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PROCOAGULANT ACTIVITY OF ADHERENT PLATELETS

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ACADEMIC DISSERTATION

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To Mika, Otto, Joel and Aksel

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals.

I **Ilveskero S,** Siljander P, Lassila R. Procoagulant activity of platelets adhered to collagen or plasma clot. *Arterioscler Throm Vasc Biol* 2001;21:628–35.

II **Ilveskero S,** Lassila R. Abciximab inhibits procoagulant activity but not release reaction upon collagen- or clot-adherent platelets. *J Thromb Haemost* 2003;1:805–13.

III **Ilveskero S,** Lassila R. Abciximab – more than tirofiban or eptifibatide – inhibits adhesion-induced procoagulant activity of platelets under static and blood flow conditions. Submitted for publication.

IV **Ilveskero S,** Lassila R. Differential inhibitory effects of platelet glycoprotein IIb/IIIa antagonists on aggregation induced by procoagulant agonists. *Thromb Res,* in press.

ABBREVIATIONS

ACS	acute coronary syndrome
ACT	activated clotting time
ADP	adenosine 5´-diphoshate
APC	activated protein C
AT	antithrombin
BSA	bovine serum albumin
CRP	collagen-related peptide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
F	factor
F1+2	prothrombin fragment 1+2
GFP	gel-filtered platelets
GP	glycoprotein
MI	myocardial infarction
MP	microparticle
PAI-1	plasminogen activator inhibitor-1
PCI	percutaneous coronary intervention
PBS	phosphate-buffered saline
PAR	protease-activated receptor
PCA	procoagulant activity
PE	phosphatidylethanolamine
PF 4	platelet factor 4
PPACK	D-phenylalanyl-L-prolyl-L-arginine chloromethylketone
PS	phosphatidylserine
PPP	platelet-poor plasma
PRP	platelet-rich plasma
SCCS	surface-connected canalicular system
SD	standard deviation
SEM	scanning electron microscopy
TAT	thrombin-antithrombin III complex
TF	tissue factor
TFPI	tissue factor-pathway inhibitor
TRAP	thrombin receptor-activating peptide
TXA_2	thromboxane A_2
VWF	von Willebrand factor

ABSTRACT

Objective: The role of platelets in hemostasis and arterial thrombosis involves their adhesion to sites of vessel injury or ruptured atherosclerotic plaque, aggregation to form a hemostatic plug or a thrombus, and acceleration of coagulation leading to thrombin generation and subsequent formation of an insoluble fibrin clot. Activated platelets promote coagulation by interacting with several coagulation factors and the subsequent formation of thrombin and fibrin further activates platelets, thus leading to explosive amplification of thrombin generation. Despite the important functional role of platelets in directing thrombus formation at the site of vascular injury, relatively little is known about their procoagulant activity (PCA) induced by permanent adhesion to a thrombogenic surface. The aim of this study was to investigate the effect of the adhesion of platelets to a thrombogenic surface on their subsequent procoagulant activation and the role of specific adhesive receptors in mediating this activation.

Methods and Results: Our study design provided activation of platelets by their firm adhesion to collagen or a plasma clot, with subsequent procoagulant alteration of the platelet phospholipid membrane. Permanently adhered platelets showed markedly enhanced PCA when compared with maximally activated platelets in suspension. The adhesion-dependent procoagulant capacity of platelets varied inter-individually but surface-independently over 3-fold. Activation of platelets by their firm adhesion was accompanied by formation of procoagulant microparticles. A monoclonal antibody against glycoprotein (GP) Ib, SZ2, attenuated platelet-dependent thrombin generation by 40% alone and by 65% in combination with abciximab. Blockade of the GP IIb/IIIa receptor by an antibody Fab fragment, abciximab, reduced platelet PCA and formation of prothrombin fragments F1+2 by 35%. Despite equal inhibition of platelet deposition on collagen, abciximab decreased platelet-dependent PCA significantly more than the synthetic GPIIb/IIIa inhibitors tirofiban or eptifibatide both under static and blood flow conditions. None of the antagonists inhibited platelet factor (PF) 4 release from adhering platelets. The effects of the three GPIIb/IIIa antagonists on platelet aggregation induced by several procoagulant GPVI-related agonists; collagen-related peptide (CRP), convulxin, or collagen fibrils, was assessed with a turbidometric aggregometer in PPACK-anticoagulated platelet-rich plasma. At concentrations that equally inhibited ADP- and collagen-induced maximal aggregation, abciximab inhibited GPVI-mediated responses to CRP or convulxin significantly more than the low-molecular-weight antagonists. Inhibition of fibrin polymerization by Gly-Pro-Arg-Pro (GPRP) potentiated tirofiban and eptifibatide to achieve the same effect as abciximab, although GPRP did not affect platelet aggregation in the absence of GPIIb/IIIa antagonists.

Conclusions: Platelet adhesion to a thrombogenic surface causes strong procoagulant activation and formation of procoagulant microparticles. Marked inter-individual but surface-independent variation in platelet procoagulant capacity suggests a uniform platelet

procoagulant mechanism. Additive reduction of platelet-dependent coagulation activity by blockade of GPIIb/IIIa and GPIb supports the involvement of both receptors in the regulation of platelet PCA. At similar anti-aggregatory efficacy abciximab decreased the PCA of adherent platelets more than the low-molecular-weight antagonists, dissociating the inhibitory effects on platelet aggregation and PCA. All GPIIb/IIIa antagonists failed to affect PF 4 release from adhering platelets, suggesting distinct mechanisms for secretion and procoagulant activation. Furthermore, GPIIb/IIIa antagonists exhibit distinct inhibition profiles on platelet aggregation triggered by procoagulant GPVI-related agonists, depending on fibrin polymerization and divalent cations.

INTRODUCTION

Platelet adhesion to exposed subendothelial components of injured vessels, with the resulting formation of platelet-rich clots, is associated with various forms of arterial thrombosis, i.e. myocardial infarction, stroke and peripheral arterial disease. Subendothelial collagen and von Willebrand factor (VWF) are the initial adhesive ligands leading to primary platelet adhesion with subsequent activation and aggregation. Simultaneously, the coagulation system is activated by subendothelial or blood-borne tissue factor (TF). Activated platelets accelerate coagulation by releasing coagulation factor (F) Va and by exposing a procoagulant phospholipid surface for the assembly of several enzymes, cofactors and substrate molecules of the coagulation cascade, thus leading to explosive amplification of thrombin generation. Arterial thrombus propagates as a result of coagulation activation leading to the formation of thrombin and fibrin, both of which further enhance the procoagulant activation of platelets. This interplay establishes a powerful resonance loop between platelets and coagulation in which the adhered and activated platelets have a fundamental role in localizing coagulation activity at the site of vascular injury, thus preventing disseminated intravascular coagulation, and in protecting the coagulation enzymes from inactivation by several inhibitors in plasma. The platelet surface also localizes the negative-feedback reactions of coagulation, such as inactivation of factors Va and VIIIa by activated protein C. In addition to platelets, platelet-derived microvesicles provide the mandatory procoagulant phospholipid surface, thus lowering the threshold at which activated clotting factors will induce explosive thrombin formation.

Despite the important functional role of platelets in directing the coagulation system and its regulation at the site of injury, the contribution of specific platelet receptors and their adhesive ligands responsible for enhanced procoagulant activity (PCA) has only recently gained interest.

To date, relatively little is known about the procoagulant capacity of adherent platelets on a thrombogenic surface. Most studies have essentially used purified coagulation factors instead of plasma, or have been performed with plasma and platelets in suspension or in whole blood in the absence of adhesive ligands.

Due to the multiple interactions of platelets and coagulation proteins in arterial thrombosis both antiplatelet agents and anticoagulants have proved effective in the prevention and treatment of arterial thrombotic events. Antiplatelet agents primarily decrease platelet activation at the site of vascular injury, thus leading to reduced aggregation and thrombus formation. This inhibition of platelet accumulation causes a quantitative decrease in platelet-dependent coagulation activity by diminishing the access of procoagulant phospholipid membranes and platelet secretion products at the thrombus site. However, relatively little is known about the direct effects of antiplatelet agents on the procoagulant capacity of platelets.

The present study was designed to investigate the procoagulant activity of platelets induced by their permanent adhesion to a thrombogenic surface. This approach is validated

by the fact that adhesion to an injured vessel wall (or pre-formed thrombus) is the primary and most physiological route to platelet activation. Moreover, we assessed the roles of specific adhesive receptors and their antagonists on the adhesion-triggered procoagulant activation of platelets. The studies concerning the effects of glycoprotein (GP) IIb/IIIa antagonists on platelet procoagulant capacity contribute to the knowledge of clinical efficacy and safety of these antiplatelet drugs, especially in combination with anticoagulants.

REVIEW OF THE LITERATURE

1. PLATELETS

Human platelets are small anucleate cells approximately 2 μm in diameter which exhibit considerable variation in size and shape depending on their activation state. Platelets are generated by fragmentation of megakaryocytes in the bone marrow and typically circulate for 10 days before they are removed by macrophages (George 2000). Normal platelet counts in blood range from 150 to 400 × 10^9/L. Multifunctional platelets are involved in many physiological and pathophysiological processes including hemostasis and thrombosis, clot retraction, vessel constriction and repair, host defence, inflammation – including promotion of atherosclerosis – and tumor growth or metastasis (Harrison 2005).

1.1. Morphology

Platelet surface

Platelet plasma membrane is a trilaminar unit composed of a bilayer of phospholipids in which cholesterol, glycolipids, and glycoproteins are embedded (White & Clawson 1980). In resting platelets, the outer leaflet of the plasma membrane consists mainly of neutral phospholipids, whereas the negatively charged aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE), are almost exclusively present in the inner leaflet (Bevers *et al.* 1982). While these anionic phospholipids are able to accelerate several steps in plasma coagulation, their localization on the inner side of resting platelets is a crucial mechanism to prevent inappropritate coagulation (Zwaal & Schroit 1997). This asymmetric lipid organisation is maintained by various membrane enzymes acting cooperatively and syncronously (reviewed in Heemskerk *et al.* 2002). In resting platelets, the phospholipid composition is maintained by an aminophospholipid translocase (Bevers *et al.* 1999) promoting inward transport of aminophospholipids. The loss of this phospholipid transmembrane asymmetry, and the consequent surface exposure of PS and PE, is likely to be mediated by a Ca^{2+}-dependent phospholipid scramblase activity and concomitant inhibition of aminophospholipid translocase activity (Comfurius *et al.* 1996, Bevers *et al.* 1999).

Glycocalix, a fuzzy layer of membrane glycoproteins (GP), glycolipids, mucopolysaccarides, and adsorbed plasma proteins, coats the outside surface of the plasma membrane, providing a transfer point for plasma proteins as they are taken up into secretory granules by endocytosis, and producing a net negative surface charge mainly due to sialic acid residues attached to proteins and lipids (Coller 1984). This electrostatic repulsion is likely to prevent resting platelets from attaching to each other and to the negatively charged endothelium.

Platelet membranous systems

The surface-connected canalicular system (SCCS) consists of an elaborate series of conduits contiguous with the surface plasma membrane coursing throughout the platelet interior (Kaushansky & Roth 2004). It acts as an internal membrane reservoir to facilitate platelet spreading and filopodia formation upon adhesion. Additionally, it holds a storage pool of membrane glycoproteins, such as GPIIb/IIIa, that increases on the platelet surface after activation.

The dense tubular system is a closed-channel network of residual endoplasmic reticulum involved in the regulation of intracellular calcium transport (Cutler *et al.* 1978). The dense tubular system is also the main site of prostaglandin synthesis in platelets (Gerrard *et al.* 1978).

Cytoskeleton

The discoid shapes of resting platelets and their ability to contract and spread upon activation depend on a cytoplasmic network of monomers, filaments and tubules constituting the platelet cytoskeleton (Fox 2001). The membrane cytoskeleton associates with the cytoplasmic domains of several transmembrane glycoproteins and appears to regulate the surface mobility of GP receptors (Nurden *et al.* 1994). Upon platelet activation and the rise of intracellular calcium, soluble monomeric actin (G-actin) polymerizes into filaments (F-actin) at the platelet periphery, and bundles of new filaments fill the developing filopodia. Binding of myosin to actin provides the tension required for granule centralization and retraction of filopodia. Additional proteins, such as talin and actin-binding protein, further cross-link the actin filaments and link the membrane cytoskeleton with surface glycoproteins. Cytoskeletal rearrangements, with the development of a contractile ring, is also associated with secretion, although the actual site of granule membrane fusion remains unclear (Kaushansky & Roth 2004).

Secretory granules

Platelets possess secretory granules containing several molecules affecting platelet function, coagulation and fibrinolysis, vascular tone and wound healing. Some of the constituents are synthesized and segregated by megakaryocytes, others are taken up from plasma and incorporated into the granules (Kaushansky & Roth 2004). The main secretory constituents of platelet cytoplasm and granules; α-granules, dense granules, and lysosomes, are listed in Table 1. Upon activation, the granules fuse with the platelet surface membrane and extrude their contents. This granule secretion is a graded process depending on the nature, number, and concentration of the original stimulus/stimuli, either strong (i.e. thrombin and collagen) or weak (i.e. ADP and epinephrine) (Packham *et al.* 1977).

Table 1. Major contents of platelet secretory granules and cytoplasm.

α-granules
- Platelet-specific proteins
  e.g. platelet factor 4, β-thromboglobulin
- Adhesive glycoproteins and α-granule membrane-specific proteins
  e.g. fibrinogen, von Willebrand factor, thrombospondin, fibronectin, vitronectin,
  P-selectin, multimerin
- Coagulation factors, coagulation inhibitors, fibrinolysis inhibitors
  e.g. factor V, protein S, plasminogen activator inhibitor-1
- Mitogenic and angiogenic factors
  e.g. platelet-derived growth factor, vascular endothelial growth factor, transforming growth factor-β
- Other proteins
  e.g. albumin, immunoglobulins

Dense granules
- Adenosine diphosphate (ADP)
- Adenosine triphosphate (ATP)
- Calcium
- Serotonin

Lysosomes
- Acid hydrolases
  e.g. elastase, collagenase, cathepsins, heparinase

Cytoplasm
- Factor XIII, tissue factor pathway inhibitor

References: Ware & Coller 1995; Kaushansky & Roth 2004

Regulation of intracellular calcium

Unstimulated platelets maintain low cytoplasmic Ca^{2+} concentrations (100 to 500 nmol/L) by limiting Ca^{2+} transport from plasma and promoting active efflux. Two pools of calcium exist in platelets: a cytosolic pool regulated by a sodium-calcium antiporter in the plasma membrane and a more slowly exchanging pool in the dense tubular system regulated by calcium, magnesium-ATPase (Kaushansky & Roth 2004).

1.2. Adhesive platelet receptors

GPIb/IX/V

GPIb/IX/V (usually named the GPIb complex) is a set of four single-chain polypeptides (GPIbα, GPIbβ, GPIX and GPV) that mediates the shear-dependent interaction between platelets and VWF (Coller *et al.* 1983a) (Fig. 1). The binding of VWF to the GPIb complex causes platelet activation through activation of protein kinase C, phosphatidylinositol 3-kinase and tyrosine kinases, leading to an increase in intracellular calcium and to inside-out signaling through GPIIb/IIIa followed by aggregation (Berndt *et al.* 2001). Patients with Bernard-Soulier syndrome lack or have deficient levels of GPIb and GPIX and exhibit a bleeding diasthesis (Lopez *et al.* 1998). The active site within the A1 domain of the VWF chain binds GPIbα, and this structure is distinct from the Arg-Gly-Asp (RGD)-containing sequence in VWF that interacts with the GPIIb/IIIa receptor (Vicente *et al.* 1990). The initial step of platelet tethering depends on the GPIb complex and subendothelial VWF, whose affinity requires shear forces generated by arterial blood flow (Kroll *et al.* 1996). This interaction between the GPIb complex on resting circulating platelets and immobilized VWF at the site of arterial injury leads to the initial capture of platelets to subendothelium, making possible their activation and subsequent firm adhesion (Savage *et al.* 1996). However, the interaction between the GPIb complex and VWF is rapidly reversible, seen as rolling of platelets on the VWF-containing surfaces, and is insufficient for stable thrombus formation (Fredrickson *et al.* 1998). Under static conditions these components display very low

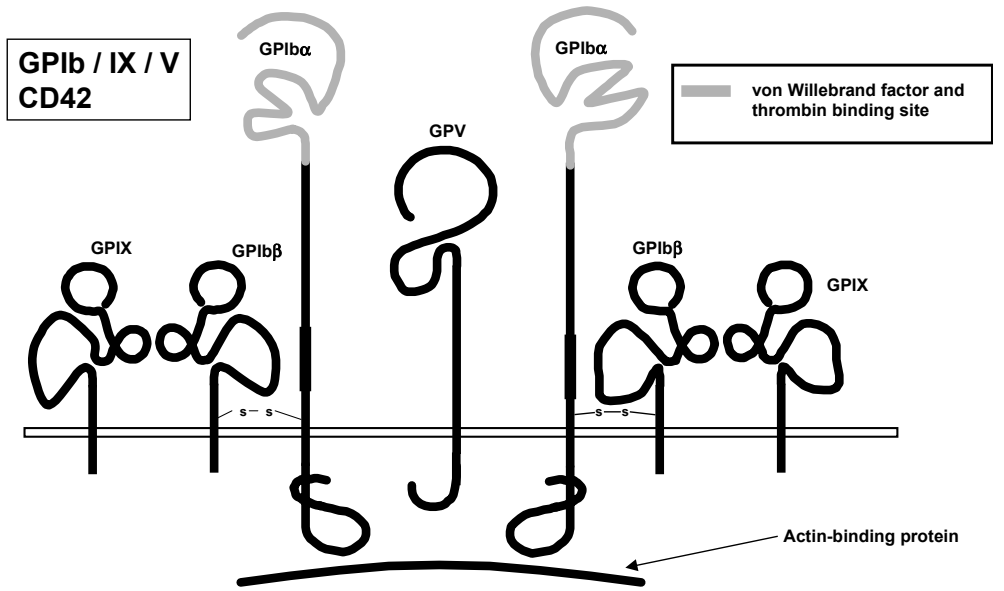

Fig. 1. Structure of platelet glycoprotein (GP) complex Ib/IX/V. Adapted from www.nibsc.ac.uk, Division of Haematology.

affinity for each other, and an exogenous stimulus, such as ristocetin, is needed to induce high affinity binding (De Luca *et al.* 2000). This shear-dependence of the initial adhesive receptor-ligand interaction of platelets explains the predisposition of platelet-rich thrombus in the arterial circulation, whereas in the venous circulation with lower shear forces clot formation is largely independent of the GPIb complex.

