Platelet signaling to procoagulant activity and heterogeneity in thrombus formation : an in vivo and ex vivo approach

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The beginning is the most important part of the work

Plato

Aan pap en mam

Contents

Chapter 1

General introduction

Platelets in haemostasis and thrombosis

Blood platelets play a crucial role in haemostasis and thrombosis. Following vascular injury, the haemostatic response is considered to be driven by platelet activation, which leads to aggregation of the cells and plug formation. Impairment in platelet function can give rise to excessive blood loss. On the other hand, undesired platelet activation upon rupture of an atherosclerotic plaque can trigger a thrombotic response, which impedes blood flow through the diseased artery and can even completely occlude the vessel[1]. Depending on the location of the blood vessel, this atherothrombotic reaction may lead to stroke or myocardial infarction. These diseases are among the most common causes of death in the Western world.

It is clear that not only platelets are involved in haemostasis and thrombosis. Complementary to platelet activation acts the process of blood coagulation or clotting, resulting in the generation of thrombin and the formation of haemostatic fibrin clots. Since thrombin is also a main platelet activator, there is increasing interest in the interactions between platelet activation and coagulation[2,3].

Thrombosis is normally classified as venous thrombosis (e.g., in deep veins) or arterial thrombosis (as in myocardial infarction and ischemic stroke). It is generally accepted that activated platelets and the coagulation system contribute in different ways to the thrombotic process in veins and arteries. Venous thrombosis is commonly associated with high coagulation activity, responsible for unwanted fibrin clot formation. On the other hand, arterial thrombosis is often linked to high platelet activation, which leads to formation of platelet-rich thrombi[4,5]. Nowadays, there are good examples of favourable combination therapy with anticoagulant and antiplatelet drugs. However, anticoagulants are still preferred in reducing venous thromboembolism, while antiplatelet agents are mostly used in the prevention of arterial thrombosis. This is in apparent paradox with the fact that thrombin is one of the most potent platelet agonists[6]. Furthermore, at least *in vitro*, antiplatelet drugs inhibit coagulation as well[7].Together, this urges for better knowledge of the functions of platelets in relation to coagulation.

To provide a general background for the activation mechanisms of platelets and the coagulation system, in this chapter, an overview will be given of: key signaling processes implicated in platelet activation; the proposed roles of platelets in thrombin and thrombus formation; and finally, of the differences among individual platelets.

Platelet signaling to calcium mobilisation and beyond

Most physiological platelet agonists evoke a sudden increase in the cytosolic free Ca^{2+} concentration. This holds for primary agonists such as collagen (exposed at a damaged vessel wall) and thrombin (the most reactive coagulation product); but also for the secondary agonists ADP and thromboxane A_2 (TxA_2), which are so-called autocoids that are produced by activated platelets themselves and enhance the platelet activation process. Elevated Ca^{2+} is implicated in almost all platelet responses, such as shape change, secretion, aggregation, development of procoagulant activity and clot retraction[8]. As illustrated in Figure 1, the key effector enzymes in the Ca^{2+} rise are members of the family of phosphoinositide-specific phospholipase C (PLC) isozymes, PLC-β and PLC-γ.

Platelets become activated in response to collagen via the immunoreceptor family member, glycoprotein VI (GPVI). Collagen binding results in the phosphorylation of two tyrosines in the the Fc receptor γ-chain of the GPVI co-receptor[9,10]. The Fc receptor γ-chain contains an immunoreceptor tyrosine-based activation motif (ITAM), which includes two conserved tyrosine residues. Initial receptor phosphorylation is accomplished by the Src family kinases, Fyn and Lyn, which associate with proline-rich regions of GPVI[11,12]. Once phosphorylated, the Fc receptor γ-chain binds the tyrosine kinase Syk via its ITAM, which then becomes phosphorylated by Src kinases and also by itself[13]. The activated Syk phosphorylates multiple signaling proteins, including the transmembrane adaptor LAT (linker for activation of T cells). Once phosphorylated, LAT serves as a docking site for proteins such as Gads, Grb2, phosphoinositide 3-kinase (PI3K) and PLC-γ2[14]. Activation of PLCγ2 causes a prolonged increase in cytosolic Ca^{2+} concentration[15].

