.

Up to now, ten types (I, II, III, IV, V, VI, VIII, XII, XIII, XIV) of collagen have been identified in the vessel wall, with fibrillar type I and III as the most abundant ones.¹⁹ As already mentioned, two platelet collagen receptors are important for direct adhesion to collagen, the integrin $\alpha_2\beta_1$ and the immunoglobulin superfamily member GP VI. At present, GP VI is believed to be the main receptor in the collagen-dependent process of generating platelet procoagulant activity. GP VI is non-covalently associated with Fc receptor (FcR) γ chain, which serves as the signal-transducing subunit of the receptor complex. Collagen activates platelets through GP VI by clustering the receptor and subsequent tyrosine phosphorylation of FcR γ chain that contains an immunoreceptor tyrosine based activation motif (ITAM). Through participation of the non-receptor tyrosine kinase SYK, phospholipase C γ 2 is activated, which causes an increase in cytosolic calcium.²⁰ With respect to the role of integrin $\alpha_2\beta_1$ in the platelet procoagulant response, Kuijpers et al.²¹ showed a reduction in both collagen induced calcium mobilization and procoagulant activity in β 1-deficient mouse platelets. Release of secondary agonists ADP and thromboxane A2, further support collagen induced platelet activation processes via G-protein coupled receptors.²²

Platelet procoagulant activity induced by thrombin

Three thrombin receptors have been identified on human platelets, namely, the GP Ib-IX-V complex and the protease activated receptors PAR-1 and PAR-4 (reviewed in ²³). Activation of PAR-receptors occurs when thrombin cleaves the N-terminus of the receptor, exposing a new N-terminus that serves as ligand for the receptor.²⁴ Since the ligand cannot diffuse away, this way of activation is highly effective. It was demonstrated that PAR-4 requires ten to hundred times higher concentrations of thrombin with respect to PAR-1, probably because the extracellular domain of PAR-4 lacks the sequences that interact with exosite 1 of thrombin.^{25,26} The current idea is that PAR-1 is the main signaling receptor at low doses of thrombin, but PAR-4 signals might be more sustained due to slower kinetics of activation and desensitization.^{27,28} Activated PAR-receptors activate G proteins. Signalling via G $_{12}$ induces shape change and launching of Gq leads to the activation of phospholipase C β , which induces an increase in the cytosolic calcium concentration. Again, release of secondary agonists ADP and thromboxane A2 enhance platelet activation processes.^{22,29} Like other G protein-coupled receptors, activated PAR-receptors are rapidly uncoupled from signalling and internalized by phosphorylation dependent mechanisms. The PAR-receptors are then delivered to lysosomes and rapidly degraded.

Recent findings suggest that GP Ib α is important for platelet activation by low dose of thrombin. It was reported that thrombin binding to GP Ib α facilitates cleavage of PAR-1³⁰, but not PAR-4.³¹ Furthermore, thrombin binding to GP Ib α is thought to induce direct signaling events via receptor crosslinking.^{32,33}

There are several conflicting reports about the role of thrombin and its receptors in the development of procoagulant platelets. In line with Bevers et al., Andersen et al. showed that thrombin is only a very weak inducer of platelet procoagulant activity. Anderson et al. identified PAR-1 as the main receptor responsible for thrombin

induced procoagulant activity and they exclude a role for PAR-4. Compatible with this notion, others showed a significant reduction in clotting time upon PAR-1 activation.^{16,34} In contrast, Dörman et al. demonstrate that upon activation with thrombin 60-80% of the platelets are procoagulant and they state that thrombin binding to GP Ib α is essential for thrombin induced procoagulant activity.³⁵ This notion is further supported by Dicker et al, who exclude a major role for PAR-1 in thrombin induced procoagulant activation.³⁶

Role of adhesive proteins and their respective receptors in platelet procoagulant activity

Current understandings indicate that adhesive receptors, like GP Ib α and integrin $\alpha_{IIb}\beta_3$, play an important role in the procoagulant activity of platelets. As already mentioned before, GP Ib α may stimulate exposure of negatively charged phospholipids in the exoleaflet of the platelet plasma membrane via its high affinity thrombin binding site.³⁵⁻³⁷ In addition, some data favor a role of the GP Ib α -VWF interaction in thrombin induced platelet procoagulant activity in coagulating plasma and platelets adhering to fibrin surfaces.^{38,39} Yet, other investigator showed in a system with gel filtered platelets that GP Ib α -VWF interaction is not important for the development of procoagulant activity.³⁵ This could mean that fibrin, but not VWF, is the important co-factor for thrombin induced platelet procoagulant activity. On the other hand, the apparent discrepancies might be explained by differences in shear rates in the various experimental setups.

Provided that platelets are activated, GP Ib α is able to interact with factor XI. Since thrombin also binds to this complex, factor XI-GP Ib α interaction facilitates the cleavage of factor XI by thrombin.⁴⁰ The cleavage of factor XI by thrombin serves in a feedback-loop: Thrombin $\rightarrow$ FXI (a) $\rightarrow$ FIX(a) $\rightarrow$ FX(a) $\rightarrow$ (pro)thrombin ($\rightarrow$ indicates activation of the zymogen by the enzyme). This might clarify why platelets of Bernard Soulier patients, which lack GP Ib α , show a diminished thrombin generation.

Another receptor thought to be important in the development of procoagulant platelets is the integrin $\alpha_{IIb}\beta_3$. It is still a matter of debate whether this receptor is involved in thrombin or collagen induced exposure of negatively charged phospholipids at the outer leaflet of the platelet plasma membrane. According to Ramström et al.^{16,41} there is no effect of integrin $\alpha_{IIb}\beta_3$ antagonists, like abciximab, on thrombin or collagen induced PtdSer exposure, while Furman et al.^{16,41} show reduced PtdSer exposure and factor V/Va binding. Nonetheless, whether it occurs via PtdSer-dependent or -independent mechanisms, several lines of investigation indicate that antagonists of integrin $\alpha_{IIb}\beta_3$ inhibit collagen and/or thrombin induced prothrombinase activity.^{35,36,42} In line with this, thrombin generation in blood of Glanzmann patients or blood of healthy individuals treated with integrin $\alpha_{IIb}\beta_3$ antagonists is delayed and thrombin generation during the propagation phase is depressed.^{34,37,43}

Inhibiting effects of integrin $\alpha_{IIb}\beta_3$ antagonists on thrombin generation disappeared with increasing concentrations of TF. For instance, when coagulation of whole blood was triggered with a relatively high TF concentration of 200 pM, there was no significant effect of abciximab on thrombin generation.³⁴ However, when heparin was present in blood triggered with 200 pM tissue factor, additive effects of abciximab on the prolongation of

the clotting time were readily detectable.⁴⁴ This implies that the influence of integrin $\alpha_{IIb}\beta_3$ becomes limited as soon as thrombin production becomes relatively high.

Several explanations for the inhibiting effect of integrin $\alpha_{IIb}\beta_3$ blockers on platelet procoagulant activation have been offered. It has been suggested that prothrombin binds to inactive integrin $\alpha_{IIb}\beta_3$ and is all set for activation when factor Xa is generated in the vicinity of the platelet surface.⁴⁵ Evidently, prothrombin activation within the prothrombinase complex is more efficient than on integrin $\alpha_{IIb}\beta_3$, but it is possible that the initial spark of thrombin is provided by activation of prothrombin bound to integrin $\alpha_{IIb}\beta_3$. Alternatively, the shedding of procoagulant microparticles from the platelet plasma membrane was reported to be reduced in the presence of integrin $\alpha_{IIb}\beta_3$ inhibitors.⁴⁶⁻⁴⁸

Scope

The studies described in the first part of this thesis are conducted to get a better understanding of the proteins and signaling pathways responsible for the procoagulant activation of platelets. A better knowledge of proteins and receptors concerning procoagulant activity might improve the understanding of excessive thrombus growth in diseased vessels. As already outlined in the previous paragraphs there are numerous conflicting data concerning platelet receptors and proteins involved in this process. We were motivated by a number of intriguing hypotheses to elucidate mechanisms involved in the generation of procoagulant platelet membranes.

In literature there are deviating results concerning the role of GP Iba in the platelet procoagulant response induced by thrombin. While some investigators⁴³ propose that the contribution of GP Iba is dependent on VWF binding, others³⁵ report that binding of thrombin, but not VWF, to GP Iba is essential for PtdSer exposure. Previous data from our group revealed that washed platelets adherent to fibrin layers generate a procoagulant surface membrane in the presence of thrombin, provided that their adhesion is mediated via GP Iba-VWF contact. In **Chapter 2** we hypothesized that in coagulating plasma shear stress potentiates the thrombin induced scrambling of phospholipids, by way of shear-dependent platelet interaction with VWF and fibrin. To study this, a system was developed in which a rotating cylinder exposed platelets in coagulating plasma to regular shear rates. It was demonstrated that platelet interaction with fibrin through shear dependent epitopes on GP Iba and by way of integrin $\alpha_{IIb}\beta_3$ plays an important role in thrombin induced platelet procoagulant activity at sufficiently high-shear rates.

The importance of VWF-fibrin contacts in platelet adhesion and procoagulant activity suggests that this interaction plays a key role in thrombus growth. We were interested in finding the fibrin binding site on VWF, because of potential therapeutic benefit: a specific blocker of the VWF-fibrin interaction might prevent excessive thrombus growth. In **Chapter 3**, the detailed localization of the fibrin binding-site on VWF is described. Our results imply that the C-domain of VWF is a critical determinant of platelet adhesion to fibrin under conditions of high shear stress.

Thrombin is a positive mediator of procoagulant activity of platelets, especially of platelets residing in a thrombus and thus in contact with adhesive substrates like collagen, VWF or fibrin(ogen). Current literature is not consistent with respect to which platelet thrombin receptor evokes scramblase activity.^{25,35} Therefore, **Chapter 4** deals with the effects of thrombin binding to GP Iba and the protease

activated receptors (PAR) 1 and 4 on calcium and procoagulant responses in platelets. According to the sustained calcium signals elicited by PAR-4, we hypothesized that this receptor is crucial for thrombin induced procoagulant activity. Yet, we established that neither the thrombin binding site on GP Iba nor the activation of PAR-4 are required for thrombin induced platelet procoagulant activity. It appeared that PAR-1 is fully responsible for procoagulant activity elicited by thrombin.

Fundamental research in relation to thrombus formation, as performed in chapters 2, 3 and 4 of this thesis, might contribute to improve treatment of patients with diseased atherosclerotic vessels. Finding a drug that prevents pathological thrombus formation without affecting normal haemostasis is the ultimate goal of thrombosis research at this moment. Currently, treatment of atherosclerotic vessels occluded or nearly occluded with thrombi involves bypass surgery with autologous or even synthetic vessels, or balloon angioplasty followed by stent implantation. The clinical manifestations of the biocompatibility of cardiovascular devices, like stents and vascular grafts, are numerous: sudden and complete obstruction of stents⁴⁹, acute and subacute thrombotic occlusion in medium sized grafts (4-6 mm)^{50,51} and bleeding problems due to anti-platelet or anticoagulant therapy. Therefore, studies in the second part of this thesis involve biocompatibility of artificial surfaces in contact with blood.

To prevent restenosis, a first criterion is a low thrombogenicity of the artificial surface. Thrombin is a prerequisite for the formation of a stable thrombus and, therefore, a possible strategy to improve the blood compatibility of artificial surfaces might be inactivation of locally generated thrombin by surface immobilized heparin. However, to date heparin coatings have not been shown to significantly reduce the number of postoperative complications.⁵² The studies described in **Chapters 5-7** were undertaken to 1) get a better understanding of the precise antithrombotic functions of immobilized heparin and, 2) to improve biocompatibility of heparin coatings.

It was established that the thromboresistant property of heparinized surfaces relies on neutralization of locally formed thrombin by surface bound heparin-antithrombin complexes, and not so much modulating protein adsorption and platelet adhesion (**Chapter 5**). A possible disadvantage of highly sulphated polysaccharides, like heparin, concerns binding of growth factors, which might positively mediate restenosis. Hence, antithrombogenic properties of immobilized heparin are compared with mildly sulphated polysaccharides, namely, chondroitin sulphate and alginate (**Chapter 6**). These polysaccharides appeared to be protein- and thromboresistant and might be used in combination with heparin on blood contacting surfaces. In **Chapter 7**, experiments with heparinized collagen are described. The low antigenicity of collagen makes it an outstanding candidate for coatings of medical devices in contact with blood, although the thrombogenic nature limits its application. It is demonstrated that heparin, already when used as a crosslinker of collagen, effectively prevents thrombus formation in collagen coated stents.

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Integrin $\alpha_{\text{Ib}}\beta_3$ and shear-dependent action of glycoprotein Ib α stimulate platelet-dependent thrombin formation in stirred plasma.

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Integrin $\alpha_{IIb}\beta_3$ and shear-dependent action of glycoprotein Ib α stimulate platelet-dependent thrombin formation in stirred plasma

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Under conditions of arterial-wall shear rates, platelets bind to von Willebrand factor (vWF) by way of the glycoprotein Ib (GP Ib) complex and integrin $\alpha_{IIb}\beta_3$. Both adhesive receptors may also play roles in the development of procoagulant activity of platelets. Here, we investigated the effect of shear stress, as provided by a rotating cylinder, on GP Ib- and integrin $\alpha_{IIb}\beta_3$ -dependent thrombin generation in coagulating platelet-rich plasma (PRP). We measured thrombin continuously with the use of fluorometry from the cleavage rate of a fluorescent low-affinity substrate. The integrin $\alpha_{IIb}\beta_3$ antagonist abciximab progressively reduced the peak of thrombin formation up to 43% when rate of stirring and shear stress were increased (estimated shear rates of 105–420 s^{-1}). Abciximab did not lower the peak of thrombin formation in stirred PRP from patients with Glanzmann's thrombasthenia lacking $\alpha_{IIb}\beta_3$ but, surprisingly, shortened the time until onset. In PRP from control subjects, antibodies specifically directed against vWF-binding epitopes on GP Ib α reduced thrombin formation, with 25% to 30% at the high but not at the low stirring rate. In combination with the anti-GP Ib antibody, abciximab retained its strong inhibitory effect only at the high stirring rate. We conclude that thrombin formation and coagulation in stirred PRP depend, to a large extent, on platelet adhesion to integrin $\alpha_{IIb}\beta_3$ and, in a shear-dependent way, on GP Ib. (J Lab Clin Med 2003;141:350-8)

Abbreviations: FITC = fluorescein isothiocyanate; GP Ib-IX-V = glycoprotein Ib-IX-V; HEPES = N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; PAR1 = protease-activated receptor 1; PFP = platelet-free plasma; PRP = platelet-rich plasma; vWF = von Willebrand factor; Z-GGR-AMC = benzoyloxycarbonyl Gly-Gly-Arg 7-amido-4-methyl-coumarin

The platelet glycoprotein Ib-IX-V (GP Ib-IX-V) complex plays an important, although not yet completely understood, role in platelet adhesion and activation. Under conditions of high shear stress,

vWf undergoes a conformational change that allows flowing platelets to bind reversibly to a surface by way of their GP Ib complex.^{1,2} This binding is immediately followed by stable platelet adhesion to a haemostatic surface as provided by collagen or fibrin fibers.^{3,4} Stabilization on fibrin is highly dependent on activated glycoprotein IIb/IIIa (integrin $\alpha_{IIb}\beta_3$), which acts as a high-affinity receptor for both vWf and fibrin.⁵⁻⁷

Activated platelets play an indispensable role in the coagulation process, mainly but not exclusively through the surface exposure of the procoagulant phosphatidylserine.⁸ Exposed phosphatidylserine dramatically stimulates the proteolytic conversion of factor X and prothrombin into the serine proteases factor Xa and thrombin, respectively. Although it is clear that platelets strongly stimulate the process of thrombin forma-

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tion in coagulating plasma in an adhesion-dependent way,⁹⁻¹¹ precisely which adhesive receptors and activation pathways are involved is still incompletely understood. Antagonists of integrin $\alpha_{IIb}\beta_3$,¹²⁻¹⁵ such as the chimeric antibody abciximab,¹⁶ effectively decrease thrombin generation in PRP. Inhibition of GP Ib-V-IX binding, whether or not in combination with anti-integrins, has a similar lowering effect on thrombin formation.^{12,17,18} Although Béguin et al¹⁸ propose that the contribution of GP Ib is also dependent on vWf, others report that GP Ib binding to thrombin but not to vWf is essential for platelet phosphatidylserine exposure.¹⁹ Because thrombin binding to GP Ib facilitates PAR1 activation,²⁰ GP Ib may thereby enhance the stimulating effect of PAR1 on procoagulant activity of platelets.²¹ The exact function of GP Ib interaction in the development of platelet procoagulant activity therefore remains to be clarified.

Knowing the shear-dependent action of vWf, we hypothesized that the presence of shear stress plays a role in stimulating the GP Ib- and integrin-dependent procoagulant platelet response (phosphatidylserine exposure)—for instance, by way of shear-dependent platelet interaction with vWF and fibrin. To provide regular shear stress to platelets, we generated a fibrin clot with aggregated platelets in coagulating PRP at the surface of a cylinder, which was rotated at defined stir rates. We then monitored thrombin generation continuously with the use of a fluorescent thrombin substrate. Antibody fragments specifically blocking shear-induced and vWf-dependent adhesion events were used to determine the involvement of this shear-dependent pathway in the procoagulant effect of platelets.

METHODS

Materials. Fluorogenic thrombin substrate Z-GGR-AMC came from Bachem (Bubendorf, Switzerland). Abciximab (c7E3 Fab) was purchased from Centocor (Leiden, Netherlands). Human α -thrombin was purified as described previously.²² F(ab')₂ fragments of monoclonal antibodies 6B4, 24G10, and 12G1 against specific vWf-binding epitopes on GPIb α were generated and characterized as described earlier.^{23,24} The PAC-1 antibody against activated integrin $\alpha_{IIb}\beta_3$ came from Becton-Dickinson (San Jose, Calif). The PAR1 receptor agonist Ser-Phe-Leu-Leu-Arg-Asn (SFLLRN) was from IHB (Leiden, Netherlands). Other materials came from sources mentioned elsewhere.²⁵

Blood donation and preparation of plasma. Blood was freshly obtained from healthy volunteers who denied having taken antiplatelet medication for at least 2 weeks. Blood was also obtained from three patients with type I Glanzmann disease, with platelets completely deficient in integrin $\alpha_{IIb}\beta_3$.²⁶ After collecting blood into 1/9 volume of 0.13 mol/L trisodium citrate, we centrifuged it at 190g for 15 minutes to obtain PRP. PFP was immediately prepared from

the PRP by means of centrifugation at 10,000 g for 10 minutes. The PRP was mixed with autologous PFP to yield the desired platelet count. The normalized PRP was used within 1 hour of venipuncture (ie, before the appearance of platelet-derived microvesicles with high procoagulant activity). All subjects gave full informed consent before donating blood.

Flow cytometry. PRP containing 2×10^8 platelets/mL was preincubated with antibodies for 10 minutes. Platelets were then activated with 15 μ mol/L SFLLRN and, after 10 minutes, evaluated on affinity modulation of integrin $\alpha_{IIb}\beta_3$ with the use of FITC-labeled PAC-1 antibody against the activated integrin.²⁷ Measurements were performed with an Epics XL flow cytometer from Coulter Electronics (Luton, UK).

Thrombin generation measurement. We measured thrombin generation in clotting PRP, usually containing 2×10^8 platelets/mL, in 10-mm fluorescence cuvettes containing a cylindrical stirring bar (diameter 9 mm, height 8 mm) using an SLM-Aminco 8100 spectrofluorometer (SLM Instruments, Rochester, NY). Four cuvettes were placed in the thermostat-equipped cuvette house of the fluorometer in such a way that fluorescence was measured in a window of 10 to 15 mm above the bottoms of the cuvettes. Stirring was done continuously at a rate of 100 to 400 rpm. The cuvettes contained 1.87 mL PRP (platelet count as indicated) and 0.93 mL HEPES buffer (136 mmol/L NaCl, 2.7 mmol/L KCl, 2 mmol/L MgCl₂, 10 mmol/L Hepes; pH 7.45). After supplementation with 0.25 mmol/L Z-GGR-AMC, coagulation was triggered with 16.7 mmol/L CaCl₂ (final concentrations). The PRP mixture was preincubated with the indicated antibodies for 10 minutes before the addition of CaCl₂. Where indicated, 10 nmol/L human α -thrombin was added immediately before CaCl₂. Fluorescence caused by cleaved amidomethyl coumarin was continuously recorded on excitation at 390 nm and an emission wavelength of 460 nm (5-nm band passes). Measurements were performed at 37°C.

To provide a defined shear stress, we set the stirring cylinder with attached platelet-fibrin clot to rotate at a given angular speed (Ω). The shear rate (γ) at the surface of the cylinder was then calculated as $\gamma = 0.5 \Omega W/e$, where W is the width of the cuvette (10 mm) and e is the distance between the cuvette wall and the cylinder (0.5 mm with centered bar). At stirring rates of 100, 200, and 400 rpm, the corresponding shear rates were 105, 210 and 420 s⁻¹, respectively. Note that 400 rpm was the highest stirring rate that could be achieved in this cuvette system without artifacts resulting from strong fluctuations in the rotation of the cylinder.

Lag times of onset of the burst of thrombin formation were deduced from the original fluorescence traces. Floating point-averaged first-derivative traces were then constructed to obtain curves of thrombin generation, as described by Hemker et al.²⁸ Thrombin generation was first expressed as arbitrary units of fluorescence intensity per minutes (AU/min). The conversion factor from fluorescence units to thrombin concentrations was determined in parallel experiments, in which thrombin was measured with S2238 substrate in samples

taken from the coagulating plasma, as described elsewhere.²⁸ Under the optical conditions in question, 1 AU/min was equivalent to 0.22 nmol/L of free α -thrombin.

Statistical analysis. To determine the statistical significance of differences, we obtained *P* values with a nonparametric test for two independent (Mann-Whitney U test) or two dependent variables (Wilcoxon signed-rank test), using the Statistical Package for the Social Sciences (SPSS, Chicago, Ill).

RESULTS

Effect of stirring on thrombin generation in clotting PRP. Thrombin generation was monitored in citrated PRP triggered with CaCl_2 at a controlled shear rate. We therefore adapted a method with which thrombin generation can be measured continuously from the cleavage of a low-affinity fluorogenic thrombin substrate, Z-GGR-AMC.²⁹ As described, in such a plasma system the major burst of thrombin formation occurs after a threshold amount of thrombin (<0.5 nmol/L) has been produced. The threshold amounts of thrombin are sufficient to activate platelets and form fibrin but cannot be detected by the low-affinity substrate. A major advantage of such substrate, however, is its low turnover; it only minimally competes with the natural thrombin substrates in plasma and therefore does not interfere with the burst of thrombin generation.^{28,30,31}

Using cuvettes stirred with a large rotating magnetic cylinder to provide regular shear stress, we continuously measured fluorescence accumulation of the cleaved Z-GGR-AMC by means of a window in the cuvette house above the bar. Under standard conditions with freshly isolated PRP from healthy subjects (2×10^8 platelets/mL), a fibrin clot trapping most of the platelets formed at the outer surface of the stirring bar after about 10 minutes of triggering with CaCl_2 . Detectable amounts of fluorescence appeared after 12 to 15 minutes, when the burst of thrombin formation started (Fig 1, A). In the presence of heparin, no fluorescent signal was observed, demonstrating that it was completely thrombin-dependent. We constructed first-derivative curves from the original fluorescence curves to better visualize the accumulation and inactivation of thrombin in time. Peak levels of thrombin were typically observed after about 10 minutes of fluorescence (Fig 1, B). When the stirring rate was increased from 0 to 200 and then 400 rpm, the onset of thrombin shortened with 3 minutes, whereas the thrombin peak increased fourfold, from 25 to 100 nmol/L. Similarly, the area under the curve, earlier designated the endogenous thrombin potential and representing the cumulative activity of thrombin during the coagulation process as a whole,^{32,33} increased about fourfold with a higher stir-

Fig 1. Effect of stir rate on thrombin generation in coagulating PRP. Citrated PRP (final platelet count 2×10^8 /mL) was triggered with CaCl_2 (16.7 mmol/L) in the presence of Z-GGR-AMC (250 μ mol/L) at various stir rates. Thrombin activity was measured from the accumulation of fluorescent amidomethyl coumarin (A). First-derivative curves were constructed to yield time courses of thrombin generation (B). The stir rate during the experiment was 0 (a), 200 (b), or 400 rpm (c), equivalent to shear rates of 105, 210, and 420 s^{-1} , respectively. Traces are from an experiment representative of three performed.

ring rate. The coagulant activity of triggered PRP thus appeared to increase with the rate of stirring.

To determine the dependence of platelets in the thrombin-generation process, we took measurements at various platelet concentrations at a stirring rate of 200 rpm. With the use of PFP (ie, in the absence of platelets), no substrate cleavage was observed for at least 60 min (Fig 2, A). Increasing the platelet count in plasma from 0.25 to 2×10^8 /mL resulted in a shortening of the lag period of thrombin formation by 40%; the thrombin peak values increased fourfold (Fig 2, B). This finding illustrates the critical role of platelets in the propagation of thrombin generation in our experimental setup.

Contribution of integrin $\alpha_{\text{IIb}}\beta_3$ to thrombin generation in stirred PRP. We next determined whether, in this rapid-stirring system, thrombin formation was dependent on integrin $\alpha_{\text{IIb}}\beta_3$ activation, as has been described in the absence of stirring or under slow-stirring conditions.^{12,16,17,34} It has been shown that antibody-derived $\alpha_{\text{IIb}}\beta_3$ inhibitors are most effective in suppressing platelet procoagulant activity. Widely used is the chimeric antibody abciximab (c7E3 Fab; Reopro), which has a consistent anticoagulant effect in other platelet-

Fig 2. Effect of platelet concentration on thrombin generation in stirred, coagulating PRP. Citrated PRP, diluted with autologous PFP to various platelet concentrations, was triggered with CaCl_2 (16.7 mmol/L) in the presence of Z-GGR-AMC (250 $\mu\text{mol/L}$) during continuous stirring at 200 rpm. Delay times of detectable thrombin generation (A) and thrombin peak values (B) are plotted as functions of platelet count. Thrombin peak values are expressed as percentages relative to standard conditions (platelet count $2 \times 10^8/\text{mL}$). Data are from an experiment representative of three performed.

plasma systems and established antithrombotic action in clinical practice.^{16,34–36} When used in the thrombin-generation assay under nonstirring conditions, abciximab has a more prominent effect on the thrombin peak level than on endogenous thrombin potential. This indicates that platelet activation affects to a larger extent the rate of thrombin formation than the total amount of active thrombin formed during the clotting process.

When added to stirred PRP before the CaCl_2 trigger, abciximab at concentrations of 5 $\mu\text{g/mL}$ and greater caused a consistent lengthening of the lag time of thrombin formation (Fig 3, A) and a clear decrease in the thrombin peak value (Fig 3, B). At 10 $\mu\text{g/mL}$, abciximab prolonged the time of onset to sudden thrombin production from 13.2 ± 2 to 27.3 ± 11.6 minutes (mean $\pm$ SD; $n = 5$, $P < .001$) but decreased the thrombin peak to $57.3\% \pm 21\%$ ($P < .001$) with respect to control conditions. Higher levels of abciximab did not further increase these effects (Fig 3, A and B).