GPIbα also possesses a high-affinity binding site for thrombin and the GPIb complex serves as a thrombin receptor after cleavage of the GPV chain (Ramakrishnan *et al.* 2001). Recent studies also suggest a relationship between thrombin binding to the GPIb complex and activation of the protease-activated thrombin receptor PAR-1 (De Candia *et al.* 2001), as well as accelerated procoagulant activation of platelets (Dörmann *et al.* 2000). Previously, Loscalzo *et al.* (1986) observed that VWF serves as a link between polymerizing fibrin and platelet GPIb. Beguin *et al.* (1999, 2004) have reported that fibrin-associated platelet procoagulant activity is mediated via the GPIb complex and VWF. In addition, Factor XI binds to the GPIbα chain on the surface membrane of activated platelets (Baglia *et al.* 2002), where it is activated by thrombin (Baglia & Walsh 1998) and then activates FIX in an alternative pathway to TF-FVIIa (Gailani *et al.* 2001). The GPIb complex can also be considered as an indirect collagen receptor while VWF is attached to subendothelial collagen and this binding together with shear conditions leads to conformational changes of VWF and high-affinity binding to the GPIb complex. In addition, the GPIb complex was found to interact with the Fc receptor (FcR) γ chain, and activation of the GPIb complex induced similar, though weaker, tyrosine phorphorylation as collagen (Wu *et al.* 2001). Recently, Cruz *et al.* (2005) demonstrated that interaction of platelet GPIb with VWF mediates the activation of GPIa/IIa, thus increasing its affinity for collagen.

Collagen receptors

Several types of collagens have been localized in the vessel wall. Fibrillar types I and III are the major constitutes of the vessel wall, although the network-forming type IV collagen is the principal form in basement membranes (Kehrel 1995). The concentrations of these collagens have been shown to increase in the intima of atherosclerotic lesions (Barnes 1985). Collagen types I, III and IV are the most platelet-reactive, capable of inducing both adhesion and aggregation (Sixma *et al.* 1997). However, fibrillar collagen usually consists of a mixture of several collagen types along with other matrix components. Recently, Bernardo *et al.* (2004) proposed that fibrillar collagen may contain a varying quantity of VWF, enhancing platelet adhesion to collagen and subsequent platelet activation. Horm collagen, the most commonly used commercial preparation in platelet studies, consists mainly of equine collagen type I along with a small amount of type III collagen and low levels of other extracellular matrix proteins.

While searching for platelet-reactive sites of collagen, Morton *et al.* (1995) found that a collagen mimetic peptide containing 10 repeats of the Gly-Pro-Hyp sequence induced platelet aggregation independent of GPIa/IIa. This collagen-related peptide (CRP) strongly activates platelets, especially when cross-linked, stimulates tyrosine phosphorylation in platelets in a similar manner as collagen (Asselin *et al.* 1997), and is inhibited by an

antibody against GPVI (Knight *et al.* 1999). Convulxin, a venom protein from the tropical rattlesnake *Crotalus durissus terrificus,* has been shown to be an agonist of GPVI (Polgar *et al.* 1997), but also to bind to GPIbα (Kanaji *et al.* 2003).

The mechanisms of signal transduction underlying the activation of platelets by collagen have been actively investigated for over three decades, but the functional significance of the receptors involved remains incompletely understood. At high shear rates platelet adhesion to collagen requires VWF (Sixma *et al.* 1997). Cho *et al.* (2003) demonstrated that the secondary mediators thromboxane (TXA_2) and adenosine diphosphate (ADP) are required for collagen-induced platelet activation in response to low collagen concentrations (2.5 μg/mL) and that GPIIb/IIIa-mediated outside-in signaling is responsible, at least in part, for this TXA_2 production and ADP secretion. However, large concentrations of collagen (50 μg/mL) induce GPIIb/IIIa activation and weak aggregation response even in the absence of the secondary mediators. In contrast, GPVI-specific agonists induce activation of GPIIb/IIIa without any significant involvement of ADP or TXA_2 (Nieswandt *et al.* 2001a, Quinton *et al.* 2002).

GPIa/IIa

GPIa/IIa (integrin $\alpha_2\beta_1$) was the first collagen receptor to be identified on platelets (Santoro 1986). It is an α-β heterodimer mediating platelet adhesion to collagen in a Mg^{2+}-dependent manner (Fig. 2). According to the classic "two-site, two-step" model, GPIa/IIa mediates firm adhesion to collagen, thereby allowing platelet interaction with the low-affinity receptor GPVI, mainly responsible for collagen-induced platelet activation (Farndale *et al.* 2004). However, the role of GPIaIIa in mediating platelet-collagen interactions is still

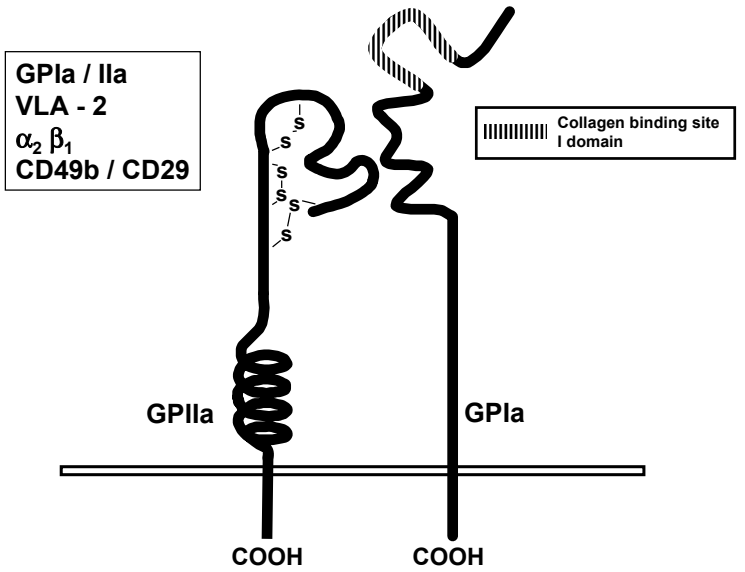

Fig. 2. Structure of platelet glycoprotein (GP) Ia/IIa. Adapted from www.nibsc.ac.uk, Division of Haematology.

controversial. The previous model has been challenged by observations that propose initial interaction between GPVI and collagen leading to subsequent conformational activation of GPIa/IIa (as well as GPIIb/IIIa), reinforced by the released secondary mediators (ADP and TXA_2) causing stable adhesion to collagen and thereby further platelet activation via GPVI (Nieswandt & Watson 2003). Jung and Moroi (2000) first showed that the affinity of GPIa/IIa for collagen is regulated by intracellular signals, suggesting that under resting conditions the affinity of GPIa/IIa for collagen is low, whereas several platelet agonists, including ADP, TXA_2, and GPVI-specific stimuli, remarkably increase this binding affinity (Moroi *et al.* 2000). Recently, Cruz *et al.* (2005) demonstrated that interaction of platelet GPIb with VWF mediates the activation of GPIa/IIa, thus increasing its affinity for collagen. On the contrary, data from a GPVI knock-out mouse model (Kato *et al.* 2003) and from studies with human platelets using a novel single chain antibody fragment against GPVI (Siljander *et al.* 2004) showed that flow conditions in the absence of GPVI lead to loss of aggregation but not primary adhesion, suggesting that GPIa/IIa (together with the GPIb/VWF axis) indeed plays a dominant role in platelet adhesion to collagen. In addition, recent data have provided evidence about signaling through GPIa/IIa (Keely & Parise 1996, Inoue *et al.* 2003).

GPVI

Although GPVI was identified on the platelet surface over 20 years ago (Clemetson *et al.* 1982), its role in collagen-mediated platelet activation has been resolved much later. Quite recently cloned and characterized, GPVI is a member of the immunoglobulin (Ig) receptor superfamily containing two IgC2-like extracellular domains formed by disulphide bonds, a transmembrane region, and a short cytoplasmic tail (Clemetson *et al.* 1999), and exclusively expressed on platelets and megakaryocytes (Fig. 3). The strength of intracellular

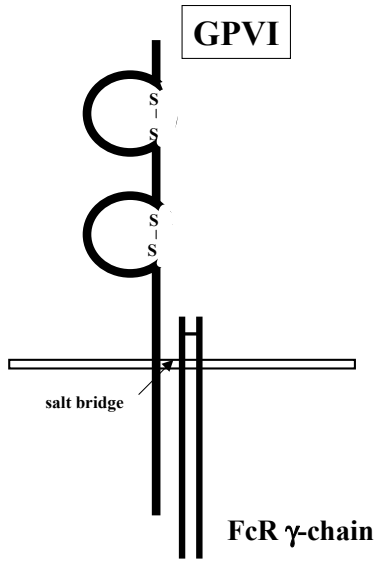

Fig. 3. Structure of platelet glycoprotein (GP) VI. Adapted from Clemetson et al. 1999.

signals arising from ligand binding to GPVI seem to depend on the ability of the agonist to cross-link and induce clustering of GPVI (Farndale *et al.* 2004). The expression of GPVI on platelet membranes is closely associated with the Fc receptor (FcR) γ-chain, which also serves as the main signaling unit for GPVI (Tsuji *et al.* 1997, Nieswandt *et al.* 2000). Only a few GPVI-deficient patients have been reported to date, suffering from a mild bleeding tendency and lacking platelet aggregation in response to collagen (Moroi & Jung 2004). A number of polymorphic variations at the GPVI locus have been identified, but their clinical significance remains unclear (Joutsi-Korhonen *et al.* 2003, Yee & Bray 2004). Furihata *et al.* (2001) observed a 5-fold variability in human platelet GPVI content, correlating with the GPVI-mediated prothrombinase activity.

It is now recognized that stable platelet plug formation on collagen under high shear rates requires signaling through GPVI, reinforced by the release of soluble secondary mediators such as ADP and TXA_2 or by generation of thrombin (Kato *et al.* 2003, Moroi & Jung 2004, Siljander *et al.* 2004). Nieswandt *et al.* (2000) observed that FcRγ-deficient and GPVI-depleted mouse platelets showed no adhesion to collagen under static or flow conditions in the presence of normal levels of GPIa/IIa, GPIb complex, and GPIIb/IIIa. However, this defective adhesion was restored in the presence of Mn^{2+}, which directly activates GPIa/IIa (Nieswandt *et al.* 2000), or agonists that can activate GPIa/IIa through inside-out signaling (Inoue *et al.* 2003). This suggests a role for GPVI in generating the intracellular signal to promote integrin (GIa/IIa and GPIIIb/IIIa) activation rather than in serving as an adhesion receptor. In contrast, Kato *et al.* (2003) observed in a mouse knock-out model that absence of GPVI did not affect primary adhesion of platelets to collagen whereas platelet aggregation upon the initial layer was inhibited. Studies with human platelets have provided controversial results. Goto *et al.* (2002) recently reported almost complete abolition of platelet deposition to collagen with two GPVI-deficient patients. Blocking of GPVI with a single-chain antibody fragment 10B12 abolished aggregation, Ca^{2+} signaling, and procoagulant response of human platelets without affecting primary adhesion to collagen in a whole blood perfusion model at high shear (Siljander *et al.* 2004). Blocking of GPIb or GPIa/IIa, or both receptors, markedly reduced primary adhesion and aggregate formation, whereas combined inhibition of GPVI and GPIb or GPIa/IIa completely abolished primary adhesion to collagen.

Suzuki *et al.* (2003) demonstrated that platelets have internal pools of GPVI on the membranes of SCCS and α-granules, and that GPVI is transported to the platelet surface membrane and to microparticles during activation. A recently identified mechanism for regulating GPVI function on platelets is metalloproteinase-mediated ectodomain shedding in response to binding of collagen, CRP, or convulxin to GPVI and followed by release of a soluble 55-kDa GPVI fragment (Gardiner *et al.* 2004). This process involving the calmodulin-binding site may represent a control mechanism for GPVI-dependent platelet adhesion and activation.

GPIIb/IIIa

GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) is the major surface receptor, present in 40 000 to 80 000 copies, on the plasma membrane of a resting platelet (Fig. 4). It is a transmembrane heterodimer consisting of a two-chain disulphide-linked α subunit with four divalent cation-binding domains and a disulphide bond-rich β subunit (McEver *et al.* 1982). The short cytoplasmic domains of the two subunits are critical for transmembrane signaling and anchor the receptor to the cytoskeletal elements. Another 20 000 to 40 000 receptor copies are localized inside the platelets, mainly in the membranes of α-granules and SCCS, capable of joining the platelet surface membrane upon platelet secretory activation (Wencel-Drake *et al.* 1986, Phillips *et al.* 1988). Cycling of GPIIb/IIIa between the platelet surface and α-granule membrane has been described (Wencel-Drake *et al.* 1993). Previous data suggest that this movement could be involved in fibrinogen transport into α-granules (Harrison 1992). Platelet activation causes mobilization of these intracellular fibrinogen-GP IIb/IIIa complexes to the platelet surface in a secretion-dependent manner (Legrand *et al.* 1989, Nurden *et al.* 1996). GPIIb and GPIIIa require Ca^{2+} to maintain their heterodimeric structure (Jennings *et al.* 1982), and the receptor complex undergoes a Ca^{2+}-dependent conformational change after platelet activation, strongly facilitating binding of fibrinogen and VWF (Sims *et al.* 1991). The outcome is cross-linking of GPIIb/IIIa receptors on adjacent platelets and subsequent platelet aggregation.

GPIIb/IIIa binds to a variety of RDG-containing ligands, including fibrinogen, fibronectin, VWF, vitronectin, and thrombospondin (Plow *et al.* 1985). However, the major ligand site of fibrinogen on the C-terminus of the γ domain lacks the RGD sequence (Farrell *et al.* 1992). The binding affinity depends on the activation state of the GPIIb/IIIa complex and varies both between ligands as well as between soluble and immobilized forms of a ligand. In addition, the plasma coagulation protein prothrombin was shown to act as a ligand for a resting GPIIb/IIIa complex (Byzova & Plow 1997). Prothrombin bound to GPIIb/

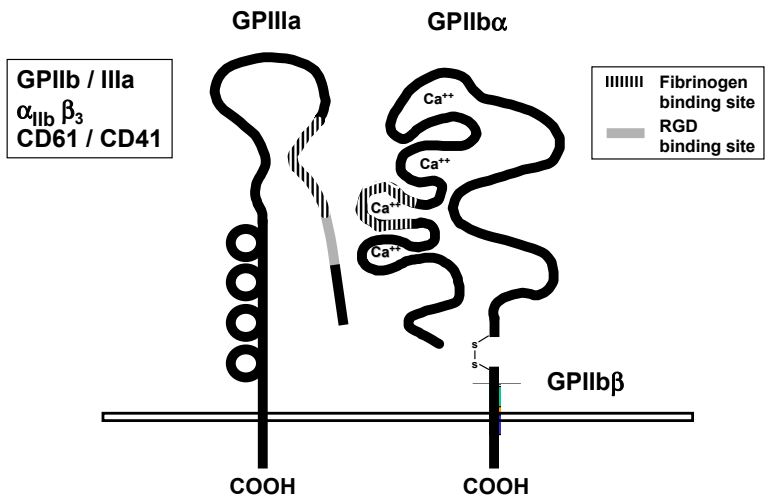

Fig. 4. Structure of platelet glycoprotein (GP) IIb/IIIa. Adapted from www.nibsc.ac.uk, Division of Haematology.

IIIa on a resting platelet can be cleaved by coagulation factor Xa resulting in the release of thrombin. Thrombin in turn can activate platelets through specific receptors (PAR-1 and -4) (Coughlin 1999) causing activation of GPIIb/IIIa receptors. The activated receptor then prefers binding fibrinogen over prothrombin. This early formation of soluble thrombin through resting GPIIb/IIIa receptors is suggested to cause initial procoagulant activation of the platelets, leading to further thrombin generation by the prothrombinase complex on the activated platelet membranes (Byzova & Plow 1997).

Platelet agonists such as ADP, collagen, or thrombin activate GPIIb/IIIa through a complex inside-out signaling process leading to confomational changes of the receptor to a high affinity state (affinity modulation), and clustering of the heterodimers to oligomers through lateral diffusion (avidity modulation) (Shattil 1999). In addition, more GPIIb/IIIa is translocated onto the platelet surface from the membranes of α-granules and SCCS upon platelet shape change and secretion. After ligand binding, the subsequent signaling process, termed outside-in signaling, modulates platelet spreading and stabilizes platelet-platelet interactions (Casserly & Topol 2002).

In addition to its roles in platelet adhesion, aggregation, and procoagulant activity, GPIIb/IIIa is also crucial in mediating clot retraction (Coller *et al.* 1983b), although the exact mechanism and clinical significance of this process remains controversial. Like the GPIb complex, GPIIb/IIIa can be considered as an indirect collagen receptor because subendothelial VWF acts as bridging molecule between platelets and collagen. This interaction between activated GPIIb/IIIa and subendothelial collagen via VWF supports stable platelet adhesion and thrombus formation.

Glanzmann thrombasthenia is a rare hereditary bleeding disorder characterized by decreased or functionally abnormal platelet GPIIb/IIIa causing a substantial reduction in platelet aggregation (Nurden & Caen 1974, Phillips & Agin 1977). Early studies on the procoagulant capacity of platelets from patients with Glanzmann thrombasthenia yielded conflicting data, but Reverter *et al.* (1996) observed a decreased rate and amount of platelet-dependent thrombin generation in these patients. Gemmel *et al.* (1993) reported that platelets of a Glanzmann thrombasthenia patient showed markedly reduced microparticle formation in response to thrombin stimulation, whereas Nomura *et al.* (1993) observed normal MP generation in response to several platelet agonists.

Additional receptors related to adhesion

Other integrin receptors for specific intracellular matrix components include receptors for fibronectin ($\alpha_5\beta_1$) (Piotrowicz *et al.* 1988), laminin ($\alpha_6\beta_1$) (Sonnenberg *et al.* 1988), and vitronectin ($\alpha_v\beta_3$) (Lam *et al.* 1989). PECAM-1 binds to heparin-like molecules and may contribute to platelet-heparin interactions and the interactions of platelets with other cells (Albelda *et al.* 1991). P-selectin is translocated from the membranes of platelet α-granules to the surface membrane upon activation and mediates adhesion of neutrophils and monocytes to activated platelets, thus connecting platelet activation and inflammation (Larsen *et al.* 1989). In addition to P-selectin and the recently discovered CD40/CD40 ligand-pathway on platelets (reviewed in Danese & Fiocchi 2005), receptors for the Fc fragment of IgG

(FcγR) are considered to be a contact link in the immune system. The only FcγR expressed on platelets is FcγRIIa, an integral membrane protein with two extracellular Ig-like domains (Rosenfeld *et al.* 1985).

1.3. Platelet receptors for soluble agonists

Thrombin receptors

In addition to the GPIb complex (after cleavage of GPV) described above, two G-protein-coupled protease-activated receptors (PAR-1 and PAR-4) serve as thrombin receptors on human platelets (Coughlin 1999). On these receptors, the ligand is already present as an amino acid sequence SFLLRN (residues 42–47) on the extracellular chain and becomes unmasked after thrombin cleavage of a peptide bond between Arg 41 and Ser 42. Thereafter this sequence irreversibly binds to the body of the receptor to affect transmembrane signaling. A synthetic peptide mimicking the PAR-1 ligand sequence, thrombin receptor-activating peptide (TRAP), is capable of activating the receptor independent of the cleavage of the peptide bond. The PAR-1 receptor is connected to a significant number of intracellular signaling pathways causing rapid platelet shape change, secretion, aggregation, and calcium mobilisation. Agonists of the PAR-1 receptor induce relatively weak procoagulant responses, whereas agonists to PAR-4 do not stimulate PS exposure (Andersen *et al.* 1999). After activation, PAR-1 is rapidly uncoupled from signaling and internalized into platelet lysosomes for degradation (Hoxie *et al.* 1993). Several studies show physiologic differences between PAR-1 and PAR-4 receptors on human platelets. Blocking the PAR-4 receptor alone has no effect on thrombin-mediated platelet activation, whereas with antibodies against the thrombin interaction site of PAR-1 the PAR-4 receptor is functional only at high thrombin concentrations (Kahn *et al.* 1999).