Human platelets express two receptors for thrombin, PAR1 and PAR4[16,17]. Cleavage of these receptors by the protease thrombin exposes a short auto-activating peptide ligand, known as a tethered ligand[18]. Both activated receptors couple to the G proteins, Gq and G13, of which the former is a strong activator of PLC-β isoforms, mediating increases in cytosolic Ca^{2+} concentration[19]. The ability of thrombin to interact with PAR1 and PAR4 is facilitated by its ability to also bind to platelet GPIb-IX-V[20,21].

Secondary mediators, of which ADP and TxA_2 are the most important, are released by activated platelets, and act as paracrine agents that reinforce the platelet activation process. ADP is released by exocytosis of dense granules stored in the platelets. The key receptors for ADP are $P2Y_1$ and $P2Y_{12}$[22]. The first, $P2Y_1$, also couples to Gq[19], which again stimulates PLC-β and causes Ca^{2+} mobilisation[22,23].

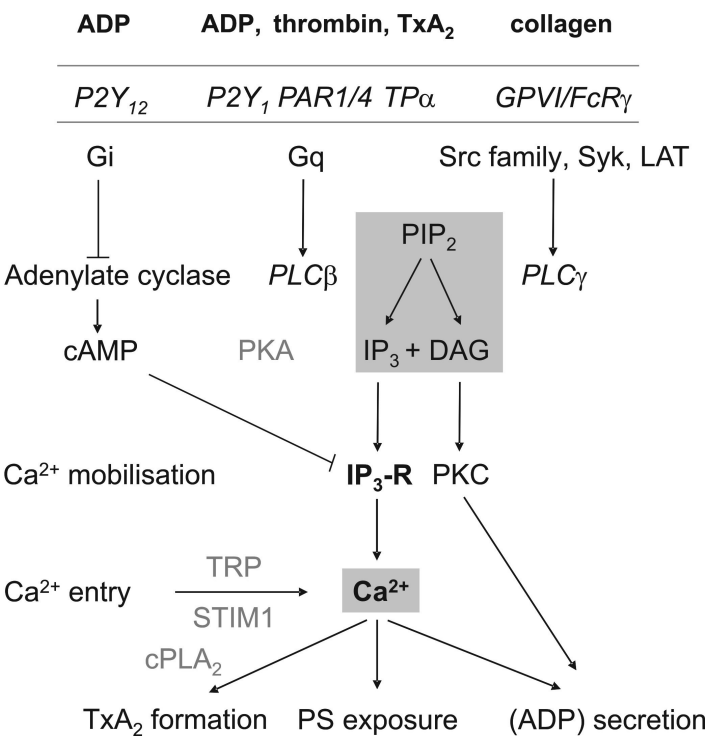

Figure 1 Overview of intracellular platelet signaling via phospholipase C (PLC) isoforms and Ca^{2+} signaling. The ADP receptor $P2Y_{12}$ activates the G-protein Gi, which couples to and inhibits adenylate cyclase. This results in reduced cAMP level and inactivated protein kinase A (PKA), relieving the PKA-mediated suppression of IP_3 receptors (IP_3-R). The ADP receptor $P2Y_1$, the thrombin receptors PAR1 and PAR4, and the TxA_2 receptor TPα all activate Gq, which couples to PLCβ. Collagen activation of GPVI, via tyrosine phosphorylation of the Fc receptor γ-chain (FcRγ) triggers a cascade of phosphorylation reactions via Src and Syk kinases. The phosphorylated adaptor protein LAT mediates activation of PLCγ2. Both PLC isoforms cleave PIP_2 into IP_3 and DAG. The former second messenger opens IP_3 receptor channels which releases Ca^{2+} from intracellular stores, while the latter activates protein kinase C (PKC), involved in secretion. Ca^{2+} release is followed by store-regulated Ca^{2+} influx, likely involving TPRC channels and the adaptor protein STIM1. Elevated Ca^{2+} can lead to phospholipase A_2 ($cPLA_2$) activation, arachidonate release and TxA_2 formation; further to PS exposure at the platelet surface; and to secretion. Mechanisms of Ca^{2+} lowering are not indicated. For further explanations, see text.

On the other hand, $P2Y_{12}$ couples to Gi, which G protein inhibits adenylate cyclase and thus reduces the cyclic AMP level[24].