To confirm that integrin $\alpha_{\text{IIb}}\beta_3$ was involved in the

Fig 3. Dose-dependent effect of abciximab on thrombin generation in stirred PRP. Thrombin generation was measured from fluorescence accumulation in PRP stirred at 200 rpm, as described in the legend to Fig 1. PRP from control subjects ($n = 5$) was preincubated for 5 minutes with the indicated abciximab concentration, then triggered with CaCl_2 (16.7 mmol/L). A, Lag times of onset of detectable thrombin generation and B, and thrombin peak values of thrombin generation, each expressed as percentages of the control conditions without abciximab. Data expressed as mean $\pm$ SD ($n = 5$); * $P \leq .05$ with respect to control conditions (Wilcoxon's test). C, Representative thrombin generation with PRP from a control subject and a Glanzmann's disease patient in the presence (+) or absence (–) of 10 $\mu\text{g/mL}$ abciximab.

inhibitory effect of abciximab under these stirring conditions, we conducted further experiments with plasma from three patients with type I Glanzmann disease, whose platelets completely lack this integrin. Thrombin generation was markedly altered in the stirred PRP from the patients. For two patients, lag times of thrombin formation after triggering of the coagulation were 39.4 and 40.9 minutes, much longer than the lag time of 13.2 ± 2 minutes in PRP from control subjects. Throm-

Fig 4. Thrombin generation in preactivated stirred PRP. Citrated PFP (a) or PRP (b and c) containing Z-GGR-AMC (250 $\mu\text{mol/L}$) was preactivated with α -thrombin (10 nmol/L) and, after 10 seconds, triggered to coagulate with CaCl_2 (16.7 mmol/L). The stir rate was 100 (b) or 400 rpm (a and c), equivalent to shear rates of 105 and 420 s^{-1} , respectively. Traces are representative first-derivative curves of thrombin generation (four experiments performed).

bin peak values in patient plasma were approximately half the normal level (Fig 3, C). In PRP from the third Glanzmann patient, no detectable thrombin was formed during the 60-minute measurement period. Addition of abciximab (10 $\mu\text{g/mL}$) to stirred PRP from the first two Glanzmann patients did not affect the thrombin peak value but, surprisingly, shortened lag time from 39.4 and 40.9 minutes to 18.2 and 20.0 minutes. In the third patient, lag time remained greater than 60 minutes in the presence of abciximab. These data indicate that in the stirred-PRP system, integrin $\alpha_{\text{IIb}}\beta_3$ -mediated interactions, most likely with in situ-generated fibrin, prominently contribute to the thrombin-forming process.

Enhanced thrombin generation in preactivated, stirred PRP. In the experiments described so far, clots in PRP were produced at the rotating cylindrical bar after about 10 minutes of recalcification. We reasoned that regular shear-dependent effects along the GP Ib-vWF axis are only obtained when the platelets immediately adhere to the rotating cylinder. We achieved this by accelerating the processes of platelet activation and clot formation, adding human α -thrombin. The citrated PRP was preincubated with a single dose of thrombin (10 nmol/L) for 10 seconds before the start of stirring and addition of CaCl_2 . Within 20 seconds, this resulted in the appearance of a clot, rapidly contracting at the surface of the rotating cylinder and trapping aggregating platelets in the plasma. Two or 3 minutes after recalcification, depending on the stir rate, fluorescent amidomethyl coumarin started to accumulate, pointing to the start of the burst of thrombin formation (Fig 4). Control experiments incorporating PFP showed that the added thrombin here also caused clot formation but not a fluorescent signal (Fig 4), indicating that it was rapidly inactivated,

most likely by plasma antithrombin. In the thrombin-preactivated PRP, the time of onset of thrombin generation was slightly shortened when the stir rate was increased from 100 to 400 rpm, and the thrombin peak value increased by about 25% (Fig 4). In most incubations, the platelet-fibrin clot remained fixed at the stirring cylinder during the entire measurement period. In a few cases, the clot was released from the cylinder, resulting in artificial fluorescence signals (not shown). We concluded that because of the continuous rotation at the cylinder surface, the adhering platelets are subjected to more or less regular shear stress during the period of thrombin-generation measurement.

Effect of specific anti-GPIIb/IIIa antibodies on thrombin generation in PRP with preformed fibrin clots under stirring conditions. To examine the involvement of shear stress-dependent GP Ib interactions in the process of thrombin generation, we used F(ab')_2 fragments from three monoclonal antibodies raised against specific epitopes of the GP Ib complex. Earlier studies have indicated that the antibodies 6B4 and 24G10 each bind to a different epitope on the GP Ib α chain, but both inhibit the shear- and ristocetin/biotroctin-induced binding of GP Ib to vWf.²⁴ Antibody 12G1 does not prevent or only weakly prevents ristocetin/biotroctin-induced binding of GP Ib to vWf.²⁴ Only 24G10 appeared to have a minor effect on GP Ib interaction with thrombin.^{23,24}

Effects of the three antibody fragments (each 10 $\mu\text{g/mL}$) were determined in thrombin-preactivated PRP that was stirred at 100 (low stir rate) or 400 rpm (high stir rate). We conducted these tests in the presence or absence of abciximab (10 $\mu\text{g/mL}$) to determine possible cumulative effects of GP Ib and integrin $\alpha_{\text{IIb}}\beta_3$ inhibition. As shown in Table I, none of the three anti-GP Ib α antibody fragments influenced the thrombin generation when the preactivated PRP was stirred at 100 rpm. In contrast, at the high stir rate of 400 rpm, the 6B4, 12G1, and 24G10 antibody fragments all reduced the thrombin peak level by 25% to 30%. The effects of 6B4 ($P = .01$, Wilcoxon's test) and 12G1 ($P = .049$) at a high stir rate were statistically significant.

Again, abciximab caused a partially inhibitory effect on thrombin generation in the thrombin-preactivated PRP (Table I). At the low stir rate, abciximab (10 $\mu\text{g/mL}$) reduced the thrombin peak value by 23%, but at 400 rpm it caused a significantly greater reduction of 43% ($P = .03$, Mann-Whitney U test). Only at 400 rpm did abciximab reduce thrombin generation in the presence of the 6B4 fragment; the difference between treatment with 6B4 and with 6B4/abciximab was statistically significant ($P = .01$).

However, we noted no significant difference between

Table 1. Inhibitory effects of antibodies against shear-dependent epitopes on GP Iba and integrin $\alpha_{IIb}\beta_3$ on thrombin generation in stirred PRP

Antibody	Thrombin peak (% of control)		P (Mann-Whitney)*
	Low stir rate	High stir rate	
6B4	108.2 $\pm$ 24.6 (5)	74.8 $\pm$ 20.7 (10)†	0.028
24G10	139.4 (1)	76.5 $\pm$ 7.5 (3)	—
12G1	88.7 $\pm$ 24.6 (4)	64.6 $\pm$ 15.6 (4)‡	0.149
Abciximab	77.4 $\pm$ 15.2 (5)‡	56.7 $\pm$ 13.5 (7)‡	0.030
6B4 + abciximab	93.0 $\pm$ 37.9 (5)	45.5 $\pm$ 15.1 (6)†	0.030

Citrated PRP containing Z-GGR-AMC (250 μ M/L) was triggered with 16.7 mmol/L CaCl_2 , directly followed by 10 nmol/L α -thrombin. The PRP was preincubated for 10 minutes with F(ab')₂ fragments of antibody 6B4, 24G10, or 12G1 10 μ g/mL where indicated. Thrombin generation was measured continuously with stirring at 100 rpm (low stir rate) or 400 rpm (high stir rate) at 37°C. Peak values of formed thrombin are expressed as percentages of control conditions (100 or 400 rpm) without antibody. Data expressed as mean $\pm$ SD (n independent experiments).

*Significance of difference between low and high stir rates (Mann-Whitney U test); †P $\leq$ 0.01; ‡P $\leq$ 0.05 compared to control condition (Wilcoxon test).

Fig 5. Effect of the anti-GPIIb/IIIa antibody 6B4 and abciximab on PAC-1 binding to resting and activated platelets. Platelets in PRP were incubated for 10 minutes with or without 6B4 F(ab')₂ fragment (10 μ g/mL), abciximab (10 μ g/mL), or both. PAC-1 binding was determined after 10 minutes of activation of the platelets with SFLLRN (15 μ M/L) with the use of flow cytometry. Controls were run in the absence of SFLLRN as indicated. Shown are percentages of platelets staining positively for FITC-PAC-1. Data expressed as mean $\pm$ SD, n = 3).

treatment with abciximab alone and with abciximab/6B4 (P = .25). Further statistical analysis showed that the difference in thrombin levels between low and high stir rates was significant in the incubations containing 6B4 fragment (P = .028) or abciximab (P = .030; Table 1). Note that the vWf activator ristocetin could not be used in these experiments because it interferes with the coagulation activity in plasma.¹⁸

Flow cytometry was used to check whether the 6B4 F(ab')₂ fragment interferes with the binding of abciximab to platelets. Integrin $\alpha_{IIb}\beta_3$ activation of the platelets was therefore monitored with FITC-labeled antibody PAC1. As shown in Figure 5, at the concentration used in the thrombin-generation experiments, 6B4 (10 μ g/mL) did not cause activation of the integrin and did

not interfere with PAC-1 binding on platelets activated with thrombin receptor-activating peptide SFLLRN. Nor did 6B4 abolish the binding of abciximab (10 μ g/mL) to the activated integrin, which would result in restoration of PAC-1 binding.

These results provide evidence that a shear-dependent binding site for vWf on GP Iba, as accessed by the antibody fragments, plays a substantial role in platelet-dependent thrombin generation when the clot-bound platelets are subjected to a sufficiently high shear rate. Furthermore, because the abciximab effect was not further potentiated by shear-dependent anti-GP Ib antibodies, it appears that the shear/GP Ib-dependent effect to a large extent involves integrin $\alpha_{IIb}\beta_3$.

DISCUSSION

In this article, we describe a method with which thrombin generation in coagulating PRP is continuously measured under defined stirring conditions. A large, rotating magnetic cylinder, forming the site of attachment of platelet-fibrin thrombi, is used to subject the clot-bound platelets to regular shear stress. Thrombin generation is quantified from the accumulation of the fluorescent cleavage product of the low-affinity substrate Z-GGR-AMC, which interferes only a little with the thrombin-generation process per se.²⁸ In this measurement system, the process of thrombin generation appears to depend fully on the presence of platelets, and it is accelerated and increased at higher stir rates. During stirring at 400 rpm, which was the highest rate achievable, the formed thrombin reaches peaks as high as 100 nmol/L, a finding in agreement with the earlier notion that the presence of the fluorescent thrombin substrate allows full development of the thrombin-generation process.

It is difficult to precisely determine the actual shear

stresses sensed by the platelets in the stirred cuvette. It is estimated that platelets adhering at the outer surface of the stirred cylinder are exposed to a shear rate of 105 and 420 s^{-1} when stirring is performed at 100 and 400 rpm, respectively (see Methods). Other authors have shown that at low/intermediate physiologic shear rates of 150–600 s^{-1} the binding by way of GP Ib becomes increasingly important for platelet adhesion to vWf and the subsequent induction of integrin $\alpha_{IIb}\beta_3$ activation.³⁷ The shear rates generated under these conditions thus seem sufficient to promote shear-dependent vWf–GP Ib interaction and later integrin activation.

Our data confirm earlier work with unstirred or slowly stirred PRP showing that the integrin antagonist abciximab is a potent inhibitor of the thrombin-generation process.^{16–18} The results newly indicate that the inhibitory effect of abciximab on thrombin generation, at least on the thrombin peak level, increases with stir rate and thus with shear (Table I). Abciximab was inhibitory from concentrations greater than 5 $\mu g/mL$ and had a nearly maximal effect at 10 $\mu g/mL$ (Fig 3). These concentrations are similar to those used by other authors (eg, those studying the effects of abciximab on platelet-dependent coagulation with preadded fibrin clots under minimal shear stress).^{18,34} In the same concentration range, abciximab is clinically effective in the prevention of thrombotic complications after percutaneous coronary angioplasty—that is, a bolus injection of 0.25 mg/kg, equivalent to a peak value in plasma of approximately 3.5 $\mu g/mL$ followed by a 12-hour infusion at 0.6 mg/h (approximately 8.5 $\mu g/mL/h$).³⁸ Note that similar coagulation-decreasing effects of integrin-blocking agents other than abciximab (eg, other antibodies, eptifibatide, and MK-383) have been obtained by other authors, although only in the absence of stirring or at a low stir rate.^{13,16,17}

Experiments with PRP from patients with Glanzmann disease confirm a role of integrin $\alpha_{IIb}\beta_3$ in thrombin formation. Thrombin formation in patient PRP was delayed and reduced, and abciximab no longer inhibited this process. However, surprisingly, abciximab shortened the lag time of thrombin generation in PRP of two of three patients with Glanzmann disease. Because abciximab did not influence the thrombin-generation process in (patient) plasma in the absence of platelets, the effect must be platelet-dependent. As a possible explanation, we suggest that abciximab, at least in the absence of integrin $\alpha_{IIb}\beta_3$, can interact with other (perhaps low-affinity) binding sites on platelets, which then act in a platelet-stimulatory fashion.

As reviewed elsewhere, the mechanism by which integrin $\alpha_{IIb}\beta_3$ blocking influences the procoagulant activity of platelets in plasma is unclear.⁸ It has been suggested that binding of prothrombin to inactivated

integrin $\alpha_{IIb}\beta_3$ triggers the generation of traces of thrombin, which in turn induce (by way of cleaved thrombin receptors) platelet procoagulant activity.³ Blocking of integrin $\alpha_{IIb}\beta_3$ with abciximab may delay initial thrombin generation and thus prolong the lag period of thrombin generation. Alternatively, integrin $\alpha_{IIb}\beta_3$ antagonism may influence coagulation by inhibiting the formation of procoagulant platelet-derived microvesicles.^{34,39}

To obtain immediate shear-dependent effects, we activated the citrated PRP with human α -thrombin shortly before adding $CaCl_2$. This resulted in rapid formation of a rotating platelet-fibrin clot before the calcium-dependent burst of thrombin generation. It should be noted that the added thrombin is rapidly inactivated in plasma (half-life of approximately 7 seconds), and therefore only the later massive burst of thrombin formation is detectable with the low-affinity fluorescent thrombin substrate. With three different antibody F(ab')₂ fragments, 6B4, 24G10, or 12G1 (10 $\mu g/mL$), each specifically directed against platelet GP Ib α and each inhibiting shear-dependent binding of GP Ib to vWf, we observed reduced thrombin formation only at a high (400 rpm) but not at a low (100 rpm) stir rate. This implies that the antibodies are only capable of influencing the thrombin-generation process at the higher shear rate of 420 s^{-1} . Additional evidence that GP Ib–vWf interactions are involved in platelet adhesion under these circumstances comes from flow studies in which whole blood from baboons was perfused over a collagen/vWf surface. It appeared that the 6B4 fragment (10 $\mu g/mL$) decreases platelet deposition by about 50% at a wall-shear rate of 650 s^{-1} .²⁴ The physiologic significance of the antibody effect is apparent from the observation that a bolus infusion of 6B4 antibody of 640 $\mu g/kg$ (equivalent to a plasma level of about 10 $\mu g/mL$) reduced the platelet-deposition arterial thrombosis in this primate model by 65%.²⁴

A typical observation was that the reduction of thrombin formation by the 6B4 anti-GP Ib fragment was enhanced by abciximab, but only at a high stir rate (Table I). Given that 6B4 does not affect the inhibitory effect of abciximab, we can conclude that 6B4 under high-stir-rate conditions interferes with the same (or similar) platelet-activation processes as abciximab. Thus the shear-dependent effect on platelet thrombin formation seems to be at least in part upstream of integrin $\alpha_{IIb}\beta_3$ -mediated effects. This GP Ib effect, like that of integrin $\alpha_{IIb}\beta_3$, most likely involves increased expression of phosphatidylserine on the platelet surface; this is a predominant parameter determining thrombin formation in platelet-containing plasma.^{12,18,19,40} The shear-dependent effect we observed may therefore explain the apparently deviating results

of Dörmann et al,¹⁹ who reported that vWf binding to GP Ib is not essential for the procoagulant response of thrombin-stimulated platelets, because the work was conducted in the absence of high-shear conditions.

We conclude that platelet interaction with fibrin through shear-dependent epitopes on GP Ib and by way of integrin $\alpha_{IIb}\beta_3$ plays an important role in the platelet-dependent coagulation process as assayed under sufficiently high shear conditions. The work thereby suggests that the interaction of platelet GP Ib with immobilized vWf, such as that which occurs under moderate and high wall-shear rates, has a more diverse effect on hemostasis than previously thought. It not only serves to tether platelets at sites of vWf deposition but supports the subsequent platelet-dependent stimulation of the coagulation process.

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Von Willebrand factor C1C2 domain is involved in platelet adhesion to polymerized fibrin at high shear rate

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Fibrin is actively involved in platelet reactions essential for thrombus growth, in which von Willebrand factor (VWF) might be an important mediator. The aim of this study was to localize VWF domains that bind to fibrin and to determine their relevance in platelet adhesion. VWF binds specifically to fibrin with an apparent K_d of 2.2 $\mu\text{g/mL}$. Competition in the presence of 2 complementary fragments, SpIII (residues 1-1365) and SpII (residues 1366-2050), indicated that the high affinity binding

site for fibrin is located in the C-terminal part, thus distinct from the A domains. Comparison of 2 deleted rVWF ($\Delta\text{D4B-rVWF}$, $\Delta\text{C1C2-rVWF}$) suggested that the C1C2 domains contained a fibrin binding site. This site is distinct from RGD, as shown by binding of D1746G-rVWF to fibrin. Perfusion studies at high shear rate demonstrated that C1C2 domains were required for optimal platelet adhesion to fibrin. With the use of a VWF-deficient mouse model, it was found

that plasma VWF is critical for platelet tethering and adhesion to fibrin. These results suggest a dual role of fibrin-bound VWF in thrombus formation: fibrin-bound VWF is critical in the recruitment of platelets by way of glycoprotein (GP) Ib, and, second, it contributes to stationary platelet adhesion by way of binding to activated $\alpha_{IIb}\beta_3$. (Blood. 2003;103:1741-1746)

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Introduction

The process of hemostasis and pathologic thrombus formation at an injured vessel wall is initiated when platelets are captured from flowing blood by way of a rapid bond formation between their glycoprotein (GP) Ib receptor and von Willebrand factor (VWF) immobilized on collagen. Platelets subsequently roll over the damaged area prior to irreversible binding through integrin $\alpha_{IIb}\beta_3$.¹⁻³ GP Ib and $\alpha_{IIb}\beta_3$ binding sites on VWF are localized on the A1 and C1 domain, respectively.⁴⁻⁸ Immobilized VWF present on the platelet membranes then serves for further platelet recruitment and thrombus growth.⁹

In parallel with the platelet adhesion process, coagulation is initiated through release of tissue factor from the damaged vessel wall.¹⁰ Propagation of blood coagulation occurs by localized enzymatic complexes assembled on the plasma membrane of adherent platelets that expose negatively charged phospholipids (reviewed in Heemskerk et al¹¹). The thrombin thus formed further activates platelets and stabilizes the growing thrombus by the formation of fibrin. Previous work from our laboratory^{12,13} and others¹⁴ suggests that in addition to its nonspecific trapping of platelets, fibrin could be actively involved in regulating thrombus growth. It is well accepted that under conditions of high shear stress VWF plays a critical role in the recruitment of circulating platelets to the fibrin network.^{1,15} The initial evidence indicating that VWF binds to fibrin from a plasma milieu was provided by Hada et al.¹⁶ Those investigators demonstrated that factor XIIIa mediated the cross-linking between fibrin and VWF. In contrast, Loscalzo et al¹⁷ demonstrated that VWF interacts noncovalently with immobilized

fibrin monomers. Other investigators extended this finding demonstrating that VWF interaction with fibrin depends on presence of high-molecular-weight VWF multimers.¹⁸ More recently, it has been shown that VWF binds to fibrin only in a purified protein system and not from plasma.¹⁵ It was postulated that of plasma proteins compete with VWF for binding to fibrin. According to these investigators, interactions between VWF and fibrin could only be detected when platelets were present. Altogether these conflicting observations initiated the present study in which we addressed the following questions: Does VWF contain a specific fibrin-binding site and does plasma VWF contribute to tethering platelets to fibrin under physiologically relevant conditions? Our results imply that the C-domain of VWF is a critical determinant of platelet adhesion to fibrin under conditions of high shear stress.

Materials and methods

Proteins

High-purity VWF concentrate was a kind gift from Dr C. Mazurier (L.F. Lille, France). Digestion of purified VWF by *Staphylococcus aureus* V8 protease and purification of 2 dimeric complementary fragments, i.e., N-terminal SpIII (residues 1-1365) and the C-terminal SpII (residues 1366-2050) were performed as described.⁶ Human fibrinogen, devoid of VWF, fibronectin, and plasminogen was obtained from Kordia (Leiden, The Netherlands). Human α -thrombin was purified as described.¹⁹ D-Phe-Pro-Arg chloromethylketone (PPACK) came from Calbiochem (San Diego, CA).

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CA). Synthetic peptide Arg-Gly-Asp-Ser (RGDS) and bovine serum albumin (BSA) were from Sigma (St Louis, MO). Factor VIII-depleted plasma came from Stago Diagnostica (Asnières-sur-Seine, France) and contained less than 1% VWF:Ag. Recombinant VWF (rVWF) and its mutants rVWF-ΔC1C2 and rVWF-D1746G were purified by immunoaffinity chromatography by using antibody RU8.²⁰ rVWF-ΔD4B was purified by conventional chromatographic methods by using heparin-Sepharose and monoQ-Sepharose. The disintegrin kistrin, an RGD-containing cysteine-rich peptide, was purified from the venom of the viper *Agkistrodon rhodostoma* as described.²¹

Animals

The wild-type and VWF-deficient mice²² were on a C57BL/6J background and were used between 10 and 16 weeks of age. Housing and experiments were done as recommended by French regulations and experimental guidelines of the European Union.

Antibodies

A polyclonal antibody against VWF was from Dako (Carpinteria, CA). Polyclonal antibodies to human SpII or SpIII fragments were raised in rabbits by immunization using the purified fragments. The immunoglobulin G (IgG) fractions were rendered monospecific by adsorption onto VWF-deficient plasma coupled to Sepharose 2B,²³ then the pass-through was immunoadsorbed onto purified VWF coupled to Sepharose 2B.²⁴ Specific antibodies were eluted with 0.1 M glycine, 0.5 M NaCl, pH 2.4.

SDS-agarose gel electrophoresis

Multimeric composition of ¹²⁵I-VWF added to fibrin and that of the radioactive fibrin-bound material, eluted from 15 filters with a 2% sodium dodecyl sulfate (SDS) solution, was analyzed by 0.1% SDS-1% agarose (Amersham, Uppsala, Sweden) gel electrophoresis under nonreducing conditions as described earlier.²⁵

¹²⁵I-VWF binding to fibrin

VWF was labeled with Na¹²⁵I (Amersham) and Iodo-gen.²⁶ Specific radioactivity varied from 1 to 4 μCi/μg (0.037–0.148 MBq μg). Labeled protein was stored at 4°C and used within 1 week. Fibrin was formed in spin-X filters (2-mL, cellulose acetate, 0.45 μm; Corning BV, Schiphol-Rijk, The Netherlands) according to Hall et al.²⁷ Briefly, thrombin (10 nM) was added to fibrinogen in 30 mM Tris (tris(hydroxymethyl)aminomethane)-HCl, pH 7.4, 150 mM NaCl, 0.1% BSA (TBS) containing 3 mM CaCl₂ to a final volume of 50 μL. The clot was allowed to form for 15 minutes at 37°C and washed with 500 μL TBS by spinning the filter cup at 14 000 rpm during 3 minutes at room temperature. Thrombin bound to the fibrin was neutralized with 5 μM PPACK during a 5-minute incubation, and the fibrin clot was then washed as described earlier. To reduce nonspecific binding, the filter with fibrin was incubated for 30 minutes with TBS containing 3% BSA. VWF binding was determined by incubating the fibrin clot on the filter with ¹²⁵I-VWF in TBS (50 μL) for 2 hours at 37°C. The filter was rinsed 3 times with 500 μL TBS containing 0.1% Tween-20 as described earlier. The amount of bound VWF was calculated from the radioactivity associated with the filter measured in a gamma-counter (Multiganima II counter; LKB Instruments SA, Bromma, Sweden). Specific binding was obtained by subtracting the amount of VWF bound to the filter in the absence of fibrin. Nonspecific filter binding ranged in all experiments from 3% to 5% of protein added. Free ¹²⁵I-VWF was calculated by subtracting the amount of ¹²⁵I-VWF bound to fibrin from the total amount of ¹²⁵I-VWF added. Mean values ± SEM were calculated for 6 independent experiments.

Preparation of plasma-free human blood cells

Preparation of washed blood cells was adapted from Savage et al.³ Briefly, blood was taken from healthy volunteers who had not taken antiplatelet medication for the preceding 2 weeks. Five volumes of blood were taken into 1 volume of acid-citrate dextrose (ACD). Blood was then spun down at 2100g for 15 minutes at 23°C. The supernatant plasma was discarded and

replaced by a double volume of HEPES buffer (10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 0.1% glucose, 0.1% BSA, pH 6.5) containing 5 mM EDTA (ethylenediaminetetraacetic acid). The cell suspension was spun down, and the cell-free supernatant was removed. This procedure was repeated twice. Finally, washed blood cells were resuspended into HEPES buffer, pH 7.45. The blood cell counts were about 95% of the original whole blood values.

Preparation of mouse fibrin-coated and human fibrin-coated coverslips

Nine volumes of blood were obtained by way of retro-orbital venous plexus sampling into 1 volume of 108 mM sodium citrate, and plasma was prepared by centrifugation of the blood at 2500g for 15 minutes. One volume of citrated mouse plasma was mixed with 7 volumes of human thrombin and CaCl₂ to final concentrations of 20 nM and 8 mM, respectively. Subsequently, 0.6 mL of that mixture was placed between 2 glass coverslips (24 × 60 mm). After 10 minutes, the coverslips were separated, and the presence of fibrin was confirmed by observation under the microscope. Coverslips containing fibrin layers were mounted in a flow chamber.²⁸ Fibrin was incubated for 30 minutes with 40 μM PPACK in TBS containing 3% BSA and then rinsed with TBS. Incubation of these fibrin layers with S2238 (0.2 mM) in HEPES buffer for 30 minutes did not result in a change in optical density measured at 405 nm, indicating that virtually all thrombin activity was neutralized.

For human fibrin formation, purified human fibrinogen and thrombin were mixed to final concentrations of 0.5 mg/mL and 10 nM, respectively, in TBS containing 3 mM CaCl₂ (final volume of 0.2 mL) and immediately transferred onto a glass coverslip (24 × 60 mm). All subsequent steps were as described for the preparation of mouse fibrin-coated coverslips. All procedures were performed at ambient temperature (20°C–22°C).

Flow experiments with mouse blood

Blood (1 mL) was collected in 20 μL heparin (1000 U/mL; unfractionated, porcine intestinal mucosa; Sigma). Prior to perfusion, 0.25 mL anticoagulated blood was mixed with 0.75 mL HEPES buffer (pH 7.45) containing 3 mM CaCl₂ and 20 U/mL heparin. Perfusion of wild-type (WT) or VWF-deficient blood over WT or VWF-deficient fibrin layers was performed at a flow rate of 125 μL/min (wall shear rate of 1000 s⁻¹) for 7 to 10 minutes at room temperature (20°C–22°C) and visualized by using video microscopy.²⁹ During the perfusion one field was recorded on videotape. At the end of the perfusion, the flow chamber was perfused with buffer at the same shear rate, and 10 fields were recorded for 2 minutes. Videotapes were analyzed off line to determine the number of stationary platelets and the number of translocating platelets on the fibrin surface.³⁰ Platelets forming transient adhesion contacts (< 30 seconds) with fibrin surface are scored as translocating platelets. Stationary adhesion was defined as cells not moving more than a single cell diameter over a 30-second period. The translocation velocity was calculated as the average distance traveled per unit time of 10 platelets/field. Data are expressed as mean values ± SEM of 3 independent experiments.

Perfusion experiments with plasma-free human blood cells

Human fibrin layers were incubated for 2 hours at room temperature with plasma-derived VWF, VWF fragments, mutated rVWF, or combinations of these proteins. Perfusion was then performed with plasma-free blood at a shear rate of 1500 s⁻¹ for 3 minutes. Surface was rinsed with HEPES buffer (pH 7.45), and the number of adherent platelets per field (14 400 μm²) was quantified as described for mouse platelets in "Flow experiments with mouse blood." Data are expressed as mean values ± SEM of 3 independent experiments.