ADP receptors

Although considered as a weak platelet agonist, ADP has a critical role in amplifying the effects of other platelet agonists. It is synthesized by megakaryocytes, stored at high concentrations in platelet dense granules and released upon platelet stimulation. ADP can directly induce platelet shape change, aggregation, and secretion, although the last event depends on the formation of TXA2 (Meyers *et al.* 1979). ADP interacts with P2 receptors which are divided into two groups; the G protein-coupled P2Y family and the ligand-gated ion channel P2X family (reviewed in Cachet 2001). Two P2Y receptors, P2Y1 and P2Y12, contribute to platelet activation. The primary role of the P2Y12 receptor is to act as a co-stimulus in the presence of low concentrations of other agonists, whereas the P2Y1 receptor has a specific role early in platelet activation. P2X1 is the third platelet receptor for ADP, but its exact role remains unclear. Inhibition of ADP-associated platelet activation with clopidogrel has proven effective in the prevention of arterial thrombosis (CAPRIE 1996). This antithrombotic effect of clopidogrel's active metabolite is thought to be mediated through covalent modifications of the P2Y12 receptor (Savi *et al.* 2000).

Receptors for other soluble agonists

Platelet responses to epinephrine are mediated through α_2 adrenergic receptors. Unlike other platelet agonists, epinephrine does not induce shape change, although it induces aggregation and secretion (Siess *et al.* 1984). Epinephrine enhances platelet activation induced by other agonists in a synergistic fashion, whereas it may reverse the effects of aspirin on thrombus formation (Mustonen & Lassila 1996, Mustonen *et al.* 2001). Activation of platelets by various agonists liberates arachidonic acid which then is converted into thromboxane A_2 by cyclo-oxygenase-1, itself irreversibly inhibited by aspirin through acetylation of a serine residue near its C-terminus (Roth & Calverley 1994). After its release into plasma TXA_2 is capable of activating other platelets although its half-life is only 30 seconds. The TXA_2 receptor is a member of the transmembrane G protein-coupled receptor family localized on platelet plasma membranes. In addition, platelets have G protein-coupled receptors for serotonin (5HT2A), vasopressin, and platelet-activating factor, although the clinical significance of these receptors *in vivo* remains unclear (Kaushansky & Roth 2004).

2. COAGULATION

A cascade or waterfall model of coagulation was originally proposed by two groups in 1964 (Davie & Ratnoff 1964, MacFarlane 1964), and subsequently refined to a scheme consisting of intrinsic and extrinsic coagulation pathways (Fig. 5) reflected by the clinical laboratory tests activated partial thromboplastin time (aPTT) and prothrombin time (PT), respectively. However, several groups have questioned the significance of separate intrinsic

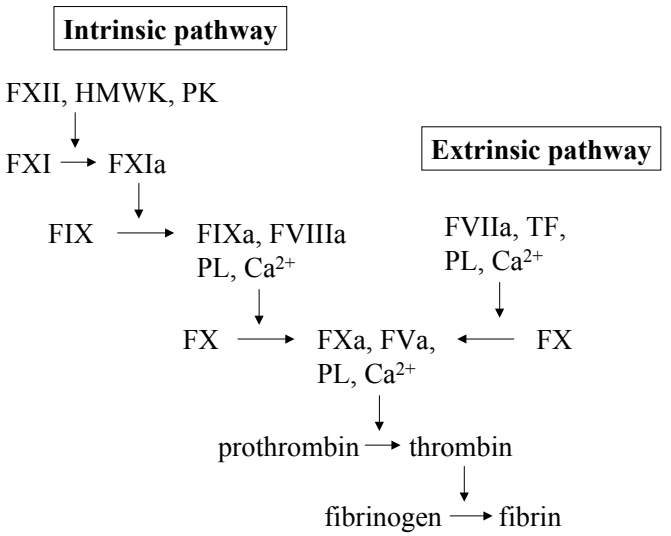

Fig. 5. Former model of the coagulation cascade with intrinsic and extrinsic pathways. F: factor; HMWK: high-molecular-weight kininogen; PK: prekallikrein; PL: phospholipid; TF: tissue factor.

and extrinsic pathways and emphasized the role of cellular components in the regulation of coagulation (Gailani & Broze 1991, Hoffman & Monroe 2001, Butenas & Mann 2002).

2.1. Initiation of coagulation

Tissue factor (TF), also known as thromboplastin or CD142, is a 47 kDa transmembrane cell surface glycoprotein and the principal initiator of the coagulation cascade *in vivo* (Nemerson 1966, Bach 1988). It is expressed at extravascular sites, particularly in richly vascularized organs, forming a protective procoagulant envelope around the vasculature. Pathological expression of TF occurs in macrophage-derived foam cells and smooth muscle cells of atherosclerotic lesions, in certain cancer cells, and in monocytes of patients with Gram-negative endotoxemia/sepsis (reviewed in Eilertsen & Østerud 2004). Recently, several studies have demonstrated the existence of circulating TF in blood (reviewed in Giesen *et al.* 1999). TF antigen has recently been reported to exist intravascularly in multiple forms: as a soluble protein, as a membrane protein in microparticles, or in monocytes, granulocytes, and platelets. One explanation is that TF-bearing procoagulant membrane particles (microparticles) shed from one cell type – possibly monocytes – could bind to the surface of other cells via adhesive receptors (Rauch *et al.* 2000, Conde *et al.* 2003, Eilertsen & Østerud 2004). Moreover, activated platelets upregulate TF expression in monocytes (Celi *et al.* 1994) or decrypt the TF of circulating monocytes, and this blood-borne TF is thought to be essential in the propagation phase of coagulation (Breimo & Østerud 2003). In contrast to microparticle-associated TF in blood, soluble TF seems to be unable to induce FVIIa-mediated thrombin generation (Sturk-Maquelin *et al.* 2003).

According to the revised models of coagulation (Fig. 6) (Hoffman & Monroe 2001, Butenas & Mann 2002), the initiation phase of blood coagulation occurs when TF is ex-

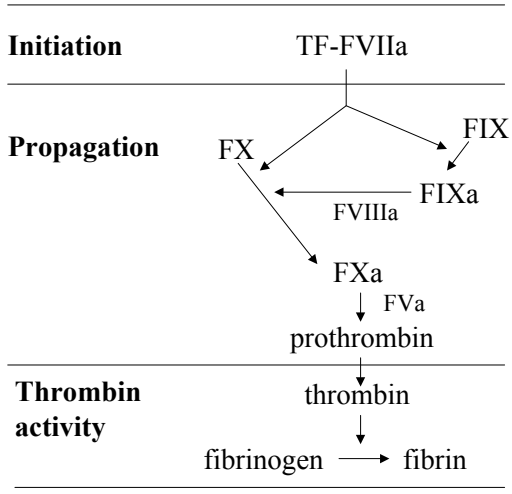

Fig. 6. Current model of coagulation. F: factor; TF: tissue factor.

posed due to endothelial damage or systemic or local proinflammatory activation of TF-expressing cells in the vasculature. An active TF-FVIIa is formed due to trace amounts of activated factor VII (FVIIa) already present in circulating blood (Morrisey *et al.* 1993). FVIIa is a very weak serine protease on its own but binding to TF enhances its catalytic activity more than 10^6-fold. However, the activity of the FVIIa-TF complex is dependent on the presence of a negatively charged phospholipid surface (e.g. phosphatidylserine). The FVIIa-TF complex (known as extrinsic factor Xase) activates vitamin K-dependent zymogens FIX and FX. Associated with PS on the surface of activated platelets (or platelet-derived microparticles), FXa forms a complex with partially activated FV exposed on activated platelets, thus generating minute amounts of thrombin (Monkovic & Tracy 1990, Monroe *et al.* 1996). Traces of thrombin subsequently activate plasma factors V and VIII in positive feedback reactions and enhance platelet activation in cooperation with exposed subendothelial collagen (Butenas *et al.* 1997).

2.2. Propagation of coagulation

In the propagation phase of blood coagulation FVIIIa associates with the serine protease-activated FIXa on the anionic phospholipid surface of activated platelets to form the calcium-dependent intrinsic tenase complex (Rosing *et al.* 1985). This complex activates FX at a significantly higher rate than does the FVIIa-TF complex. Subsequently, FXa interacts with FVa and prothrombin on a phospholipid surface to form the Ca^{2+}-dependent prothrombinase complex, promoting proteolytic cleavage of prothrombin and resulting in a burst of thrombin generation. It has been estimated that the presence of phospholipids stimulates the prothrombinase complex to convert prothrombin to thrombin 1000- to 100 000-fold faster than a physiological concentration of FXa alone (Nesheim *et al.* 1979, Rosing *et al.* 1980). Thrombin propagates the coagulation cascade through further amplification of its own generation by activating FXI (which subsequently generates more FIXa), FV, FVIII, and platelets, thus leading to sustained interplay between coagulation activity and platelets (Pieters *et al.* 1989, Gailani & Broze 1991, Butenas *et al.* 1997). The subsequent massive thrombin generation ultimately results in an insoluble cross-linked fibrin clot by cleavage of fibrinogen (Bailey *et al.* 1951) and FXIII (Naski *et al.* 1991).

2.3. Intrinsic pathway of coagulation

The role of the intrinsic or accessory pathway in normal hemostasis is poorly understood, and deficiencies of FXII, prekallikrein and high-molecular-weight kininogen are not associated with bleeding problems (Brummel-Ziedins *et al.* 2004). However, FXI has a prominent role in coagulation while it can be activated by thrombin (without activation through the intrinsic pathway) and thereafter accelerate coagulation via the extrinsic pathway (Gailani & Broze 1991, Oliver *et al.* 1999).

2.4. Termination of coagulation

Coagulation activity is regulated by several physiological inhibitors which inactivate either serine proteases or their cofactors (Fig. 7). Tissue factor-pathway inhibitor (TFPI) constitutes the major inhibitor of the initiation phase of coagulation (Golino *et al.* 2002). It is present at relatively low concentrations in blood and inactivates FXa and the TF-FVIIa-FXa complex (Rapaport 1991). The most abundant physiological anticoagulant, antithrombin (AT) III, effectively neutralizes several serine proteases, especially thrombin and FXa, unless they are bound to a protective phospholipid surface, and its effect is strongly promoted by heparins (Olson *et al.* 1993). In addition to its strong procoagulant effects, thrombin also exhibits anticoagulant properties. After binding to thrombomodulin, thrombin loses its anticoagulant capacity and inhibits coagulation by activating the vitamin K-dependent protein C on a negatively charged phospholipid surface. Activated protein C (APC) forms a complex with protein S to inhibit activated coagulation factors Va and VIIIa, thus leading to inhibition of thrombin generation (Kisiel *et al.* 1977, Esmon *et al.* 1982). In contrast to TFPI, the protein C pathway and AT III have only minor effects on the initiation phase of blood coagulation, whereas they constitute the major inhibitors in the propagation phase (van't Veer & Mann 1997a, van't Veer *et al.* 1997b). In addition to activating protein C, the thrombin-trombomodulin complex protects clot formation by inhibiting fibrinolysis through the action of thrombin activatable fibrinolysis inhibitor (TAFI) (reviewed in Bajzar 2000).

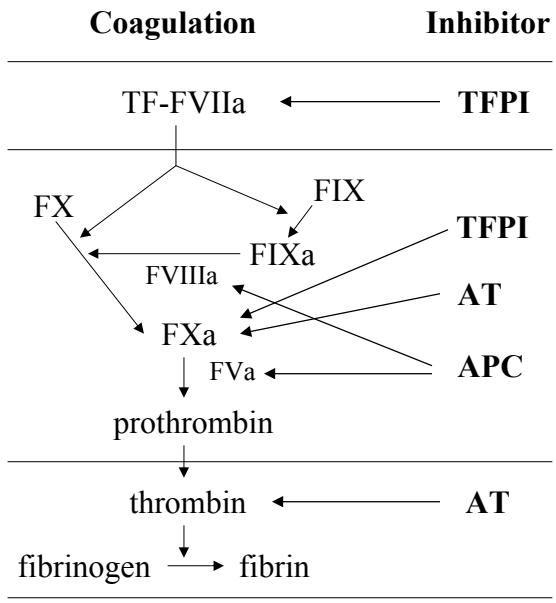

Fig. 7. Regulation of coagulation. In addition to FXa and thrombin, antithrombin also inhibits several other serine proteases. APC; activated protein C; AT: antithrombin (III); F: factor; TF: tissue factor; TFPI: tissue factor-pathway inhibitor.

3. PLATELET FUNCTION IN HEMOSTASIS AND THROMBOSIS

3.1. Adhesion, aggregation and secretion

Platelet response to exposed subendothelial matrix is fundamental to initiation of the hemostatic cascade or thrombosis at the higher shear rates found in arteries. Platelet function in hemostasis and thrombosis is a complex process consisting of platelet tethering, adhesion, activation, spreading, aggregation, degranulation, procoagulant activation, and microparticle generation.

The rapid interaction between the GPIb complex on resting platelets and VWF immobilized on subendothelial collagen is crucial for the capture of platelets from circulation. This interaction is rapidly reversible, seen as rolling of platelets on the surface, and thus insufficient for stable adhesion. During this initial tethering other direct and indirect collagen receptors come into contact with their adhesive ligand sites on subendothelial collagen. According to current knowledge, primary adhesion to collagen is mediated through the interplay of the GPIb complex, GPIaIIa, and GPVI. Signaling through GPVI is crucial for conversion of the glycoproteins Ia/IIa and IIb/IIIa to the high-affinity states needed for the formation of a stable platelet plug. Following adhesion, rapid signal transduction leads to platelet activation causing platelet shape change and spreading on the adhesive surface with extrusion of filopodia, resulting in formation of a fully spread, flattened platelet with central eminence, resembling a fried egg (Ware & Coller 1995). The extent of degranulation of platelet secretory α- and dense granules is dependent on the strength of the agonist, and leads to enhancement of platelet activation and aggregation through release of secondary platelet activators, fibrinogen, and VWF. Upon activation, platelet surface membrane GPIIb/IIIa receptors are activated through inside-out signaling to bind soluble fibrinogen and VWF with high affinity. Additional GPIIb/IIIa receptors from the SCCS and α-granules, possibly in a ligand-bound state, are transported to the platelet surface upon activation. Adjacent platelets are thus connected to each other to form aggregates.

3.2. Physiologic inhibition of platelet activation

Platelet activation is inhibited by several mechanisms including the intact endothelial barrier, brief half-life of certain platelet activators (such as TXA_2), inhibition of generated thrombin by AT, and tendency of blood flow to wash away unbound platelet activators. Endothelial cells generate nitric oxide (NO) in response to shear stress and other platelet activators, and this molecule inhibits platelet activation through cyclic guanyl monophosphate (Calverley & Maness 2004). Prostaglandins PGE_1, PGE_2 and PGI_2, inhibit platelet activation through G protein-coupled receptors that regulate cyclic adenylate monophosphate generation (Calverley & Maness 2004). Moreover, desensitization of activated receptors by biochemical modification or their internalization from the platelet surface regulate ongoing platelet activation.

3.3. Platelet procoagulant activity (PCA)

Membrane phopholipids

In an advanced stage of activation platelets stimulate blood coagulation by providing a surface at which the coagulation factors are activated to generate thrombin (Bevers *et al.* 1982, Rosing *et al.* 1985, Bevers *et al.* 1991, Zwaal *et al.* 1992, Zwaal & Schroit 1997). In resting platelets the negatively charged phospholipids PS and PE are situated on the inner leaflet of the plasma membrane and this asymmetric distribution is maintained by an aminophopholipid translocase (reviewed in Heemskerk *et al.* 2002). The loss of transmembrane asymmetry is mediated by a Ca^{2+}-dependent phopholipid scramblase activity and concomitant inhibition of the aminophospholipid transferase (Comfurius *et al.* 1990, Williamson *et al.* 1995). The signaling pathways that regulate platelet procoagulant activity are largely unknown, but both PS/PE exposure and bleb formation are shown to require a prolonged rise in intracellular Ca^{2+} in response to mobilization of intracellular Ca^{2+} stores and influx of extracellular Ca^{2+} (reviewed in Heemskerk *et al.* 2002). Recently, Bahou *et al.* (2004) described a novel Ca^{2+}-independent but α-granule secretion-dependent intracellular signaling pathway involving platelet IQGAP1 that specifically regulates the development of platelet PCA under high shear conditions in a mouse model. However, the mechanisms leading to IQGAP1 activation remain unknown.

Generation of microparticles

During the process of PS and PE exposure, activated platelets change their shape and form membrane blebs that are easily shed from the remnant platelet as microvesicles with similar procoagulant phospholipid surfaces (Sims *et al.* 1989, Zwaal *et al.* 1992, Chang *et al.* 1993). The molecular basis of microvesiculation remains poorly characterized, but it appears to be Ca^{2+}-dependent and involves calpain and caspase-3 activation, and cytoskeletal proteolysis (Sims *et al.* 1989). In the rare hereditary Scott syndrome, impairment of phospholipid scramblase leads to the absence of both procoagulant surface and microparticle (MP) generation upon platelet activation, causing bleeding problems (Sims *et al.* 1989, Zwaal *et al.* 2004). In contrast, patients with Wiscott-Aldrich syndrome have platelets with enhanced exposure of procoagulant phospholipids and increased MP release (Shcherbina *et al.* 1999).

Platelet procoagulant agonists

Initially, it was recognized that Ca^{2+}-ionophores such as A23187 or ionomycin are especially potent stimulators of platelet PCA and microvesiculation (Bevers *et al.* 1982). Other agents that can induce exposure of procoagulant phopholipids on the platelet surface include Ca^{2+}-mobilizing compounds such as inhibitors of the sarco/endoplasmic reticulum Ca^{2+}-ATPases (Dachary-Prigent *et al.* 1995) and the complement proteins C5-9 (Sims *et al.* 1988). Exposure of PCA can also be caused by high shear stress (Miyazaki *et al.* 1996) or experimental cell damage subsequent to freeze-thawing or ultrasonication.

With isolated platelets in suspension, collagen is incapable of inducing strong procoagulant activation to support thrombin generation upon activated platelets in the absence of thrombin (Bevers *et al.* 1982, Zwaal *et al.* 1992). In contrast, platelet adhesion to fibrillar collagen, even in the absense of thrombin, can trigger the procoagulant response (Thiagarajan & Tait 1991, Heemskerk *et al.* 1997). The procoagulant activation induced by collagen is mediated by GPVI (Nieswandt *et al.* 2001a, Siljander *et al.* 2001) and adhesion via GPIa/IIa serves to potentiate the signaling effects of GPVI (Heemskerk *et al.* 1999). Accordingly, platelet adhesion to the GPVI-specific agonist CRP induces procoagulant activation of platelets (Heemskerk *et al.* 1999).