Calcium-dependent activation of cytosolic phospholipase A_2 cleaves arachidonic acid from membrane phospholipids. The shortly living autocoid TxA_2 is synthesised from arachidonic acid by the enzymes cyclooxygenase and TxA_2 synthase[25]. After diffusion across the plasma membrane, TxA_2 binds to the TPα receptors on the same or adjacent platelets. TPα also couples to Gq and thus activates PLC-β[26].

Both PLC-γ and PLC-β isoforms cleave membrane-bound phosphatidyl inositol-4,5-biphosphate to form the second messengers inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol[27]. Once released from the membrane, the hydrophilic IP_3 binds to IP_3-operated Ca^{2+} channels in the endoplasmic reticulum membrane, surrounding intracellular Ca^{2+} stores. These mediate rapid release of Ca^{2+} into the cytosol, in a typically Ca^{2+}-enhanced way[28,29]. Phosphorylation of the IP_3 receptors by cyclic AMP-dependent protein kinase down-regulates their Ca^{2+} channel activity, and thereby suppresses Ca^{2+} mobilisation[30,31]. The second PLC product, 1,2-diacylglycerol, remains membrane-associated and activates the serine-threonine protein kinase C, which is implicated in secretion.

A direct effect of depletion of the Ca^{2+} stores is triggering of the process of store-regulated Ca^{2+} entry, by which Ca^{2+} from the extracellular medium enters into the cell. In platelets, this leads to substantial amplification of the Ca^{2+} signal[8,32]. The ion channels mediating Ca^{2+} entry and implicated in the linkage mechanism between store

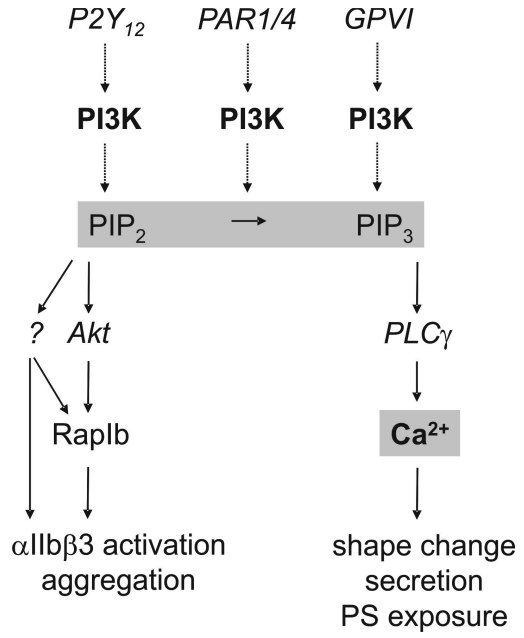

Figure 2 Overview of the roles of phosphoinositide 3-kinase (PI3K) in platelet activation. The ADP receptor $P2Y_{12}$, the thrombin receptors PAR1 and PAR4, and the collagen receptor GPVI activate PI3K, which converts PIP_2 to the second messenger PIP_3. PI3K activated downstream of GPVI stimulation plays a role in recruiting PLCγ to the plasma membrane. As shown in Figure 1, elevated Ca^{2+} following PLCγ2 activation, leads to PS exposure at the platelet surface and to secretion. Platelet secretion products reinforce the activation process through positive feedback mechanisms. Separately, $P2Y_{12}$ and PAR1/4 stimulation activates the PI3K, Akt and RapIb pathway, which leads to integrin αIIbβ3 activation and subsequent platelet aggregation.

depletion and Ca^{2+} entry likely include the TRPC channels, as these can interact with platelet IP_3 receptors[28,33]. Adaptor proteins in the endoplasmic reticulum membrane like STIM 1 are identified as putative reticular Ca^{2+} sensors that regulate the Ca^{2+} entry[34]. In particular, Ca^{2+} entry is required to achieve the prolonged, high cytosolic Ca^{2+} concentration for exposure of procoagulant phosphatidylserine (PS) at the platelet surface[35].

The PI3K enzyme plays a role in signaling from the major classes of surface receptors, including ADP, thrombin and collagen receptors. PI3K converts PIP_2 to the phospholipid derivative phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Figure 2). The Gi and Gq protein-coupled pathways starting from $P2Y_{12}$ and PAR1/4 lead to activation of PI3K, which is involved in the activation of integrin αIIbβ3 via the GTP-binding protein, RapIb[36]. In addition, PI3K is also activated following GPVI stimulation, and plays a role in recruiting PLCγ2 to the plasma membrane and, hence, in mediating Ca^{2+} mobilisation[37].