Statistical analysis

To determine the statistical significance of differences, *P* values were obtained with a nonparametric test for 2 independent variables (Mann-Whitney test). Error bars indicate SEM.

Figure 1. VWF binding to fibrin. Increasing concentrations of fibrinogen were mixed with 10 nM thrombin for 15 minutes at 37°C on the membrane of spin-X tubes. The fibrin strands were washed, and thrombin bound to fibrin was neutralized with PPACK. ¹²⁵I-VWF (250 ng/mL) was then added to the filter cups and incubated for 2 hours at 37°C. After washing, binding percentages were determined by gamma counting of the filters and the supernatants. Error bars indicate SEM.

Results

Characterization of ¹²⁵I-VWF binding to fibrin

In an initial experiment, a fixed amount of purified human ¹²⁵I-VWF (0.25 µg/mL) was added to varying amounts of thrombin-clotted fibrinogen. Figure 1 shows that relative to total VWF, the percentage of bound ¹²⁵I-VWF increased nonlinearly with increasing fibrinogen concentrations. It was observed that when fibrinogen concentrations higher than 1.5 mg/mL were used, some retention of buffer occurred on the filter membrane, leading to ineffective washing of the fibrin. Therefore, all subsequent experiments were performed with clots prepared from incubations of 0.75 mg/mL fibrinogen with thrombin.

Figure 2 reveals no difference in the multimeric composition of purified human VWF (lane A) and that of VWF eluted from the fibrin clot (lane B). Thus, both high-molecular-weight and low-molecular-weight multimers are capable of binding to fibrin under the present experimental conditions, using a VWF concentration that is about 40-fold below the VWF plasma concentration.

Figure 3 shows the amount of fibrin-bound ¹²⁵I-VWF as a function of the concentration of free ¹²⁵I-VWF. It is apparent that the binding data fits the Langmuir model with an apparent dissociation constant of 2.2 µg/mL. The maximal binding capacity of fibrin (37.5 µg) on the filter was 76 ng VWF. Thus, under saturating conditions about 2 ng VWF binds per 1 µg thrombin-formed fibrin.

To examine the specificity of the ¹²⁵I-VWF binding to fibrin, a competition experiment was performed with nonlabeled VWF. ¹²⁵I-VWF (0.25 µg/mL) was added to amounts of nonlabeled VWF that varied from 0 to 0.2 mg/mL and incubated with fibrin present on the filter membrane. It is clearly shown in Figure 4 that nonlabeled VWF can displace nearly all fibrin-bound ¹²⁵I-VWF.

Figure 2. Multimeric composition of fibrin-bound VWF. ¹²⁵I-VWF (lane A) and SDS-eluted fibrin-bound ¹²⁵I-VWF (lane B) from 15 spin-X tubes were subjected to SDS-agarose electrophoresis. Gels were loaded with approximately 1 µg protein.

Figure 3. VWF dose dependency. Increasing amounts of ¹²⁵I-VWF were added to fibrin strands formed from 0.75 mg/mL fibrinogen (37 µg fibrinogen on filter) and 10 nM thrombin. Specific binding, calculated from total binding and nonspecific binding (in the absence of fibrin), is plotted versus the free VWF concentration. The solid line is the result of a nonlinear regression analysis that fits the data points of 3 independent experiments to a Langmuir binding model. Error bars indicate SEM.

To investigate whether plasma proteins compete with VWF for binding to fibrin, we performed serial dilutions of VWF-deficient plasma in HEPES buffer and determined the specific binding of ¹²⁵I-VWF (0.25 µg/mL). Compared with a specific binding of ¹²⁵I-VWF of 26% in the absence of VWF-deficient plasma, it was found that binding percentages varied around this value when the plasma protein concentration increased from 1% to 100%. It is concluded that other plasma proteins do not interfere with the binding of VWF to fibrin.

Identification of the fibrin binding sites on VWF

Figure 5 shows that the binding of ¹²⁵I-VWF (0.25 µg/mL) to fibrin (0.75 mg/mL) is completely abrogated in the presence of a polyclonal antibody against VWF (2.5 µg/mL), whereas polyclonal antibodies against the SpII fragment inhibited the binding of VWF dose dependently, down to 5% of the amount obtained in the absence of antibody. In contrast, in the presence of anti-SpIII (5 µg/mL), binding reached 50% of that in the absence of antibody. Next, purified VWF fragments SpII and SpIII were examined for their ability to compete with ¹²⁵I-VWF for binding to fibrin. Low concentrations of SpII effectively inhibited binding of VWF to fibrin (Figure 6). In contrast, SpIII did not appear to inhibit ¹²⁵I-VWF binding to fibrin in an efficient way. Collectively, these results imply that SpII contains VWF's major binding site for fibrin.

Competition experiments performed with ¹²⁵I-rVWF in the presence of mutated rVWF lacking the domains D4 and B (ΔD4B-rVWF) or C1 and C2 (ΔC1C2-rVWF) revealed that both rVWF and the ΔD4B-rVWF were capable of competing with ¹²⁵I-rVWF for interaction with fibrin (Figure 7). However, the ΔC1C2-rVWF hardly affected the binding of ¹²⁵I-rVWF, indicating

Figure 4. Competition of nonlabeled VWF with ¹²⁵I-VWF. ¹²⁵I-VWF (250 ng/mL) and increasing concentrations of unlabeled VWF were incubated in filters without and with fibrin (37 µg) for 2 hours at 37°C. Nonspecific binding in the absence of fibrin was subtracted from the total binding to obtain the specific binding. Results are expressed as percentages of specific binding of ¹²⁵I-VWF in the absence of nonlabeled VWF. Error bars indicate SEM.

Figure 5. Inhibition of ^{125}I -VWF binding to fibrin by polyclonal antibodies against VWF and its proteolytic fragments SpII and SpIII. Polyclonal antibodies to VWF (□), SpII (●), or SpIII (▲) were preincubated with 250 ng/mL ^{125}I -VWF before addition to filters without and with fibrin (37 μg) for 2 hours at 37°C. For each point nonspecific binding (< 3% of total binding) was subtracted from the total binding to obtain the specific binding. Results are expressed as percentages of specific binding of ^{125}I -VWF measured in the absence of antibody. Error bars indicate SEM.

Figure 7. Effect of VWF mutants on the binding of ^{125}I -VWF to fibrin. ^{125}I -WT-rVWF (250 ng/mL) and increasing concentrations of WT-rVWF (●), ΔC1C2-rVWF (▲), ΔD4B-rVWF (□), and D1746G-rVWF (Δ) were incubated on filters without and with fibrin (37 μg) for 2 hours at 37°C. For each point nonspecific binding (< 3% of total binding) was subtracted from the total binding to obtain the specific binding. Results are expressed as percentages of specific binding of ^{125}I -VWF in the absence of competitor. Error bars indicate SEM.

that this mutant lacks the fibrin binding site. In addition, Figure 7 also shows that D1746G-rVWF could compete with rVWF for binding to fibrin, showing that the primary binding site in the C domains does not require an intact RGD sequence. Moreover, neither the RGD peptide nor the disintegrin kistrin competed with VWF for binding to fibrin (data not shown).

Platelet adhesion on fibrin layers incubated with human VWF, VWF fragments, or mutated rVWF under high shear rate conditions

Incubation of fibrin layers with VWF was also evaluated for its relevance in platelet adhesion at high shear rate. Three minutes after the start of a high shear rate (1500 s^{-1}) perfusion of washed blood cells over a fibrin surface, preincubated with purified plasma-derived VWF (5 μg/mL), 379 ± 62 stationary platelets/field were measured. This is 2.6-fold higher than the control fibrin layers that were not preincubated with VWF (Figure 8A). These results confirm the role of VWF in platelet adhesion to fibrin under high shear rate conditions.

The platelet adhesion data from the perfusion experiments with SpII (0.2 mg/mL) and SpIII (0.2 mg/mL) in the presence of VWF (5 μg/mL) are in accordance with their competing potencies as established in the direct binding experiments. That is, SpIII did not affect ($P = .53$) the VWF-mediated platelet adhesion to fibrin, whereas SpII significantly ($P < .002$) reduced the number of adherent platelets to the value found in the absence of VWF (Figure 8A). Furthermore, the results of platelet adhesion experiments with fibrin layers that were incubated with SpII or SpIII in the absence of VWF are compatible with those obtained with the competition experiments. That is, fibrin layers incubated with SpII showed even a lower number of adherent platelets when compared with the control, whereas fibrin incubated with SpIII showed a partly

Figure 6. Inhibition of ^{125}I -VWF binding to fibrin by SpII and SpIII. ^{125}I -VWF (250 ng/mL) and increasing concentrations of unlabeled SpII (●) and SpIII (▲) were incubated on filters without and with fibrin (37 μg) for 2 hours at 37°C. For each point nonspecific binding (< 3% of total binding) was subtracted from the total binding to obtain the specific binding. Results are expressed as percentages of specific binding of ^{125}I -VWF in the absence of competitor. Error bars indicate SEM.

reduced platelet adhesion when compared with fibrin incubated with VWF. The effect of SpII can be explained by assuming that SpII prevents the interaction between fibrin and platelet- or plasma-derived VWF that apparently contaminates the plasma-free blood preparation. Because SpII lacks the GP Ib binding site, SpII cannot support platelet adhesion under conditions of high shear stress. SpIII, however, does contain the GP Ib binding site but apparently binds to fibrin with a lower affinity when compared with VWF.

As expected from the competition binding experiments (cf Figure 7), the mutant ΔD4B-rVWF (5 μg/mL) supported platelet adhesion, whereas the mutant ΔC1C2-rVWF (5 μg/mL) did not (Figure 8B). This finding strongly supports the notion of a functional fibrin-binding site on the C-domains of VWF. To examine whether VWF is also necessary for stationary platelet adhesion to fibrin by way of the platelet integrin $\alpha_{\text{IIb}}\beta_3$, fibrin surface was incubated with the mutant D1746G-rVWF. This VWF mutant is capable of interacting with fibrin but cannot interact with integrin $\alpha_{\text{IIb}}\beta_3$ because it lacks the RGD sequence. Platelet adhesion to fibrin incubated with D1746G-rVWF (Figure 8B) did not differ from that observed with WT-rVWF ($P = .63$).

Figure 8. Effect of VWF, VWF fragments, and VWF mutants on platelet adhesion to fibrin. (A) Human fibrin surfaces were incubated with SpII (200 μg/mL), SpIII (200 μg/mL), plasma-purified VWF (5 μg/mL), and plasma-purified VWF (5 μg/mL) in the presence of 200 μg/mL VWF fragments SpII or SpIII. (B) Human fibrin surfaces were incubated with WT-rVWF (5 μg/mL), ΔD4B-rVWF (5 μg/mL), and ΔC1C2-rVWF (5 μg/mL) and D1746G-rVWF (5 μg/mL) for 2 hours at ambient temperature. After removing nonbound material, plasma-free blood was perfused at a shear rate of 1500 s^{-1} over the fibrin surfaces for 3 minutes. Platelet adhesion data are calculated relative to the mean value obtained with fibrin that was incubated with plasma-purified VWF (A) or WT-rVWF (B). Controls are nontreated fibrin layers. Error bars indicate SEM.

Contribution of plasma-derived VWF to platelet adhesion on fibrin

We have previously reported that fibrin layers formed from recalcified, platelet-free plasma perfused at high shear rate over a surface of phospholipid and tissue factor contain sufficient amounts of VWF to support platelet adhesion.¹³ To find support for the notion that VWF adsorbs to fibrin from circulating plasma rather than is being trapped during clot formation, perfusion experiments were performed with blood and fibrin layers from wild-type and VWF-deficient mice at a shear rate of 1000 s^{-1} . During the perfusion of wild-type blood over wild-type fibrin, the number of platelets that made contact with the fibrin surface increased in time (Figure 9). The majority of these platelets showed stop-and-go movements with an average translocation velocity of $21 \pm 9 \text{ } \mu\text{m/s}$. A few adherent platelets (about 5%-10% of total) attained a stationary adhesion. The rather slow initial phase of platelet adhesion suggests that optimal platelet adhesion requires the uptake of VWF from flowing blood. To confirm this notion, wild-type fibrin was perfused with VWF-deficient blood. Under these conditions, platelets did not contact the surface, suggesting that no functional VWF remained attached to the fibrin during its preparation. To further confirm this notion, the complementary experiment was performed in which VWF-deficient fibrin was perfused with healthy blood. Like platelet adhesion on healthy fibrin, a slow increase in the number of translocating platelets (mean translocation velocity of $24 \pm 13 \text{ } \mu\text{m/s}$) was observed during the initial phase of the experiment, followed by a more rapid and linear increase in platelets that translocated on the fibrin surface (Figure 9). In addition, a few firmly adherent platelets were observed at the end of the perfusion.

Discussion

The main findings of this study are the following: (1) VWF binds specifically and in a saturable manner to fibrin, (2) the fibrin high-affinity binding site on VWF is located on the C-domain, and (3) plasma VWF binding to fibrin by way of its C-domain is essential for platelet adhesion under high shear rate conditions.

We found that VWF binds to polymerized, noncross-linked fibrin with an apparent K_d of $2.2 \text{ } \mu\text{g/mL}$. This value is lower than the K_d of $15 \text{ } \mu\text{g/mL}$ reported previously.¹⁷ The difference may be attributed to the fact that the present study examined the interaction of VWF with polymerized fibrin, whereas Loscalzo et al¹⁷ used soluble fibrin monomer covalently linked to acrylonitrile beads. Specificity of VWF binding to fibrin was confirmed in competition experiments, showing that nonlabeled VWF could displace ^{125}I -VWF bound to fibrin.

Some of the characteristics of the VWF-fibrin interaction reported here seem to contradict earlier findings. First, in contrast

Figure 9. Dependency of platelet adhesion to mouse fibrin on plasma VWF. Platelet-surface contacts are depicted as a function of perfusion time for wild-type mouse blood perfused over wild-type mouse fibrin (●), VWF-deficient mouse blood perfused over wild-type mouse fibrin (▲), and wild-type mouse blood perfused over VWF-deficient mouse fibrin (□). Perfusions were performed at a shear rate of 1000 s^{-1} . Values are mean $\pm$ SEM of 3 independent experiments. Error bars indicate SEM.

to a previous report¹⁸ we did not find any indication that fibrin binds preferentially the high-molecular-weight multimers of VWF. An explanation of this difference is probably related to difference in the source of VWF (released from endothelial cells versus plasma-derived) and binding conditions (plasma clot versus preformed fibrin). Second, whereas we found that binding of radio-labeled VWF in a plasma environment was not different from that in a buffers system, others¹⁵ reported that no binding of VWF could be detected when plasma was perfused over a fibrin surface or when immobilized fibrinogen was incubated with control plasma in an enzyme-linked immunosorbent assay. We note, however, that conditions of our binding assay differed markedly from that used by others,¹⁵ which could explain our apparent conflicting observation.

To establish the localization of the fibrin binding site on the V_I subunit (1-2050), competition experiments of ^{125}I -VWF binding were performed in the presence of unlabeled complementary SpIII and S fragments, with mutated rVWF containing deletions or single residue substitution, as well as inhibition studies in the presence of polyclonal antibodies prepared against each of these fragments. Our results point to the C domain of VWF as the major binding site for fibrin, because SpII (residues 1366-2050) was a better competitor of VWF binding to fibrin than SpIII (residues 1-1365), because binding was reduced 50% in the presence of approximately $6 \text{ } \mu\text{g/mL}$ SpII, whereas SpIII at this concentration hardly prevented the binding of VWF; (2) preincubation of VWF with a polyclonal antibody against SpII inhibited binding of VWF to fibrin by 95%, whereas anti-SpIII had very little effect; and (3) unlike $\Delta\text{D4B-rVWF}$, $\Delta\text{C1C2-rVWF}$ did not compete with r-VWF for binding to fibrin. Because the RGD sequence is present in the C domain, we verified using the D1746G-rVWF that binding did not involve RGD, a finding confirmed by the absence of inhibition of VWF binding to fibrin by an RGDS peptide or the disintegrin kistrin.

We have further substantiated the role of VWF binding to fibrin by way of its C-domain by showing its importance in platelet adhesion at a high shear rate. A 2- to 3-fold increase in platelet adhesion was measured when fibrin was preincubated with full-length rVWF or $\Delta\text{D4B-rVWF}$, whereas a preincubation with $\Delta\text{C1C2-rVWF}$ did not result in a significant increase ($P = .21$). In separate experiments, we verified that $\Delta\text{C1C2-rVWF}$ contained a functional GP Ib binding site, because both WT-rVWF and $\Delta\text{C1C2-rVWF}$ competed equally well with ^{125}I -VWF for binding to GP Ib (data not shown). The fact that after a 3-minute perfusion with plasma-free blood cells a substantial number of adherent platelets were observed on the control fibrin surface suggests that the plasma-free blood preparation contains either plasma VWF or platelet VWF that is released during the washing procedure.

It has been reported that VWF competes with fibrin for the occupancy of activated integrin $\alpha_{\text{IIb}}\beta_3$.³¹ To re-investigate the role of VWF interaction with the integrin $\alpha_{\text{IIb}}\beta_3$ in platelet adhesion at high shear rate, the VWF mutant D1746G-VWF was used. D1746G-VWF was as effective as rVWF in platelet adhesion on fibrin under conditions of high shear. This finding is compatible with the notion that the fibrin binding site on VWF does not overlap with its integrin $\alpha_{\text{IIb}}\beta_3$ binding site and, therefore, allows fibrin-bound VWF to compete with fibrin for binding to platelet integrin $\alpha_{\text{IIb}}\beta_3$.

To establish the role of plasma VWF in platelet adhesion to fibrin at high shear rates, experiments were performed with blood and fibrin from wild-type and VWF-deficient mice. Indeed, when VWF-deficient blood was perfused at high shear rate over a fibrin layer prepared from VWF-deficient plasma, no translocating or firmly adherent platelets were detected on fibrin. In contrast, numerous translocating platelets were observed when wild-type blood was perfused over wild-type fibrin. Surprisingly, the number

of surface-contacting platelets increased relatively slowly during the first minutes of the perfusion. It appeared that fibrin prepared from wild-type mouse plasma does not contain functional VWF, because no platelets adhered to this fibrin when perfused with blood from VWF-deficient mouse. As expected, a similar delay in platelet adhesion was observed when fibrin prepared from VWF-deficient mouse plasma was perfused with wild-type mouse blood. It is apparent that under high shear rate conditions optimal platelet adhesion is achieved when sufficient amounts of plasma VWF are adsorbed on fibrin.

The perfusion experiments with mouse blood and mouse fibrin also disclose that, besides increasing numbers of translocating platelets in time, the number of stationary platelets increased, as well. The finding that the majority of platelets show translocation, suggests that under the conditions of this experiment, integrin $\alpha_{IIb}\beta_3$ is not activated and, thus, refers to an earlier observation that integrin $\alpha_{IIb}\beta_3$ needs to be activated before platelets can irreversibly bind to fibrin.³²

In conclusion, this study demonstrates that the major binding site on VWF for fibrin resides in the C-domains between amino acids 1637 and 1899 at a site that does not overlap the RGD sequence. The relevance of the VWF-fibrin interaction in platelet

adhesion at a high shear rate was demonstrated in perfusion experiments with human and mouse blood over fibrin layers in the presence and absence of VWF. Collectively, our results point at a dual role of fibrin-bound VWF during thrombus growth, which might add to a better understanding of the contribution of VWF to the formation of fibrin-rich thrombi at a high shear rate.³³ We suggest that fibrin-bound VWF present at an injured vessel wall or disrupted atherosclerotic plaque may critically contribute to thrombus growth by tethering (nonactivated) platelets from fast flowing blood. Subsequently, VWF bound to fibrin would stabilize the fibrin-rich thrombus by bridging more platelets through activated integrin $\alpha_{IIb}\beta_3$. Therefore, drugs that hamper fibrin-VWF interaction could be effective antithrombotic agents, not causing hemorrhagic tendency because platelet interactions with extracellular matrix proteins would be preserved.

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Synergistic effect of thrombin on collagen-induced platelet procoagulant activity is mediated through PAR-1

Synergistic effect of thrombin on collagen-induced platelet procoagulant activity is mediated through PAR-1

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In the blood coagulation process, the rate of thrombin formation is critically dependent on the presence of phosphatidylserine (PtdSer) at the surface of activated platelets. Whereas thrombin alone is only a weak agonist to induce platelet procoagulant activity, it synergistically enhances the collagen-induced platelet procoagulant response. The objective of this study is to elucidate the mechanism of this synergistic action of thrombin with a focus on the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and the various platelet receptors for thrombin. We demonstrate that procoagulant activity is related to a sustained increased $[\text{Ca}^{2+}]_i$, rather than peak levels of $[\text{Ca}^{2+}]_i$. Increased PtdSer exposure coincides with increased $[\text{Ca}^{2+}]_i$ and was observed in a subpopulation of the platelets for each of the various agonists used. Sustained increased $[\text{Ca}^{2+}]_i$ levels appeared to depend on the influx of extracellular Ca^{2+} . Fab-fragments of the monoclonal antibody 2D2 against the thrombin binding site on GPIIb made clear that this receptor did not signal for platelet procoagulant activity. Inhibition of protease-activated receptor 1 (PAR-1) and PAR-4 by selective intracellular inhibitors and selective desensitization of these receptors revealed that PAR-1 activation is a prerequisite for both sustained elevations in $[\text{Ca}^{2+}]_i$ and procoagulant activity induced by collagen plus thrombin. It remains to be elucidated why activation of PAR-1 by thrombin, but not by the PAR-1 activation peptide SFLRN, amplifies collagen-induced procoagulant activity.

Introduction

Platelet membranes have an asymmetric distribution of phospholipids over the two membrane leaflets with aminophospholipids almost exclusively present in the cytoplasmic leaflet. This asymmetric distribution can be dissipated by the action of a phospholipid scramblase activity, resulting in surface exposure of phosphatidylserine (PtdSer). Presence of PtdSer in the exoleaflet of platelet plasma membrane is of physiological importance because it enhances thrombin formation, which is essential to the formation of a stable haemostatic plug or which contributes to the formation of stable thrombi that may occlude blood vessels.¹

Several decades ago, it became clear that a combination of collagen and thrombin is a far more potent agonist in generating procoagulant platelet surfaces than each of the individual agonists alone.² The mechanism behind this synergistic action of thrombin and collagen, however, remains to be elucidated. A key element in the process is the level of the cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$). For instance, thrombin alone is a poor platelet agonist in terms of inducing a procoagulant response, but when used together with an inhibitor of sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) a strong procoagulant response was observed that is associated with a high and sustained increase in $[\text{Ca}^{2+}]_i$.³ It has been suggested that the synergistic effect of thrombin and collagen in exposing PtdSer is based on the ability of this combination of agonists to induce a high calcium influx that persists during a sufficiently long period to stimulate scramblase that transfers PtdSer from inner leaflet to outer-leaflet and to inhibit aminophospholipid translocase that transports PtdSer from the outer leaflet to the inner leaflet.^{4,5} We hypothesize that thrombin and collagen must act together through distinct platelet receptors and signalling pathways that reinforce their individual capacities in elevating the platelet intracellular calcium level.

Glycoprotein (GP)VI is the major collagen-signalling receptor that induces platelet procoagulant activity (reviewed in ⁶). However, in spite of the increasing knowledge of receptor-related platelet activation, the contribution of the different thrombin-dependent signal pathways leading to a net transport of PtdSer to the exoleaflet is still a matter of debate. The platelet thrombin receptors, PAR-1, PAR-4 and GPIb-IX-V are all identified as potential contributors to the thrombin induced procoagulant response. Whereas, it was claimed that PAR-1 is the primary mediator of thrombin-induced procoagulant activity, thereby excluding a role for PAR-4,⁷ other reports stressed the importance of the thrombin binding site on the GPIb α chain of the GPIb-IX-V complex.⁸⁻¹⁰ The importance of PAR-1 was further emphasized in work that showed a shorter clotting time of whole blood in the presence of the PAR-1 agonist SFLRN.¹¹ An explanation for these different findings could be the neglected role of GPIb α as a cofactor in the thrombin-catalyzed activation of PAR-1.¹² Interaction of thrombin with GPIb α may enhance the efficiency of thrombin to activate PAR-1, hence lowering its concentration required to evoke platelet procoagulant activity. It has been demonstrated that GPIb α is not a cofactor in the thrombin-catalyzed activation of PAR-4.¹³ Although at high thrombin concentrations an increased and sustained $[\text{Ca}^{2+}]_i$ is induced via PAR-4,^{14,15} this thrombin receptor appears insignificant in the generation of procoagulant platelet surfaces.⁷ In one study it was shown that PAR-4 activation peptide acts synergistically with PAR-1 activation peptide in the generation of collagen-mediated procoagulant activity.¹⁶

The objective of this study is to delineate the relative contributions of PAR-1, PAR-4 and GPIb-IX-V in thrombin and thrombin plus collagen-induced platelet procoagulant activity and to further explore the relationship between increases in intracellular calcium and the generation of a procoagulant surface. We have found that the interaction of thrombin with its receptors GPIb α , PAR-1 and PAR-4

resulted in very rapid elevations of $[Ca^{2+}]_i$, but none of the interactions resulted in a significant increase in platelet procoagulant activity. Only in combination with collagen, thrombin, but not SFLLRN or AYPGKF, caused a high and sustained $[Ca^{2+}]_i$, as well as a high procoagulant activity. We also established that intracellular PAR-1 and PAR-4 antagonists, but not a MoAb against the thrombin binding site on GPIIb α , inhibited the synergistic effect of thrombin on the collagen-induced platelet procoagulant response.

Experimental Procedures

General reagents and Antibodies

Bovine serum albumin (BSA) and apyrase were from Sigma. Chromogenic substrate for thrombin (S2238) was from Chromogenix. Fibrillar HORM-type collagen was from Nycomed. Alexa Fluor 647-conjugated Annexin A5, Fura Red-AM and Fura-2-AM were from Molecular Probes. Lactadherin was a kind gift of Prof. Jan Trige Rasmussen (University of Aarhus, Denmark) and was conjugated with FITC using a standard procedure.¹⁷ Human factor Xa, human prothrombin, human thrombin and bovine factor Va, were purified as described before.¹⁸ MoAb 2D2 is directed against the N-terminal globular domain of GPIIb α and completely blocks thrombin interaction with glycosialicin (data not shown). The palmitoylated peptides pal-RCLSSSAVANRS (PAR-1 antagonist; P1pal-12^{19,20}) and pal-SGRRYGHALR (PAR-4 antagonist; P4pal-10^{19,20}) and the PAR-1 activating peptide SFLLRN-NH₂ and the PAR-4 activating peptide AYPGKF-NH₂ were prepared in our laboratory by solid-phase peptide synthesis using the *in situ* neutralization/HBTU activation procedure for tBoc chemistry as previously described.²¹

Platelet preparation

Blood (1 volume) was collected into 1/6 volume of acid/citrate/dextrose (80 mmol/L trisodium citrate, 52 mmol/L citric acid and 180 mmol/L glucose). Platelet-rich plasma (PRP) was obtained by centrifugation at 190 $\times$ g for 15 minutes. For calcium measurements platelets were loaded with Fura-2 (3 μ mol/L) or Fura-Red (20 μ mol/L) during 45 minutes at 37 $^{\circ}$ C. Platelets were then washed and finally resuspended in Hepes buffer (136 mmol/L NaCl, 2.7 mmol/L KCl, 5 mmol/L Hepes, 2 mmol/L MgCl₂, 10 mmol/L glucose, 0.1% BSA, pH 7.45), as described before.²²

Measurement of $[Ca^{2+}]_i$ in platelet suspension

Changes in $[Ca^{2+}]_i$ of Fura-2 loaded platelets were measured at 37 $^{\circ}$ C under continuous stirring by dual excitation fluorometry in an SLM-Aminco 8100 spectrofluorometer (SLM Instruments). Ratio values of fluorescence at 340 and 380 nm were converted to levels of $[Ca^{2+}]_i$ as described.²³ Experiments were performed in triplicate. Peak amplitudes of the calcium signal were defined as $\Delta[Ca^{2+}]_{max}$, representing the maximal increase in $[Ca^{2+}]_i$ with respect to $[Ca^{2+}]_i$ of non-stimulated cells. The increase in $[Ca^{2+}]_i$ with respect to $[Ca^{2+}]_i$ of non-stimulated cells after 5 minutes of activation with agonist is defined as $\Delta[Ca^{2+}]_{4-5}$.