Controversial data exist on the mechanisms of thrombin-induced platelet PCA. Although a potent agonist for platelet aggregation and secretion, thrombin was proposed to cause only little PS exposure when added to isolated, washed platelets (Bevers *et al.* 1983). This weak procoagulant response in the absence of plasma proteins was similar to that induced by agonists of the PAR-1 receptor, whereas agonists of PAR-4 failed to stimulate PS exposure (Andersen *et al.* 1999). However, in the study of Dicker *et al.* (2001) α-thrombin was a strong inducer of procoagulant activity of gel-filtered platelets (GFP) in suspension whereas the procoagulant capacity induced by the PAR-1 receptor agonist SFLLRN (at concentrations causing maximal aggregation and Ca^{2+} mobilization) was only one fifth of that induced by 10 nM thrombin. In their study, several antibodies against PAR-1 had no effect on thrombin-induced platelet PCA. Dörmann *et al.* (2000) postulated that GPIb-bound thrombin is mainly responsible for procoagulant activation of GFP after stimulation with thrombin in suspension and that this activation requires both PAR-1 and GPIIb/IIIa as well as platelet-platelet interaction. Additionally, other groups (Miyata *et al.* 1996, Jamieson 1997) have postulated that thrombin-induced platelet PCA is mediated through binding to GPIb, although these experimental conditions were devoid of fibrin(ogen). In sharp contrast, Liu *et al.* (1997) suggested that platelet activation by thrombin is exclusively mediated via G-coupled receptors rather than by GPIb. In addition, thrombin may stimulate the prothrombinase reaction of isolated platelets by activating factor V, secreted from platelets during activation (Briede *et al.* 2001).

Procoagulant activity of platelets in plasma

In the presence of plasma proteins, adhesive platelet receptors seem to play a crucial role in the procoagulant activation of platelets. Reverter *et al.* (1996) first described inhibition of platelet-mediated thrombin generation by the 7E3 antibody against GPIIb/IIIa and $\alpha_v\beta_3$, whereas an antibody against GPIb, 6D1, had no effect on thrombin generation in the absence of fibrin. Additionally, platelets from patients with Glanzmann thrombasthenia lacking GPIIb/IIIa receptors supported significantly less thrombin generation than normal platelets (Gemmel *et al.* 1993, Reverter *et al.* 1996, Weiss & Lages 1997).

In platelet-rich plasma (PRP) under static conditions thrombin generation strongly stimulates platelet procoagulant response (Beguin *et al.* 1989), acting in concert with forming fibrin and VWF (Beguin & Kumar 1997, Sanders *et al.* 1998). In the study of Beguin *et al.* (1997), platelet-dependent thrombin generation in PRP was suppressed by antibodies

against VWF, while coagulation activity in platelet-poor plasma (PPP) persisted. Kumar *et al.* (1994 and 1995) demonstrated the important role of clot-bound thrombin on further platelet activation and observed an additional activating effect of fibrin on the procoagulant action of platelets. Using fibrin clots prepared by snake venom protease and devoid of thrombin, Beguin *et al.* (1999) observed a marked increase of thrombin generation in PRP in response to fibrin, while coagulation in PPP remained unaltered. This additional procoagulant effect was not affected by inhibition of GPIIb/IIIa, whereas antibodies blocking VWF binding to GPIb prevented the procoagulant effect of fibrin but failed to inhibit platelet-dependent thrombin generation in the absence of added fibrin. In accordance, thrombin generation in PRP of a patient with Bernard-Soulier syndrome was not increased after addition of thrombin-free fibrin (Beguin *et al.* 2004). Prevention of fibrin polymerization with antibody A11 or with DRGDW inhibited thrombin generation in PRP despite the presence of thrombin (Beguin *et al.* 2004). Loscalzo *et al.* (1986) previously reported that VWF serves as a link between polymerizing fibrin and platelet GPIb receptor. It has been suggested that under static conditions polymerizing fibrin could induce a conformational change in VWF similar to that brought about by shear, immobilization, or ristocetin (Soslau *et al.* 2001, Beguin *et al.* 2004). Briede *et al.* (2003) demonstrated that shear-dependent platelet binding to VWF via the GPIb complex significantly potentiated the procoagulant effect of thrombin on platelets deposited on fibrin surface.

VWF-GPIIb/IIIa interaction also seems to play a role in platelet procoagulant activation, but only after platelet activation by thrombin (Goto *et al.* 1995). Decreasing VWF activity with a neutralizing antibody inhibited procoagulant activity of PRP both in the presence and absence of added fibrin, without affecting thrombin generation in plasma. Two separate VWF-dependent mechanisms of platelet procoagulant activation can be suggested: one associated with GPIb and fibrin, and the other with GPIIb/IIIa (Beguin *et al.* 1999, Beguin *et al.* 2004).

In summary, at a site of arterial injury, platelet-mediated thrombin generation is initiated on the first platelets adhering to collagen. Subsequently, TF-induced thrombin generation further accelerates the procoagulant activation of platelets. After formation of a hemostatic plug or thrombus, adhesive collagen is sealed and further procoagulant activation of platelets is supported by the fibrin clot, thus promoting a sustained interaction between platelets and coagulation. In addition, blocking of purinergic ADP receptors $P2Y_1$ and $P2Y_{12}$ has been shown to reduce platelet procoagulant activity and microparticle release (Herault *et al.* 1999, Storey *et al.* 2000), and platelets from patients with a storage pool deficiency lacking ADP release have reduced platelet-dependent procoagulant capacity (Weiss & Lages 1997). Thus, ADP released from platelets upon activation is able to potentiate the procoagulant effects of other platelet agonists.

Platelet receptors for coagulation factors

There is accumulating evidence that in addition to negatively charged phopholipids, protein components of the platelet membrane also have specific roles in platelet-dependent coagulation. Several coagulation factors interact with membrane glycoproteins on resting

or activated platelets, thus contributing to platelet PCA, especially in the initiation phase of coagulation. Factor XI binds to the GPIbα chain on the surface membrane of activated platelets (Baglia *et al.* 2002), where it is activated by thrombin (Baglia & Walsh 1998) and affects thrombin generation induced by TF (Keularts *et al.* 2001). Gailani *et al.* (2001) observed that FIX can bind to one chain of the dimeric FXIa on the platelet surface and become activated by this alternative pathway. There is also evidence for a specific, non-lipid activation-induced binding site for FVIII on platelets (Ahmad *et al.* 2000). Byzova and Plow (1997) demonstrated that prothrombin binds to GPIIb/IIIa on resting platelets. Prothrombin bound to GPIIb/IIIa on a resting platelet was cleaved by FXa resulting in the release of thrombin, which in turn can activate platelets through specific receptors leading to activation of the GPIIb/IIIa receptors. Thus, this pathway of thrombin generation also contributes to platelet-dependent coagulation, especially during the initiation phase. It is possible, that the inhibititory effects or GPIb and GPIIb/IIIa antagonists on platelet procoagulant activity may in part rely on blocking the binding of FXI or prothrombin, respectively.There are also some data on the existence of a FXa receptor, effector protease receptor-1 (EPR-1), on the platelet surface and on its role in regulating prothrombinase activity (Bouchard *et al.* 1997).

Platelet-derived coagulation factors

In addition to providing the procoagulant phospholipid surface and binding sites for several coagulation factors, platelets contribute to coagulation activity by releasing several compounds, such as FV, FXIII, fibrinogen, VWF, and protein S. Approximately 20% of FV contained in whole blood is found in the α-granules of platelets (Tracy *et al.* 1982) and can be secreted upon platelet activation (Chesney *et al.* 1981). Several observations indicate that platelet-derived membrane-bound FV has a pivotal role in promoting hemostasis at sites of vascular injury (Allen *et al.* 2004). Platelet FV Quebec (Tracy *et al.* 1984) and New York (Weiss *et al.* 2001) are bleeding disorders characterized by fully functional plasma FV but defective platelet FV, suggesting that platelet-derived FV may be more important for normal hemostasis than plasma FV. Accordingly, a patient with an acquired inhibitor against both plasma and platelet FV presented with bleeding problems (Grigg *et al.* 1989), whereas another patient with inhibitor against only plasma FV did not suffer from abnormal bleeding tendency (Nesheim *et al.* 1986). Camire *et al.* (1998) observed that platelet-derived but not plasma-derived FV is protected from proteolytic inactivation by APC on the platelet surface. A proportion of platelets (enriched in young platelets) stimulated with both collagen and thrombin (COAT platelets) express high levels of α-granule-derived factor V on their surface membrane and this expression parallels the surface exposure of procoagulant phospholipids (Alberio *et al.* 2000). Factor Xa preferentially binds to these platelets, leading to enhanced prothrombinase activity when compared with other platelets (Alberio *et al.* 2000). COAT platelets also retain serotonin-conjugated procoagulant proteins on their surface (Dale *et al.* 2002) by interaction with specific receptors or by serotonin-binding sites on fibrinogen or thrombospondin (Szasz & Dale 2002).

4. EFFECTS OF ANTIPLATELET AGENTS ON PLATELET PROCOAGULANT ACTIVITY (PCA)

The dominant effect of antiplatelet agents is the inhibition of platelet activation at the site of vascular injury under higher shear rates, thus leading to reduced aggregation and thrombus formation. This inhibition of platelet accumulation naturally leads to a quantitative decrease in platelet-dependent coagulation activity by diminishing the access of procoagulant phospholipid membranes and platelet secretion products at the thrombus site. However, antiplatelet agents may also exert direct qualitative effects on the procoagulant capacity of platelets. Theoretically, this inhibition of platelet procoagulant capacity could be achieved by reduced release of 1) negatively charged procoagulant phospholipids on the outer surface of activated platelets, 2) procoagulant agents such as FVa from platelet secretory granules, 3) microparticles, or 4) plasminogen activator inhibitor (PAI)-1 (which in turn could result in enhanced fibrinolysis).

4.1. Aspirin, dipyridamole and clopidogrel

To date, relatively little is known about the direct effects of the most widely used antiplatelet drugs on platelet-dependent activation of coagulation. Szczeklik *et al.* (1986) first showed that aspirin induced delayed generation of thrombin, whereas Reverter *et al.* (1996) reported only a modest decrease in thrombin generation and peak thrombin level induced by aspirin, both *in vitro* and *in vivo*, at concentrations that completely inhibited arachidonic acid-triggered aggregation. Butenas *et al.* (2001) observed no significant affect of ingested aspirin (325 mg × 2/d for 3 days) on TF-induced thrombin generation in whole blood, assessed by thrombin-antithrombin III (TAT) complex formation or fibrinopeptide A release. Also, addition of 5 μM dipyridamole to blood had no effect on platelet-dependent coagulation activity in their study. Vanschoonbeek *et al.* (2004) obtained similar results using the calibrated thrombogram method. Blocking of the purinergic ADP receptor P2Y12 has been shown to reduce platelet PCA in an animal model (Herault *et al.* 1999) and in human platelets (Storey *et al.* 2000, Vanschoonbeek *et al.* 2004).

4.2. Glycoprotein IIb/IIIa antagonists

Three parenteral GPIIb/IIIa antagonists – abciximab, tirofiban, and eptifibatide – are currently available for clinical use. These agents have been used for the short-term treatment of patients undergoing percutaneous coronary intervention (PCI) or with acute coronary sydromes (ACS). The goal of treatment is to aggressively inhibit GPIIb/IIIa-mediated platelet aggregation for a short time (24–72 h) during the period of highly elevated risk of platelet thrombus formation. To achieve beneficial effects, doses of GPIIb/IIIa antagonists target 80% receptor occupancy of unstimulated platelets, whereas more extensive occupancy causes marked prolongation of bleeding time (Coller *et al.* 1989 and 1991). In addition to blocking platelet aggregation, GPIIb/IIIa antagonists also inhibit the outside-in

signaling that follows the binding of adhesive ligands to the activated receptor (Shattil *et al.* 1998). This signaling results in further platelet spreading, granule secretion, clot retraction, GPIIb/IIIa receptor activation, and expression of procoagulant activity (Casserly & Topol 2002).

Abciximab

Abciximab (c7E3 Fab, ReoPro^{R}, EliLilly/Centocor) is a parenteral chimeric antibody fragment currently approved for use in patients undergoing PCI, although it has been studied in patients with ACS as well. It is a high-affinity GPIIb/IIIa antagonist with a dissociation constant (K_d) of 5 nmol/L, resulting in a predominantly receptor-bound distribution following administration (Kleiman 1999). The binding site of abciximab on the GPIIb/IIIa receptor seems to be distinct from the ligand-binding site, since abciximab is able to bind to the receptor even if it is occupied by a RGD peptide. Abciximab also interacts with the vitronectin receptor $\alpha_v\beta_3$ on platelets, endothelial cells, and smooth muscle cells, as well as with the activated MAC-1 ($\alpha_M\beta_2$) receptor on leukocytes (Coller 1999). Abciximab has a slow, up to 4 h, half-time rate of dissociation from the GPIIb/IIIa receptor and it redistributes to other platelets in vivo (Mascelli *et al.* 1998). While plasma levels of abciximab fall quickly after infusion, platelet-bound abciximab can be detected for up to 15 days after treatment (Mascelli *et al.* 1998), and shear-induced platelet deposition remains abnormal for up to one week after treatment (Osende *et al.* 2001).

Tirofiban

Tirofiban (Aggrastat^{R}, MSD) is a synthetic parenteral non-peptide tyrosine derivative that mimics the RGD integrin recognition sequence (Hartmanet *et al.* 1992, Egbertson *et al.* 1994). It is highly selective for the GPIIb/IIIa receptor and competitively inhibits platelet aggregation by occupying the binding pocket of the receptor (Kleiman 1999). Tirofiban is approved for use in patients with ACS, although it has been used in several studies in patients undergoing PCI. It is a lower affinity antagonist with a K_d of 15 nmol/L, and the unbound fraction of the infused drug accounts for approximately 35% of the total circulating pool. It has a rapid off-rate with a dissociation half-time of only about 10 s, and normal platelet function is restored within 3–4 h after discontinuation of infusion (Kereiakes *et al.* 1999).

Eptifibatide

Eptifibatide (Integrilin^{R}, Schering-Plough/COR Therapeutics) is a synthetic parenteral cyclic heptapeptide demonstrating a high specificity for the GPIIb/IIIa receptor by its KGD sequence and binding to the ligand-binding pocket of the receptor (Phillips & Scarborough 1997). It is a low-affinity antagonist with a K_d of 120 nmol/L and has a rapid onset of action. Due to the rapid dissociation of the drug, restoration of normal platelet function occurs within 4 h of cessation of infusion. Eptifibatide is currently approved for use both in patients with ACS and in patients undergoing PCI. Low levels of Ca^{2+} in *ex vivo* or *in vitro* studies conducted in trisodium citrate-anticoagulated blood dimish the fibrinogen-binding affinity of the

GPIIb/IIIa receptor and facilitate binding of eptifibatide, thus leading to an enhanced inhibitory effect of eptifibatide compared to its clinical efficacy *in vivo* (Phillips *et al.* 1997).

Clinical efficacy

The greatest clinical impacts of parenteral GPIIb/IIIa antagonists have been demonstrated in patients undergoing PCI. In a pooled analysis of all placebo-controlled PCI trials (Fig. 8), the absolute and relative risk reduction in the composite 30-day endpoint of death and myocardial infarction (MI) was 2.9 and 34%, respectively (Casserly & Topol 2002). Placebo-controlled trials have suggested a more pronounced reduction in MI and urgent revascularization after PCI with abciximab (EPIC 1994, EPILOG 1997, EPISTENT 1998) when compared with eptifibatide (IMPACT 1997, ESPRIT 2000) or tirofiban (RESTORE 1997) in patients undergoing angioplasty or stenting. The 30-day outcome of the TARGET trial (Topol *et al.* 2001a) confirmed the superiority of abciximab over tirofiban in patients undergoing PCI. Reduction of mortality has been reported only with abciximab, and becomes manifest only during long-term follow-up.

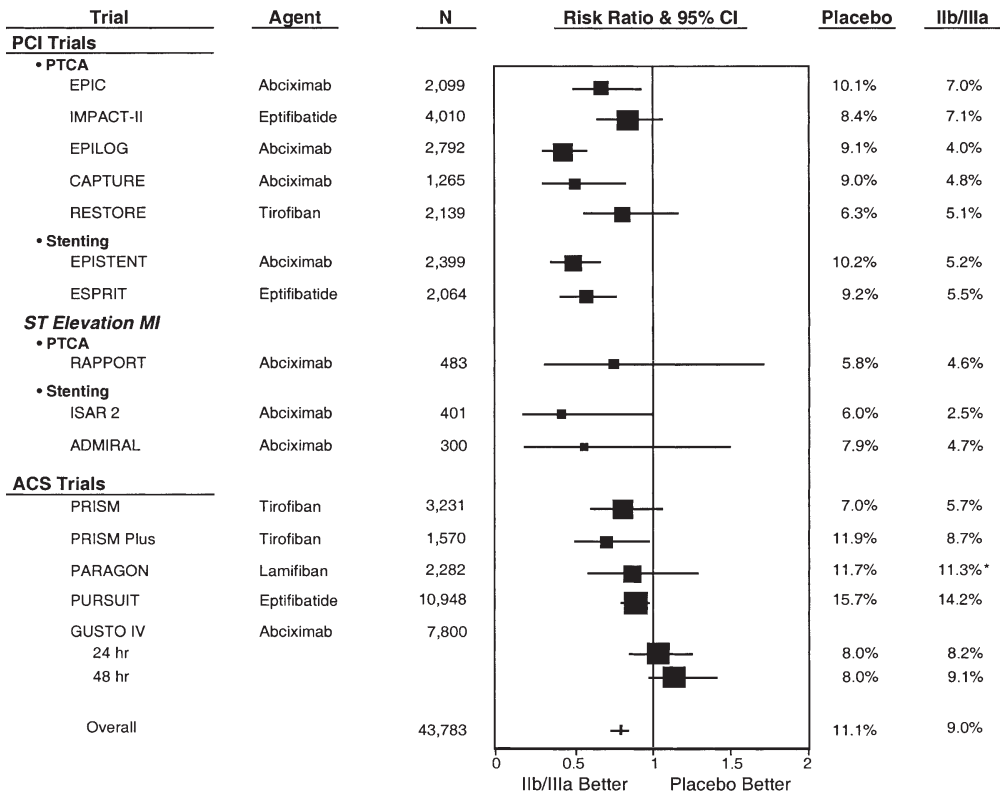

Fig. 8. Death or non-fatal myocardial infarction (MI) outcomes at 30 days in randomized placebo-controlled trials of parenteral GPIIb/IIIa antagonists. Risk ratio (RR) with 95% CI, size of RR box being proportional to total sample size. Frequency of death or non-fatal MI in columns 5 and 6. Reproduced with permission from Casserly & Topol 2002. ACS: acute coronary syndromes; PCI: percutaneous coronary intervention; PTCA: percutaneous transluminal coronary angioplasty.