$P2Y_{12}$ plays a critical role in supporting platelet activation, because it serves as the major receptor for the secondary feedback agonist ADP, and also because it signals in a distinct way to other receptors. It is the ability of the $P2Y_{12}$ receptor to activate Rap1b towards αIIbβ3 activation, that underlies its ability to act in synergy with other receptors. Although there are still missing links in this pathway, it is clear that a reduction in cyclic AMP is not required[38-40]. Important components in the signaling pathway to integrin activation are PI3K isoforms and the downstream mediator, Akt/PKB.

It has been reported that platelets contain various PI3K isoforms, i.e. the class IA isoforms PI3Kα, β and δ, and the class IB isoform PI3Kγ[41]. Both class IA and IB forms consist of a catalytic and regulatory subunit. In platelets, class IA PI3K forms comprise to date three p110 catalytic subunits (α, β and δ) and three regulatory subunits (85α, 85β, and 55γ). In addition, the p85α protein possesses two alternatively spliced variants, p55α and p50α. Of the various regulatory subunits, p85α is the most abundantly expressed, and it interacts with the phosphorylated adaptor protein, LAT. The class IA regulatory subunits are characterized by the presence of SH2 and SH3 domains, by which the protein can interact with growth factor-like receptor complexes, such as the GPVI signaling complex.

The class IB PI3Kγ comprises a complex of a p110γ catalytic subunit and a unique p101 regulatory subunit. Thus, the p101 protein specifically binds to the N-terminal domain of p110γ[42]. The regulatory subunit lacks SH2 and SH3 domains and is, in general, considered to interact with the β/γ subunits of heterotrimeric G proteins[42]. Whether this is also the case for platelets, has not yet been investigated. It is also not

completely sure which regulatory subunit can bind to which catalytic subunit. As far as known, the inter-SH2 domains of p85α, p55α, p50α, p85β and p55γ constitutively interact with the N-terminal domain of p110α, β and δ. Binding of the dual SH2 domains to tyrosine-phosphorylated adaptor proteins activates the kinase function of the p110 subunits. The precise signaling and functions of the various isoforms in platelets are unclear.

Platelets and coagulation

That platelets have an active role in the coagulation process appears from thrombin generation experiments in platelet-rich plasma. Here, thrombin generation strongly relies on the presence of activated platelets, as inhibition with several platelet antagonists has an anticoagulant effect by slowing down the formation of thrombin and fibrin[2,7,43]. Indeed, binding of several coagulation factors to the platelet surface has been reported[38,44]. The recognition that platelet activation and coagulation are mutually interacting processes has stimulated the research to find 'receptors' or binding components for coagulation factors on the platelet surface. Current evidence shows that these binding components comprise both surface phospholipids and glycoproteins (Table 1).

For long, it is known that resting platelets have an outer membrane phospholipid surface that is not active in coagulation[45]. Due to the activity of an aminophospholipid translocase, platelets and other cells sequester the aminophospholipids PS and phosphatidylethanolamine at the inner membrane leaflet[46,47]. In most cell types, ABC transporters or P_4-ATPases are found to be responsible for the translocation reaction[48]. For platelets, Ca^{2+} ionophores and potent Ca^{2+}-mobilizing agonists (collagen plus thrombin) are capable to evoke surface exposure of PS due to the so-called scramblase reaction[35,45]. However, the molecular identity of this scramblase has remained unclear[48,49].

Activated platelets that expose PS bind vitamin K-dependent coagulation factors (prothrombin and factors VII, IX and X) as well as vitamin K-dependent anticoagulation factors (protein C and S) via their Gla residues. Most of the blood coagulation enzymes belong to the serine protease family. They are primarily synthesized in the liver and require post-ribosomal modifications for proper functioning. The γ-carboxylation of N-terminal glutamic acid residues to γ-carboxyglutamic acid (Gla) under the influence of a vitamin K-dependent carboxylase is considered of key importance for adequate function in coagulation.