Combined assay of $[Ca^{2+}]_i$ and PtdSer exposure in single platelets

Analyses were performed with a FACScan flow cytometer (Beckton Dickinson). Fura-Red was used to determine changes in $[Ca^{2+}]_i$. Upon calcium binding fluorescence intensity of Fura-Red decreases. Alexa Fluor 647-conjugated Annexin A5 (1:250 dilution) was used to measure PtdSer exposure. Fura-Red is excited with the argon laser tuned at 488 nm. Alexa Fluor 647-annexin A5 is

excited with the HeNe laser at 633 nm. The instrument was set to measure forward angle scattered light (FS), side angle scattered light (SS), Fura-Red (FL3, 670 nm long pass filter) and Alexa Fluor 647-annexin A5 (FL4, 661 $\pm$ 16 nm bandpass filter). Platelets (1.3 $\times$ 10⁷/mL) were activated with agonists in the presence of 3 mmol/L calcium for 5 min at 37 $^{\circ}$ C under continuous stirring (200 rpm) and then incubated for two minutes at 37 $^{\circ}$ C with FITC-lactadherin or Alexa Fluor 647-annexin A5 to reach saturation of the lactadherin and annexin A5 binding sites on the platelet surface. Platelets were identified by using analytical gates based on FS and SS log signals. To quantify PtdSer exposure of platelets in the absence of extracellular calcium, FITC-lactadherin (1:50 dilution) was used. Fluorescence was collected in FL1 through 530 $\pm$ 30 nm bandpass filter. Fluorescence intensities of 10,000 individual platelets in FL1, FL2 and FL3 was determined. Data were analysed using WinMDI software (<http://facs.scripps.edu/software.html>).

Prothrombinase assay

Platelets (1.3 $\times$ 10⁷/mL) in a volume of 50 μ L were stimulated with agonists for 5 minutes at 37 $^{\circ}$ C under continuous stirring with a small stirring bar (200 rpm). A solution (45 μ L) of factor Xa and factor Va in Hepes buffer containing 3 mmol/L Ca²⁺ was added and after 30 s thrombin generation was started by the addition of 5 μ L prothrombin. The final concentrations were: 40 pmol/L Xa, 100 pmol/L Va, 3 mmol/L CaCl₂, 100 nmol/L prothrombin and 6.5 $\times$ 10⁶ platelets/mL. Thrombin generation was stopped after 1 min by addition of Hepes buffer containing 20 mmol/L-EDTA and assayed as described.²⁴ When thrombin was used as an agonist, changes in OD at 405 nm were corrected for substrate conversion by this exogenous thrombin. The assay conditions were chosen such that the rate of thrombin formation was linear in time (up to 5 min) and linear with both the factor Xa and platelet concentration (up to 7 $\times$ 10⁶ sonicated platelets/mL). Exogenous factor Va was added, to make the rate of thrombin formation independent on release and activation of platelet factor V. A reference curve was constructed using washed platelets that were sonicated for three minutes on ice. Data are expressed as percentage of the rate of thrombin generation obtained for 6.5 $\times$ 10⁶ sonicated platelets/mL.

Statistics

To determine the statistical significance of differences, P-values were obtained with a non-parametric test for two independent variables (Mann-Whitney Test). Differences were defined significant when $P < 0.05$. Data are expressed as mean values $\pm$ SD of at least 3 independent experiments.

Results

Thrombin-induced intracellular calcium response and platelet prothrombinase activity.

Low amounts of thrombin (0–2 nmol/L) cause a rapid and transient rise in platelet $[Ca^{2+}]_i$, whereas higher thrombin concentrations (> 2 nmol/L) induce a rapid but more sustained rise in $[Ca^{2+}]_i$ (Fig. 1A). The dose-dependent differences in calcium mobilisation probably arise from differences in the kinetics of activation and desensitization of the thrombin receptors PAR-1 and PAR-4. At low thrombin concentrations, PAR-1 is more readily activated than PAR-4. However, the half-life of activated PAR-1 was reported to be shorter than that of PAR-4.^{15,25} Consequently, the more sustained Ca²⁺ flux seen at higher thrombin concentrations likely reflects the slower kinetics of

activation and inactivation of PAR-4. Indeed, when used at concentrations that gave a maximal response^{16,26}, the specific PAR-1 activating peptide SFLLRN (10 $\mu\text{mol/L}$) induced a sharp increase in $[\text{Ca}^{2+}]_i$ that was followed by an immediate and rapid decrease of $[\text{Ca}^{2+}]_i$. Platelets treated with the specific PAR-4 activating peptide AYPGKF (0.5 nmol/L), showed a similar steep rise in $[\text{Ca}^{2+}]_i$ but with a significant slower decline (Fig. 1A). Table I depicts the parameters $\Delta[\text{Ca}^{2+}]_{\text{max}}$ and $\Delta[\text{Ca}^{2+}]_{t=5}$ (defined under Experimental Procedures). $\Delta[\text{Ca}^{2+}]_{\text{max}}$ values of about 1 $\mu\text{mol/L}$ were obtained with thrombin concentrations ≥ 5 nmol/L . The $\Delta[\text{Ca}^{2+}]_{t=5}$ value increased proportionally with the thrombin concentration, reflecting the role of PAR-4 in sustaining $\Delta[\text{Ca}^{2+}]_i$ levels. Our data also reveal that thrombin induces a higher $\Delta[\text{Ca}^{2+}]_{t=5}$ than each of the specific PAR-1 and PAR-4 activation peptides. This difference might be attributed to the presence of a third thrombin receptor, namely GPIb-IX-V. To study the participation of GPIb α , we made use of Fab-fragments of 2D2 MoAb (2D2-Fab). 2D2 MoAb and 2D2-Fab completely inhibited low dose (0.2-0.6 nmol/L) α -thrombin-induced platelet aggregation and had no inhibitory effect on platelet aggregation induced by higher α -thrombin concentrations (2 nmol/L). Aggregation by ADP, collagen, ristocetin, botrocetin or the PAR-1 agonist SFLLRN was unaffected by MoAb 2D2 (data not shown). 2D2-Fab (10 $\mu\text{g/mL}$) inhibited the thrombin (2 nmol/L)-induced $\Delta[\text{Ca}^{2+}]_{\text{max}}$ with 40%, but not $\Delta[\text{Ca}^{2+}]_{t=5}$. Because higher concentrations of 2D2-Fab did not result in a more reduced calcium flux, it is unlikely that the affinity of 2D2-Fab for GPIb α is too low to compete with thrombin (2 nmol/L) for the binding site on GPIb α (data not shown). When platelets were stimulated with 0.5 nmol/L thrombin in the presence of 2D2-Fab (10 $\mu\text{g/mL}$), we found a 70% reduced $\Delta[\text{Ca}^{2+}]_{\text{max}}$ and a 40% reduced $\Delta[\text{Ca}^{2+}]_{t=5}$ value. It is apparent that the relative contribution of GPIb α in the calcium response increases with decreasing thrombin concentrations. The capacity of thrombin-treated platelet suspensions to support prothrombin activation by the factor Xa-factor Va complex is plotted in Fig. 1B and 1C as a function of $\Delta[\text{Ca}^{2+}]_{\text{max}}$ and $\Delta[\text{Ca}^{2+}]_{t=5}$, respectively. It is clearly shown that while $\Delta[\text{Ca}^{2+}]_{\text{max}}$ and $\Delta[\text{Ca}^{2+}]_{t=5}$ values increase with thrombin concentrations above 0.5 nmol/L , prothrombinase activity (closed circles) does not increase more than 2-fold ($P < 0.05$) compared to resting platelets (closed square). PAR-1 and PAR-4 activation peptides SFLLRN (open square) and AYPGKF (open triangle) were unable to generate procoagulant platelet surfaces. Both SFLLRN and AYPGKF generate $\Delta[\text{Ca}^{2+}]_{\text{max}}$ values that are higher than the threshold thrombin concentration of 0.5 nmol/L . Furthermore, AYPGKF, but not SFLLRN, produces

Figure 1: Effect of thrombin, SFLLRN and AYPGKF on platelet $[\text{Ca}^{2+}]_i$ responses and platelet supported prothrombinase activity (PPA). Fura-2 loaded washed platelets were incubated with thrombin (0.2-10 nmol/L), SFLLRN (10 nmol/L) and AYPGKF (0.5 nmol/L). Changes in $[\text{Ca}^{2+}]_i$ during stimulation under static conditions at 37 °C are depicted in panel A. The calcium responses represented by $[\text{Ca}^{2+}]_{\text{max}}$ (panel B) and $[\text{Ca}^{2+}]_{t=5}$ (panel C) were plotted versus corresponding prothrombinase activity expressed as percentage with respect to sonicated platelets (details see Experimental Procedures). The symbols indicate unstimulated platelets (■), platelets activated with SFLLRN (□)

and $\Delta[\text{Ca}^{2+}]_{t=5}$ values comparable with 0.5 nmol/L thrombin. These data indicate that neither SFLLRN nor AYPGKF mimics thrombin with respect to generation of procoagulant platelet membranes, in spite of relatively high calcium responses. Because of the demonstrated participation of GPIb α in the thrombin (≤ 0.5 nmol/L)-induced calcium response, platelet procoagulant activity was also measured under the same conditions but in the presence of 2D2-Fab (10 $\mu\text{g/mL}$). GPIb α seems not involved in platelet procoagulant activity induced by low thrombin concentrations, since the blocking 2D2-Fab does not inhibit (data not shown).

Thrombin receptor-mediated calcium flux synergistically enhances collagen-receptor mediated generation of platelet procoagulant activity. Collagen induces a rise in $[\text{Ca}^{2+}]_i$ via a GPVI-mediated pathway²⁷, which appears to be associated with an increase in platelet procoagulant activity.²⁸ Whereas thrombin alone

Table I. Changes in intracellular calcium peak values $[\text{Ca}^{2+}]_{\text{max}}$ and intracellular calcium concentration after 5 min incubation $[\text{Ca}^{2+}]_{t=5}$ with thrombin, SFLLRN and AYPGKF

Agonist	Concentration	$\Delta[\text{Ca}^{2+}]_{\text{max}}$ (nmol/L)	$\Delta[\text{Ca}^{2+}]_{t=5}$ (nmol/L)
Thrombin	0.2 nmol/L	159±22	34±4
	0.5 nmol/L	286±121	65±18
	2 nmol/L	544±30	229±70
	5 nmol/L	917±111	290±34
	10 nmol/L	988±26	360±21
SFLLRN	10 $\mu\text{mol/L}$	1002±36	36±1
AYPGKF	500 $\mu\text{mol/L}$	450±75	71±11

Table II. Changes in intracellular calcium peak values $[Ca^{2+}]_{max}$ and intracellular calcium concentration after 5 min incubation $[Ca^{2+}]_{t=5}$ with collagen plus either thrombin, SFLLRN or AYPGKF.

Agonist	Concentration	$\Delta[Ca^{2+}]_{max}$ (nmol/L)	$\Delta[Ca^{2+}]_{t=5}$ (nmol/L)
Collagen	10 μ g/ml	441 $\pm$ 4	198 $\pm$ 33
plus thrombin	0.2 nM	441 $\pm$ 123	265 $\pm$ 44
	0.5 nM	484 $\pm$ 51	374 $\pm$ 41
	2 nM	589 $\pm$ 94	459 $\pm$ 21
	5 nM	718 $\pm$ 20	549 $\pm$ 27
	10 nM	748 $\pm$ 6	626 $\pm$ 71
plus SFLLRN	10 μ M	829 $\pm$ 136	227 $\pm$ 48
plus AYPGKF	500 μ M	598 $\pm$ 142	265 $\pm$ 58

hardly induces procoagulant activity, it acts synergistically in combination with collagen, but the precise mechanism of this synergism remains to be elucidated. We therefore investigated the effect of increasing thrombin concentrations on collagen-mediated $[Ca^{2+}]_i$ changes and generation of platelet procoagulant activity. In the absence of thrombin, collagen (10 μ g/ml) causes a modest, but rather sustained $[Ca^{2+}]_i$ level (Fig. 2A, bottom trace). Low concentrations of thrombin greatly increase the collagen-mediated initial rate of the calcium flux. In addition, thrombin dose dependently increases the sustained $[Ca^{2+}]_i$ levels (Table II). Because of the relatively short half-life of both activated PAR-1 and PAR-4, this synergistic action of thrombin as demonstrated by sustained and elevated calcium levels, is remarkable. Neither SFLLRN nor AYPGKF in combination with collagen could induce a similar sustained calcium influx (Fig. 2A). Fig. 2B and 2C depict the capacity of thrombin plus collagen activated platelets to support prothrombinase activation as a function of $\Delta[Ca^{2+}]_{max}$ and $\Delta[Ca^{2+}]_{t=5}$, respectively. In the absence of thrombin and compared with non-treated platelets, collagen generates a five times higher prothrombinase activity, while in the presence of thrombin concentrations ≥ 0.5 nmol/L a 15-fold increase was found (Fig. 2B). While this increase is associated with a significant increase in $\Delta[Ca^{2+}]_{t=5}$, the $\Delta[Ca^{2+}]_{max}$ is not affected when the thrombin concentration is increased from 0 to 0.5 nmol/L. This strongly suggests that $\Delta[Ca^{2+}]_{t=5}$ but not $\Delta[Ca^{2+}]_{max}$ is closely related to the platelet procoagulant response. Increasing the thrombin concentration above 0.5 nmol/L significantly increased $\Delta[Ca^{2+}]_{t=5}$ and $\Delta[Ca^{2+}]_{max}$, but did not further increase procoagulant activity.

SFLLRN increased the $\Delta[Ca^{2+}]_{max}$ of collagen-induced calcium response ($P < 0.05$) and increased prothrombinase activity 1.5-fold ($P = 0.05$) (Fig. 2B, open square). The PAR-4 activation peptide AYPGKF had less effect on $\Delta[Ca^{2+}]_{max}$ and prothrombinase activity (Fig. 2B, open triangle). Interestingly, even at saturating concentrations of SFLLRN and AYPGKF, the $\Delta[Ca^{2+}]_{t=5}$ values remained below the value found with thrombin (0.5 nmol/L) plus collagen.

Calcium influx and generation of procoagulant platelet surfaces in individual cells

Our interpretations, thus far, did not take into account the possibility that treatment of platelets in suspension with collagen plus thrombin results in the appearance of subpopulations with different calcium fluxes and variable extent of PtdSer exposure.^{5,29,30} Flow cytometry analysis (Fig. 3) showed that treatment of platelets with the calcium ionophore, ionomycin, yielded a single population of annexin A5 positive platelets which all showed an increased

$[Ca^{2+}]_i$ level. Stimulation of platelets with collagen (10 μ g/mL) plus thrombin (0.5 nmol/L) resulted in two populations: one large population of annexin A5 negative platelets, low in $[Ca^{2+}]_i$, and a small population with increased $[Ca^{2+}]_i$ that did bind annexin A5. In addition, Fig. 3 shows that in platelets activated with thrombin or collagen alone, the subpopulation of platelets that bind annexin A5 and have increased $[Ca^{2+}]_i$ is substantially smaller when compared to collagen plus thrombin activated platelets. Table III shows the distribution of high and low $[Ca^{2+}]_i$ in annexin A5 positive platelets for the different agonists. These data indicate that the generation of procoagulant platelet surfaces, probed by annexin A5 binding, is closely associated with a sustained high $[Ca^{2+}]_i$. It should be emphasized that the $[Ca^{2+}]_i$ levels and annexin A5 binding

Figure 2: Effect of collagen plus thrombin, SFLLRN and AYPGKF on platelet $[Ca^{2+}]_i$ responses and platelet supported prothrombinase activity. Fura-2 loaded washed platelets were incubated with collagen (10 μ g/mL) in the presence of thrombin (0.2–10 nmol/L), SFLLRN (10 μ M) and AYPGKF (0.5 μ M). Changes in $[Ca^{2+}]_i$ during stimulation under stirring conditions at 37 °C are depicted in panel A. The calcium responses represented by $[Ca^{2+}]_{max}$ (panel B) and $[Ca^{2+}]_{t=5}$ (panel C) were plotted versus changes in $[Ca^{2+}]_i$ during stimulation under stirring conditions at 37 °C are depicted in panel A. The calcium responses represented by $[Ca^{2+}]_{max}$ (panel B) and $[Ca^{2+}]_{t=5}$ (panel C) were plotted versus corresponding prothrombinase activities, expressed as percentage with respect to sonicated platelets (for details see Experimental Procedures). The symbols indicate unstimulated platelets (■), platelets activated with collagen plus SFLLRN (□), collagen plus AYPGKF (Δ) and collagen plus thrombin at the indicated concentrations (●). Values are means and SD of three independent experiments.

Figure 3: Relation between $[Ca^{2+}]_i$ and PtdSer exposure in single platelets. Washed, Fura-Red loaded platelets were activated during 5 min at 37 °C under continuous stirring with ionomycin (5 mmol/L), thrombin (0.5 nmol/L), collagen (10 mg/ml) and collagen plus thrombin in the presence of $CaCl_2$ (3 mM). $[Ca^{2+}]_i$ and PtdSer exposure were analyzed by flow cytometry as described under Experimental Procedures. Shown is one representative experiment out of four performed. Quadrants were chosen so that >97% of control platelets was present in the lower-right quadrant.

Table III. Distribution of annexin A5 positive platelets with high and low $[Ca^{2+}]_i$.

Agonist	Annexin A5 binding (% of total)*	
	High $[Ca^{2+}]_i$ **	Low $[Ca^{2+}]_i$ ***
Thrombin	1.2±0.3	0.2±0.1
Collagen	2.7±0.1	0.3±0.2
Collagen plus thrombin	12.2±1.3	0.7±0.2
Ionomycin	87.9±1.4	2.4±0.4

* Corrected for control platelets. Values were taken from corresponding dot plots shown in Fig. 3

** Upper-left quadrant in Fig. 3

*** Upper-right quadrant in Fig. 3

were measured 5 min after the addition of the agonist. Thus, the apparent relationship between high calcium levels and annexin A5 binding is only true for those platelets that are not transient in their calcium response.

The relative contributions of thrombin receptors to the collagen plus thrombin-stimulated generation of procoagulant platelet surfaces.

The relative contribution of thrombin-activated PAR-1 and PAR-4 to the thrombin plus collagen stimulation of platelet procoagulant activity was investigated by using the intracellular PAR-1 and PAR-4 antagonists, P1pal-12 and P4pal-10, at a concentration (5 μ mol/L) that was sufficient to completely inhibit the calcium response induced with 0.5 nmol/L thrombin. P1pal-12 and P4pal-10 decreased the thrombin plus collagen induced $\Delta[Ca^{2+}]_{max}$ (Fig. 4A) and $\Delta[Ca^{2+}]_{5}$ values (Fig. 4B). $\Delta[Ca^{2+}]_{max}$ values were reduced to the level of that induced by collagen alone. The collagen-induced calcium response was not inhibited by P1pal-12 and P4pal-10 (data not shown).

Fig. 4C shows that the inhibition of the thrombin induced calcium flux is associated with a decreased ability of the stimulated platelets to support prothrombin activation. Both P1pal-12 and P4pal-10 reduce the prothrombinase activity to the level of that induced by collagen alone. It should be emphasized that P1pal-12 and P4pal-10 have an inhibiting effect on prothrombinase as such, as measured with vesicles of 20 mol% PtdSer and 80 mol% PtdCho (data not shown). These data suggest that both the PAR-1 and PAR-4 receptors are involved in the synergistic stimulation of platelets by collagen and thrombin. However, P4pal-10 has been reported to inhibit platelet functions also signal through activated PAR-1.^{19,20}

In an attempt to further delineate the role of PAR-1 and PAR-4, experiments were performed with PAR-1 and PAR-4 desensitized platelets. Desensitization of the receptors was achieved by incubation of platelets with SFLLRN (10 μ M) or AYPGKF (500 μ M) and was confirmed by the inability of these platelets to increase $[Ca^{2+}]_i$ when challenged again with SFLLRN or AYPGKF (data not shown). When PAR-1 desensitized platelets were challenged with collagen (10 μ g/ml) plus thrombin (0.5 nmol/L), prothrombinase activity was not higher than that of collagen stimulated platelets (Fig. 4C). In contrast, PAR-4 desensitization had no significant effect on the thrombin

Figure 4: Involvement of thrombin receptors GPIIb/IIIa, PAR-1 and PAR-4 in calcium and procoagulant responses induced by collagen plus thrombin. Washed platelets were pre-incubated during 10 min under continuous stirring with P1pal-12 (5 mM), P4pal-10 (5 mM), SFLLRN (10 mM), AYPGKF (0.5 mM) or 2D2-Fab (10 mg/ml) before addition of $CaCl_2$ (3 mM) and collagen (C) (10 mg/ml) plus thrombin (T) (0.5 nmol/L). Activation with agonist was done during 5 min at 37 °C under continuous stirring. Calcium signals were analysed for $[Ca^{2+}]_{max}$ (panel A) and $[Ca^{2+}]_5$ (panel B). Prothrombinase activity (panel C) was measured after 5 min and expressed as percentage with respect to the activity measured with sonicated platelets. Values are means and SD of three independent experiments. *Significantly increased with respect to collagen ($P < 0.05$).

plus collagen-induced generation of platelet procoagulant activity.

As reported in a previous section, blocking the interaction of 0.5 nmol/L (or less) thrombin with GPIIb/3 with the 2D2-Fab significantly reduced the thrombin-induced calcium flux. The calcium response of platelets that were stimulated with collagen plus thrombin was not inhibited by 2D2-Fab (Fig. 4A and B). Also, the generation of platelet procoagulant surfaces was not inhibited by 2D2-Fab. These findings suggest that thrombin-GPIIb/3 interaction is not required for the synergistic effect of thrombin on the collagen-induced procoagulant response.

It is becoming increasingly apparent that collagen-mediated platelet activation is enforced by secreted ADP.^{31,32} From the impaired prothrombinase activity in collagen plus thrombin stimulated platelets from patients with storage pool deficiency, it was concluded that secreted ADP may play an important role in the generation of prothrombinase activity by contributing to the maintenance of a critical $[Ca^{2+}]_i$.³³ We confirm that also under the controlled conditions of our calcium flux and prothrombinase assays, secreted ADP reinforces the platelet calcium response and prothrombinase activity induced by thrombin, collagen and the combination of thrombin and apyrase. In spite of the presence of apyrase, the generation of procoagulant activity by collagen plus thrombin was not affected by 2D2-Fab³ ($P=0.85$), further emphasizing that GPIIb/3-thrombin interaction does not contribute to the thrombin plus collagen induced procoagulant platelet response.

Thrombin-mediated influx of extracellular calcium enhances collagen-induced procoagulant response

To establish the role of extracellular calcium influx in the collagen plus thrombin induced generation of procoagulant platelet surfaces, $[Ca^{2+}]_i$ changes and platelet prothrombinase activity were determined in the presence of

Figure 5: Calcium signals of platelets activated with collagen, thrombin and collagen plus thrombin in the absence of extracellular calcium and after recalcification. Fura-2 loaded platelets were activated in the absence of extracellular calcium with 0.5 nM thrombin (T), 10 mg/ml collagen (C) or a combination of both agonists (C+T). Five minutes after activation 1 mM $CaCl_2$ was added. Shown is one representative experiment out of three performed.

Table IV. Effect of extracellular calcium on generation of platelet prothrombinase activity (PPA) and changes in intracellular calcium peak values $[Ca^{2+}]_{max}$ and intracellular calcium concentration after 5 min incubation $[Ca^{2+}]_{t=5}$ with thrombin, collagen and collagen plus thrombin.

Agonist	$\Delta[Ca^{2+}]_{max}$ (nmol/L)		$\Delta[Ca^{2+}]_{t=5}$ (nmol/L)		Prothrombinase activity (%)*	
	Ca^{2+}	EGTA	Ca^{2+}	EGTA	Ca^{2+}	EGTA
Thrombin	379±28	146±35	84±5	0±0	4.6±0.9	2.5±0.8
Collagen	373±84	122±18	195±16	9±3	9.7±2.9	7.6±2.1
Collagen and Thrombin	491±69	183±15	383±83	19±11	26.4±2.6	10.1±1.2

* Prothrombinase activity is expressed as % of maximal, which was obtained with sonicated platelets

EGTA. The calcium flux in the presence of EGTA was greatly reduced. $\Delta[Ca^{2+}]_{max}$ and $\Delta[Ca^{2+}]_{t=5}$ values were 491±69 and 383±83 in the presence of 1 mmol/L Ca^{2+} and 183±15 and 19±11 in the presence of 1 mmol/L EGTA. These reductions were also observed when platelets were activated with thrombin or collagen alone (Table IV). Interestingly, there was no significant reduction in collagen induced procoagulant activity in the absence of extracellular calcium, in spite of strongly reduced $\Delta[Ca^{2+}]_{max}$ and $\Delta[Ca^{2+}]_{t=5}$. The ability of thrombin plus collagen-activated platelets to support prothrombin activation was greatly reduced when platelet stimulation was performed in the presence of EGTA. In the absence of extracellular calcium the synergistic effect of thrombin seems to disappear (Table IV). In this respect it is of interest to note that the procoagulant activity assay is performed in the presence of extracellular calcium (3 mmol/L) and, as shown in Fig. 5, addition of calcium to platelets, stimulated in the presence of EGTA, caused a rapid and strong increase in $[Ca^{2+}]_i$ which could have resulted in an additional exposure of PtdSer and thus in an overestimation of the platelet procoagulant activity.

To investigate the effect of recalcification on expression of PtdSer, we conducted the following experiment. Platelets (1.3×10^7 /ml) were stimulated for 5 min with thrombin plus collagen in the presence of EGTA. At 5 min, a sample was taken from the activation mixture, diluted 1:10 and supplied with FITC-lactadherin, a calcium-independent anionic phospholipid binding protein.¹⁷ Five minutes after the start of the reaction, extracellular calcium was added (3 mmol/L) and PtdSer exposure was determined again with FITC-lactadherin. The percentage of lactadherin positive cells before ($16.3 \pm 4.5\%$, $n=3$) and 1.5 min after the addition of extracellular calcium ($19.7 \pm 4.2\%$, $n=3$) did not differ significantly, indicating that in case of thrombin plus collagen stimulated platelets influx of extracellular calcium did not further increase PtdSer exposure in the outer leaflet of the platelet membrane.

Discussion

The importance of procoagulant platelet surfaces for normal hemostasis is evident from the relatively severe bleeding tendency of patients that have impaired platelet prothrombinase activity.³³⁻³⁵ It is generally assumed that platelets contribute to the generation of thrombin by providing a procoagulant surface through the rearrangement of their membrane phospholipids resulting in the exposure of negatively charged PtdSer in the exo-leaflet of the platelet plasma membrane and that such a rearrangement is initiated by an increase in the intracellular calcium concentration.^{3,33} In spite of several studies on the relationship between

intracellular calcium concentration and generation of platelet procoagulant activity and the platelet thrombin receptors that are involved, a number of questions are still unanswered. For example, in the presence of collagen, thrombin becomes a much more potent inducer of platelet procoagulant activity. Whether this synergistic effect is related to changes in intracellular calcium and to what extent the thrombin receptors GPIb-V-IX, PAR-1 and PAR-4 are involved remains to be elucidated.