In ACS (unstable angina and non-ST elevation MI), the small-molecule GPIIb/IIIa antagonists eptifibatide, tirofiban, and another non-peptide antagonist lamifiban, have demonstrated efficacy as a component of medical therapy alone or when combined with PCI or surgical revascularization (PRISM 1998, PRISM-PLUS 1998, PURSUIT 1998, PARAGON 1998), although the benefit has been more modest than that observed in PCI trials (an overall absolute and relative risk reduction in incidence of death and MI at 30 days of 1.4% and 11%, respectively) (Chew & Moliterno 2000). The role of abciximab in the treatment of patients with ACS is obscure. In one study, abciximab has shown a benefit in patients with ACS who undergo PCI following a prolonged infusion both during the medical treatment phase and following PCI (CAPTURE 1999). In contrast, the more recent GUSTO IV study demonstrated no benefit of abciximab when used as medical therapy alone for patients with ACS (Simoons 2001).

In patients with acute ST-elevation MI, a combination of GPIIb/IIIa antagonists to full-dose thrombolytic therapy led to increased bleeding complications. In the GUSTO V study with abciximab plus half-dose reteplase versus full-dose reteplase alone, significant reductions in reinfarction, recurrent ischemia and urgent revascularization were observed with combination therapy, but without any mortality benefit (Topol 2001b). The ASSENT-3 trial also demonstrated a marked reduction in reinfarction, no mortality benefit, and excess of bleeding complications with the combination of abciximab and tenecteplase (ASSENT-3 2001).

Oral GPIIb/IIIa antagonists

Several oral GPIIb/IIIa antagonists have been evaluated in phase III clinical studies: sibrafiban (SYMPHONY 2000), lotrafiban (Topol *et al.* 2000), orbofiban (Cannon *et al.* 2000), and xemilofiban (O'Neill *et al.* 2000). These all are non-peptide prodrugs that have to undergo one or more entzymatic steps to become an active drug, and generally elicit low bioavailability. To date, all studies with oral GPIIb/IIIa have failed to demonstrate a benefit in the settings of PCI and ACS. A meta-analysis reported a significantly increased risk in mortality and major bleeding without any effect on MI, whereas the need for urgent revascularization was reduced (Chew *et al.* 2001).

Parenteral GPIIb/IIIa antagonists and platelet procoagulant activity

The first observation of the effect of platelet GPIIb/IIIa blockade on coagulation activity came from evaluation of the EPIC study. Moliterno *et al.* (1995) reported that activated clotting times (ACT), used to monitor heparin dosing in the interventional laboratory, were significantly prolonged in patients receiving abciximab compared to placebo during PCI. This finding was confirmed *in vitro* by Ammar *et al.* (1997), both in heparinized blood and in blood anticoagulated with the direct thrombin inhibitors hirudin and PPACK. Moreover, thrombin generation induced by platelets from patients with Glanzmann thrombastenia is also reduced when compared with normal platelets (Gemmel *et al.* 1993, Reverter *et al.* 1996, Weiss & Lages 1997). Several *in vitro* and *ex vivo* studies have demonstrated that GP IIb/IIIa antagonists reduce platelet-dependent thrombin generation (Reverter *et al.* 1996,

Dangas *et al.* 1998, Keularts *et al.* 1998, Pedicord *et al.* 1998, Rao *et al.* 1999, Li *et al.* 2000), expression of negatively charged phospholipids and binding of FV/Va on the platelet surface (Furman *et al.* 2000), and procoagulant microparticle formation (Gemmel *et al.* 1993, Reverter *et al.* 1996). All *in vitro* studies have utilized isolated platelets in suspension, PRP, or whole blood and activated platelets with soluble agonists rather than with permanent adhesion to a thrombogenic surface. While one study suggested that the anticoagulant effect of abciximab was due to blockade of both GPIIb/IIIa and the vitronectin receptor $\alpha_v\beta_3$ (Reverter *et al.* 1996), other investigators have reported that the effect is exclusively mediated by blockade of GPIIb/IIIa (Ammar *et al.* 1997, Furman *et al.* 2000, Lages & Weiss 2001). Byzova and Plow (1997) reported a novel link between platelets and blood coagulation by showing that prothrombin binds to platelet GPIIb/IIIa in a cation-dependent manner and this interaction accelerates prothrombin activation to thrombin. This binding was inhibited by different peptide ligands and monoclonal antibodies against GPIIb/IIIa, suggesting another possible mechanism underlying the anticoagulant effect of GPIIb/IIIa antagonists.

The contents of platelet secretory granules mediate several important effects on platelet activation, coagulation, fibrinolysis, inflammation, and atherogenesis. Given the central role of GPIIb/IIIa in platelet activation, the blockade of this receptor was initially assumed to elicit beneficial effects on thrombus propagation in part through inhibition of platelet secretion. So far, studies have provided controversial results. Reverter *et al.* (1996) initially observed reduced release of platelet factor (PF) 4 and platelet-derived growth factor in response to c7E3. Several investigators have reported mild or no antisecretory effects of abciximab (Tsao *et al.* 1997, Furman *et al.* 2000, Klinkhardt *et al.* 2000), tirofiban (Furman *et al.* 2000), or eptifibatide (Furman *et al.* 2000), whereas others have suggested paradoxically accelerated secretion induced by abciximab and eptifibatide (Schneider *et al.* 2000).

AIMS OF THE STUDY

Despite the important functional role of platelets in directing thrombus formation at the site of arterial injury, relatively little is known about their procoagulant activity induced by adhesion to a thrombogenic surface. The aim of this thesis was to investigate the effect of firm adhesion on the procoagulant activity of platelets and the role of specific platelet glycoprotein receptors mediating this activation. We set out to develop a new adhesion-dependent assay to assess platelet-mediated activation of coagulation upon two thrombogenic surfaces: 1) collagen, as an initial platelet-adhesive surface in arterial injury; and 2) a plasma clot, as a model of a preformed thrombus promoting sustained interaction between platelets and the coagulation system.

The study focused on the following specific issues:

1. Characteristics and interindividual variability of adhesion-induced platelet procoagulant activity.
2. The roles of glycoprotein IIb/IIIa and Ib receptors in adhesion-induced platelet procoagulant activity and release of PF 4.
3. Effects of the three clinically administered GP IIb/IIIa antagonists – abciximab, tirofiban, and eptifibatide – on adhesion-dependent platelet PCA and aggregation induced by various procoagulant platelet agonists.

METHODS

1. BLOOD COLLECTION AND ANTICOAGULANTS

Blood was obtained from healthy non-smoking volunteers who had not taken any medication during the preceeding 14 days. Free-flowing blood was collected via venipuncture from a cubital vein with a 16- or 17-gauge polytetrafluoroethylene cannula (BOC Ohmeda AB) in supine position after a 20-minute rest. The first 3 ml were collected in ethylenediaminetetraacetic acid (EDTA) for blood cell counts and hematocrit assessed with a Thrombocounter Coulter T-540 (Coulter Electronics Inc, USA). For plasma separation, nine volumes of blood were collected into one volume of 0.109 or 0.129 M trisodium citrate. For platelet-rich plasma (PRP) blood was anticoagulated with 0.109 M trisodium citrate (**II-IV**) or with 40 μmol/L PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, Calbiochem-Novabiochem Co) (**III, IV**). For gel-filtered platelets (GFP), blood was collected into polypropylene tubes containing 131 mmol/L acidic citrated dextrose (pH 4.5), at a ratio of one volume to six volumes of blood. For whole blood perfusions with subsequent procoagulation studies (**III**) only minimal anticoagulation, 1 μmol/L D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (PPACK), was used to allow initial thrombin generation.

2. POOLED AND DEFIBRINATED PLASMA

Pooled citrated platelet-poor plasma (PPP) from 10 donors was prepared by repeated centrifugation (1500 g, 10 min, 22°C). Plasma fibrinogen (assessed by the functional method of Clauss; normal range, 2.0 to 4.0 mg/mL), prothrombin fragments (F1+2) (Enzygnost F1+2 micro, Behring; normal, <1.1 nmol/L), thrombin-antithrombin complexes (TAT) (Enzygnost TAT micro; Behring; normal, ≤4.1 μg/L), and D-dimer (Asserachrom D-Di, Diagnostica Stago; normal, <400 ng/mL), as well as the global coagulation tests (aPTT, thrombin time, and PT) in the pooled plasma were all within normal ranges.

Pooled plasma was defibrinated essentially as described in Hemker *et al.* (1993), and used as the source of pro- and anticoagulant factors to avoid clot formation and uncontrolled loss of soluble thrombin during the procoagulation assay (Liu *et al.* 1979, Kumar *et al.* 1994). One volume of reptilase (STA-reptilase, Diagnostica Stago) was added to 49 volumes of citrated plasma, incubated for 5 min at 37°C and for 10 min on ice. Then the clot was centrifuged (2100 g, +4°C) for 10 min to separate the plasma. In this reptilase-treated plasma F 1+2, TAT, and D-dimer were all within normal ranges. Ristocetin (1mg/ml, Sigma)-induced aggregation of GFP in defibrinated plasma indicated normal function of VWF.

3. PLATELET PREPARATION

PRP was separated from whole blood after centrifugation (180 g, 12 min, 22ºC), and platelet count was adjusted with PPP when needed. GFP were prepared from PRP as reported previously by Timmons and Hawiger (1989). To control platelet activation during the filtration, small doses of apyrase (1 U/mL, Sigma) and PGE1 (25 ng/mL, Sigma) were added to PRP. Platelets suspended in HEPES buffer without cations were eluted through a Sepharose CL-2B column (Pharmacia LKB Biotechnology Inc, Sweden). Isolated platelets were diluted in HEPES buffer to different densities (10–300 × 10^6/mL) and divalent cations were added just before experiments. For specific adhesion experiments (**II**) GFP were supplemented with 1) pooled citrated plasma, 2) fibrinogen-depleted pooled plasma, 3) commercial FVIII- and VWF-deficient plasma (Sigma), or 4) antithrombin III-immunodepleted plasma (American Diagnostica Inc) to a final density of 100 × 10^6/mL.

4. ADHESIVE SURFACES (I–III)

4.1. Collagen

Collagen-coated coverslips for static experiments (**I–III**) were prepared by applying Horm collagen-reagent (Nycomed) at 25 µg/mL in the buffer provided (isotonic glucose solution, pH 2.7 to 2.9, 100 µL/coverslip) on round plastic Thermanox coverslips (area 1.77 cm^2, Nunc), incubated for 1 h at 37°C in a humid chamber and washed three times in PBS. For perfusion studies (**III**) 9 µL of Horm collagen suspension was repeatedly smeared on plastic Permanox coverslips (25×75 mm, Nunc), washed with a neutralizing buffer (Williams *et al.* 1978), and incubated for 30 min in a moist chamber at 37ºC. Bovine serum albumin (2% BSA, Sigma) in phosphate-buffered saline (PBS) was used as a control substrate as well as to block the collagen-coated coverslips.

4.2. Plasma clots

Plasma clots (**I–III**) were generated from pooled citrated plasma (diluted 1:2 in PBS, 100 µL/coverslip) by recalcification (5 mmol/L $CaCl_2$) and addition of bovine α-thrombin (Dade, 0.1 U per coverslip) on Thermanox coverslips. The final concentration of ionized calcium in this 1:2 diluted citrated plasma was physiological, 1.1 mmol/L (Microlyte 6, Kone Instruments, Finland, reference values, 1.1–1.3 mmol/L). The plasma clots were incubated for 30 min at 37°C in a humid chamber and for 30 min at room temperature. Thereafter, the clot-coated coverslips were washed six times in PBS.

4.3. Fibrin surfaces

Fibrin surfaces (**II**) were prepared from a 100 µL solution of plasminogen- and VWF-free human fibrinogen (Enzyme Research Laboratories) (2 mg/mL in PBS) by either 0.5 U of α-thrombin or 0.5 BU of reptilase (Diagnostica Stago) in the presence of 2 mmol/L $CaCl_2$, incubated for 1 h at 37°C, and washed in PBS.

5. PLATELET DEPOSITION ON ADHESIVE SURFACES (I–III)

5.1. Labeling of platelets

For quantitating platelet deposition on a thrombogenic surface in the static adhesion experiments (**I-III**), PRP or GFP were incubated with 10 nmol/L ^{3}H-serotonin (5-hydroxy (G-^{3}H)tryptamine creatinine sulphate, Amersham) at 37°C for 15 min (Mustonen & Lassila 1996). At this serotonin concentration platelet membranes are labeled without contribution of the 5-HT_{2A}-receptor, controlled by a 5-HT_{2A}-receptor blocker ketanserin (unpublished data). In whole blood perfusion experiments (**III**), PRP was first separated and platelets were labelled with ^{3}H-serotonin before reconstituting with the remaining blood cells.

5.2. Adhesion assay

In an adhesion assay (**I–III**) ^{3}H-serotonin-labeled GFP (supplemented with 2 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$) were allowed to adhere on a thrombogenic surface in the presence or absence of inhibitors at 37ºC for 30 min under slow rotation. In adhesion experiments with PRP or plasma-supplemented GFP (**II**), $MnCl_2$ (2 mmol/L) was used instead of $CaCl_2$ and $MgCl_2$ to ensure functional GPIIb/IIIa receptors without inducing coagulation.

The deposited ^{3}H-activity was measured by liquid scintillation counting. All data on platelet deposition are expressed in numbers of ^{3}H-positive platelets per cm^2 of coverslip area. In the case of plasma clots the number of deposited ^{3}H-positive platelets was corrected with the amount of ^{3}H-serotonin released: ^{3}H-scintillation activity in the platelet suspension and release of serotonin into plasma were measured in tubes with imipramine-formaldehyde on ice (centrifuged at 9 500 g for 2 min) (Holmsen & Dangelmaier 1989).

In addition, adhesive surfaces were occasionally studied with scanning electron microscopy (SEM) (JEOL-820 or Zeiss DSM 962) as previously reported (Siljander *et al.* 1996).

5.4. Effect of thrombin on platelet adhesion to plasma clots

To study the effect of thrombin on platelet (GFP 100 × 10^9/L) adhesion to plasma clots (**II**) we inhibited thrombin with its physiological antagonist antithrombin III (Atenativ, a kind gift from Octapharma, 0.05–10 U/mL). Alternatively, 1 U/mL unfractionated heparin (Leo Pharma), 1 U/mL enoxaparin (Klexane, Aventis Pharma), 25 U/mL recombinant hirudin

(Calbiochem) or 10 μmol/L PPACK (Calbiochem) were preincubated with GFP (100 × 10^9/L) for 10 min before adhesion. The selected concentrations of these thrombin inhibitors prolonged thrombin time (with 1 U/mL thrombin) to over 300 s. Optionally, plasma clots were preincubated with the thrombin inhibitors before the adhesion experiment.

5.5. Effect of fibrin polymerization on platelet adhesion to plasma clots

The effect of fibrin polymerization during platelet adhesion to clots (**II**) was assessed with GPRP (0.5 or 1 mmol/L, Bachem) added to GFP (100 × 109/L) 10 min before adhesion. These concentrations of GPRP prolonged thrombin time without affecting thrombin-induced aggregation of GFP (PACKS-4, Helena Laboratories) or the activity of thrombin on a chromogenic substrate (S-2238, Chromogenix).

6. PLATELET PROCOAGULANT ACTIVITY

6.1. Two-stage PCA assay: Generation of thrombin on adhesion-activated platelets (I–III)

Adhesion-induced platelet procoagulant activity (Fig. 9) was studied upon two thrombogenic surfaces: 1) collagen, as an initial platelet-adhesive surface in arterial injury, and

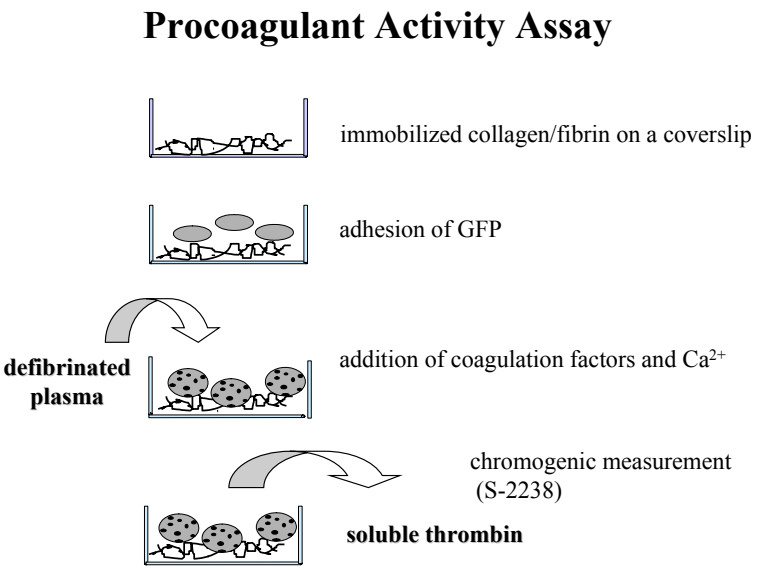

Fig. 9. Two-stage procoagulant activity assay. Gel-filtered platelets (GFP) were first allowed to adhere to a thrombogenic surface in the presence of 2 mM $CaCl_2$ and 1 mM $MgCl_2$. Coagulation was initiated on the adhered platelets by incubating with 1:20 diluted defibrinated pooled plasma and traces of tissue factor, and thrombin generation was assessed chromogenically (S-2238).

2) plasma clots, to model a preformed thrombus promoting sustained interaction between platelets and coagulation.

In the first phase, GFP (in 2 mM $CaCl_2$- and 1 mM $MgCl_2$-supplemented HEPES buffer) were allowed to adhere to a thrombogenic surface in the presence or absence of inhibitors for 30 min at 37°C under slow rotation, and the coverslips were washed in PBS to remove unattached platelets.

In the second phase, coagulation was initiated on the adhesion-activated platelets by incubating with defibrinated 1:20 diluted pooled plasma (500 μL) with or without tissue thromboplastin (1:5000 final concentration of Thromborel S, Behringwerke AG). Defibrinated plasma was used as a source of clotting factors and to prevent clot formation with unpredictable binding of thrombin during the assay. Thrombin generation was stopped with an equal volume of stopping buffer (125 mM NaCl, 50 mM Tris-HCl, 2 mM EDTA; pH 7.9) (Heemskerk *et al.* 1999) and measured with a chromogenic substrate S-2238 (For details, see **I**). Time-dependence of the thrombin generation induced by the adhered platelets was assessed at various time-points ranging between 2 and 30 min. The amount (U/mL) of soluble thrombin formed was assessed with a standard curve of α-thrombin. This thrombin generation was then adjusted with the number of deposited platelets, as assessed simultaneuosly with radiolabeled platelets.

6.2. PCA of adhered platelets in comparison with PCA of activated platelets in suspension (I)

The procoagulant activity of collagen-adherent platelets was compared with that of an equal number of GFP in suspension after their activation with 10 μg/mL thrombin receptor activating peptide (TRAP, SFLLRN, Bachem) and 10 μg/mL collagen, or 10 μmol/L calcium ionophore A23187 (Sigma). Platelets were first activated either by adhesion to a collagen surface or by the soluble agonists. Coagulation was initiated with defibrinated plasma and tissue thromboplastin, and thrombin generation was measured with S-2238 at the time when maximal thrombin generation was reached.