A transient increased $[Ca^{2+}]_i$ is not associated with the generation of a procoagulant surface

We found that a transient increase of $[Ca^{2+}]_i$ induced by thrombin resulted in a relatively small increase in platelet prothrombinase activity of about 2-fold. In addition, saturating amounts of the thrombin receptor agonists SFLLRN and AYPGKF also transiently increased $[Ca^{2+}]_i$, but did not affect prothrombinase activity. Thus, at this point, the generation of platelet procoagulant activity could not be related to the changes in $[Ca^{2+}]_i$ according to peak values ($\Delta[Ca^{2+}]_{max}$) and persistence ($\Delta[Ca^{2+}]_{t=5}$). While collagen alone increased prothrombinase activity 5-fold, saturating levels of thrombin caused an additional 3-fold increase. Again, compared with thrombin, saturating levels of SFLLRN and AYPGKF were unable to enhance the collagen-induced generation in platelet procoagulant activity. Thrombin, SFLLRN and AYPGKF, had a strong additional effect on collagen-induced $\Delta[Ca^{2+}]_{max}$. Increasing concentrations of thrombin, but not SFLLRN and AYPGKF, greatly increased $\Delta[Ca^{2+}]_{t=5}$. These findings suggest that the generation of platelet procoagulant activity requires sustained high $[Ca^{2+}]_i$ values and that a rapid, but transient increase during 1–2 minutes is not sufficient. This notion is in agreement with the observations of Dachery-Prigent et al.³⁶ using flow cytometry to establish a relationship between Ca^{2+} fluxes and PtdSer exposure. However, our data are in disagreement with those reported by Andersen et al.⁷ These investigators speculated in their studies with PAR activation peptides that transient $[Ca^{2+}]_i$ peak values of 0.8 $\mu\text{mol/L}$ and higher are required to maximally generate platelet procoagulant activity. It should however be noted that their conclusion was based only on calcium peak values of single concentrations thrombin, SFLLRN and AYPGKF, while we analysed both $\Delta[Ca^{2+}]_{max}$ and $\Delta[Ca^{2+}]_{t=5}$ of SFLLRN, AYPGKF and increasing thrombin concentrations in the presence and absence of collagen. We found that even in the presence of collagen, in spite of high $\Delta[Ca^{2+}]_{max}$ values, SFLLRN and AYPGKF did not generate platelet procoagulant activity. The synergistic effect of thrombin was already maximal at 0.5 nmol/L giving rise to a $\Delta[Ca^{2+}]_{t=5}$ between 0.3 and 0.4 $\mu\text{mol/L}$. Thus, in spite of increasing $\Delta[Ca^{2+}]_{max}$ and $\Delta[Ca^{2+}]_{t=5}$ values with increasing thrombin concentrations above 0.5 nmol/L, the procoagulant activity did not further increase.

Thrombin receptors and their role in the generation of platelet procoagulant activity.

The marked difference in both calcium and prothrombinase responses induced by the different thrombin receptor agonists suggests a differential involvement of thrombin on the one hand and PAR activation peptides on the other hand. The apparent key element appears to be the ability of the agonist to maintain sufficient elevated $[Ca^{2+}]_i$ for at least 5 min. We clearly demonstrated that when PAR-1 signalling is inhibited, collagen plus thrombin-induced calcium signal and generation of platelet procoagulant activity are reduced to the level of that obtained with collagen alone. This probably also explains why a relatively low thrombin

concentration of 0.5 nmol/L was already sufficient to obtain maximal prothrombinase activity, since PAR-1, but not the lower affinity receptor PAR-4, is readily activated by this thrombin concentration. We note that this concept is in contrast with recently published work¹⁶ suggesting that a combination of PAR-1 and PAR-4 activation peptides is as active as thrombin to enhance collagen-induced generation of platelet procoagulant activity. Yet, in that study, the differences between collagen induced procoagulant activity in the absence and presence of SFLLRN and AYPGKF were only marginal and therefore its significance can be questioned. We also measured the platelet prothrombinase activity induced by SFLLRN plus AYPGKF in the presence and absence of collagen. It was found that this combination of PAR-1 and PAR-4 agonists did not significantly enhance the platelet procoagulant response when compared with that of thrombin (data not shown).

Unlike thrombin, SFLLRN and AYPGKF do not interact with the high affinity thrombin receptor GPIb α . Differences in the response of these agonists are therefore likely to reflect the interaction of thrombin with GPIb α .^{8,10,37–39} In a recent study by Liu et al.,⁴⁰ no evidence was found for a role of GPIb α in thrombin mediated platelet activation. Using 2D2-Fab directed against the thrombin exosite II binding site on GPIb α , we confirmed that this receptor is involved in calcium signaling and platelet aggregation induced by low thrombin concentrations (≤ 0.5 nmol/L). However, saturating amounts of the 2D2-Fab did not affect thrombin or thrombin plus collagen-induced generation of procoagulant activity, excluding a significant role of thrombin-GPIb α interaction in this particular platelet function.

Weiss and Lages³³ established that platelets from patients with storage pool deficiency showed a diminished calcium and procoagulant response upon activation with collagen plus thrombin. These investigators disclosed that the decrease in calcium mobilisation and the impaired prothrombinase activity could be corrected by the addition of ADP after stimulation. It is further revealed that the P_2Y_{12} receptor is likely involved in the ADP-induced exposure of PtdSer.^{16,41} We confirmed that when platelets were activated with collagen and/or thrombin in the presence of apyrase, calcium influx and prothrombinase activity decreased about 25%. This indicates that in addition to PAR-1, ADP release is required to obtain maximal PPA in response to collagen plus thrombin. Notably, other platelet activation processes like calcium responses and aggregation, induced via the collagen receptor GPVI or the thrombin receptor PAR-1, but not PAR-4, are known to be dependent on ADP release.^{13,42}

In view of a recent finding⁴³ that the role of GPIb α in the activation of the GTPase Rap1B is masked by ADP receptor signaling, we examined whether this is also true for the function of GPIb α in generating platelet procoagulant activity. The finding that platelets in the presence of the ADP scavenger apyrase responded equally in the thrombin plus collagen-induced generation of platelet procoagulant activity, either in the presence or absence of 2D2-Fab, however excludes the possibility that under the conditions of our experiments signaling via GPIb α remains unnoticed because of ADP receptor signaling.

Platelets are heterogeneous with respect to calcium and procoagulant response.

Earlier published work demonstrated a heterogeneity among individual platelets in responding to agonist-induced increases in $[Ca^{2+}]_i$ and PtdSer exposure.^{5,36,46} Thus far, our observations were made with platelet suspensions and could therefore be subjected to erroneous interpretations when

only a small fraction of the whole platelet population responds. Dachary-Prigent et al.³⁶ specifically demonstrated that after ten minute activation under non-stirring conditions with collagen (20 µg/ml) and thrombin (10 nmol/L) only a small subpopulation of the platelets exposed PtdSer and that all these platelets had an elevated $[Ca^{2+}]_i$. We have performed the same experiments but at much lower thrombin concentrations. In addition, our measurements were performed under stirring conditions, which appears to be necessary for optimal collagen induced calcium signalling and prothrombinase activity.³³ We found that after 5 min stimulation with collagen (10 µg/ml) or thrombin (0.5 nmol/L) the percentage of annexin A5-binding platelets with elevated calcium levels is about 1-3%. When platelets are activated with collagen plus thrombin the percentage of these platelets increased to about 12% of the total population. Because these increases correlated nicely with the increase in procoagulant activity measured in platelet suspension, we conclude that only a small fraction of thrombin plus collagen treated platelets is responsible for coagulation amplification. We also note an excellent relationship between the percentage of single platelets with elevated $[Ca^{2+}]_i$ after 5 min activation with collagen and thrombin as measured by flow cytometry and the $[Ca^{2+}]_{i=5}$ values measured in platelet suspensions under the same conditions. This again confirms that sustained elevations in $[Ca^{2+}]_i$ are required for platelets to loose their phospholipid membrane asymmetry. Further studies are necessary to determine why only a specific subpopulation of platelets is able to maintain high $[Ca^{2+}]_i$ and expose PtdSer after activation with collagen plus thrombin. A likely explanation could be that older platelets become less responsive due to reduced receptor functioning.

Influx of extracellular calcium is essential to generate platelet procoagulant activity.

A number of studies have indicated that influx of extracellular calcium is necessary for PtdSer exposure.^{33,47,48} The mechanism by which this influx is regulated remains to be elucidated. In view of the rapid desensitization of PAR-1 and the need of a sustained high $[Ca^{2+}]_i$, we examined the temporal relationship between extracellular calcium concentration and generation of platelet procoagulant activity. When platelets were challenged with collagen, thrombin or a combination of both in the absence of extracellular calcium, $[Ca^{2+}]_i$ slightly increased and calcium levels rapidly returned to those of resting platelets (Fig. 5), indicating that extracellular calcium influx is required to obtain sustained $[Ca^{2+}]_i$. When the procoagulant activities of platelets that were treated with collagen, thrombin and collagen plus thrombin in the presence or absence of extracellular Ca^{2+} are compared, it becomes clear that the absence of extracellular calcium does not affect significantly the collagen-induced procoagulant response ($P=0.25$) but reduced the collagen plus thrombin-induced response significantly to that of collagen alone ($P<0.01$). The combined findings suggest a stronger extracellular Ca^{2+} -dependency for the collagen plus thrombin-induced procoagulant response than for the collagen-induced response. The notion that collagen might stimulate platelet procoagulant activity in a calcium independent manner is supported by a recent study demonstrating that the GPVI/FcγR3/SLP-76 signal pathway, essential for calcium signalling, is not essential for platelet procoagulant activity induced by collagen.⁴⁹ These investigators showed that fibrillar collagen, but not the GPVI specific agonist convulxin, stimulates procoagulant activity in murine SLP-76 null platelets. From these findings it is tempting to

speculate that there is redundancy of GPVI/calcium-dependent⁵⁰ and calcium-independent pathways contributing to collagen induced platelet procoagulant activity. We conclude that influx of extracellular calcium is necessary to obtain a sufficiently high sustained $[Ca^{2+}]_i$ for a maximal procoagulant response and that of the two agonists collagen plus thrombin, thrombin is the essential determinant.

The validity of the interpretations of our findings could be questionable when the extracellular calcium that was added to perform the prothrombinase measurements induces the generation of platelet procoagulant activity. If true, platelets stimulated in the presence of EGTA are expected to have initially lower procoagulant activity values. However, determination of PtdSer exposure using the calcium-independent PtdSer binding protein lactadherin by flow cytometry, prior to and after recalcification of platelet suspensions treated with thrombin plus collagen, revealed no differences in the amount of bound lactadherin, suggesting that the influx of extracellular calcium under the conditions of the prothrombinase assay did not increase platelet procoagulant activity. We can think of several reasons why the rapid increase of $[Ca^{2+}]_i$ caused by the influx of extracellular calcium does not result in an additional procoagulant surface generation. Firstly, although the temporal relationship between changes in $[Ca^{2+}]_i$ and exposure of PtdSer cannot be easily made because of the relatively slow binding kinetics of PtdSer-binding proteins like annexin A5,⁵ it is evident that the generation of platelet procoagulant activity is a delayed response to a sudden increase of $[Ca^{2+}]_i$. It is feasible that a more prolonged incubation period than used in the prothrombinase assay is required to measure a significant increase in platelet procoagulant activity. However, analysis of FITC-labeled lactadherin-binding by a flow cytometric time drive during 5 min after recalcification of the platelet suspension failed to demonstrate an increase in lactadherin binding. Secondly, it is an interesting thought that during the incubation of platelets with collagen plus thrombin, PAR-1 became desensitized, which in turn prevents somehow the preservation of a high thrombin-mediated sustained $[Ca^{2+}]_i$ level.

In conclusion, treatment of platelets with collagen plus thrombin causes a sustained increase in $[Ca^{2+}]_i$ in 12% of the whole platelet population. Only these platelets had exposed PtdSer at their cell surfaces and supported prothrombin activation. The sustained elevation in $[Ca^{2+}]_i$ is the result of an increased influx of extracellular calcium, which in turn is the result of a thrombin-dependent signaling pathway mediated through PAR-1 activation. The proposed function of PAR-1 is apparently incompatible with its rather short half-life.⁵¹ We speculate that the interaction of collagen with GPVI alters in some way the kinetics of inactivation of PAR-1. It is also possible that thrombin, via PAR-1, stimulates collagen receptor signaling. Since it has been suggested that lipid rafts orchestrate signaling by platelet collagen receptor GPVI⁵³, thrombin could stimulate recruitment of GPVI to lipid rafts. It remains to be elucidated why PAR-1 activation by thrombin, but not by PAR-1 activation peptide SFLLRN, mediates collagen and thrombin induced platelet procoagulant activity. In contrast to the PAR-1 activating peptide SFLLRN, thrombin is a rather a-specific protease of multiple substrates. Besides cleaving the N-terminal region of PAR-1 resulting in the release of a 41-amino acid peptide, it also cleaves GPV at the platelet surface. Although the exact roles of the 41-amino acid peptide and cleaved GPV in hemostasis are largely unknown, both cleavage products were shown to positively mediate platelet adhesion and activation

reactions.⁵²⁻⁵⁴ Therefore, it is tempting to hypothesize that in addition to PAR-1 signalling, other thrombin proteolytic actions are crucial in enhancing the collagen-induced generation of procoagulant platelet surface.

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Fibrinogen Adsorption, Platelet Adhesion and Thrombin Generation at Heparinized Surfaces Exposed to Flowing Blood

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Keywords

Heparin coating, artificial surface, fibrinogen, platelets, thrombin

Summary

Thrombus formation at an artificial surface in contact with blood is a complex process that encompasses accretion of platelets from flowing blood and fibrin deposition. Platelet adhesion and fibrin formation are intimately intertwined reactions that are triggered by different sets of surface adsorbed plasma proteins. To dissect the contribution of protein adsorption and platelet adhesion to thrombin formation, a coherent study was performed with non-coated (NC) and heparin-coated (HC) surfaces. Thrombin production in whole blood, platelet adhesion and protein adsorption were studied using an amidolytic thrombin assay, a dynamic platelet adhesion assay and ellipsometry, respectively. Thrombin generation in flowing whole blood exposed to HC surfaces was greatly diminished when compared with NC surfaces. However, separate platelet adhesion and protein adsorption studies with anticoagulated whole blood revealed that platelets do not adhere because fibrinogen is not available in the protein layer that was deposited during the perfusion. These findings indicate that the *in vitro* thrombogenicity of a material cannot be predicted from platelet adhesion and protein adsorption data when these measurements are performed with anticoagulated blood or platelet rich plasma. Preincubation of NC and HC surfaces with fibrinogen or 2000-fold diluted plasma resulted in similar amounts of surface-bound fibrinogen and mediated massive platelet adhesion from flowing whole blood. These results indicate that a) platelet adhesion correlates with the availability of surface-bound fibrinogen and b) NC and HC surfaces are indistinguishable with respect to protein (fibrinogen) adsorption and platelet adhesion. It is apparent that the heparinized surface used in our studies exerts its anti-thrombogenic properties by neutralizing locally formed thrombin and not by reducing fibrinogen-dependent platelet adhesion.

Introduction

The initial response of blood exposed to an artificial surface is adsorption of blood proteins. This event triggers a number of biological reactions like inflammation and blood coagulation (1). It is widely

accepted that the nature of the adsorbed protein layer determines w biological systems and blood cells respond and to what ex Thrombus formation at an artificial surface in contact with bloo thought to occur when platelets adhere via (activated) integrins on platelet plasma membrane to a selective group of adsorbed pla proteins (e.g. fibrinogen) deposited at an artificial surface (2). However, because of the intimately intertwined nature of platelet blood coagulation reactions, thrombin formation is also essentia surface thrombogenicity (5, 6). Thrombin generation in this case is n likely initiated by the contact system that in turn is triggered by adsorption of specific plasma proteins (factor XII, HMWK, prekallikrein) (7). Therefore, activation of platelets and coagula might be initiated by the adsorption of two different classes of pla proteins.

In spite of numerous studies on the relationship between surf chemistry and protein adsorption on the one hand (8-10) and betw protein adsorption and platelet adhesion on the other hand (11-14), it still remains to be clarified what precise molecular and cell interactions contribute, and to what extent, to surface thrombogenicity under conditions that are physiologically relevant. Moreover, prediction of the *in vivo* thrombogenic properties of artificial surfa from isolated protein adsorption studies per se might be questioned for several reasons (15). It is well known that the adsorption and desorpt kinetics of the plasma proteins that are involved in the thromboge processes at artificial surfaces are strongly influenced by protein concentration and surface affinity of other plasma proteins. In addition, the conformation of adsorbed proteins and thus its biologi functioning might also change in a time dependent manner (16-20).

In this study we compared the thrombin generating potential of nc coated and heparin-coated surfaces in relation to protein adsorption a platelet adhesion properties of these surfaces under flow conditions. Thrombin generation and platelet adhesion experiments were performed with flowing whole blood, while quantitative protein adsorption in particular that of fibrinogen, was studied from buffer systems a from a plasma environment also under flow conditions. The findings of this study confirmed the reduced thrombogenicity of heparin-coat surfaces. However, the potential of this particular heparin-coat surface with respect to protein adsorption and platelet adhesion was n different from that of a non-coated surface.

Materials and Methods

Materials

Glass coverslips and glass capillaries were obtained from Menzel-Gläs (Braunschweig, Germany). Silicon slides were from Aurel GmbH (Landsber Germany). Polyethylenimine (PEI) was from Sigma (St. Louis, USA). Sodium heparin (165 U/mg) was supplied by Biolberica (Barcelona, Spain). Calce

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acetoxymethyl ester was obtained from Molecular Probes (Leiden, The Netherlands). D-Phe-Pro-Arg chloromethylketone (PPACK) came from Calbiochem (San Diego, USA). All other reagents were of analytical grade.

Proteins: Bovine serum albumin (BSA) and aprotase were from Sigma (St. Louis, USA). Human fibrinogen, not contaminated with von Willebrand factor and fibronectin, was from Kordia (Leiden, The Netherlands). Rabbit anti-human fibrinogen and rabbit IgG were purchased from DAKO (Glostrup, Denmark).

Surface modification of glass coverslips and silicon slides: After a primary rinse by sonication in isopropanol, glass coverslips and silicon slides were subjected to glow-discharge air plasma cleaning. Briefly, treatment was performed in a class 10,000 clean room, using a capacitively coupled parallel-plate plasma reactor (Gambetti Kenologia, Binasco, Italy) with the samples located on the water-cooled grounded electrode. The reactor volume is about 3 l, and the distance between the electrodes 10 cm. Flow rate was 20 ml/min and the pressure inside the chamber before the onset of the discharge was 2 Pa. The power discharge was 50 W and the treatment time 180 s. These surfaces will be referred as NC surfaces. After this cleaning step, glass cover slips and silicon slides were dipped in 2 ml of a 0.5% (w/v) aqueous solution of PEI for two h. After a prolonged rinsing in doubly distilled water, samples were dried in a laminar flow hood. The PEI treated samples were then immersed in 5 ml of a periodate treated heparin in 0.4 M acetate solution, pH 4.7. Periodate heparin was prepared by dissolving 20 mg sodium periodate into 100 ml of a 0.5% sodium heparin solution in 0.05 M phosphate buffer (pH 6.9). Periodate oxidation was performed for 12 h in the dark. NaCNBH₃ was added to the coupling solution, and the reaction was carried on overnight, while stirring. The heparin-coated surfaces were extensively rinsed, stored overnight in doubly distilled water and dried in a laminar flow hood. The coupling of heparin was confirmed by toluidine blue staining.

Flow chamber: The parallel plate flow chamber used in this study was previously described (21). Briefly, a cavity with a depth of 0.2 mm, a width of 5.0 mm and a length of 42.5 mm was made in polymethyl-methacrylate. Glass coverslips (24 × 60 mm) serve as the bottom of the chamber. The surface of the coverslip in contact with perfusion solution is 2 cm².

Thrombin generation: The inlet of the tubular flow reactor (glass capillary; length 65 mm and inner diameter of 0.5 mm) was connected to two syringes; one containing citrated whole blood and the other containing 0.2 M CaCl₂ in HEPES buffer. The pump-driven syringes were set at flow rates of 54 and 6 µl/min, respectively. Timed samples of 25 µl were taken and added to 675 µl HEPES buffer containing 20 mM EDTA and 0.2 mM S2238. The diluted blood sample was centrifuged for 1 min at 4000 rpm and the supernatant was assayed for thrombin activity as previously described (22).

Loading platelets with calcein: Blood was drawn from healthy volunteers, who had not taken any anti-platelet medication in the preceding two weeks, into a 40 µM PPACK solution. Platelet-rich plasma (PRP) was prepared by centrifugation at 190 g for 15 min and incubated during 45 min at 37°C with 2.5 µM calcein acetoxymethyl ester in the presence of aprotase (0.1 U/ml). Calcein-treated PRP and the red cells were mixed to reconstitute whole blood.

Fibrinogen-coated glass coverslips: Glass coverslips were exposed to a solution of 100 µg/ml human fibrinogen for 20 min at room temperature. The coverslips were then rinsed in HEPES buffer (136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 10 mM HEPES, 1 mg/ml BSA) and incubated with this buffer for 15 min.

Platelet adhesion: The flow chamber with NC or HC glass coverslip was connected with silicone tubing (inner diameter 0.5 mm) to a syringe filled with blood containing calcein-loaded platelets. Perfusion experiments under low shear stress conditions (wall shear rate of 50 s⁻¹) were performed at 100 µl/min in flow chambers with a slit depth of 200 µm. During the perfusion fluorescent real-time images were recorded as described before (23). Briefly, the flow chamber was placed on the stage of an inverted microscope (Diaphot 200, Nikon, Tokyo, Japan) and fluorescence images are recorded via a 40× quartz oil-immersion objective coupled to a high-resolution and a low-light level intensified, charge-coupled device camera. Calcein fluorescence measurements were performed with a 485 nm excitation filter, a 505 nm dichroic long-pass filter, and a 530 nm emission filter. Four fluorescence images were taken every

10 s, digitized and averaged after subtraction of background images. Image analysis was performed off-line using Quantecell 700/900 software (Visitech, Sunderland, UK). The platelet adhesion results are expressed as the percentage of total area covered by calcein-loaded platelets calculated from at least 5 different microscopic fields. All procedures were performed at room temperature.

Protein adsorption measurements by ellipsometry: Protein adsorption was measured by ellipsometry under flow conditions as described before (24). The ellipsometer is an optical instrument that measures the changes in polarization of light due to reflection. These changes are influenced by the amount of protein that binds on the silicon slide. In short, circularly polarized light from a helium-neon laser ($\lambda = 632.8$ nm) passes a polarizer (P), then a compensator and is reflected by the silicon slide, which is mounted in a quartz cuvette filled with buffer. The reflected light then passes another polarizer, the analyzer (A), and is detected by a photodiode. Computer-controlled stepping motors on the polarizer and analyzer automate the instrument. The positions of P and A are adjusted in a way that the resulting light intensity is kept minimal (null ellipsometry). Adsorption of protein on the reflecting silicon surface changes the P and A values. The adsorbed protein mass can be calculated from these changes using the Lorentz-Lorenz equation (24). However, under the conditions of our experiments it can simply be estimated from $\Gamma = 0.085 \times \Delta P$ µg/cm², with ΔP expressed in degrees. Measurements were performed at room temperature.

Results

Thrombin generating potential of non-coated and heparin-coated surfaces: Thrombin generation at the inner surface of NC and HC glass capillaries exposed to flowing recalcified whole blood was monitored in timed samples, taken at the outlet of the capillary, with an amidolytic assay for thrombin activity. A marked difference in the thrombin generating potential of NC and HC surfaces was observed (Fig. 1): whereas NC surfaces showed a rapid onset of thrombin generation 5 min after the start of the perfusion, only small amounts of thrombin were detected in the effluent of HC capillaries at the end of the perfusion experiment. It is apparent that on the NC surfaces shortly after the initiation of the blood coagulation system the thrombin-dependent positive feedback reactions are responsible for the sudden onset of a steady state thrombin production. The greatly diminished thrombin production at HC surfaces is likely attributed by the heparin-mediated inhibition of these thrombin-dependent feedback reactions (25). Alternatively, a platelet repelling property of immobilized heparin could also be responsible for the diminished thrombin generation because adherent platelets have been shown to be critical to the propagation of thrombin generation at artificial surfaces (26). Thus, the thromboresistance of a heparinized surface could also be explained by a reduced platelet adhesion because of a diminished fibrinogen adsorption. We, therefore, next examined adsorption of fibrinogen on NC and HC surfaces.

Fibrinogen adsorption on NC and HC surfaces: Ellipsometric measurements were performed to obtain quantitative data on fibrinogen adsorption for NC and HC silicon surfaces exposed to purified fibrinogen and platelet free plasma. We found that an incubation of NC and HC surfaces with 10% diluted platelet free plasma resulted in a rapid adsorption of 0.47 and 0.48 µg/cm² protein, respectively (Fig. 2). To examine whether the adsorbed protein layer also contained fibrinogen, rabbit anti-human fibrinogen IgG was added after the plasma was replaced by HEPES buffer (Fig. 2). The plasma depletion step resulted in a slightly decreased mass of adsorbed protein, indicating that some protein desorbed from the surfaces. Addition of anti-human fibrinogen IgG, however, did not result in a significantly increased protein adsorption. The two possible explanations for this

Fig. 1 Thrombin generation at non-coated and heparin-coated surfaces. Flowing citrated whole blood (wall shear rate 50 s^{-1}) was recalcified just before the entrance of non-coated (●) and heparin-coated (○) glass capillary (length 6.5 cm, inner diameter 0.5 mm) as described in the text. Samples were taken at the outlet of the capillary and assayed for thrombin activity. Values are mean $\pm$ SD ($n = 3$)

Fig. 2 Time dependency of protein adsorption from 10% plasma. Non-coated (curve a) and heparin coated (curve b) silicon slides were mounted in the ellipsometer and exposed to 10% plasma. After 30 min incubation the surfaces were rinsed with HEPES buffer and anti-fibrinogen (16 μg/ml) was added

Fig. 3 Detection of adsorbed fibrinogen by anti-fibrinogen. Non-coated silicon slide was mounted in the ellipsometer and exposed to 1 μM human fibrinogen. After 20 min incubation the surface was rinsed with HEPES buffer, and non-specific rabbit IgG (16 μg/ml) followed by rabbit anti-human fibrinogen (16 μg/ml) were added

Fig. 4 Time dependency of protein adsorption from 0.05% plasma. Non-coated (curve a) and heparin coated (curve b) silicon slides were mounted in the ellipsometer and exposed to 0.05% plasma. After 30 min incubation the surfaces were rinsed with HEPES buffer and anti-fibrinogen (16 μg/ml) was added

finding are: 1) the anti-human fibrinogen antibody does not recognize fibrinogen in the protein layer or 2) fibrinogen is not present or exposed in the protein layer. To explore these possibilities, the following control experiments were performed. Firstly, NC surface was exposed to 1 μM purified human fibrinogen. A rapid adsorption of fibrinogen was observed, yielding a maximum mass of adsorbed fibrinogen of 0.76 μg/cm^2 (Fig. 3). The same experiment with HC surface gave a slightly lower

Fig. 5 Time dependency of platelet adhesion from whole blood. Non-coated (A) and heparin-coated (B) glass coverslips were pretreated with 0.3 μM human fibrinogen (▲), 10% plasma (◆), 0.05% plasma (●) or non-treated (□), and subsequently exposed to PPACK anticoagulated whole blood in a flow chamber at a shear rate of 25 s^{-1}

Table 1 Platelet adhesion on non-coated (NC) and heparin-coated (HC) glass coverslips

Pre-incubation	Area coverage (%) ^a		Rate of platelet adhesion (% area coverage/min)	
	NC surface	HC surface	NC surface	HC surface
None	0.1 ± 0.1	0.1 ± 0.1	0.01	0.01
Fibrinogen	27.3 ± 0.6	18.1 ± 7.9	2.93	2.15
0.05% plasma	12.0 ± 3.1	9.3 ± 3.2	1.18	1.01
10% plasma	0.2 ± 0.2	0.2 ± 0.2	0.03	0.02

^a Values are means ± SD (n=10) after 10 minutes perfusion with whole blood anti-coagulated with PPACK.