6.3. One-stage PCA assay: Generation of thrombin on PRP clots (I, II)

The procoagulant effect of platelets (**I**) and contribution of GPIIb/IIIa or GPIb on this activity (**II**) was also studied in a one-stage procoagulation assay with PRP clots (Fig. 10). PRP was adjusted to 100–300 × 10^9 platelets/L with PPP and diluted 1:2 in PBS. PRP clots (100μL, corresponding finally to 5–15 × 10^6 platelets in the clot) were prepared on a Thermanox coverslip by recalcification (15 mmol/l $CaCl_2$) and treatment with 0.1 U of bovine α-thrombin. The platelet-containing clots were incubated at 37°C for 15 min and washed three times in PBS. Plasma clots without platelets were prepared identically from PPP. To assess the procoagulant activity of the clots, the PRP clots and their PPP controls were incubated with 1:20 diluted defibrinated pooled plasma and traces of tissue thromboplastin (1:5000 final concentration of Thromborel S) at 37°C for 10 min under slow rotation. Clots

were also incubated with PBS buffer only to estimate the amount of soluble thrombin originating from the clot. These absorbance values were subtracted from the results obtained with plasma. Thrombin generation was stopped and determined as described above.

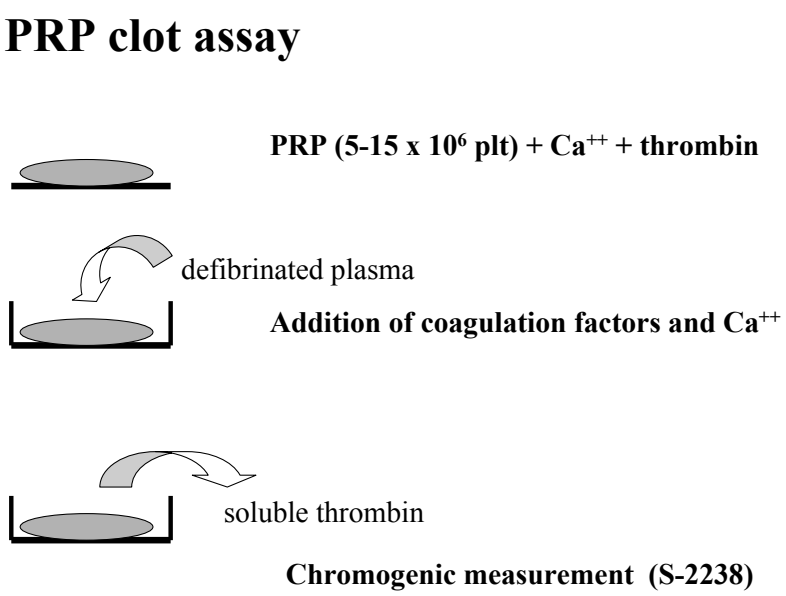

Fig. 10. Platelet-rich plasma (PRP) clot assay. PRP clots were prepared on a coverslip by recalcification and treatment with thrombin. Coagulation was initiated by incubating PRP clots and their platelet-poor plasma (PPP) controls with 1:20 diluted defibrinated pooled plasma and traces of tissue thromboplastin, and thrombin generation was assessed chromogenically at 10 min. For details, see text.

6.4. Prothrombin fragments (F1+2) (I–III)

The capacity of surface-adherent platelets to promote thrombin generation was also estimated with F1+2 after adhesion of GFP to a thrombogenic surface. Subsequently, GFP suspensions (9 vol) were collected in a platelet-inhibitory anticoagulant (Teitel *et al.* 1982) (one vol), centrifuged immediately (11 000 g, 5 min, 4°C), and the supernatant subjected to ELISA.

6.5. Platelet-derived microparticles (MP) in PCA of adhered platelets (I)

MP generation during adhesion-induced platelet activation was studied in GFP supernatant after the adhesion assay. After removal of non-adhered platelets by centrifugation in the presence of apyrase (1 U/mL) and PGE_1 (25 ng/mL) to avoid platelet activation, the TF-triggered procoagulant activity of the supernatant was studied after addition of defibrinated

pooled plasma and tissue thromboplastin. To confirm the role of soluble platelet-derived microparticles in supporting coagulation activation after the adhesion-induced platelet activation, the PCA of the supernatant was measured both in the presence of MP and after their filtration (0.1 μm Millipore, Ireland). Furthermore, the procoagulant activity of the MP-supernatant was adjusted with the number of adhered platelets on collagen or clots (studied with radiolabel).

6.6. Whole blood perfusions (III)

The effects of different GP IIb/IIIa antagonists on adhesion-induced platelet PCA was further assessed by whole blood perfusions using the Turitto perfusion chamber with defined rheological characteristics (Hall *et al.* 1998). The concentrations of the GPIIb/IIIa antagonists were selected to inhibit 80% of ADP-induced aggregation response in PPACK-PRP. PPACK (1 μM)-anticoagulated blood was divided into 7.5 mL aliquots and incubated with 10 μg/mL abciximab (Reopro^{R}, EliLilly/Centocor), 100 ng/mL tirofiban (Aggrastat^{R}, MSD), 1 μg/mL eptifibatide (Integrilin^{R}, Schering-Plough), or a buffer control at 37°C for 5 min before single passage for 1 min over a collagen-coated coverslip at a shear rate of 1600 s^{-1}. Afterwards, the coverslip was briefly perfused with PBS, then rinsed once with buffer to flush the unattached cells. Platelet deposition was assessed with ^{3}H-serotonin labeled platelets as described above and occasionally with SEM. Surfaces obtained during simultaneous perfusions without platelet labeling were subjected to coagulation assay as in the two-stage assay described above.

7. PLATELET FACTOR 4 (II,III)

To further assess adhesion-induced platelet activation, we measured α-granule release of platelet factor (PF) 4 (ELISA, Asserachrom PF 4, Diagnostica Stago) after adhesion of GFP to collagen or clots. After the adhesion assay samples were collected into a platelet-inhibitory anticoagulant (Teitel *et al.* 1982), centrifuged (11 000 g, 5 min, 4°C), and the supernatant assayed.

8. AGGREGATION (III–IV)

Platelet aggregation was assessed with a PACKS-4 turbidometric aggregometer (Helena Laboratories) to define equivalent concentrations of different GPIIb/IIIa antagonists (**III**) or to study the effects of these antagonists on platelet activation induced by various procoagulant agonists (**IV**). Citrated or PPACK-anticoagulated PRP, or GFP (in HEPES with 2 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$) were stirred with various concentrations of GPIIb/IIIa

antagonists or buffer control at 37°C for 1 min before adding the agonist. The effect of fibrin polymerization during platelet aggregation was controled with GPRP (1 mmol/L) added 10 min before the aggregation. The rate of primary aggregation (min^{-1}) and extent of maximal aggregation (%) at 5 min were assessed. The inhibition of aggregation brought about by the GPIIb/IIIa antagonists was calculated from these data.

9. STATISTICAL ANALYSIS

N refers to the number of blood donors. The experiments were performed in duplicates or triplicates. Data are expressed as mean ± standard deviation (SD), unless indicated otherwise. Differences amongst independent groups were tested using unpaired student's *t*-test, whereas differences between paired data were evaluated by paired student's *t*-test. The threshold for statistical significance was set at a p-value <0.05.

RESULTS

1. PROCOAGULANT ACTIVITY (PCA) OF ADHERENT PLATELETS

1.1. Effect of adhesion on platelet PCA

To assess the effect of permanent adhesion to a thrombogenic surface on PCA we compared the procoagulant capacity of collagen-adherent platelets to that of activated platelets in suspension (**I**). Thrombin generation was initiated on collagen-adherent platelets or equal number of maximally activated platelets in suspension by incubation with 1:20 diluted pooled defibrinated plasma and a trace amount of tissue thromboplastin, and finally determined chromogenically after 10 min. To avoid uncontrolled binding of thrombin to the newly forming fibrin we used defibrinated plasma as the source of coagulation factors and natural anticoagulants. When adjusted for platelet number, collagen-adhered GFP supported 30% more thrombin generation than maximally, with TRAP and collagen or with calcium ionophore, activated GFP in suspension under equal conditions ($p<0.01$ for TRAP plus collagen, $p<0.05$ for calcium ionophore) (**I**, Fig.6). Thus, firmly adhered platelets on a thrombogenic surface showed markedly enhanced PCA when compared with platelets in suspension, even after their strong activation.

1.2. Platelet adhesion to a thrombogenic surface

To dissociate platelet adhesion to a thrombogenic surface and the subsequent platelet aggregation upon the initial adherent layer, GFP were labeled with ^{3}H-serotonin and thereafter diluted in HEPES buffer at different platelet counts (10–300 × 10^9/L) (**I**). At platelet densities of up to 100 × 10^9/L, mainly single platelets were attached on collagen surfaces or plasma clots, whereas higher platelet densities supported further aggregation on the initially adhered platelets. Thus, at the lower density scanning electron micrographs depict GFP (100 × 10^9/L) as a single adhesive layer on collagen or plasma clots and show fully spread "fried egg" forms with pseudopod extensions, whereas at the higher density (300 × 10^9/L) extensive platelet aggregation was triggered under equal conditions (**I**, Fig. 4).

1.3. Individual variation in platelet adhesion and subsequent PCA

Individual differences in platelet deposition to collagen or a plasma clot were studied with platelets from 6 healthy donors (**I**, Figs. 3A and 3B). At platelet densities of up to 100 × 10^9/L only minor interindividual differences in deposition occurred on both substrates, but higher densities showed over 3-fold variability. This individual variability in platelet deposition was triggered mainly by varying aggregation, especially on plasma clots. Equally, the platelet counts of the supernatants after deposition on collagen or plasma clots were diminished owing to platelet aggregation (**I**, Figs. 3C and 3D, respectively).

Individual variation in adhesion-induced platelet PCA was studied with unlabeled platelets obtained from the same donors. Again, thrombin generation was initiated on the collagen- or clot-adherent platelets by 1:20 diluted pooled defibrinated plasma with tissue thromboplastin and determined chromogenically after 10 min. Although platelet deposition on collagen or plasma clots did not markedly vary at platelet densities up to 100×10^9/L, thrombin generation induced by these adhered platelets varied almost 4-fold (**I**, Figs. 5A and 5B).

1.4. PCA of platelets deposited on collagen versus on plasma clot

At all densities, platelet deposition to plasma clots was 2–3 times higher than on collagen surfaces (**I**, Figs. 3A and 3B). Data from the adhesion and subsequent procoagulation experiments were used to adjust platelet-induced thrombin generation to the number of adhered platelets on the thrombogenic surface (**I**, Fig. 5C). This platelet adhesion-quantitated generation of soluble thrombin was up to 5-fold greater on collagen than on plasma clots ($p<0.05$), although it attenuated with increasing platelet densities, suggesting the importance of the initial adherent layer for adhesion-induced platelet PCA.

The rate of thrombin generation after adhesion-triggered platelet activation and the effect of tissue thromboplastin were studied in a two-stage procoagulation assay at several time-points ranging between 2 and 30 min (**I**, Fig. 2). Thrombin was formed significantly faster upon clot-adherent platelets than upon platelets bound to collagen. After initial activation over the plasma clots, tissue thromboplastin did not improve the maximal rate or extent of platelet-dependent thrombin generation. In contrast, thrombin generation upon collagen-bound platelets progressed slowly, but tissue thromboplastin accelerated it almost 4-fold.

1.5. PCA of soluble platelet-derived microparticles

MP formation during platelet adhesion and subsequent MP-induced procoagulant activity were studied in GFP supernatants after adhesion to collagen or a plasma clot, with BSA as a control surface (**I**, Fig.7). After a 30 min adhesion of GFP (100×10^9/L), the non-adherent platelets were removed from the supernatant by centrifugation, and the tissue thromboplastin-triggered thrombin generation in 1:20 diluted pooled defibrinated plasma was measured in the presence of soluble MP and after their removal by filtration. On collagen, the thromboplastin-induced thrombin generation after MP filtration decreased by 32±6% ($p<0.05$). After clot-induced platelet activation, MP filtration reduced the PCA of the supernatant by 43±14% ($p<0.05$). When the PCA of the soluble MP was adjusted by the number of initially adhered platelets on collagen or a clot, MP-induced thrombin generation per 10^6 adhered platelets on collagen was over 3-fold more than on clots ($p=0.01$).

2. GPIIb/IIIa ANTAGONISTS AND ADHESION-DEPENDENT PLATELET PCA

2.1. Effect of abciximab on platelet deposition on adhesive substrates

Platelet deposition on collagen or a plasma clot was studied with ^{3}H-serotonin labeled GFP (100 or 300 × 10^9/L, supplemented with 2 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$) in the presence and absence of abciximab (c7E3) (**II**). Abciximab decreased platelet deposition on collagen, especially at higher concentrations allowing aggregation (**II**, Fig. 1A). However, deposition of isolated platelets on plasma clots remained unaltered (GFP 100 × 10^9/L) or even increased (GFP 300 × 10^9/L) in the presence of abciximab (**II**, Fig.1B). In the scanning electron micrographs the typical platelet aggregation upon clots was diminished by abciximab and instead the clot surface appeared completely covered with single platelets (**II**, Fig.2).

To disclose this accelerating effect of abxicimab on the deposition of GFP on plasma clots, we assessed the roles of 1) soluble or clot-bound thrombin, and 2) platelet-derived fibrin(ogen) during the adhesion assay (**II**). In the presence of the physiological plasma anticoagulant antithrombin (AT), or either indirect (unfractionated heparin or low-molecular-weight heparin) or direct (hirudin or PPACK) thrombin inhibitors, platelet adhesion to plasma clots declined equally in the presence and absence of abciximab (**II**, Fig. 5B). The impact of the newly forming soluble thrombin on deposition of GFP on clots was evidenced in the presence of increasing concentrations of AT (**II**, Fig. 5A). In the absence of AT abciximab failed to inhibit platelet deposition, but even at low concentrations (0.1 U/mL) AT markedly improved the inhibitory effect of abciximab ($p<0.001$). While inhibition of fibrin polymerization with GPRP did not reduce adhesion of GFP (100 × 10^6/mL), the accelerating effect of abciximab was completely abolished ($p<0.001$) (**II**, Fig. 5C). In contrast, inhibitors of thrombin or fibrin polymerization did not affect platelet adhesion to collagen in the presence of abciximab (data not shown). The concentrations of GPRP used in these experiments prologed thrombin time without affecting thrombin-induced platelet aggregation in GFP or chromogenic (S-2238) thrombin activity.

The role of clot-bound thrombin on platelet deposition and the abciximab-induced acceleration of platelet deposition on plasma clots was confirmed upon thrombin- or reptilase-induced fibrin surfaces (prepared from isolated fibrinogen in the absence of other plasma proteins) (**II**). In parallel with the plasma clots, abciximab increased deposition of GFP (300 × 10^6/mL) on thrombin-induced fibrin surfaces (12.2 ± 1.1 vs 18.9 ± 6.9 × 10^6 platelets/cm^2, $p<0.01$, n=3), and again GPRP abolished this abciximab-triggered acceleration (9.3 ± 1.1 vs 8.1 ± 1.3 × 10^6 platelets/cm^2, n=3). In the presence of a thrombin inhibitor (10 μmol/L PPACK) or with reptilase-induced fibrin surfaces platelet deposition attenuated 5-fold, fully neutralizing the influence of abciximab (2.6 ± 0.2 vs 2.8 ± 0.4 × 10^6 platelets/cm^2 in the presence of PPACK, and 2.3 ± 0.3 vs 2.4 ± 0.5 × 10^6 platelets/cm^2 on reptilase-treated fibrin surfaces).

Next, deposition of GFP to clots was assessed in the presence or absence of plasma in the platelet suspension (**II**). To allow functional GPIIb/IIIa without activation of coagulation and subsequent clot formation platelets were supplemented with 2 mM $MnCl_2$ (instead of calcium and magnesium). GFP (100×10^6/mL) deposited on plasma clots to the same extent in the presence of different cations (data not shown). Upon addition of normal or defibrinated plasma to the platelet suspension, abciximab clearly decreased deposition of GFP to clots, as it did in PRP (100×10^6/mL), suggesting an inhibitory aid by some plasma proteins (other than fibrinogen). In the presence of VWF- and FVIII-deficient plasma abciximab also decreased platelet deposition. In AT-depleted plasma deposition of GFP increased and the inhibitory effect of abciximab was reduced (**II**, Fig. 6).

2.2. Effect of abciximab on adhesion-induced platelet PCA

Thrombin generation was initiated on collagen- or plasma clot-adherent platelets by incubation with 1:20 diluted pooled defibrinated plasma and traces of thromboplastin. When adjusted by the number of adhered platelets on collagen or a clot, abciximab reduced platelet-associated thrombin generation by 35% on average (range 10–75%, $p<0.03$), irrespective of the thrombogenic surface (**II**, Figs. 3A and 3B). Thrombin generation was also measured by soluble F1+2 during platelet (GFP 100×10^6/mL) adhesion to plasma clots. Compatible with the chromogenic thrombin activity, the levels of F1+2 were 38% lower (51.3 ± 5.1 vs. 32.1 ± 2.8 nmol/L, $p<0.005$) in the presence of abciximab.

2.3. Procoagulant activity of PPP and PRP clots

The roles of platelet GPIIb/IIIa and GPIb receptors on platelet-derived PCA were studied in a one-stage assay of PPP and PRP clots (**II**). Abciximab or SZ2, a monoclonal antibody against GPIb, did not affect the procoagulant activity of PPP clots, but the 5-fold higher thrombin generation upon PRP clots (with 15×10^6 platelets) was reduced by 38% or 44% when abciximab or SZ2, respectively, was present in the clot ($p<0.03$). This inhibitory effect of abciximab on platelet-dependent thrombin generation was equal to that observed in the two-stage assay and with F1+2 after platelet adhesion to plasma clots. In the presence of both abciximab and SZ2 platelet-dependent generation of soluble thrombin on PRP clots was reduced by 65%.

2.4. Effects of different GPIIb/IIIa antagonists on adhesion-dependent platelet PCA

Next, we compared the effects of the three clinically available GPIIb/IIIa receptor antagonists – abciximab, tirofiban and eptifibatide – on the procoagulant activity of platelets adhered to a collagen surface or plasma clot (**III**). To determine the equipotent concentrations of the different GPIIb/IIIa antagonists for further PCA experiments, we first studied the dose-responses of the antagonists upon maximal aggregation to 5 μM ADP in PPACK-PRP (**III**, Fig. 1A-C) or to 0.05 U/mL thrombin in GFP (**III**, Fig. 1D-F).

For static PCA experiments with isolated platelets the concentrations of the GPIIb/IIIa antagonists were selected to maximally inhibit 1) thrombin-induced aggregation of GFP (**III**, Fig. 1 D-F), and 2) the procoagulant activity of collagen-adherent platelets in the two-stage PCA assay (**III**, Fig. 2). In the static assay GFP (100×10^9/L) were first allowed to adhere to collagen for 30 min under slow rotation, and thrombin generation was triggered on the adhesion-activated platelets as described above. Deposition of ^{3}H-serotonin-labeled GFP (100×10^9/L) to collagen ($0.57 \pm 0.2 \times 10^6$ plt/cm^2 in control) was equally inhibited ($p<0.001$ for all antagonists) in the presence of 10 μg/mL abciximab (40% reduction), 50 ng/mL tirofiban (51% reduction), or 1 μg/mL eptifibatide (46% reduction) (**III**, Fig. 3 A). Despite this relatively equal inhibition of platelet deposition to collagen, the adhesion-dependent procoagulant capacity of these same platelets (without radiolabel) (0.74 ± 0.18 U/mL, mean ± SD) was differentially inhibited by the antagonists. In the two-stage PCA assay, platelet-dependent thrombin generation was attenuated most with abciximab, by 49%, in comparison to 31% with tirofiban ($p<0.05$), or 27% with eptifibatide ($p<0.005$) (**III**, Fig. 3 B).