Table 2 Protein adsorption on non-coated (NC) and heparin-coated (HC) silicon surfaces

Pre-incubation	Γ ($\mu\text{g}/\text{cm}^2$) ^a		$\Delta\Gamma$ ($\mu\text{g}/\text{cm}^2$) ^b	
	NC	HC	NC	HC
Fibrinogen	0.76	0.60	0.46	nd ^c
0.05% plasma	0.49	0.41	0.27	0.34
10% plasma	0.47	0.48	0	0

^a Protein mass before rinsing.
^b Increase in protein mass after rinsing and addition of anti-human fibrinogen antibody.
^c Not determined.

mass of adsorbed fibrinogen ($0.60 \mu\text{g}/\text{cm}^2$) (data not shown). Fig. 3 further shows that replacement of the fibrinogen solution with HEPES buffer did not result in desorption of immobilized fibrinogen from the NC surface. The addition of purified rabbit IgG ($16 \mu\text{g}/\text{ml}$) resulted in no further increase in protein adsorption, indicating that the surface was already saturated with protein and that this non-specific rabbit IgG did not recognize adsorbed human fibrinogen. In contrast, addition of rabbit anti-human fibrinogen IgG resulted in an additional increase of about $0.46 \mu\text{g}/\text{cm}^2$ of adsorbed protein. Evidently, this increase in protein adsorption can be attributed to the binding of anti-human fibrinogen IgG with surface-bound fibrinogen. These results thus clearly indicate that the rabbit anti-human fibrinogen IgG is a suitable tool for the detection of adsorbed fibrinogen. We therefore conclude from the protein adsorption data for NC and HC surfaces preincubated with 10% diluted plasma (Fig. 2) that fibrinogen is not detectable in the protein layers thus obtained.

The absence of detectable fibrinogen in a protein layer adsorbed from whole blood could indicate a fast replacement of that protein by other plasma proteins (16, 27). This replacement will not occur when protein adsorption is conducted from a highly diluted plasma solution (28, 29). Exposure of NC and HC surfaces to 0.05% plasma resulted in the adsorption of 0.49 and $0.41 \mu\text{g}/\text{cm}^2$ protein, respectively. Remarkably, these amounts are only little less than those found with 10% diluted plasma, indicating that under these conditions again saturation of protein adsorption is obtained. In contrast to the results

seen for both NC and HC surfaces with 10% diluted plasma (Fig. 2), the addition of rabbit anti-human fibrinogen IgG yielded a significant increase in protein adsorption (Fig. 4), indicating the presence of fibrinogen in that protein layer.

Platelet adhesion from flowing whole blood to non-coated and heparin-coated surfaces: The next question to address is how important is the availability of fibrinogen for the platelet adhesive properties of surfaces in contact with flowing blood. PPACK anticoagulated whole blood of which the cells were loaded with calcein was passed during 10 min through flow chambers with mounted NC and HC surfaces. The surface coverage with fluorescent platelets during the perfusion was monitored in real-time and off-line analyzed. Nearly no platelet-associated fluorescence could be detected on both surfaces during a 10-min perfusion (Fig. 5). If this finding is compatible with our observation that fibrinogen is not available on surfaces preincubated with 10% diluted plasma (Fig. 2), it also implies that fibrinogen is already unavailable from the start of the perfusion. This points at a very rapid dislocation of fibrinogen during the process of protein adsorption.

To find support for this notion, platelet adhesion studies were performed on NC and HC surfaces that were preincubated with purified fibrinogen and highly diluted (2000-fold) plasma. Previous experiments already established that NC and HC surfaces treated in this way adsorbed fibrinogen that was recognized by a rabbit anti-human fibrinogen antibody (Figs. 3 and 4). A subsequent perfusion of these surfaces with whole blood resulted in a linear increase in the number of

adherent platelets with perfusion time (Fig. 5). After a perfusion of 10 min the fibrinogen-pretreated NC and HC surfaces were covered for $27.3 \pm 0.6\%$ (mean $\pm$ SD, $n = 5$) and $18.1 \pm 7.9\%$ (mean $\pm$ SD, $n = 5$), respectively, with adherent platelets. The coverage of NC and HC surfaces pretreated with 0.05% diluted plasma was $12.0 \pm 3.1\%$ and $9.3 \pm 3.3\%$, respectively. It is apparent that the rate at which the surfaces were covered with platelets increased proportionally with the amount of available fibrinogen (cf. Table 1 and Table 2).

Discussion

It is generally believed that surface thrombogenicity is associated with the potential of a surface to adhere blood platelets. Platelet adhesion, in turn, is thought to depend on the ability of a surface to adsorb fibrinogen. Therefore, protein adsorption and platelet adhesion experiments are frequently performed to evaluate the thrombogenicity of artificial surfaces. This study confirms the dependency of platelet adhesion on the availability of fibrinogen in the protein layer deposited at an artificial surface under conditions that mimic the physiological situation, i.e. flowing whole blood or plasma. However, a relationship between the level of thrombin generation and platelet adhesion could not be established. Our experimental data indicate that platelet adhesion data do not predict the thrombogenic nature of artificial surfaces in contact with blood, raising the questions: 1) what factors prevent platelets from adhering to these surfaces and 2) should the mediating role of adherent platelets in thrombin generation be reconsidered? Answers to these questions would be helpful in understanding why heparinization of artificial surfaces reduces the intrinsic thrombogenicity of such surfaces.

Fibrinogen adsorption and platelet adhesion: Ellipsometric measurements showed that NC and HC surfaces exposed to 10-fold diluted plasma adsorbed similar amounts of plasma protein (around $0.5 \mu\text{g}/\text{cm}^2$), suggesting that heparinized surfaces are not protein repelling. In spite of the presence of a protein layer, surface-bound fibrinogen could not be detected with rabbit anti-human fibrinogen (Table 2). Exposure of NC and HC surfaces to 2000-fold diluted plasma resulted in 0.49 and $0.41 \mu\text{g}/\text{cm}^2$ adsorbed protein, respectively, which is similar to the amounts found with 10-fold diluted plasma. This relatively small amount of protein in 0.05% plasma (about $40 \mu\text{g}/\text{ml}$) is apparently sufficient to saturate NC and HC surfaces. In the protein layer adsorbed from 2000-fold diluted plasma, we readily detected an abundant presence of fibrinogen in contrast to our findings with 10-fold diluted plasma. It is of interest to note that the platelet area coverage of both NC and HC surfaces parallels the amount of fibrinogen that was detected in the protein layer on the artificial surface after exposure to 10-fold diluted and 2000-fold diluted plasma (Table 1). This finding once more indicates that with respect to fibrinogen adsorption and platelet adhesion, the heparinized surface used in this study is indistinguishable from a non-coated hydrophilic surface.

While fibrinogen absorbs from a buffer solution to NC and HC surfaces and such pretreated surfaces accrete readily platelets from flowing whole blood, only a few platelets adhered during the perfusion of non-pretreated surfaces with flowing whole blood. These data confirm that the deposition of a protein layer from flowing whole blood is followed by a rearrangement of that layer in which fibrinogen is very rapidly displaced. This rearrangement, also called the Vroman effect (16, 30), is apparently slowed down or absent when the protein concentration in the fluid phase is greatly decreased (29).

Platelet adhesion and thrombin generation: We have previously demonstrated that, under flow conditions, the presence of surface-bound anionic phospholipids are mandatory to the propagation of

thrombin generation at an artificial surface (25). Similarly, significant thrombin generation at artificial surfaces was only seen when exposed to platelet rich plasma but not when exposed to platelet poor plasma (26). The apparent platelet-independent thrombin generation now reported seems to contrast with these earlier observations. However, we must emphasize that the platelet adhesion study was performed in flowing PPACK-anticoagulated blood and that thrombin generation was measured in flowing citrated blood that was recalcified just before it entered the glass capillary. Thus it is feasible that a few free flowing platelets are tethered by fibrinogen that adsorbed to the surface before it is displaced by other plasma proteins. The first traces of thrombin generated in recalcified citrated blood, at the surfaces of these adherent platelets probably induce additional platelet adhesion and so amplify surface-localized thrombin generation, which in turn results in an explosive increase in the rate of thrombin generation. The thrombin generation data with heparinized surfaces strongly support this notion. Because HC surfaces are indistinguishable from NC surfaces with respect to protein adsorption and platelet adhesion a diminished thrombin generation on HC surfaces can only be explained by the rapid heparin-dependent inhibition of thrombin-mediated positive feedback reactions, e.g. platelet activation and generation of the critical cofactors Va and VIIIa.

In conclusion, our work suggests that protein adsorption and platelet adhesion data from anticoagulated whole blood are inappropriate parameters to assess the thrombogenic properties of biomaterials because anticoagulation prevents a key feature of thrombin, i.e. its positive feedback function in platelet adhesion and activation and thus surface thrombus formation. Predictions for the adverse haemostatic reactions at the blood-material interface could therefore be better based on thrombin generation measurements in blood passing by an artificial surface. Our findings also imply that surface thrombogenicity is probably most effectively reduced by immobilized agents, like heparin, that cause enhanced inhibition of surface-localized, thrombin-dependent positive feedback reactions.

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Thrombogenicity of
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Thrombogenicity of polysaccharide-coated surfaces

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Abstract

Heparinization of artificial surfaces has been proven to reduce the intrinsic thrombogenicity of such surfaces. The mechanism which immobilized heparin reduces thrombogenicity is not completely understood. In the present study heparin-, alginic acid-chondroitin-6-sulphate-coated surfaces were examined for protein adsorption, platelet adhesion and thrombin generation. protein-binding capacity from solutions of purified proteins was significantly higher for heparin-coated surfaces when compared with alginic acid- and chondroitin sulphate-coated surfaces. Yet, when the surfaces were exposed to flowing plasma, only heparinized surface adsorbed significant amounts of antithrombin. None of the surfaces adsorbed fibrinogen under these conditions and as a result no platelets adhered from flowing whole blood. Our results indicate that protein adsorption and platelet adhesion from anticoagulated blood cannot be used to assess the thrombogenicity of (coated) artificial surfaces. Indeed, the thrombin generation potentials of the different surfaces varied remarkably: while non-coated surface readily produced thrombin, alginic acid- and chondroitin sulphate-coated surfaces showed a marked reduction and virtually no thrombin was generated in flowing whole blood passing by heparinized surfaces.

Keywords: Polysaccharide coating; Artificial surface; Fibrinogen; Platelets; Thrombin and antithrombin

1. Introduction

Artificial surfaces in contact with blood trigger a number of biological systems through the adsorption of proteins and cells. It is generally believed that the nature of the adsorbed protein layer determines all adverse events that impair the use of artificial materials in medical devices: thrombus formation as a result of platelet adhesion, platelet activation and initiation of coagulation [1–3] and activation of the complement system that in turn results in leukocyte adhesion and activation [4]. The most widely used method to prevent thrombus formation at artificial surfaces is the administration of heparin (for a review see Ref. [5]).

Heparin is a sulphated polysaccharide with a repeating disaccharide of alpha-D-glucosamine and uronic acid [6]. The repeating disaccharide units contain clusters of O- and N-sulphated groups. The antithrombotic action of heparin is based on its strong stimulatory effect on the neutralization of thrombin and factor Xa by antithrombin [7,8], a member of the serine protease inhibitor (serpin) superfamily [9,10]. However, the anticoagulant response to heparin is unpredictable because the heparin-antithrombin complex is unable to inhibit fibrin-bound thrombin [11]. This finding explains why in experimental animals heparin is less effective than direct thrombin inhibitors [12]. Yet, immobilization of heparin on the artificial surface is successfully used to reduce surface thrombogenicity [13]. Moreover, heparin-coated surfaces are thought to significantly improve in vitro haemocompatibility because of reduced adsorption of procoagulant and proinflammatory enzymes [14].

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Immobilization of negatively charged sulphated polysaccharides could make surfaces more biocompatible because of the electrostatic repulsion of negatively charged blood components. Furthermore, an increased wettability as a result of the presence of negatively charged groups may lead to a diminished or more reversible protein adsorption [15]. Yet, highly sulphated polysaccharides like heparin, have been reported to stimulate the process of neo-intima formation. This stimulation is probably the result of an interaction between growth factors and these polysaccharides [16–18]. Interestingly, fucosylated chondroitin sulphate has a strong inhibitory effect on smooth muscle cell proliferation [19]. Thus, it should be questioned whether other sulphated polysaccharides could be an attractive alternative for heparin to decrease the thrombogenic activity of artificial surfaces.

In this study we have compared the antithrombogenic properties of immobilized heparin with that of immobilized chondroitin-6-sulphate and alginic acid. Chondroitin-6-sulphate is a sulphated polysaccharide in which N-acetyl β -D-galactosamine and β -D-glucuronic acid are the alternating saccharide units [6]. The sulphate group is at the 6-position of the galactosamine residue. Alginate contains mannuronic (M) and glucuronic (G) groups [20]. It was reported that the G-groups are prothrombotic and that the M-groups have an anticoagulant effect [21]. The alginic acid used in this study is a non-sulphated hydrophilic polyuronic acid composed primarily of anhydro- β -D-mannuronic acid residues. An additional advantage using this polysaccharide in humans could be its non-mammalian source, which eliminates the risk of contamination with pathogenic agents [8]. The thrombogenic parameters that were evaluated in this study are: (1) adsorption of the purified plasma proteins albumin, fibrinogen, high molecular weight kininogen (HMWK), IgG and antithrombin, (2) platelet adhesion from flowing whole blood, and (3) thrombin generation at the artificial surfaces in flowing whole blood.

2. Materials and methods

2.1. Materials

Glass cover slips ($24 \times 60 \text{ mm}^2$) and glass capillaries (length 60 mm and inner diameter 1.2 mm) were obtained from Menzel-Gläser (Braunschweig, Germany). Silicon slides were from Aurel GmbH (Landsberg, Germany). Polyethylene-imine (PEI), Chondroitin-6-sulphate and alginic acid came from Sigma (St. Louis, USA). Sodium heparin (165 U/mg) was supplied by Bioiberica (Barcelona, Spain). D-Phe-Pro-Arg chloromethylketone (PPACK) came from Calbiochem (San Diego, USA). All other reagents were of analytical grade.

2.2. Proteins

Human serum albumin was obtained from Sigma (St. Louis, USA). Human fibrinogen, not contaminated with von Willebrand factor and fibronectin, and HMWK were from Kordia (Leiden, The Netherlands). Rabbit IgG was purchased from DAKO (Glostrup, Denmark). Human IgG was purified by affinity chromatography on Protein A Sepharose. Antithrombin was purified according to the method of Thaler and Schmer [22].

2.3. Surface modification

Glass coverslips, glass capillaries and silicon slides were rinsed and coated with the polysaccharides as previously described [23]. Briefly, glass coverslips and silicon slides were dipped in 2 ml of a 0.5% (w/v) aqueous solution of PEI for 2 h. After a prolonged rinse in double distilled water, samples were dried in a laminar flow hood. The PEI treated samples were then immersed in 5 ml of a periodate treated polysaccharide in 0.4 M acetate solution, pH 4.7. NaCNBH_3 was used to couple the polysaccharides to the PEI-coated surface.

2.4. Protein adsorption measurements by ellipsometry

The ellipsometer is an optical instrument that measures the changes in the polarization of light due to reflection. These changes are strongly influenced by the amount of protein that adsorbs to the silicon slide. In short, circularly polarized light from a helium–neon laser ($\lambda = 632.8 \text{ nm}$) passes a polarizer (P), then a compensator and is reflected by the silicon slide, which is mounted in a quartz cuvette filled with buffer. The reflected light then passes another polarizer, the analyzer (A), and is detected by a photodiode. Computer-controlled stepping motors on the polarizer and analyzer automate the instrument. The positions of P and A are adjusted in a way that the resulting light intensity is kept minimal (null ellipsometry). Adsorption of protein on the reflecting silicon surface changes the P and A values. The adsorbed protein mass Γ can be calculated from these changes using the Lorentz–Lorenz equation [24]. However, under the conditions of our experiments it can simply be estimated from $\Gamma = 0.085 \times \Delta P \mu\text{g}/\text{cm}^2$, with ΔP expressed in degrees. Measurements were performed at ambient temperature ($20\text{--}22^\circ\text{C}$) under vigorously stirring conditions.

2.5. Detection of plasma antithrombin in surface-adsorbed protein layer

Heparin-coated and non-coated silicon slides were incubated with diluted plasma (10%) for 10 min. The

plasma was then removed and replaced by Hepes buffer. Rabbit antihuman antithrombin antibody (16 µg/ml) was added and protein adsorption was monitored by ellipsometry. To confirm that antithrombin adsorbed on non-coated and heparinized surfaces is equally detectable by the rabbit antihuman antithrombin antibody, heparin-coated and non-coated silicon slides were incubated with Hepes buffer containing 0.5 µM antithrombin, rinsed with Hepes buffer and then incubated with rabbit antihuman antithrombin antibody (16 µg/ml).

2.6. Thrombin generation in flowing blood

The inlet of a capillary was connected to two syringes: one containing citrated whole blood and the other containing 0.2 M CaCl₂ in Hepes buffer pH 7.45 (20 mM Hepes, 140 mM NaCl). Pump-driven syringes were set at flow rates of 54 and 6 µl/min, respectively. Timed samples of 25 µl were taken at the outlet and added to 675 µl Hepes buffer containing 20 mM EDTA and 0.2 mM S2238. The erythrocytes were removed by centrifugation and the supernatant was assayed for thrombin activity as previously described [25].

2.7. Preparation of fibrinogen coated coverslips

Glass coverslips were exposed for 60 min at room temperature to a solution of 1 mg/ml fibrinogen. After that the surfaces were rinsed in Hepes buffer and incubated with this buffer for 15 min.

2.8. Platelet adhesion studies

Blood (9 vol) was collected into 0.13 M trisodium citrate (1 vol) or into a small aliquot of 40 µM PPACK. Platelet-rich plasma (PRP) was separated from the red and white blood cells by centrifugation at 190g for 15 min. PRP was 45 min incubated at 37°C with 2.5 µM calcein acetoxymethyl ester in the presence of apyrase (0.1 U/ml). Reconstituted whole blood (1 volume PRP mixed with 1 volume red blood cell suspension from the first centrifugation step) was used for the platelet adhesion study using a parallel plate flow chamber [23]. Flow experiments are performed at a flow rate of 50 µl/min, (wall shear rate of 25 s⁻¹). Platelet adhesion was monitored in real-time using an inverted microscope (Nikon diaphot 200; Nikon, Tokyo, Japan). Surface coverage with calcein-loaded platelets was measured with a combined fluorescence imaging and microphotometric system (FIMS) connected to the microscope and commanded by a UNIX/Quanticell-driven computer system (Applied Imaging, Sunderland, UK) [26].

3. Results

3.1. Protein adsorption

Protein adsorption was measured by ellipsometry from solutions containing various concentrations of purified albumin, IgG, fibrinogen, antithrombin + HMWK. The amount of protein adsorbed per unit area (µg/cm²) of the non-coated, PEI-, chondroitin sulphate-, alginic acid-, and heparin-coated surfaces as a function of their concentration in free solution is depicted in Fig. 1. For all proteins examined, adsorption on chondroitin-6-sulphate-coated surfaces was 10–20% of the amounts found on non-treated surfaces. Slightly more protein was adsorbed by alginic acid-coated surfaces. In comparison to chondroitin-6-sulphate-coated surfaces, heparin-coated surfaces bound relatively high amounts of fibrinogen and antithrombin. The relative high adsorption of antithrombin was expected for a heparinized surface; the high adsorption of fibrinogen was however rather surprising. A protein adsorption from plasma was higher for a heparin-coated surface when compared with the alginic acid- and chondroitin-6-sulphate-coated surfaces. The data indicate that chondroitin-6-sulphate and alginic acid are more protein repelling polysaccharides than heparin.

3.2. Specificity of the antithrombin binding to heparin-coated surfaces

Fig. 1 also demonstrates that a non-coated and a heparin-coated surface exposed to 1 µM antithrombin binds about similar amounts of antithrombin: 0.47 and 0.52 µg/cm², respectively. To investigate the specificity of this binding, a heparin solution was added after maximal adsorption of antithrombin was achieved. The amount of adsorbed antithrombin prior to and after the addition of heparin are summarized in Table 1. The data clearly show that in case of the heparinized surface 35% of initially adsorbed antithrombin desorbed from the surface, suggesting that only part of antithrombin is reversibly bound to immobilized heparin. Most of the antithrombin (about 65%) binds apparently irreversibly and non-specifically to the heparin-coated surface. The non-coated surface apparently adsorbs antithrombin irreversibly and thus not specifically.

3.3. Availability of antithrombin on heparin-coated surfaces

We earlier demonstrated that fibrinogen, adsorbed from plasma on heparin-coated surfaces, is rapidly displaced by other plasma proteins [23]. Because the thromboresistance of heparin-coated surfaces relies at least in part on the ability of adsorbed antithrombin to

Fig. 1. Protein adsorption on non-coated, polyethylene-imine (PEI)-treated, and immobilized heparin (HEP), alginic acid (ALG) and chondroitin-6-sulphate (CS) silicon surfaces.

Table I
Displacement of surface-bound antithrombin by free heparin

Addition	Conc.	Protein adsorption	
		Non-coated surface (µg/cm ²)	Heparin-coated surface (µg/cm ²)
Antithrombin	1 µM	0.43	0.41
+ heparin	10 U/ml	0.43	0.28
+ heparin	20 U/ml	0.43	0.26

inactivate coagulation enzymes, it is important to know what fraction of antithrombin bound to immobilized heparin can be displaced by other plasma proteins. Fig. 2A shows that a non-coated surface that was first exposed to plasma and then incubated with anti-antithrombin antibody apparently did not adsorb more protein (curve a). In contrast, a significant amount of IgG from the anti-antithrombin antibody solution adsorbed to a heparinized surface. These findings indicate that the protein layer deposited from plasma on a heparinized surface retains antithrombin, while the protein layer on non-coated surfaces does not. A control experiment as shown in Fig. 2B reveals that a non-

specific rabbit IgG did not adsorb to heparin-coated and non-coated surfaces that were pretreated with 0.5 µM antithrombin. However, when the antithrombin pretreated surfaces were incubated with rabbit antihuman antithrombin IgG, the amount of adsorbed protein significantly increased in both cases. It is our conclusion that if antithrombin adsorbs from plasma on non-coated surfaces it is rapidly displaced by other plasma proteins. We found that antithrombin was also readily displaced on alginic acid- en chondroitin-6-sulphate-coated surfaces when these surfaces were exposed to plasma (data not shown). Together, these findings imply that antithrombin is only available in plasma protein layers when the surface is coated with heparin. This notion is in agreement with an earlier report that demonstrated that histidine rich glycoprotein competes with antithrombin for immobilized dermatan sulphate and chondroitin sulphate but not on heparan sulphate with high affinity for antithrombin [27].

3.4. Platelet adhesion for flowing whole blood

We next examined platelet adhesion from flowing whole blood that was anticoagulated with the

(A)

(B)

Fig. 2. Antithrombin adsorption. (A) Non-coated and heparin-coated silicon slides were exposed to 10% plasma. After 30 min incubation, the surfaces were rinsed with HEPES-buffer and rabbit antihuman antithrombin (16 $\mu\text{g}/\text{ml}$) was added (arrow). Curves a and b represent the rabbit antihuman antithrombin antibody deposition on the non-coated and heparin-coated surface, respectively. (B) Non-coated and heparin-coated silicon slides were exposed to 0.5 μM antithrombin. After 30 min incubation, the surface was rinsed with HEPES-buffer, non-specific rabbit IgG (16 $\mu\text{g}/\text{ml}$) and subsequent rabbit antihuman antithrombin (16 $\mu\text{g}/\text{ml}$) were added. Curves a and b represent the antibody deposition on the non-coated and heparin-coated surface, respectively.

thrombin inhibitor PPACK. Fig. 3A shows that platelet adhesion was almost negligible for all surfaces except for the positively charged PEI-coated surface. We previously demonstrated that the protein layer adsorbed from undiluted plasma on hydrophilic surfaces does not contain fibrinogen [23]. To confirm the notion that fibrinogen adsorbed from plasma is readily displaced by other plasma proteins, polysaccharide coated surfaces were first exposed to purified fibrinogen and then to flowing PPACK-anticoagulated whole blood. The increase in surface coverage with adherent platelets as a function of the perfusion time is shown in Fig. 3B. Non-coated surfaces bind the most platelets, followed by heparin-, PEI- and alginic acid-coated surfaces. No platelet adhesion was observed on chondroitin-6-sulphate-coated surfaces. Preadsorption with fibrinogen did not affect the platelet adhesion capacity of PEI-coated surfaces, suggesting that platelet adhesion to this surface is fibrinogen independent.

(A)

(B)

Fig. 3. Platelet deposition: (A) PPACK-anticoagulated whole blood was passed at a wall shear rate of 25 s^{-1} over non-coated and coated glass coverslips; (B) Same experiment as in (A) but non-coated and coated coverslips were pretreated with fibrinogen (0.1 mg/ml during 60 min). Symbols: (▲) non-coated; (■) PEI; (Δ) heparin; (□) alginic acid; and (●) chondroitin-6-sulphate.

3.5. Thrombin generation in flowing whole blood

Overall thrombogenicity was assessed in a global assay that measures thrombin generation at the artificial surface in contact with flowing recalcified whole blood. The highest thrombin production was observed for non-coated surfaces (Fig. 4). As a matter of fact, in this case clot formation at the outlet of the tube was seen 10 min after the start of the perfusion. Significant thrombin generation was also measured for PEI-coated surfaces, albeit at a much slower rate. Polysaccharide-coated surfaces showed the least thrombogenic activity. In case of the heparinized surface thrombin generation was virtually absent during a 1-h perfusion with recalcified whole blood. Interestingly, both alginic acid- and chondroitin-6-sulphate-coated surfaces also show very little thrombin generation. Because these polysaccharides do not catalyze thrombin inactivation, it is assumed that thromboresistance of these surfaces is associated with their protein and platelet repelling properties.

4. Discussion

This study demonstrates that polysaccharide coatings greatly reduce the thrombogenicity of artificial surfaces when measured as surface-associated thrombin

Fig. 4. *Thrombin generation*: Thrombin was measured in recalcified whole blood flowing through (▲) non-coated; (■) PEI; (△) heparin; (□) alginic acid; and (●) chondroitin-6-sulphate-coated glass capillaries.

generation. Protein adsorption and platelet adhesion properties of these polysaccharides, however, markedly differ. Heparin-coated surfaces adsorbed the highest amount of (plasma) proteins, whereas chondroitin-6-sulphate and the non-sulphated polysaccharide alginic acid adsorb relatively small amounts of plasma proteins. Platelet adhesion was closely associated with the fibrinogen binding capacity of these surfaces but only when the surfaces were incubated with purified fibrinogen prior to exposure to flowing whole blood. Under these conditions non-coated, PEI-coated and heparin-coated surfaces accreted much more platelets than chondroitin-6-sulphate and alginic acid-coated surfaces. Surfaces that were directly exposed to flowing blood did, with the exception of the PEI-coated surface, not adhere platelets. Thus, the fibrinogen adsorption capacity of artificial surfaces apparently does not predict their platelet adhesion property. The rapid displacement of fibrinogen in the protein layer at the surface-blood interface apparently prevents the accretion of platelets [23,28].

In spite of the apparent absence of platelet adhesion, the thrombogenic activities of the examined surfaces differ greatly when assessed from their thrombin generating potential. Taking into account that platelets are indispensable for thrombin generation and thrombus formation (reviewed in [29]), the physiological sense of *in vitro* platelet adhesion studies, even when flowing whole blood is used, should be questioned. It is apparent that evaluation of the thrombogenicity of artificial surfaces should be performed under conditions that allow thrombin generation. Presently, we can only speculate about how coatings of immobilized polysaccharides reduce the thrombogenic activity of artificial surfaces. We previously reported that a rapid inactivation of surface-localized thrombin by antithrombin bound to immobilized heparin is likely the most effective way to prevent further thrombin generation and thus

thrombus formation at artificial surfaces [30]. The present work strongly supports this notion. Antithrombin adsorbs readily to all surfaces, but, with the exception of heparin-coated surfaces, no antithrombin could be detected in the protein layer that was deposited from flowing plasma. It is apparent that in these cases antithrombin is readily displaced or masked by other plasma proteins. Importantly, antithrombin bound to immobilized heparin can apparently not be displaced and retains its anticoagulant activity. It appeared that a small fraction of total bound antithrombin (about 35%) could be displaced by adding free heparin, indicating that most of the antithrombin is not heparin bound. Yet, this fraction of heparin-bound antithrombin seems to be extremely efficient to abrogate thrombin formation at artificial surfaces. The two important features that can explain this efficiency are: (1) thrombin plays a critical role in its own generation (reviewed in [31]), and (2) localizing antithrombin near the site of thrombin generation greatly eliminates the transport limitations in the thrombin-antithrombin reaction.

Immobilized alginic acid and chondroitin-6-sulphate are devoid of antithrombin activity and yet makes an artificial surface thromboresistant. We note, however, that blood coagulation is initiated in a platelet-independent manner, namely by tissue factor or by activation of the contact factors at negatively charged artificial surfaces. One of the proteins involved in the initiation phase of blood coagulation is HMWK. During the propagation phase thrombin converts fibrinogen into fibrin, which in turn is essential for platelet adhesion and activation [32]. Interestingly, when compared with non-treated surfaces alginic acid and chondroitin-6-sulphate coated surfaces adsorb relatively small amounts of both HMWK and fibrinogen. It is therefore feasible that this low protein adsorption acts synergistically in reducing thrombus formation at these surfaces.

From an interfacial physical-chemical point of view it is important to appreciate that the charged polysaccharides were immobilized by multi-point attachment directly on the PEI layer. This way of linking is expected to yield a flat, "side-on" configuration of the polysaccharides on the surface [33]. It is known that the nature of polysaccharide-surface coupling affects the architecture of the aqueous interface and, as a consequence, the properties of the surface layer [34]. Thus, the data stemming from this work can be considered as a useful first step towards the building up of a structure-properties database, aimed at the fine tuning of surface-immobilized polysaccharide layers. Our work further implies that if a high surface density of immobilized heparin exerts an adverse effect like stimulation of neointima formation, mixtures of heparin and low sulphated (chondroitin-6-sulphate) or non-sulphated polysaccharides (alginic acid) could be an interesting alternative to improve the haemocompatibility of artificial surfaces.

5. Conclusions

Numerous materials have been used to coat artificial surfaces in an attempt to reduce their inherent thrombogenicity. Coatings with naturally occurring bioactive or bioinert substances like heparin and phosphorylcholine, respectively, improve biocompatibility by preventing the activation of blood components such as platelets and coagulation factors. In vitro thrombogenicity testing includes often protein adsorption and platelet adhesion. A major drawback of these testing algorithms is that they are performed in the absence of thrombin generation. Our present work demonstrates that the results from protein adsorption and platelet adhesion studies not necessarily predict the thrombogenicity of materials. In contrast to the lower sulphated polysaccharides, alginic acid and chondroitin sulphate, immobilized heparin as used in this study is not protein repelling and interacts with platelets when preincubated with purified fibrinogen. Yet, when recalcified whole blood is perfused through capillaries with a heparin coating, thrombin generation remains below the detection limit. In contrast, capillaries coated with the other sulphated polysaccharides showed a partial thromboresistance. Although all surfaces adsorbed antithrombin from a purified protein solution, albeit to different extent, only heparinized surfaces adsorbed antithrombin from flowing whole plasma. It is evident that the thromboresistant property of heparinized surfaces relies on neutralization of locally formed thrombin by surface-bound heparin-antithrombin bound complexes. The thromboresistance of alginic acid- and chondroitin sulphate-coated surfaces is most likely due to reduced protein adsorption, which in turn results in a reduced contact activation of the coagulation system.

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Covalently-bound heparin makes
collagen thromboresistant.

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Covalently-Bound Heparin Makes Collagen Thromboresistant

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Objective—Blood compatibility of artificial surfaces depends on their immunogenic and thrombogenic properties. Collagen's weak antigenicity makes it an attractive candidate for stent coatings or fabrication of vascular grafts. However, the thrombogenic nature of collagen limits its application. We examined whether heparinization can make collagen more thromboresistant.

Methods and Results—Collagen was heparinized by crosslinking collagen with extensively periodate oxidized heparin and/or by covalently bonding of mildly periodate oxidized heparin. Both ways of heparinization have no effect on platelet adhesion and could not abolish induction of platelet procoagulant activity. However, thrombin generation was completely prevented under static and flow conditions. The functionality of immobilized heparin was confirmed by specific uptake of antithrombin, 13.5 ± 4.7 pmol/cm² and 1.95 ± 0.21 pmol/cm² for mildly and heavily periodate heparin, respectively.

Conclusions—These results indicate that immobilization of heparin on collagen, even as a crosslinker, is a very effective way to prevent surface thrombus formation. These data encourage the application of heparinized collagen as stent-graft material in animal and eventually human studies. (*Arterioscler Thromb Vasc Biol.* 2004;24:613-617.)

Key Words: collagen ■ heparin ■ thrombogenicity ■ thrombosis ■ blood flow

Cardiovascular disease, including vascular stenosis, is still the leading cause of death in Western society. Obstructive atherosclerotic disease, causing angina pectoris or even myocardial infarction, is currently treated by the implantation of a stent or through bypass surgery. Unfortunately, attempts to implant vascular grafts with a small diameter are not successful because of thrombotic and inflammatory reactions.¹⁻⁶ Thus, to enhance blood compatibility, a surface has to be designed that is both anti-immunogenic and thromboresistant. Collagen has been widely used in medical applications, including skin replacement, bone substitutes, and artificial valves.^{7,8} Recently, a number of investigators attempted to use modified collagen as a vascular graft material.⁹⁻¹² Potential advantages of the natural biological polymer collagen are its weak antigenicity and high tensile strength, which can resist high arterial blood pressures. Furthermore, collagen is a suitable substrate for endothelial cell growth in vitro,¹¹ which makes its application as an artificial vessel even more attractive. Nevertheless, the prothrombotic properties of collagen¹³ are a major drawback in its applicability in blood contacting devices.

To make collagen more thromboresistant, Wissink et al¹⁴ coupled the sulfated polysaccharide, heparin, to collagen. An in vitro assay showed that the immobilized heparin was

functionally active as it accelerated the thrombin antithrombin (AT) reaction. In addition, this group demonstrated that crosslinking of collagen had an adverse effect on the antigenicity and degradation rate of collagen, but stimulated endothelial cell adhesion and proliferation.^{11,12} The suitability of collagen as a vascular graft material was also emphasized in a rabbit study, which established that collagen grafts were completely endothelialized after one month of implantation.¹⁰

This study was undertaken to get a better understanding of the precise antithrombotic functions of immobilized heparin. Three types of heparinized collagen (heparin-crosslinked collagen, heparinized heparin-crosslinked collagen, and heparinized EDC/NHS-crosslinked collagen) were evaluated for their thrombogenicity by measuring platelet adhesion, platelet activation, and thrombin generation.

Methods

Preparation of Collagen and Heparinized Collagen Sheets

Four different types of type I collagen (Syntacoll) sheets were prepared; namely, noncrosslinked collagen (NC), heparin-crosslinked collagen (HC), heparinized EDC/NHS-crosslinked col-

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lagen (HEC), and heparinized heparin crosslinked collagen (HHC). For a detailed description, see <http://atvb.ahajournals.org>.

Heparin Assay

In all (static) experiments collagen and heparinized collagen sheets, mounted in the wells of a 48-well microtiter plate (Corning), were repeatedly rinsed in HEPES buffer (5 mmol/L HEPES, 136 mmol/L NaCl, 2.7 mmol/L KCl, 2 mmol/L $MgCl_2$, 1 mg/mL BSA, pH 7.45) to remove unbound heparin. The amount of heparin in the rinse-buffer was measured by adding a 30 μ L aliquot into 112.5 μ L AT (0.66 μ mol/L). After an incubation of 3 minutes at 37°C, 7.5 μ L bovine factor Xa (1 μ mol/L) was added. At timed intervals, 25- μ L samples were transferred into a cuvette containing 425 μ L HEPES buffer containing 20 mmol/L EDTA and 50 μ L chromogenic substrate S2765 (2 mmol/L, Chromogenix). The amount of heparin was calculated from the rate of disappearance of the factor Xa activity. A reference curve was constructed from heparin (190 U/mg; Diosynth) that was used to prepare heparinized collagen.

Platelet Adhesion and Procoagulant Activity

Venous blood was obtained from volunteers who denied taking any medication in the 2 weeks before sampling. Washed platelets were prepared as described.¹⁵ Wells were blocked with 20 mg/mL BSA in HEPES buffer. A 1-mL suspension of washed platelets (3×10^8 platelets) was then added to collagen and heparinized collagen films in a 48-well microtiter plate, and nonbound platelets were removed after an incubation of 45 minutes at room temperature under stationary conditions. For scanning electron microscopy (SEM) analysis, collagen sheets were treated as described.¹⁶ SEM images were analyzed for platelet surface coverage using ImagePro software (Media Cybernetics). Collagen adherent platelets were incubated with 3 mmol/L $CaCl_2$ in HEPES buffer for 15 minutes. This was followed by incubation with 1 μ g/mL Oregon Green-labeled annexin V (NeXins) and 3 mmol/L $CaCl_2$ in HEPES buffer. Nonbound annexin V was removed with HEPES buffer containing 3 mmol/L $CaCl_2$, and platelet bound annexin V was eluted in HEPES buffer containing 20 mmol/L EDTA. The amount of recovered annexin V was measured in a spectrofluorometer (SLM) with excitation wavelength 485 nm and emission wavelength 535 nm. A reference curve was constructed with known amounts of Oregon Green-labeled annexin V.

Determination of Specific Antithrombin Binding to Heparinized Collagen

Specific binding of AT to heparinized collagen was determined as described.¹⁷ See <http://atvb.ahajournals.org> for further information.

Thrombin Generation

Collagen films with adherent platelets were exposed to 300 μ L of citrated PFP or AT depleted plasma (Biopool) containing 250 μ mol/L of the fluorogenic thrombin substrate Z-GGR-AMC (Bachem). Coagulation was started by the addition of 20 mmol/L of $CaCl_2$. Fluorescence tracings as a result of thrombin generation were recorded with a temperature controlled microplate fluorometer (SPECTRAMAX GEMINI XS, Molecular Devices) at 37°C with λ_{ex} =368 nm and λ_{em} =460 nm. Floating point averaged, first derivative traces were constructed to obtain thrombin generation, as described by Hemker et al.¹⁸

Thrombin generation at collagen surfaces in flowing whole blood was measured as follows: Small pieces of the collagen sheets were cut into pieces of 15 mm length and 10 mm width. The sheets were pretreated in deionized water and folded around a stent of 15 mm length and 3.0 mm diameter (beStent Brava™, Medtronic) with a few mm overlap. After drying at room temperature, 1.5-mm silicone rings were fixed at both ends. Next, the stents were expanded in silicone tubing with inner diameter of 1.5 mm and rinsed overnight with HEPES buffer at a flow rate of 500 μ L/min to remove nonbound heparin. Flowing citrated whole blood (27 μ L/min) was mixed with flowing (3 μ L/min) HEPES buffer containing 200 mmol/L $CaCl_2$, and perfused through the stent-containing silicone tube as described.¹⁹ Timed samples (30 μ L) were taken at the outlet and mixed with 420 μ L HEPES buffer containing 20 mmol/L EDTA and 200 μ mol/L

Figure 1. Adhesion of platelets to (heparinized) collagen films. A, SEM images of noncrosslinked collagen (NC), collagen crosslinked with heparin (HC), collagen crosslinked with carboxydimide and additional heparin treatment (HEC), and collagen crosslinked with heparin and additional heparin treatment (HHC) before (top) and after platelet adhesion (middle and bottom). B, platelet surface coverage as a percentage of total area. Shown are mean $\pm$ SD of at least 10 different fields.

S2238 (Chromogenix). The diluted blood sample was stored on ice for the duration of the flow experiment and subsequently centrifuged at 10,000 rpm for 20 seconds. The supernatant was assayed for thrombin activity as described.²⁰

Statistical Analysis

The data were expressed as mean $\pm$ SD. To determine the statistical significance of differences, probability values were obtained with a nonparametric test for two (Mann-Whitney Test) or more (Kruskal-Wallis Test) independent variables.

Results

Effect of Heparinization on Platelet Adhesion

Prior to experimentation, collagen films were extensively rinsed to remove any nonbound heparin. Heparin in the effluent was assayed as described. The collagen films were then incubated with washed platelets. Platelet adhesion was assessed from SEM micrographs (Figure 1A, middle). It is clearly seen that all adherent platelets formed pseudopods (Figure 1A, bottom). No significant differences in platelet adhesion to NC, HC, HEC, and HHC were observed, indicating that heparinization of collagen does not affect platelet adhesion (Figure 1B).

Effect of Heparinization on Collagen-Induced Platelet Procoagulant Activity

Collagen-adherent platelets were examined for their loss of plasma membrane phospholipid asymmetry, utilizing the property of annexin V to bind only to cell membranes

Annexin V Binding to Collagen-Adherent Platelets

Surface	Annexin V Binding	
	Absence of Platelets (ng/surface)	Presence of Platelets (ng/surface)
NC	0	44.4 ± 2.0
HC	0	47.5 ± 10.3
HEC	0	26.9 ± 7.4
HHC	4.4 ± 4.1	28.2 ± 2.5

Data are mean ± SD of three independent experiments (n=5).

containing anionic phospholipids such as phosphatidylserine.²¹ The collagen surfaces, except HHC, did not bind annexin V. No significant difference could be observed in the amount of annexin V bound to platelets on NC and HC ($P=0.42$, Mann-Whitney test), indicating that multi-point coupled heparin, when used as a crosslinker of collagen, does not prevent the collagen-induced activation of adherent platelets (Table 1). However, annexin V binding was significantly reduced for HEC and HHC surfaces with adherent platelets ($P \leq 0.01$, Kruskal-Wallis test). It is apparent that immobilization of heparin, using mildly periodate oxidized heparin, partially inhibits collagen-induced exposure of anionic phospholipids in the outer leaflet of the platelet plasma membrane.

Antithrombin Binding

The AT binding capacities of the different collagen surfaces are shown in Figure 2. The NC surface bound hardly any AT (0.09 ± 0.08 pmol/cm²). When the collagen surfaces contained heparin, either as crosslinker (HC) or immobilized on crosslinked collagen (HHC or HEC), significant amounts of AT bound to these surfaces. HC bound 1.95 ± 0.21 pmol/cm² AT, which is about 25-fold more than NC. Interestingly, heparinization of HC greatly increased the AT binding capacity (23.8 ± 4.1 pmol/cm²). It is very likely that the mild periodate oxidation procedure used to immobilize heparin to the HC preserves the binding sites for AT to a larger extent than the modification of the crosslinker heparin. This notion is supported by the finding that HEC also binds relatively high amounts of AT (13.5 ± 4.7 pmol/cm²).

Thrombin Generation at Collagen Surfaces under Static Conditions

Collagen surfaces with adherent platelets were exposed to recalcified citrated platelet-free plasma, and thrombin gener-

Figure 2. Binding of antithrombin to immobilized heparin. NC, HC, HEC, and HHC were incubated with AT (5 μ mol/L) for one hour. Unbound AT was removed and bound AT was eluted with heparin and quantified (see <http://atvb.ahajournals.org> for more details). Data are mean ± SD of three independent experiments.

Figure 3. Thrombin generation under static conditions. Collagen and heparinized collagen films with adherent platelets were exposed to recalcified platelet-free plasma (A1-D1) or AT deficient plasma (A2-D2) containing the fluorogenic thrombin substrate Z-GGR-AMC. Thrombin formation on NC (A), HC (B), HEC (C), and HHC (D) are shown as a function of time.

ation was continuously monitored as described. It was first established that under the conditions of the assay, collagen does not generate thrombin in the absence of adherent platelets. Thus, under the conditions of the experiment, thrombin generation requires the presence of (activated) platelets (data not shown). When NC surfaces with adherent platelets were exposed to PFP, thrombin activity became detectable after a lag phase of about 12 minutes, and a peak level of thrombin was reached after about 20 minutes (Figure 3, left). In contrast, when the same experiment was performed with HC, HEC, or HHC, thrombin generation was not detected. To examine the contribution of heparin cofactor II, heparinized collagen surfaces with adherent platelets were exposed to AT-depleted plasma. In contrast with normal PFP, significant amounts of thrombin were generated on heparinized collagen films. Maximal levels of thrombin were reached after approximately 20 minutes for HC and 25 minutes for HEC and HHC surfaces (Figure 3, right). The sustained levels of thrombin activity in the case of AT-depleted plasma indicate that heparin cofactor II hardly contributes to the inactivation of thrombin in plasma. Consequently, heparinized-collagen surfaces do not inhibit thrombin generation in AT-depleted plasma.

Thrombin Generation at Collagen Surfaces under Flow Conditions

Stents were wrapped in a collagen sheet, inserted in silicone tubes (inner diameter 1.5 mm), expanded, and perfused with recalcified whole blood. Figure 4 shows the thrombin concentration measured at the outlet of the flow system as a function of the perfusion time. In the control, an empty silicone sleeve with (Figure 4, Δ) and without (Figure 4, $\blacktriangle$) an inserted stent, traces of thrombin appeared 16 and 18 minutes after the start of the perfusion, respectively. Non-

Figure 4. Thrombin generation in flowing blood in contact with collagen coated stents. Empty silicone sleeve (▲), bare stent (△), and stent wrapped in a sheet of NC (●), HC (□), HEC (■), or HHC (○) were perfused with recalcified whole blood at a flow rate of 30 μ L/min. Depicted is the thrombin concentration at the outlet of the flow reactor as a function of perfusion time. Arrows indicate time of extensive clot formation and occlusion of the flow reactor tubing.

crosslinked collagen dramatically shortened the lag phase in thrombin generation and increased the maximal thrombin concentration (Figure 4, ●). The blood perfusion had to be stopped after 40 minutes because of massive clot formation in the silicone tube, resulting in an irregular flow. Neither thrombin generation, nor blood clot formation, were detectable in silicon tubes with stents that were wrapped in collagen that had been cross-linked with heparin or heparinized after cross-linking with heparin or EDC/NHS. These findings suggest that also with flowing whole blood, multi-point attached heparin as a cross-linker by virtue of its AT binding capacity can prevent the initiation and propagation of collagen-induced thrombin generation at the surface of adherent platelets. These findings suggest that multi-point attached heparin, as a cross-linker by virtue of its AT binding capacity, can also prevent the initiation and propagation of collagen-induced thrombin generation at the surface of adherent platelets with flowing whole blood.

Discussion

Different heparinized collagen preparations, namely collagen crosslinked with extensively periodated heparin (HC), HC with immobilized mildly periodated heparin (HHC), and collagen crosslinked with EDC/NHS and treated with mildly periodated heparin-crosslink (HEC), were evaluated for their thrombogenic properties. We wished to reveal to what extent heparin as a collagen crosslinker or covalently bound to the collagen surface is able to reduce collagen's thrombogenicity. Our results demonstrate that numerous platelets adhered to collagen films, and that these platelets supported the process of thrombin generation at the collagen surface. Heparinization of collagen could not abolish the binding and collagen-induced activation of platelets. However, in contrast to nontreated collagen surfaces, no thrombin generation could be detected on heparinized surfaces.

Platelet adhesion, determined as the percentage of surface coverage, was the same for NC, HC, HEC, and HHC. This result clearly indicates that heparinization of collagen (either as a crosslinker or immobilized on crosslinked collagen) does not prevent, or even reduce, platelet adhesion. Moreover, platelets that adhere to heparinized collagen become activated, resulting in pseudopod formation and the appearance

of phosphatidylserine in the outer leaflet of the plasma membrane. This result appears to contrast with findings of other investigators, who demonstrated that heparin coatings significantly decreased platelet adhesion.^{22,23} However, our observation that platelet adhesion on collagen is not inhibited by the presence of immobilized heparin is in line with a previously reported study that demonstrated that heparin immobilization on carbodiimide crosslinked collagen even slightly increased the number of adherent platelets.¹⁴ Contradictory findings in the effects of immobilized heparin on platelet adhesion might be related to the type of surface to which heparin is bound.

Collagen-induced platelet procoagulant activity is mediated by the interaction of collagen with its platelet receptor, GPVI.²⁴ It is apparent that crosslinking collagen with heparin did not inhibit the loss in phospholipid membrane asymmetry, as monitored by annexin-V binding. In contrast, collagen-induced platelet procoagulant activity was significantly reduced when collagen films were treated with mildly periodate oxidized heparin, implying that under these circumstances, when the polysaccharide chain of heparin is almost fully intact, immobilized heparin interferes with platelet activation by collagen. Whether immobilized heparin affects the interaction of collagen with its primary platelet receptor GPVI or with a secondary one (P2Y2 and/or P2Y12) awaits further experimentation. Because these experiments were performed with adherent platelets that were extensively washed, heparin-accelerated inactivation of traces of thrombin is highly unlikely. Interference of heparin with platelet activation by collagen was previously demonstrated under both static and flow conditions.²⁵⁻²⁷ One study showed that under static conditions or at low shear rate, immobilized heparin did not affect platelet deposition to collagen, but strongly inhibited the platelet release reaction.²⁶ Another study in rats revealed that under flow conditions (low and high shear rate), heparin released by stimulated mast cells did not block $\alpha_2\beta_1$ -dependent platelet adhesion, but attenuated subsequent platelet activation as well as fibrinogen binding to platelets.²⁷ Together, these results support the notion that mildly periodate oxidized heparin may interfere with collagen-induced platelet activation (ie, release reaction and exposure of negatively charged phospholipids in outer leaflet of the plasma membrane).

Exposure of heparinized collagen films with adherent (activated) platelets to recalcified platelet-free plasma demonstrated that, independent of the method of heparinization, thrombin formation is abolished completely during the time of the experiment (60 minutes). We surmise that the amount of AT (± 2 pmol/cm²) that binds to collagen films crosslinked with highly periodated heparin is already sufficient to prevent thrombin generation at these surfaces. These results extend earlier findings from our group illustrating that AT bound to immobilized heparin is probably the most effective way to prevent thrombus formation at an artificial surface.^{19,28,29} To confirm that the thromboresistance of the heparinized collagen surfaces was indeed AT-dependent, thrombin generation was measured with AT-depleted plasma. While thrombin generation on heparinized collagen with adhering platelets was strongly inhibited when exposed to normal plasma,

significant thrombin generation was observed when the same experiment was performed with AT-depleted plasma, suggesting that the thromboresistance of heparinized collagen surfaces is fully dependent on the presence of AT. The physiological relevance of our findings was established in a perfusion setup, where stents coated with heparinized collagen films were exposed to flowing whole blood. While an explosive thrombin formation was observed in blood that passes the nonheparinized collagen-coated stents, stents coated with heparinized collagen appeared to be fully thromboresistant. We postulate that immobilization of heparin effectively catalyzes the thrombin-antithrombin reaction, whereby levels of surface-located thrombin remain below the threshold values that are needed to exert its positive feedback reactions, such as activation of the cofactors factors V and VIII and generation of procoagulant cell membranes, that regulate thrombin formation. The *in vitro* finding that heparinized collagen films are highly thromboresistant encourages further research on the application of collagen in medical devices in contact with blood.

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GENERAL DISCUSSION

Introduction

The physiological significance of thrombin generation in man is apparent from the observations that even incomplete deficiency of prothrombin or deficiency of factors VIII and IX is associated with severe bleeding disorders.^{1,2} In addition, mice deficient in prothrombin die shortly after birth from haemorrhage, indicating that thrombin deficiency is incompatible with haemostasis and post natal survival.^{3,4} Activated platelets play a critical role in thrombin generation. The surfaces of these platelets serve as binding sites for enzymatic complexes that catalyze prothrombin and factor X activation. The studies presented in the first part of this thesis (chapters 2, 3, 4) were aimed to obtain better insight in the mechanisms of thrombus formation, in particular with respect to exposure of procoagulant platelet surfaces and thrombin generation at these surfaces. Progress in understanding the process of thrombus formation can improve current antithrombotic therapies. To date, treatment of thrombotic vessels includes implantation of stents or vascular grafts to recuperate blood flow. There is, however, a high percentage of restenosis or thrombo-embolic complications occurring within months or even weeks after surgery (reviewed in⁵). Moreover, the anticoagulant therapy that is needed to prevent acute thrombus formation is frequently associated with a severe bleeding risk. The second part of this thesis (chapters 5, 6, 7) is therefore concerned with artificial surfaces in contact with blood. Because of its pivotal role in thrombus formation, we considered that surface modification with thrombin inhibitors, like heparin, could improve haemocompatibility. Although various heparin coatings have currently been developed, reports on the improvement of clinical manifestation have been mixed.⁶⁻¹¹ The studies described in this thesis were undertaken to gain more insight in the working mechanism of surface immobilized heparin.

Adhesive receptors GP Iba and integrin $\alpha_{IIb}\beta_3$ stimulate thrombin generation in platelet-rich plasma

Thrombin (and thrombus) formation is highly dependent on: 1) initiation of coagulation, via the extrinsic tissue factor pathway or through contact activation on artificial blood contacting surface and 2) procoagulant cell membranes, which catalyse tenase and prothrombinase reactions.

After endothelial damage or rupture of an atherosclerotic plaque, coagulation is initiated by TF exposed on vascular cells (i.e. fibroblasts, smooth muscle and endothelial cells). Thrombin generation is then catalysed on the membrane of procoagulant platelets adhering to matrix proteins like collagen, since fibroblasts or smooth muscle cells do not provide suitable surfaces to catalyse this process.¹² After the cells in the vessel wall are covered with platelets and fibrin, an initiator different from vessel wall derived TF is essential for thrombus propagation. Recently, it was proposed that leukocyte-derived microparticles, bearing both TF and PSGL-1, circulate in blood and accumulate in the developing platelet rich thrombus (reviewed in^{13,14}). Platelets interact with these particles via their membrane protein P-Selectin. The significance of this blood borne tissue factor for thrombus formation is however still a matter of debate.^{15,16} An alternative for TF dependent propagation of thrombus formation is activation of FXI by thrombin.^{17,18} In this process, thrombin that is initially produced via the TF pathway, activates FXI on the surface of activated platelets.¹⁹

Several investigators described that platelets in a thrombus, adherent to fibrin, required activation via the GP Iba-VWF axis and thrombin to become procoagulant.^{20,21} In contrast, Dörman et al. reported that VWF binding to GP Iba is not essential for the procoagulant response of thrombin stimulated platelets.²² In chapter 2, we demonstrate that the stimulating effect of the GP Iba-VWF interaction on thrombin induced platelet procoagulant activity is only observed when platelets are subjected to high shear rates. This likely explains the deviating results of Dörmann et al.²², since their work was conducted in the absence of high shear stress.

Besides GP Iba, another platelet adhesive receptor, namely the integrin $\alpha_{IIb}\beta_3$, was shown to contribute to procoagulant activity. Antagonists of this integrin, such as the c7e3 Fab fragment abciximab, effectively decrease thrombin generation in coagulating plasma (chapter 2). Our results indicate for the first time that the inhibitory effect of abciximab on thrombin generation increases with shear rate. This suggests that GP Iba-VWF and integrin $\alpha_{IIb}\beta_3$ signals act synergistically in platelet procoagulant activation. Experiments with blood from Glanzmann patients further confirm a role of integrin $\alpha_{IIb}\beta_3$ in thrombin generation, since thrombin formation is strongly reduced (chapter 2).²⁰ To our surprise, abciximab stimulated coagulation in Glanzmann patients. Because abciximab had no effect in platelet free plasma, the effect must be platelet dependent. Thus far, we have no explanation for this striking effect of abciximab. Overall we conclude that at arterial shear rate platelet interaction with fibrin through GP Iba-VWF and by way of integrin $\alpha_{IIb}\beta_3$ plays an important role in the procoagulant response of platelets.