Thrombin generation was also measured by soluble F1+2 during deposition of GFP (100×10^9/L) to a plasma clot in the presence of 10 μg/mL abciximab, 100 ng/mL tirofiban, 1 μg/mL eptifibatide or buffer control (**III**, Fig. 4). Again, the levels of F1+2 after platelet deposition to clots were 21% lower in the presence of abciximab than with tirofiban or eptifibatide, compatible with the thrombin generation upon collagen-adherent platelets.

2.5. Platelet PCA after whole blood perfusion over collagen

For whole blood perfusion experiments the concentrations of GPIIb/IIIa antagonists were selected to inhibit ADP-induced aggregation in PPACK-PRP by 80% (**III**, Fig 1 A-C). Platelet deposition to collagen was determined after 1 min blood perfusions at a shear rate of 1600 s^{-1} with ^{3}H-serotonin-labeled platelets in the presence of 10 μg/mL abciximab, 100 ng/mL tirofiban, 1 μg/mL eptifibatide, or a buffer control. At these concentrations all GPIIb/IIIa antagonists efficiently and equally reduced platelet deposition on collagen by approximately 90% (**III**, Figs. 5 and 6). The procoagulant capacity of these perfusion-adhered platelets (unlabeled) on the collagen coverslip was assessed as described under static conditions by incubating with diluted defibrinated plasma and tissue thromboplastin (**III**). Again, compatible with the static assay utilizing isolated platelets and either collagen or clot surfaces, abciximab reduced the generation of soluble thrombin on collagen-adherent platelets 35% more than tirofiban ($p<0.001$) or eptifibatide ($p<0.01$) (**III**, Fig. 5).

2.6. Effects of GPIIb/IIIa antagonists on PF 4 release

Release of PF 4 from platelet α-granules during adhesion of GFP (100×10^9/L) to collagen or clots was studied in the presence of GPIIb/IIIa antagonists at 10 μg/mL abciximab, 100 ng/mL tirofiban, or 1 μg/mL eptifibatide (**III**). In general, platelets depositing on clots released more PF 4 than those depositing on collagen (98±7 vs 68±15 U/mL, $p=0.02$). Irrespective of the thrombogenic surface, all three of the GPIIb/IIIa antagonists failed to modulate PF 4 release during platelet deposition (**III,** Table 1).

3. GPIIb/IIIa ANTAGONISTS AND GPVI-MEDIATED PLATELET AGGREGATION

Turbidometric aggregation with ADP as a standard agonist has been the method most used to assess the pharmacodynamics of GPIIb/IIIa antagonists. Appreciating the central role of GPVI in mediating collagen-induced platelet activation, we assessed the inhibitory effects of three currently available GPIIb/IIIa receptor antagonists – abciximab, tirofiban, and eptifibatide – on platelet aggregation induced by ADP or by various GPVI-mediated agonists (**IV**). Under conditions that equally inhibited over 80% of ADP-triggered platelet aggregation in PPACK-anticoagulated PRP, abciximab inhibited convulxin- and CRP-induced platelet aggregation significantly more effectively than the low-molecular weight antagonists ($p<0.005$) (Fig. 11). All agents inhibited convulxin-induced aggregation more effectively (abciximab 90±6%, tirofiban 64±20%, and eptifibatide 61±14%) than compatible

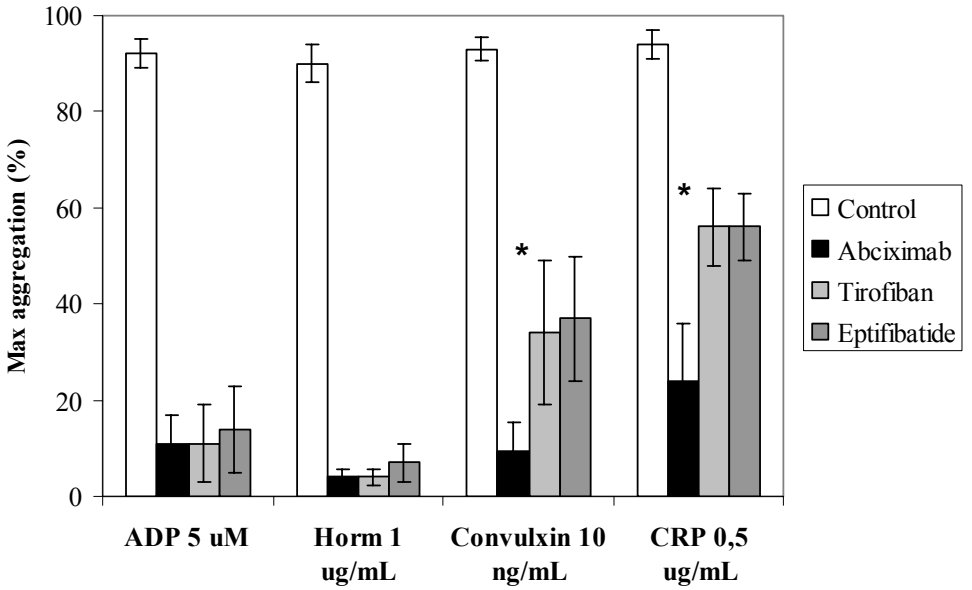

Fig. 11. Inhibition of platelet aggregation by glycoprotein (GP) IIb/IIIa antagonists. PPACK-anticoagulated platelet-rich plasma (300×10^9/L) was preincubated with buffer control, 10 μg/mL abciximab, 100 ng/mL tirofiban, or 1 μg/mL eptifibatide before triggering platelet aggregation with 5 μM ADP, 1 μg/mL Horm collagen, 10 ng/mL convulxin, or 0.5 μg/mL CRP. Maximal aggregation data of 10 donors are expressed as mean ± SD. *$p<0.005$. ADP: adenosine diphosphate; CRP: collagen-related peptide; PPACK: D-phenylalanyl-L-prolyl-L-arginine chloromethylketone; SD: standard deviation.

CRP-induced aggregation (abciximab 75±18%, tirofiban 41±7%, and eptifibatide 41±6%) ($p<0.01$ for all). In contrast, Horm collagen-induced aggregation was completely inhibited with all antagonists under equal conditions. In addition, TRAP-induced platelet aggregation in PPACK-PRP was effectively and equally inhibited by all GPIIb/IIIa antagonists at the same concentrations (data not shown).

The superior inhibitory effect of abciximab on GPVI-mediated platelet aggregation was observed at the agonist concentrations that induced maximal aggregation. However, when the concentrations of these triggering agonists were increased 5- to 10-fold, all antagonists reached a similar level of inhibition, that is only 35±5% in the case of convulxin and 31±3% in the case of CRP (**IV**, Fig. 2). In the dose-response studies of the GPIIb/IIIa antagonists (**IV**, Fig. 3) the inhibition of convulxin- or CRP-triggered aggregation in PPACK-PRP seemed to reach a plateau at the same concentration of abciximab (10 μg/mL) as in ADP-induced aggregation, but 3-fold higher concentrations of tirofiban (300 ng/mL) or eptifibatide (3 μg/mL) were needed for the same effect.

3.1. Effect of divalent cations on inhibition of platelet aggregation

The role of divalent cations in the inhibitory effects of the antagonists was studied in citrate-anticoagulated PRP (**IV**). After chelation of divalent cations all GPIIb/IIIa antagonists equally inhibited convulxin- and CRP-triggered aggregation up to 70% (abciximab 60–69%, tirofiban 65–69%, and eptifibatide 71–74%, at the concentrations described above). Higher concentrations of GPIIb/IIIa antagonists (abciximab 30 μg/mL, tirofiban 500 ng/mL, or eptifibatide 5 μg/mL) failed to potentiate this inhibitory effect.

3.2. Inhibition of fibrin polymerization during platelet aggregation

Next, we assessed the effect of an inhibitor of fibrin polymerization, i.e. GPRP (1.0 mmol/L), on the inhibitory capacity of the GP IIb/IIIa antagonists (**IV**, Fig. 4). GPRP had no effect on convulxin- or CRP-triggered aggregation in PPACK-PRP in the absence of GPIIb/IIIa antagonists. However, it significantly potentiated the inhibitory effect of tirofiban and eptifibatide, whereas the effect of abciximab remained unaltered. After preincubation with GPRP the maximal aggregation in the presence of tirofiban or eptifibatide was on average 65% (tirofiban 59%, $p=0.03$, eptifibatide 72%, $p=0.01$) or 44% (tirofiban 43%, $p=0.005$, eptifibatide 45%, $p=0.001$) less after activation of platelets with convulxin or CRP, respectively.

DISCUSSION

It is now well established that the interaction between blood platelets and coagulation factors is essential for hemostasis and thrombosis, especially at the higher shear rates found in arteries. But despite the important role of activated platelets in directing the coagulation system at the site of vascular injury, relatively little is known about platelet PCA triggered by their permanent adhesion to a thrombogenic surface. Most studies have monitored the procoagulant effects of platelets following activation in suspension. However, platelets are by nature adhesive elements which perform their hemostatic function under flow conditions. It is therefore likely that studies on platelets in suspension do not necessarily reflect the wide range of platelet functional responses to vascular injury.

Our study design provided activation of platelets by their firm adhesion, with subsequent procoagulant alteration of the phospholipid membrane. The importance of permanent adhesion for platelet PCA became evident when compared with that of platelets in suspension. Platelets activated by adhesion to a collagen surface supported 30% more thrombin generation than an equal number of platelets in suspension, even after maximal activation. Previously, Haimovich *et al.* (1993) have reported enhanced $\alpha_2\beta_1$ integrin-mediated platelet activation upon immobilized ligand compared with soluble ligand applied to platelets in suspension, and studies with immobilized monomeric collagen under flow conditions supported the same conclusion (Polanowska-Grabowska *et al.* 1993, Gear *et al.* 1997). Recently, adhesion of platelets to a monolayer of a $\alpha_2\beta_1$-specific peptide GFOGER induced spreading and shape change in the presence of inhibitors of secondary mediators, even though the peptide had no effect on platelet activation in suspension (Inoue *et al.* 2003). In concordance with our findings, Heemskerk *et al.* (1997) observed that interaction of platelets with immobilized collagen could lead to exposure of procoagulant phopholipids, whereas in previous studies collagen was unable to induce procoagulant activation when applied to platelets in suspension in the absence of thrombin (Bevers *et al.* 1982). Equally, the GPVI-specific ligand CRP was ineffective in inducing platelet PCA when added to isolated platelets resuspended in buffer, whereas adhesion of platelets to a CRP-coated coverslip caused strong procoagulant activation (Heemskerk *et al.* 1999). Further, platelets adhered to fibrinogen also showed potentiated activation by convulxin or CRP compared with platelets in suspension (Siljander *et al.* 2001). This enhanced adhesion-triggered activation could well be due to the capacity of the adhesive substrate to support platelet spreading and cytoskeletal extension, thus promoting effective signaling crucial to platelet procoagulant activation as well as providing more membrane accessibility for coagulation factors. In addition, augmented accessibility of glycoprotein receptors from the SCCS during shape change and spreading of adhering platelets (Escolar *et al.* 1989) could be related to the enhanced procoagulant capacity of platelets.

Several studies have demonstrated a significant increase in the presence of GPIb on the luminal side of spread platelets after activation under static (Escolar *et al.* 1994, White *et al.* 1995) or flow (White *et al.* 1994) conditions. Studies on GPIIb/IIIa receptor expression

after surface activation of platelets have provided contradictory results presumably due to varying experimental conditions (reviewed in Escolar & White 2000). Revealed by flow cytometric analysis, only a minor fraction (10–15%) of platelets were directly activated upon incubation with even extremely high (50 μg/mL) concentrations of fibrillar collagen in suspension, and further platelet activation was reliant on the release of secondary mediators, i.e. ADP and TXA_2 (Nieswandt *et al.* 2001b). These data and the fact that in vivo collagen is present as a monolayer further underlines the importance of adhesion for subsequent platelet activation. However, at the site of vascular injury, only the initial layer of adherent platelets are in contact with collagen, whereas further platelet activation is mediated by adhesion to the newly-forming fibrin clot with active clot-bound thrombin (Kumar *et al.* 1994 and 1995). Thus, procoagulant transformation occurs precisely at the sites where coagulation is desired (exposed subendothelial collagen) or already present (fibrin clot).

The contribution of flow conditions and other blood cells was assessed in the whole blood perfusion experiments, although the limitations of our study include the use of defibrinated and diluted plasma and the absence of vascular endothelium. Instead of purified coagulation factors, we used defibrinated pooled plasma as a source of procoagulants to eliminate uncontrolled binding of the newly-forming thrombin to the fibrin clot, and to overcome the effects of natural anticoagulants. In the presence of plasma fibrinogen under similar conditions, measurable soluble thrombin was on average 28% lower owing to clot formation and thrombin binding to the newly-forming fibrin. The report by Kumat *et al.* (1994) also indicated that ≈30% of all thrombin formed during coagulation activation is adsorbed onto fibrin. Inhibitors of activated coagulation factors in plasma, such as TFPI, APC, and AT, also play a major role in regulating and localizing the coagulation activation. Only a portion of the total amount of activated factors will be bound to cell surfaces. The factors that are surface-bound are relatively protected from plasma protease inhibitors, while soluble factors are more rapidly inactivated. Thus, those activated factors that do not remain localized on a cell surface (or bound to the forming fibrin clot) are inhibited and prevented from catalyzing coagulation reactions in the systemic circulation. When this control mechanism is overwhelmed, disseminated intravascular coagulation ensues.

At lower platelet densities, allowing mainly adhesion, the individual variation in platelet deposition on collagen and plasma clots was relatively modest. However, at higher platelet densities deposition markedly (2- to 4-fold) varied between donors, owing to increased aggregation. This varying aggregation during the first step of the assay reflected individual differences in platelet activation, since in the absence of plasma it was mediated by platelet-derived ligands: fibrinogen, VWF, or thrombospondin. Although platelet deposition on collagen or plasma clots showed only modest variation with platelet densities up to 100×10^9/L, thrombin generation induced by these adhered platelets varied over 3-fold independent of the thrombogenic surface, reflecting donor-dependent differences in the procoagulant capacity of platelets. Similar evidence of interindividual variation in platelet procoagulant activity has previously been reported (Monroe *et al.* 1994, Kumar *et al.* 1995, Sumner *et al.* 1996), although the precise pathophysiological mechanisms are still unknown. Furihata *et al.* (2001) observed a 5-fold range in platelet GPVI content in healthy subjects and

demonstrated that GPVI-mediated prothrombinase activity was directly proportional to the GPVI content of platelets. There is good evidence that platelet GP polymorphisms are linked to receptor expression and/or functional heterogeneity of platelets (reviewed in Yee & Bray 2004), but their association with platelet procoagulant capacity remains unclear. Recently, a low-frequency allele of GPVI was reported to be associated with reduced surface expression and functional responses, including significantly lower thrombin-generating potential (Joutsi-Korhonen *et al.* 2003). However, results of studies on the association of platelet GP receptor polymorphisms with the risk of arterial thrombosis have so far been contradictory (Kunicki 2002, Yee & Bray 2004).

We observed several differences in platelet deposition and subsequent PCA depending on the adhesive surface. Platelet deposition on plasma clots was significantly greater than on collagen surfaces, especially at higher platelet densities allowing aggregation. The rate of thrombin generation induced by clot-adherent platelets was markedly faster than that of collagen-adherent platelets during the initial phase of coagulation activation. As previously reported (Heemskerk *et al.* 1999), thrombin generation proceeded slowly on collagen-adherent platelets in the absence of tissue thromboplastin, while no coagulation activity was observed in the absence of both platelets and exogenous tissue thromboplastin under these experimental conditions. The presence of thromboplastin clearly enhanced platelet-dependent coagulation activity more efficiently than thrombin generation in plasma without platelets. These findings are compatible with the idea that although TF is not absolutely necessary, the interaction between TF and activated platelets induces a local rapid thrombin burst at the site of vessel injury. In vivo, endothelial damage causes simultaneous exposure of collagen and TF, an integral membrane protein of subendothelial cells, allowing optimal response to vascular injury. On the contrary, when the clot has already formed, it contains thrombin and additional clotting factors, so that the role of TF is less crucial.

When platelet-dependent thrombin generation in the two-stage assay was adjusted by the number of adhered platelets, the collagen-adherent platelets in the presence of thromboplastin generated up to 5 times more soluble thrombin than the clot-adherent platelets. However, the interindividual variation in platelet procoagulant capacity was surface-independent. Platelet-adjusted thrombin generation decreased at increased platelet densities, underlining the importance of the initial adhesive layer on coagulation activity, with restriction on excessive platelet plug formation (Coller *et al.* 1993, Heynen *et al.* 1994, Kirchhofer *et al.* 1995, Wencel-Drake *et al.* 1996). Moreover, in larger platelet aggregates, only the superficial layer of activated platelets may come into contact with plasma coagulation factors and thus effectively contribute to thrombin generation.

Activation of platelets by their firm adhesion to collagen or a plasma clot was accompanied by formation of platelet-derived procoagulant MP. Again, when MP-dependent thrombin generation was adjusted by the number of adherent platelets from which the MP originated, soluble thrombin generated by MP from collagen-adherent platelets exceeded that of the clot. This finding may be explained by the quantity and/or quality of the MP produced by collagen-adherent platelets. In addition, these surface-associated differences may be related to the retention of the newly-forming thrombin (Kumar *et al.* 1994) as well

as MP (Siljander *et al.* 1996) on the fibrin clot. Retained thrombin and MP then contribute to the rapid coagulation activity on the clots while lower amounts of soluble thrombin and numbers of MP are detected in the supernatant.

In conclusion, our findings demonstrate the major differences between collagen and plasma fibrin clots as adhesive and thrombogenic surfaces. First, the initial activation of platelets by adhesion to subendothelial collagen leads – in the presence of traces of TF – to a marked burst of thrombin and platelet-derived MP into the circulation. Second, the subsequently forming clot, through contributing to explosive platelet adhesion and coagulation activation, retains significant amounts of active thrombin and numbers of procoagulant MP, thus localizing the process at the site of vascular injury.