VWF C-domain mediates platelet adhesion to fibrin

The traditional view of haemostasis in which fibrin only functions in stabilizing the platelet plug has to be changed. First of all, fibrin was shown to be actively involved in platelet adhesion and aggregation.²⁵⁻²⁸ Its active role in haemostasis was further emphasized in patients with platelet disorders, in which fibrin formed through addition of recombinant factor VIIa, compensated for a lack of platelet adhesion.²⁹ Furthermore, fibrin stimulates thrombin induced platelet procoagulant activation, which might stimulate thrombus growth.^{30,31}

It is well accepted that under conditions of high shear stress VWF plays a critical role in the recruitment of circulating platelets to the fibrin network.^{26,32} In addition, we revealed that thrombin induced procoagulant activity of fibrin adherent platelets is dependent on GP Iba-VWF interaction when shear stress is high (chapter 2). Preventing the interaction of platelets with fibrin could therefore be a novel antithrombotic approach for several reasons: 1) only excessive thrombus growth is inhibited, implying that there is no hemorrhagic tendency as platelet interactions with extracellular matrix proteins remain unaffected, 2) fibrin is a major adhesive protein in diseased vessels, since pathologic studies revealed that there is constant formation and breakdown of fibrin within an atherosclerotic vessel wall.¹³ To specifically inhibit platelet adhesion to fibrin, it was important to investigate how fibrin and VWF interact. In chapter 3 we therefore attempted to localize VWF domains that bind to fibrin. As depicted in Fig. 1, the high affinity binding site on VWF for fibrin was found in the C-domain (amino acids 1637-1899). Perfusion experiments with Δ C-rVWF further established that this C-domain was critical for platelet adhesion to fibrin under conditions of high shear

Figure 1: Structure of von Willebrand factor. The locations of five kinds of conserved structural domains (A, B, C, D, CK) are indicated. Amino acid residues of the mature VWF subunit are numbered 1-2050. Binding sites within the subunit have been localized for several macromolecules as indicated. The location of the fibrin binding site in the C1C2-domain was determined in chapter 3 of this thesis.

stress (chapter 3). Further research is required to reveal which specific amino-acid sequence in the C-domain is responsible for fibrin binding.

Recently, the adhesive protein thrombospondin-1 was shown to be an alternative substrate for VWF under high shear stress conditions, which was able to interact directly with GP Iba. In addition, thrombospondin-1 was identified as an adhesive substrate for red blood cells to fibrin. We noted a 26% identity in 33 amino acids between thrombospondin-1 amino acids 317-409 and the VWF-C domain (amino acids 1667-1789). This suggests that thrombospondin-1 might interact with fibrin through the same amino-acid sequence as VWF and through this interaction could mediate the adhesion of red blood cells at venous shear rate. It is not feasible that thrombospondin-1 bound to fibrin acts as an adhesive protein for platelets under high shear conditions since we observed no platelet adhesion when VWF deficient mouse blood was perfused over fibrin layers made from VWF deficient plasma (chapter 4).

Role of thrombin receptors in platelet procoagulant activity

Current literature points to thrombin as a key mediator of the platelet procoagulant response. While thrombin alone appeared to be a weak stimulator of procoagulant activity,³⁴⁻³⁷ it strongly stimulates this process in platelets residing in a thrombus and thus in contact with adhesive substrates like collagen, VWF or fibrin(ogen).^{20,21,37} To date, results are rather inconsistent with respect to which thrombin receptor is implicated in platelet procoagulant activation.^{22,36,38,39} To study the role of thrombin receptors GP Iba, PAR-1 and PAR-4 in the generation of a procoagulant membrane surface, we activated platelets with collagen and thrombin in the presence of various antagonists of these receptors. When PAR-1 signalling was ceased, due to treatment of platelets with the intracellular antagonist P1pal-12⁴⁰ or receptor desensitisation, procoagulant activity was severely reduced to that of collagen activated platelets. On the other hand, blocking the interaction of thrombin with GP Iba or abolition of PAR-4 signalling did not interrupt with collagen plus thrombin induced platelet procoagulant activity (Chapter 4).

We established by flow cytometry that all platelets exposing PtdSer in their exoleaflet, had sustained elevations

in $[Ca^{2+}]_i$. In addition, interfering with PAR-1 signalling revealed that activation of PAR-1 by thrombin was crucial for the sustained elevations in $[Ca^{2+}]_i$ induced by collagen-thrombin (chapter 4). These results suggest that PAR-1 activation evokes a sustained elevation in $[Ca^{2+}]_i$, which strongly enhances a phospholipid scramblase that shuttles negatively charged phospholipids from the inner leaflet to the outer leaflet of the plasma membrane in collagen adherent platelets. Still, it is rather puzzling by which mechanism this thrombin receptor is able to maintain a high sustained intracellular level because of its short half-life. It is tempting to speculate that collagen receptor signalling alters the kinetics of inactivation of PAR-1.

The validity of current in vitro thrombogenicity tests should be questioned

To improve biocompatibility of medical devices in contact with blood, surfaces have to be designed that are both anti-immunogenic and antithrombotic.⁴¹ In developing such surfaces it is important to use assays with a high prognostic value. Currently, there is a tendency to focus on individual aspects thrombus formation rather than considering the whole process. For example, biocompatibility of artificial surfaces in contact with blood is often assessed as adsorption of purified proteins or platelet adhesion studies from anticoagulated blood, and thus in the absence of thrombin generation.⁴²⁻⁴⁴ In chapters 5 and 6 of this thesis we questioned whether such assays can predict the in vivo thrombogenic properties.

We demonstrated that the results of adsorption studies with isolated proteins are not predictive for in vivo functioning. (chapter 5). Non-coated and heparin-coated surfaces adsorbed high amounts of fibrinogen from buffer solution. However, no fibrinogen could be detected in protein layers adsorbed from plasma. On the other hand, when plasma was strongly diluted, fibrinogen bound to both surfaces. These data confirmed the findings of Vroman et al.⁴⁵, showing that the deposition of a protein layer from flowing whole blood is followed by a rearrangement, in which fibrinogen is rapidly displaced. If fibrinogen adsorption is important for platelet adhesion, the fact that adsorption and desorption kinetics of plasma proteins are strongly influenced by the concentration and surface affinity of other plasma proteins⁴⁶, makes protein adsorption studies from buffer solutions unreliable in predicting platelet adhesion to such surfaces. Purified antithrombin (AT) adsorbed readily to both non treated and heparinized surfaces. However, when these surfaces were exposed to flowing plasma, AT could be detected only in the protein layer deposited on the heparinized surface. Apparently, it is readily displaced or masked by other proteins on non-coated surfaces, but on heparinized surfaces AT cannot be dislodged due to a specific interaction with heparin. This again indicates that adsorption assays with purified plasma proteins have a low prognostic value with respect to the in vivo situation.

With respect to platelet adhesion from flowing anticoagulated whole blood, surfaces with immobilized heparin were indistinguishable from uncoated hydrophilic surfaces, suggesting that heparin coatings do not affect thrombus formation (chapter 4, 5). Nevertheless, thrombin production and the formation of occluding thrombi was greatly diminished in recalcified whole blood passing by heparinized surfaces when compared with non-coated surfaces. Overall, our findings indicate that platelet adhesion

studies from anticoagulated blood not necessarily predict thrombogenicity of materials, since anticoagulation prevents thrombin formation and its positive feedback reactions necessary for thrombus formation. In vitro predictions of blood compatibility could therefore better be based on thrombin generation measurements in blood passing by the artificial surface.

Working mechanism of immobilized heparin

Heparin is widely used to prevent thrombosis at blood contacting devices (reviewed in ⁴⁷). The antithrombotic action of heparin is based on the stimulatory effect on the neutralization of thrombin and factor Xa by antithrombin.⁴⁸ A disadvantage of systemic heparin is its rather narrow therapeutic window. In addition, heparin treatment is associated with an increased bleeding risk. Immobilization of heparin on the artificial surface overcomes this problem while reducing surface thrombogenicity.⁴⁹ We found that no significant amounts of thrombin could be generated on heparinized surfaces exposed to flowing blood (chapters 5-7) and while some investigators proposed that heparinized surfaces are protein and platelet repelling⁵⁰⁻⁵³, we (chapters 5 and 6) and others⁵⁴ demonstrated that there is no difference in protein adsorption and platelet adhesion on non-coated and heparinized surfaces. Yet, immobilized heparin specifically interacted with AT and in this way thrombin generated near the blood contacting surface could be neutralized. Evidence for this was provided in chapter 5, showing that AT could only be detected in protein layers deposited on heparinized surfaces. Chapter 6 further confirmed that binding of AT to immobilized heparin was fully responsible for the antithrombotic actions of heparinized surfaces, since surface heparinization abolished thrombin generation in normal, but not AT deficient blood. Surface heparinization even prevented coagulation in blood exposed to collagen surfaces with adherent procoagulant

platelets (chapter 7). Figure 2 schematically depicts our idea how immobilized heparin prevents thrombus formation on blood contacting materials.

Are heparin coatings desirable?

Several heparin coatings have reached the commercial stage in cardiopulmonary bypass and in coronary stents.⁵⁵ Yet, reports on the improvement of in vivo biocompatibility are ambiguous^{6-10,56} and to date, heparin coatings have not yet been shown to significantly improve patient outcome.⁵⁶⁻⁵⁸ This could be due to the fact that heparin is a powerful anticoagulant, but it only partially reduces the inflammatory response associated with artificial surfaces in contact with blood.^{59,60} To improve biocompatibility a surface has to be designed that affects both coagulation and inflammatory processes. Because of its weak antigenicity and its proven biocompatibility in other medical applications⁶¹ (skin replacement, bone substitutes, artificial valves), we proposed to use collagen as stent coating or vascular graft material (chapter 7). In addition, collagen was shown to be a suitable substrate for endothelial cell growth, which make its application as an artificial vessel even more attractive.⁶² A drawback of using collagen in contact with blood is its active role in thrombus formation via platelet adhesion and activation. Yet, the data presented in chapter 7 of this thesis revealed that immobilized heparin, used as a crosslinker of type I collagen, was able to fully prevent occlusive thrombus formation in flowing whole blood passing through stents coated with heparinized-collagen.

On the other hand, highly sulphated polysaccharides, like heparin, could stimulate the process of neo-intima formation by forming complexes with growth factors.^{11,63,64} Interestingly, less sulphated polysaccharides were shown to inhibit smooth muscle cell proliferation.⁶⁵ We therefore compared the antithrombotic properties of immobilized heparin with those of the less sulphated polysaccharides chondroitin-6-sulphate and alginic acid (chapter 6). We revealed that the thrombin generation potential of the low sulphated polysaccharide coatings was greatly diminished with respect to non treated surfaces. This antithrombotic potential is probably explained by the protein repelling property of these surfaces, which in turn results in a reduced contact activation of the coagulation system. Still, in comparison with the heparinized surface, where thrombin generation remains below the detection limit, surfaces coated with chondroitin-6-sulphate and alginic acid showed only partial thromboresistance. It thus appears that heparin bound AT most efficiently abrogates thrombin formation. These findings imply that if a high surface density of immobilized heparin exerts adverse effects, like neo-intima stimulation, mixtures of heparin and lowly sulphated polysaccharides could be an interesting alternative to improve haemocompatibility of artificial surfaces. Other alternatives might be mixtures of immobilized heparin and local drug delivery systems to prevent platelet activation. For instance, platelet thrombin receptor antagonist PIPal-12, which was shown to fully prevent platelet procoagulant activation induced by thrombin (chapter 4).

Figure 2 : When an artificial surface comes in contact with blood, coagulation is triggered via activation of factor XII or via inflammatory monocytes that release tissue factor (initiation). Thrombin formation is then catalysed on the membranes of adherent platelets, potentiating thrombus formation via fibrin deposition and by positive feedback reactions, e.g. platelet activation and generation of the critical cofactors Va and VIIIa (propagation). When heparin is immobilized on the blood contacting surface, heparin-bound AT readily neutralizes the first traces of thrombin, and thus all further positive feedback reaction necessary for thrombus formation (Termination).

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NEDERLANDSE SAMENVATTING

Bloedplaatjes vervullen een belangrijke rol in de bloedstolling (hemostase). De binnenbekleding van een intact bloedvat bestaat uit endotheelcellen die antitrombotische eigenschappen hebben. Als het endotheel beschadigd raakt gaan bloedplaatjes zich hechten aan subendotheliale matrix eiwitten zoals collageen (adhesie). Bij gezonde personen is er een evenwicht dat ervoor zorgt dat bloedingen snel gestopt kunnen worden en er niet onnodig stolsels gevormd worden. Wanneer er spontaan een trombus ontstaat die een bloedvat afsluit noemen we dit trombose. Bij een arteriële trombose komt er in het weefsel achter de trombose geen bloed meer waardoor dit weefsel kan afsterven na verloop van tijd. Arteriële trombose is een acute complicatie die zich kan ontwikkelen in atherosclerotische (verkalkte) bloedvaten. Gebeurt dit in het hart, dan spreken we van een hartinfarct en als dit in de hersenen plaatsvindt, noemen we dit een herseninfarct.

Fundamentele kennis omtrent het ontstaan en groei van een trombus vormt de basis voor de ontwikkeling van effectieve antitrombotische middelen. Dit is dan ook de achterliggende gedachte van het onderzoek dat beschreven is in dit proefschrift. De studies in het eerste gedeelte van dit proefschrift (hoofdstukken 2-4) zijn uitgevoerd om een beter begrip te krijgen van de eiwitten, receptoren en signaaltransductie processen die verantwoordelijk zijn voor de vorming van plaatjes met stollingsbevorderende (procoagulante) activiteit.

Bij behandeling van vernauwde of door een trombus verstopte bloedvaten wordt vaak lichaamsvreemd materiaal gebruikt wordt, wat veelvuldig leidt tot klinische complicaties. Een voorbeeld is het plaatsen van een stent na percutane transluminale angioplastie (dotteren). Bekende problemen zijn plotselinge occlusie van stents door trombi, ook wel restenose genoemd, of bloedingproblemen als gevolg van antiplaatjes- of antistollingstherapie. Omdat trombine onontbeerlijk is in het proces van trombusvorming veronderstellen wij dat het coaten met trombine remmers, zoals heparine, de biocompatibiliteit van lichaamsvreemde oppervlakken in contact met bloed zal verbeteren. De resultaten van klinische studies met heparine coatings zijn echter niet consistent positief. Het tweede deel van dit proefschrift (hoofdstukken 5-7) bestudeert contact van bloed met lichaamsvreemde oppervlakken. Het doel van deze studies is het verkrijgen van meer inzicht in het werkingsmechanisme van heparine coatings.

GP Iba en integrine $\alpha_{IIb}\beta_3$ stimuleren trombine generatie in plaatjes-rijk plasma

Onder arteriële stromingscondities is plaatjesadhesie afhankelijk van de binding aan von Willebrand factor (VWF) via glycoproteïne (GP) Iba en integrine $\alpha_{IIb}\beta_3$. Beide receptoren zouden ook een rol kunnen spelen in de vorming van plaatjes met procoagulante activiteit. In hoofdstuk 2 hebben wij gekeken naar het effect van afschuifsnelheden, veroorzaakt door een roterende cilinder, op GP Iba en integrine $\alpha_{IIb}\beta_3$ afhankelijkke trombine generatie in stollend plaatjes-rijk plasma (PRP). Trombinevorming werd continu gemeten met behulp van een fluorescerend trombinesubstraat. Antilichamen tegen VWF bindingsepitopen op GP Iba reduceerden trombinevorming in PRP met 25-30% bij een hoge (420 s^{-1}), maar niet bij een lage (120 s^{-1}), afschuifsnelheid. De integrine $\alpha_{IIb}\beta_3$ antagonist abciximab reduceerde de plaatjes-afhankelijke trombinevorming tot 43%. Verder nam het remmende effect van abciximab af bij hogere afschuifsnelheden. De rol van integrine $\alpha_{IIb}\beta_3$ werd verder bevestigd in experimenten met PRP van Glanzmann

patiënten, die geen functioneel integrine $\alpha_{IIb}\beta_3$ tot expressie brengen. Trombinevorming in het PRP van deze patiënten was sterk gereduceerd. Verassend genoeg stimuleerde de remmer van de plaatjes aggregatie (abciximab) trombinevorming in PRP van Glanzmann patiënten. Omdat abciximab geen effect had op de trombinevorming in plaatjes-vrij plasma, moet dit effect plaatjesafhankelijk zijn. Tot nu toe hebben we nog geen verklaring gevonden voor dit fenomeen. We concluderen dat bij arteriële afschuifsnelheden de vorming van een procoagulant bloedplaatje en trombinevorming afhankelijk zijn van plaatjesadhesie (aan fibrine) via GP Iba en integrine $\alpha_{IIb}\beta_3$.

VWF-C domein is verantwoordelijk voor plaatjesadhesie aan fibrine

Fibrine stimuleert de procoagulante activiteit van plaatjes door trombine en is op deze manier belangrijk voor de groei van een trombus. Onder arteriële stromingscondities is VWF nodig voor plaatjes interactie met fibrinedraden. Het doel van de studie in hoofdstuk 3 was het lokaliseren van de domeinen op VWF die verantwoordelijk zijn voor de binding aan fibrine. Wij hebben gevonden dat VWF specifiek bindt aan fibrine. Met behulp van VWF mutanten, die bepaalde domeinen missen, hebben we aangetoond dat de bindingsplaats voor fibrine zich bevindt in het C-domein. In een perfusiemodel waarin bloed onder hoge afschuifsnelheden (1500 s^{-1}) over fibrine oppervlakken stroomt hebben we verder laten zien dat het C-domein op VWF essentieel is voor plaatjesadhesie op fibrine. Het specifiek blokkeren van de interactie van plaatjes met fibrine zou een doorbraak kunnen zijn voor het ontwikkelen van een nieuw soort antitromboticum. Het voordeel is dat alleen excessieve trombusgroei geremd wordt. Dit betekent waarschijnlijk dat het risico op bloedingen beperkt blijft omdat plaatjes interactie met extracellulaire matrix eiwitten niet verstoord is. Verder onderzoek is echter nodig om uit te zoeken welke specifieke aminozuur sequentie in het VWF-C domein verantwoordelijk is voor de binding aan fibrine.

PAR-1 is verantwoordelijk voor het synergistisch effect van trombine op de collageen geïnduceerde procoagulante respons van plaatjes

In het bloedstollingsproces is de snelheid van trombinevorming afhankelijk van de aanwezigheid van negatief geladen fosfatidylserine aan het oppervlak van geactiveerde bloedplaatjes. Een bloedplaatje dat fosfatidylserine tot expressie brengt noemen we daarom een procoagulant bloedplaatje. Over het algemeen wordt aangenomen dat de vorming van een procoagulant bloedplaatje geïnitieerd wordt door een verhoging van de intracellulaire calciumconcentratie. Eerder onderzoek heeft aangetoond dat trombine een zwakke agonist is met betrekking tot de vorming van plaatjes met deze procoagulante activiteit. Trombine bleek echter synergistisch te werken met collageen bij het induceren van procoagulante activiteit. In hoofdstuk 4 van dit proefschrift werd onderzocht of dit synergistisch effect gerelateerd is aan veranderingen in intracellulair calcium en wat de bijdrage is van de trombine receptoren GP Iba, PAR-1 en PAR-4.

We toonden aan dat trombine slechts een tijdelijke verhoging geeft in intracellulair calcium en nauwelijks effect heeft op de procoagulante respons van bloedplaatjes. Echter, in aanwezigheid van collageen bleef het calciumsignaal na activering met trombine langdurig op een

hoog niveau, wat resulteerde in een procoagulante activiteit die 15 keer zo hoog was als die bij controle plaatjes. Hieruit concluderen we dat de procoagulante activiteit van een plaatje veroorzaakt wordt door een langdurige verhoging in intracellulair calcium. Dit werd bevestigd met behulp van flow cytometrie, waarin aangetoond werd dat de aanwezigheid van fosfatidylserine aan de buitenkant van het plaatjesmembraan altijd samengaat met een verhoogd intracellulair calcium niveau. Deze verhoging bleek afhankelijk te zijn van de influx van extracellulair calcium. Verder werd slechts een subpopulatie van de plaatjes ($\pm 15\%$) procoagulant na activering met collageen en trombine. Deze heterogene respons zou te maken kunnen hebben met een verminderde receptor functie bij oudere bloedplaatjes, maar dit moet verder uitgezocht worden.

Met behulp van een antilichaam (MoAb 2D2) tegen de trombine-bindingsplaats op GP Iba werd duidelijk dat deze receptor niet betrokken was bij de vorming van plaatjes met procoagulante activiteit. Remming van de trombinereceptoren PAR-1 en PAR-4 met selectieve intracellulaire remmers en selectieve receptor desensitisatie toonde aan dat PAR-1, maar niet PAR-4, activering noodzakelijk is voor zowel de langdurig verhoogde intracellulaire calcium niveaus, als de procoagulante respons geïnduceerd door trombine en collageen. De bevinding dat PAR-1 activering verantwoordelijk is voor de langdurig verhoogde intracellulaire calcium niveaus, is niet in overeenstemming met de korte levensduur van de receptor na activering met trombine. Wij speculeren dan ook dat de interactie van collageen met het bloedplaatje de inactiveringskinetiek van de PAR-1 receptor verandert.

Deze studie impliceert dat antagonisme van PAR-1 een drastisch remmend effect heeft op de procoagulante activiteit van collageen geadhereerde bloedplaatjes. Dit suggereert dat specifieke remming van de PAR-1 receptor een effectieve strategie is om trombinevorming aan het oppervlak van een vormende trombus, en dus trombusgroei, te beperken. Verder onderzoek in trombose modellen is nodig om dit te bevestigen.

Fibrinogeen adsorptie, plaatjesadhesie en trombine vorming aan gehepariniseerde oppervlakken

Thrombus vorming aan een artificieel oppervlak in contact met bloed is een complex proces dat begint met eiwit adsorptie vanuit plasma en eindigt met plaatjesadhesie en fibrinevorming. Om te kijken wat de bijdrage is van eiwitadsorptie en plaatjesadhesie aan trombinevorming hebben we een studie uitgevoerd met niet-gecoate (NC) en gehepariniseerde (HC) oppervlakken (hoofdstuk 5). Eiwit adsorptie, plaatjesadhesie en trombinevorming in volbloed werden bestudeerd met respectievelijk ellipsometrie, een dynamische plaatjesadhesie assay en een amidolytische trombine assay. De trombinevorming in bloed dat stroomt (afschuifsnelheid 50 s^{-1}) over HC oppervlakken was sterk gereduceerd in vergelijking met NC oppervlakken. Aan de andere kant lieten plaatjesadhesie en eiwitadsorptie metingen vanuit ontsteld bloed zien dat plaatjes niet hechten aan NC en HC oppervlakken omdat geen fibrinogeen aanwezig was in de eiwitlaag die gevormd is aan het oppervlak vanuit plasma. Deze bevindingen indiceren dat, in vitro, de trombogeniciteit van een materiaal niet kan worden voorspeld met plaatjesadhesie en eiwitadsorptie data vanuit ontsteld bloed. Pre-incubatie van NC en HC oppervlakken met fibrinogeen of 2000x verdund plasma resulteerde in vergelijkbare hoeveelheden geadsorbeerd fibrinogeen, wat resulteerde in plaatjesadhesie vanuit stromend bloed. Deze

resultaten geven aan dat plaatjesadhesie correleert met de beschikbaarheid van fibrinogeen aan het oppervlak en dat er geen verschil is tussen NC en HC oppervlakken met betrekking tot eiwit adsorptie en plaatjesadhesie vanuit ontsteld bloed. Het HC oppervlak is anti-trombogene vanwege de binding van antitrombine, dat lokaal gevormde trombine direct neutraliseert. Bewijs voor deze stelling wordt geleverd in hoofdstuk 6, waarin we aantoonen dat antitrombine alleen aanwezig is in eiwitlagen, gevormd vanuit plasma, aan HC oppervlakken. Hoofdstuk 7 bevestigt verder dat antitrombine gebonden aan HC oppervlakken volledig verantwoordelijk is voor de antitrombotische eigenschappen, omdat heparinisatie de trombine vorming platlegde in normaal, maar niet in antitrombine deficient bloed.

Klinische studies laten zien dat heparine coatings op stents of synthetische omleidingen (vascular graft) de biocompatibiliteit van deze lichaamsvreemde oppervlakken niet significant verbeteren. Een reden dat heparine coatings tot nog toe geen verbetering opleverden in patientenstudies kan zijn dat gesulfateerde polysaccharides, zoals heparine, interacties aangaan met groeifactoren en op deze manier neo-intima vorming kunnen stimuleren. Aangetoond is echter dat minder gesulfateerde polysaccharides gladde spiercel proliferatie remmen. In hoofdstuk 6 hebben we daarom de trombogene eigenschappen van geïmmobiliseerd heparine vergeleken met minder gesulfateerde polysaccharides chondroitine sulfaat en alginaat. We hebben laten zien dat trombine vorming in bloed dat stroomt over de oppervlakken gecoat met chondroitine sulfaat en alginaat sterk gereduceerd is in vergelijking met het NC oppervlak. Deze reductie kan verklaard worden uit de verminderde eiwitadsorptie aan deze oppervlakken. Echter, in vergelijking met HC oppervlakken waren de oppervlakken gecoat met de minder gesulfateerde polysaccharide slechts gedeeltelijk tromboresistent. Deze bevindingen geven aan dat als een hoge oppervlakte dichtheid van geïmmobiliseerd heparine negatieve effecten heeft, een mengeling van heparine en alginaat of chondroitin sulfaat een interessant alternatief kan zijn.

Geïmmobiliseerd heparine maakt collageen tromboresistent

Bloedcompatibiliteit van lichaamsvreemde oppervlakken hangt af van immunologische en trombogene eigenschappen van het materiaal. Het is bekend dat heparine weinig effect heeft op de ontstekingsreactie die gepaard gaat met de bloed-materiaal interactie. Wij stellen voor om collageen te gebruiken als stent of vascular graft coating vanwege zijn zwakke antigeniciteit, de bewezen biocompatibiliteit bij verschillende medische toepassingen (artificiële hartkleppen, huidvervanger) en het geven van een goede ondergrond voor endotheelcel groei. Het nadeel van collageen is uiteraard de actieve rol in plaatjesadhesie en -activering. In hoofdstuk 7 hebben wij onderzocht of het immobiliseren van heparine op collageen de trombogeniciteit omlaag kan brengen. Het bleek dat, zowel onder statische, als onder stromingscondities, trombinevorming aan het gehepariniseerde collageen oppervlak werd voorkomen. Dit indiceert dat het immobiliseren van heparine op collageen een effectieve maatregel is om trombusvorming aan het oppervlak te verhinderen. Deze bevindingen zijn een aanmoediging om gehepariniseerd collageen te testen als stent coating in diervormen en uiteindelijk in humane studies.

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Curriculum vitae

Jeffrey Keuren werd geboren op 28 maart 1978 te Roermond en kreeg de doopnamen Jozef Franciscus Wilhelmus. Hij volgde zijn middelbare schoolopleiding aan het Stedelijk Lyceum te Roermond, waar in 1996 het VWO diploma behaald werd. In datzelfde jaar werd aangevangen met de studie Gezondheidswetenschappen aan de Universiteit Maastricht (UM). In 1997 behaalde hij het certificaat Scheikunde aan het James Boswell Instituut, Universiteit Utrecht. Tijdens de studie Gezondheidswetenschappen werd de status van artikel 9-deskundige volgens de Wet op de Dierproeven behaald en slaagde hij voor het examen Stralingshygiëne, deskundigheidsniveau 5^b. Na zijn afstudeerstage bij de vakgroep Biochemie (UM), werd in 2000 het doctoraal diploma Gezondheidswetenschappen met als afstudeerrichting Biologische Gezondheidskunde behaald. Van september 2000 tot april 2004 was hij werkzaam als assistent in opleiding bij de vakgroep Biochemie (UM) onder begeleiding van Dr. T. Lindhout. De resultaten van dit vierjarig promotieonderzoek zijn beschreven in dit proefschrift en gepresenteerd op congressen in onder andere Parijs, Aachen en Birmingham. Tijdens deze periode werd onder andere onderzoek verricht bij het Institute National de la Santé et de la Recherche Médicale (INSERM) in Parijs, Frankrijk. Na het succesvol volgen van een driejarig cursusprogramma aan de Cardiovasculaire Research School Maastricht-Amsterdam (CARMA), werd hem in 2003 het CARMA-PhD-Training Certificate uitgereikt. Sinds april 2004 is de schrijver van dit proefschrift werkzaam als onderzoeker bij de Unit Onderzoek en Onderwijs van Sanquin Bloedbank Regio Zuidoost.

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