The contribution of specific adhesive platelet receptors and their ligands responsible for enhanced PCA has only recently gained interest. GPVI is considered the principal receptor mediating the procoagulant activation induced by platelet adhesion to collagen (Heemskerk *et al.* 1999, Goto *et al.* 2002, Siljander *et al.* 2004), and platelet adhesion *per se* potentiates the procoagulant activation mediated by GPVI (Siljander *et al.* 2001), possibly by triggering additional activation steps or by ensuring optimal GPVI-ligand contact. In this study, we set out to assess the roles of GPIIb/IIIa and GPIb receptors on adhesion-induced platelet PCA. As expected, blockade of GPIIb/IIIa with abciximab lowered platelet deposition to collagen by inhibiting aggregation upon the initial layer of adherent platelets. In addition, abciximab reduced the procoagulant capacity of these remaining collagen-adherent platelets by 30%. Surprisingly, platelet deposition to plasma clots was not reduced in the presence of abciximab, although the formation of typical aggregates seemed inhibited. However, platelet-dependent thrombin generation was reduced by almost 40% despite the full surface coverage of single platelets. A similar inhibitory effect of abciximab on platelet-dependent coagulation activity was confirmed by reduced generation of soluble F1+2 during platelet adhesion to plasma clots and also in the one-stage procoagulation assay, where the procoagulant capacity of PRP clots – but not of PPP clots – was attenuated. This supports a true effect on platelet-mediated thrombin generation, not a direct influence on the coagulation system. These findings are in accordance with previous studies with platelets in suspension or whole blood. It has been reported that GPIIb/IIIa antagonists reduce platelet-dependent thrombin generation (Reverter *et al.* 1996, Byzova & Plow 1997, Keularts *et al.* 1998, Furman *et al.* 2000, Li *et al.* 2000, Dicker *et al.* 2001), expression of procoagulant phopholipids and binding of FXI and FV/Va on the platelet surface (Furman *et al.* 2000), and formation of procoagulant microparticles (Gemmel *et al.* 1993, Reverter *et al.* 1996). In these studies the inhibitory effect of abciximab on platelet-dependent thrombin generation ranged between 25 and 60%. In our hands, abciximab reduced adhesion-triggered platelet-dependent thrombin generation by 30–40%, although we have previously observed that the procoagulant capacity of firmly adhered platelets on a thrombogenic surface exceeds that of even maximally activated platelets in suspension.

The monoclonal antibody against GPIb, SZ2, clearly inhibited adhesion-induced platelet PCA, without affecting platelet deposition to collagen or plasma clots. Similar results have been obtained in previous studies with platelets in suspension. Beguin *et al.*

(1999) showed that GPIb and VWF are required in fibrin-associated platelet PCA during TF-induced coagulation. In a report by Dörmann *et al.* (2000) the thrombin-binding site of GPIb was essential for thrombin-induced platelet procoagulant activity, which also required PAR-1, GPIIb/IIIa, and platelet-to-platelet contact. In addition, Bernard-Soulier platelets lacking GPIb have reduced procoagulant capacity (Dicker *et al.* 2001, Beguin *et al.* 2004). When combining SZ2 and abciximab the procoagulant capacity of PRP clots in the one-stage assay was further inhibited, supporting the involvement of both GPIb and GPIIb/IIIa in platelet procoagulant activation. However, this combined inhibition was not complete, since 35% of the maximal platelet procoagulant capacity remained, suggesting alternative mechanisms for platelet PCA. Beguin *et al.* (2004) have recently reported similar results, whereas in the study of Dicker *et al.* (2001) co-inhibition of GPIb and GPIIb/IIIa completely blocked procoagulant activity of normal platelets. This discrepancy is likely due to different experimental conditions, since Dicker *et al.* assessed thrombin-induced PCA of isolated platelets with purified coagulation factors in the absence of fibrin(ogen), whereas we, as well as Beguin *et al.*, used PRP. Indeed, Beguin *et al.* (2004) suggest that, in addition to thrombin, polymerizing fibrin is crucial for GPIb-mediated platelet procoagulant activity in a VWF-dependent manner. In addition, binding of FXI to GPIb (Keularts *et al.* 2001, Baglia *et al.* 2002) may also affect GPIb-mediated platelet PCA.

Accelerated deposition of GFP to plasma clots in the presence of abciximab was related to clot-bound thrombin and polymerization of platelet-derived fibrin(ogen). In the presence of AT or thrombin inhibitors, deposition of GFP to clots decreased both in the presence and absence of abciximab. Accordingly, abciximab inhibited platelet deposition on fibrin surfaces prepared with reptilase but increased deposition on thrombin-induced fibrin clots. Inhibition of fibrin polymerization in GFP suspension abolished the accelerating effect of abciximab. Legrand *et al.* (1989) found that thrombin concentrations allowing fibrin polymerization induces accelerated binding of secreted fibrinogen to the platelet surface, and this is markedly decreased when fibrin formation is inhibited with GPRP. Furthermore, upon platelet activation with thrombin, a ligand-occupied internal pool of α-granule GPIIb/IIIa receptors is expressed on the platelet surface in a secretion-dependent manner (Nurden *et al.* 1996, Gawaz *et al.* 2000). Compatible with our results, abciximab fails to inhibit this expression of fibrinogen-bound GPIIb/IIIa receptors from the internal pool (Kleiman *et al.* 1995, Bihour *et al.* 1999). Thus, during platelet adhesion to plasma clots containing remarkable amounts of fibrin-bound thrombin, the internal GP IIb/IIIa -bound fibrin(ogen) may directly polymerize with the fibrin surface. Indeed, platelet adhesion to collagen and subsequent fibrin thickness were enhanced during an ex vivo perfusion with non-anticoagulated blood from patients with Glanzmann thrombasthenia (Hainaud *et al.* 2002), as previously reported with a GPIIb/IIIa antagonist lamifiban (Kirchhofer *et al.* 1995).

The roles of adhesive platelet receptors and their ligands in platelet procoagulant activation are summarized in Fig. 12. Initially, platelet adhesion to subendothelial collagen triggers the procoagulant alteration of this initial layer of adherent platelets at the site of vascular injury. According to current data, GPVI is the principal receptor mediating collagen-induced procoagulant activation. Arterial thrombus progresses as a result of coagulation

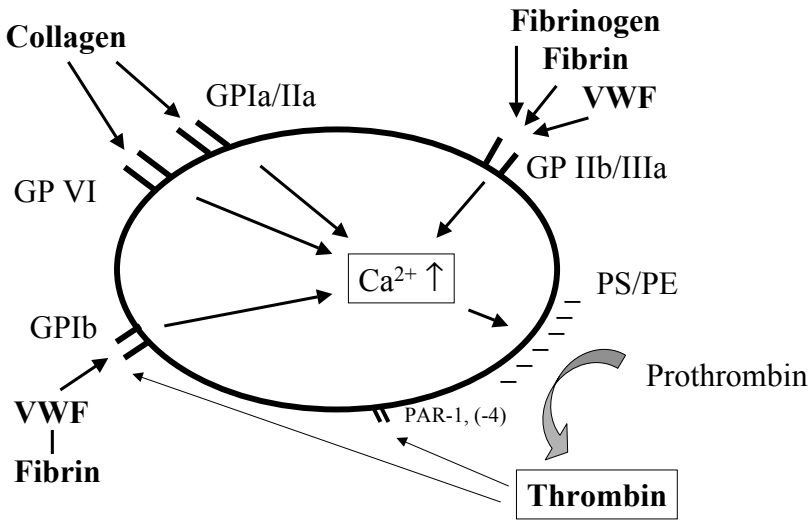

Fig. 12. From platelet adhesion to procoagulant activation. GP: glycoprotein; PAR; protease-activated receptor; PE: phosphatidylethanolamine; PS; phosphatidylserine, VWF; von Willebrand factor.

activation leading to formation of thrombin and fibrin, and further platelet activation occurs on the surface of the fibrin clot through GPIIb/IIIa and GPIb receptors. In addition to the fibrin-VWF-GPIb pathway (Loscalzo *et al.* 1986, Beguin *et al.* 1999, Beguin *et al.* 2004), there is evidence that fibrin interacts with platelets with a greater affinity than fibrinogen (Tuszynski *et al.* 1984, Legrand *et al.* 1989, Harfenist *et al.* 1985), and that this interaction occurs via GPIIb/IIIa receptors (Hantgan 1988). Recently, Phillips *et al.* (2004) observed that polymerized fibrin can function as a platelet co-stimulus by up-regulating expression of binding sites for FVIIIa in a GPIIb/IIIa-dependent manner. The resulting thrombin further accelerates procoagulant activation of platelets through binding to GPIb, PAR-1 and PAR-4. Thus, the procoagulant activation of adherent platelets requires the concerted action of several platelet receptors at the site of arterial injury. It it likely that *in vivo* the pathways of platelet activation are dependent on the actual conditions, such as shear rate, level of tissue factor, and presence of thrombin or fibrin. Equally, the dominant mechanisms observed *in vitro* may be strongly related to the experimental conditions used.

Next, we compared the effects of the three currently available GPIIb/IIIa receptor antagonists on the release reaction and PCA subsequent to deposition-induced activation of platelets both under static and blood flow conditions. To achieve beneficial clinical effects, doses of GPIIb/IIIa antagonists target 80% receptor occupancy of unstimulated platelets, whereas excessive occupancy causes marked prolongation of bleeding time (Coller 1995). Turbidometric aggregometry is the most widely used method to assess the pharmacological effects of GPIIb/IIIa antagonists, and to select dosing for clinical trials. In fact, receptor occupancy studies with synthetic antagonists have been considered unreliable due to their lower affinity and rapid off-rate (Casserly & Topol 2002). At concentrations that equally

inhibited platelet aggregation, all GPIIb/IIIa antagonists decreased the generation of new thrombin on the surface-deposited platelets. However, under all experimental set-ups adherent platelets generated the least amount of thrombin in the presence of abciximab when compared with tirofiban or eptifibatide.

The existing data on the relative effects of different GPIIb/IIIa antagonists on platelet PCA are inconsistent. Pedicord *et al.* (1998) found equal inhibition of platelet PCA with abciximab and low-molecular-weight antagonists, whereas Furman *et al.* (2000) reported abciximab to be a stronger inhibitor of platelet surface factor V/Va and annexin V binding than tirofiban and eptifibatide. Lages and Weiss (2001) showed that abciximab consistently inhibited (40–60%) collagen-activated or thrombin- and collagen-coactivated GFP, whereas the non-antibody antagonists were weaker inhibitors of thrombin and collagen-induced PCA and unable to inhibit collagen-induced platelet PCA. In the few clinical trials comparing different GPIIb/IIIa antagonists during PCI, abciximab was superior to tirofiban (Topol *et al.* 2001a) and to eptifibatide (Lincoff *et al.* 2003). Different kinetics or binding sites of the antagonists on the GPIIb/IIIa receptor (Cierniewski *et al.* 1999, Kereiakes *et al.* 2000, Jennings *et al.* 2000) may be associated with differences in platelet PCA or generation of platelet-derived MP. In later studies (Furman *et al.* 2000, Lages & Weiss 2001) the enhanced effect of abciximab was not mediated via the $\alpha_v\beta_3$ receptor, in contrast to a previous report by Reverter *et al.* (1996). Lages and Weiss (2001) postulate that the inhibitory effect of different GPIIb/IIIa antagonists relates to the agonist studied, a finding supported by GPIIb/IIIa-deficient platelets (Weiss & Lages 1997). Also in our hands, the relative inhibitory effect of abciximab was stronger on collagen-adherent platelets than on platelets adhering to thrombin-induced plasma clots.

Independent of the thrombogenic surface, all GPIIb/IIIa antagonists failed to affect PF 4 release from the adhering GFP, suggesting distinct mechanisms for procoagulant activation and the release reaction. Thus, the activated platelets at the site of thrombus formation preserve the ability to neutralize heparin therapy and also likely provide local stimulation of smooth muscle cell growth via platelet-derived growth factor. Previous reports on the effects of GPIIb/IIIa antagonists on α-granule release have provided contradictory results. Initially, Reverter *et al.* (1996) observed reduced PF 4 secretion in response to abciximab. In agreement with our results, Dickfeld *et al.* (2001) reported that abciximab, tirofiban, and eptifibatide failed to inhibit P-selectin expression during platelet activation with ADP or TRAP. Bihour *et al.* (1999) showed that during strong activation of isolated platelets with TRAP abciximab had little effect on the secretion of P-selectin. Accordingly, secreted P-selectin was not significantly reduced during abciximab infusion in patients undergoing PCI, when compared with samples taken before the medication. Equally, Neumann *et al.* (2001) found no significant differences in the percentage of P-selectin positive platelets in patients undergoing PCI with abciximab, tirofiban, or eptifibatide. In addition, several other studies have demonstrated mild or no antisecretary effects of abciximab (Klinkhard *et al.* 2000, Furman *et al.* 2000), tirofiban (Furman *et al.* 2000, Schneider *et al.* 2000), and eptifibatide (Furman *et al.* 2000), whereas Schneider *et al.* (2000) reported paradoxical potentiation of platelet secretion with abciximab and eptifibatide.

Recent studies have placed the immunoglobulin superfamily member GPVI in a central position in the complex processes of collagen-induced platelet activation, aggregation, degranulation and procoagulant activation (Nieswandt & Watson 2003, Farndale *et al.* 2004, Siljander *et al.* 2004, Moroi & Jung 2004). High-affinity conformation of GPIIb/IIIa in response to collagen-induced inside-out signalling seems to be mediated by GPIa/IIa and GPVI(-FcRγ), and further reinforced by the release of soluble mediators (Nakamura *et al.* 1999). We observed that inhibition of maximal platelet aggregation induced by various GPVI-related agonists depends on the agonist used, and that abciximab more effectively than tirofiban or eptifibatide reduced GPVI-mediated aggregation. Since GPVI is now considered as the key receptor in collagen-induced platelet procoagulant activation, these results give one explanation to previous observations of ours and others of the distinct inhibitory effects on platelet-dependent coagulation activity by various GPIIb/IIIa antagonists. This difference in the inhibitory capacity of these antagonists was dependent on fibrin polymerization and divalent cations. Legrand *et al.* (1989) have shown that intracellular fibrinogen from α-granules is expressed on activated platelets under conditions where binding of plasma fibrinogen is prevented by GPIIb/IIIa receptor antagonists. It has been suggested that the internal pool of GPIIb/IIIa on membranes of α-granules and the open surface canalicular system plays a role in this early and irreversible binding of platelet fibrinogen to its receptor, and that upon strong platelet activation this ligand-occupied pool of internal GPIIb/IIIa receptors is expressed on the platelet surface in a secretion-dependent manner (Legrand *et al.* 1989, Nurden *et al.* 1996). Furthermore, Legrand *et al.* (1989) demonstrated with thrombin-activated isolated platelets that rebinding of secreted fibrinogen to GPIIb/IIIa on the platelet surface occurred only in the presence of thrombin concentrations allowing fibrin polymerization, and was abolished in the presence of GPRP. These data are in accordance with our new findings on the effect of GPRP on platelet aggregation in the presence of GP IIb/IIIa antagonists. Upon platelet activation with strong procoagulant agonists in the presence of plasma, fibrinogen is exposed on the platelet surface and may polymerize in response to thrombin generation upon the activated platelets. Abciximab seems to inhibit the procoagulant response to GPVI-mediated stimuli more effectively than the synthetic GPIIb/IIIa antagonists, leading to reduced fibrin polymerization and better inhibition of platelet aggregation. With weak procoagulant agonists, soluble collagen, TRAP and ADP, platelet aggregation was equally inhibited by all GPIIb/IIIa antagonists. In addition, they all reduced aggregation of GFP in suspension (devoid of coagulation activity) to the same level. To maintain normal levels of divalent cations during these experiments we used PPACK-anticoagulated PRP. In citrated PRP the inhibitory effects of various GPIIb/IIIa antagonists did not differ from each other, reflecting the importance of physiological concentrations of divalent cations for normal binding of the antagonists to the receptor (Hourdille *et al.* 1985, Legrand *et al.* 1989, Phillips *et al.* 1997). Furthermore, although calcium does not accelerate thrombin-catalyzed fibrinopeptide release, it has a major role in the further polymerization of fibrin (Boyer *et al.* 1972).

Our findings are relevant to several clinical issues. The data on the procoagulant activation of adherent platelets add to the awareness that platelets are not equal to phospholipid

vesicles in coagulation. In addition to procoagulant phospholipids, non-lipid receptors for coagulation factors (Bouchard *et al.* 1997, Byzova & Plow 1997, Baglia & Walsh 1998, Baglia *et al.* 2002, Keularts *et al.* 2001, Ahmad *et al.* 2000) and platelet-derived FVa have been shown to influence platelet-dependent thrombin generation (Alberio *et al.* 2000). Due to the individual variation in platelet PCA, the role of platelet activation through adhesive ligands should be considered in monitoring overall coagulation activity. The succesful clinical use of GPIIb/IIIa antagonists may in part rely on their anticoagulant effects, which in turn could affect the safety profile of these drugs, especially in combination with anticoagulants and fibrinolytics. Because of this dual role of platelets in hemostasis and thrombosis, it is evident that dosage of these drugs should not be based on platelet aggregation measurements alone. We demonstrated that abciximab particularly well controls the thrombogenicity of collagen surfaces. This is compatible with the excellent efficacy of abciximab during percutaneous interventions upon exposure of fresh collagen and other subendothelial matrix during angioplasty and stenting, and its somewhat limited efficacy with preformed thrombotic surfaces as in the conservative treatment of unstable angina and myocardial infarction (Casserly & Topol 2002). The ability of a strong agonist to expose GPIIb/IIIa on the platelet surface in a preactivated, ligand-bound state is of considerable importance when evaluating the effects of antithrombotic drugs that inhibit fibrinogen binding to GPIIb/IIIa. Finally, we observed that GPIIb/IIIa antagonists exhibit distinct characteristic inhibition profiles. Careful evaluation of the specific effects of GPIIb/IIIa antagonists on platelet activation and procoagulant action in comparative clinical settings will undoubtedly identify the optimal applications and combinations with anticoagulants for these agents in the future.

CONCLUSIONS

Permanently adhered and activated platelets on a thrombogenic surface display markedly enhanced procoagulant activity in comparison with maximally activated platelets in suspension. Thus, adherent platelets strongly promote thrombus formation but, on the other hand, regulate the hemostatic integrity by localizing coagulation activity at the site of vascular injury. Marked interindividual but surface-independent variation in adhesion-induced platelet PCA suggests a uniform platelet procoagulant mechanism.

Additive reduction of platelet-dependent coagulation activity by blockade of GPIIb/IIIa and GPIb receptors supports the involvement of both GPIIb/IIIa- and GPIb-mediated mechanisms of platelet procoagulant activity. However, that 35% of the maximal platelet procoagulant capacity remains suggests additional mechanisms for platelet procoagulant activity, likely GPVI and involvement of fibrin polymerization.

At similar anti-aggregatory efficacy abciximab decreased thrombin generation on adherent platelets more than tirofiban or eptifibatide both under static and blood flow conditions, dissociating aggregation and procoagulant activity. Independent of the thrombogenic surface, all GPIIb/IIIa antagonists failed to affect PF 4 release from the adhering platelets, suggesting distinct mechanisms for procoagulant activation and secretion.

GPIIb/IIIa antagonists exhibit distinct inhibition profiles in platelet aggregation triggered by procoagulant GPVI-related platelet agonists. This difference was dependent on fibrin polymerization and divalent cations. During CRP- or convulxin-induced platelet activation, inhibition of fibrin polymerization by GPRP potentiated the effects of tirofiban and eptifibatide to reach that of abciximab, although GPRP did not affect platelet aggregation in the absence of GPIIb/IIIa antagonists. Thus, the ability of strong procoagulant platelet agonists to expose pre-activated and ligand-bound GPIIb/IIIa from the internal pool seems important.

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