Table 1 Procoagulant entities at the platelet surface

Surface component	Factor(s) bound	Function	Refs.
Aminophospholipids (PS, PE)	vitamin K-dependent prothrombin, factors VII(a), IX(a), X(a)	assembly of tenase and prothrombinase complex	[45]
Tissue factor or another receptor	factor VII(a)	initiation of coagulation	[58,59]
GPIb-V-IX	thrombin	facilitates thrombin activity	[79]
	factor XI	facilitates factor XI activation	[63]
	factor XII	factor XI activation	[64]
Integrin αIIbβ3	fibrin(ogen)	platelet aggregation	[114,115]
	prothrombin	facilitates prothrombin activation	[65]
Serotonin groups	fibrinogen, factor Va, thrombospondin	formation of coated platelets	[111,116]
Elusive EPR1	factor Xa	prothrombin activation EPR1 is non-existent	[67-70]
Unknown receptor	factor V(a)	facilitates prothrombin activation	[38]
	factor XIIIa	cross-linking of fibrin	[72]

The N-terminal Gla modules of the coagulation factors prothrombin, VII, IX, X, protein C and protein S contain between 9 and 11 Gla residues that are pivotal for Ca^{2+} ion binding. The Ca^{2+}-bound conformation of Gla modules is essential for binding of these coagulation proteins to negatively charged PS-containing (platelet) surfaces, and thus for initiation and propagation of blood coagulation. Accordingly, the PS-exposing membranes, by acting as assembly sites for the tenase and prothrombinase complexes, strongly accelerate the process of thrombin generation and, hence, clotting. In the tenase reaction, the protease factor IXa, assisted by factor VIIIa, together with its cofactor factor Va, cleaves factor X into its active form, factor Xa[50]. In the prothrombinase reaction, factor Xa catalyzes the cleavage of prothrombin into thrombin. Of relevance for this thesis, procoagulant (platelet) phospholipids (PS) stimulate these two reactions by several orders of magnitude[51].

Abnormal regulation of platelet phospholipid asymmetry is incidentally seen in patients. In the rare Scott syndrome, impairment of phospholipid scrambling is accompanied by an increased bleeding tendency. Activated platelets from such patients are strongly impaired in their ability to support prothrombin and factor X activation[52]. In addition, Scott platelets produce less microparticles[53,54], which normally also propagate the coagulation process. Important for this thesis, it has been proposed that the store-regulated Ca^{2+} entry is defective in the Scott syndrome[55]. An alternative view is that

platelets from Scott patients lack a Ca^{2+}-dependent phospholipid scramblase activity, but no missing proteins have been found in these patients[56].

It has been recognized that PS exposure is required, but not sufficient to explain the stimulation of coagulation by platelets[35,57]. In recent years, evidence has been presented that (activated) platelets can also bind several coagulation factors via protein interactions (Table 1). Platelets interact with factor VIIa, which is a serine protease initiating the coagulation process[58]. Some authors have reported that (activated) platelets contain tissue factor, which is the common cellular receptor for factor VIIa[59]. However, others have challenged the significance of this finding, because platelets store large amounts of tissue factor pathway inhibitor[60,61].

The GPIb-V-IX complex not only acts as a shear-dependent receptor for von Willebrand factor (vWF), but also provides binding sites for coagulation factors. For instance, GPIb-V-IX binds two thrombin molecules. Furthermore, this complex immobilises factor XI which, once activated by thrombin, converts factor IX to factor IXa which subsequently cleaves factor X to factor Xa, thus providing a pathway of thrombin generation independently of tissue factor[62,63]. Others have demonstrated that biotinylated factor XII binds to GPIbα in a Zn^{2+}-dependent fashion[64].

Clearly, integrin αIIbβ3 is the major fibrin(ogen)-binding protein on activated platelets. However, this integrin also binds prothrombin even on non-stimulated platelets, thus providing a local pool for thrombin generation under coagulant conditions[65]. Coagulation factor binding can also be achieved by serotonin residues on the platelet surface. Dale and co-workers show that stimulation of platelets with collagen and thrombin, but also with Ca^{2+} ionophores, results in so-called COAT (collagen- and thrombin) stimulated platelets, currently also indicated as coated platelets[66]. These platelets can expose serotonin-conjugated proteins that interact with serotonin binding sites on fibrinogen and thrombospondin. The same platelets may also bind fibronectin and vWF, as well as granule-derived factor Va, which is involved in coagulation. It is suggested that the coated platelets also express surface PS and support prothrombinase activity[66].

Some authors have suggested that platelets bind factor Xa via a receptor consisting of anionic phospholipids, factor Va and effector cell protease receptor-1 (EPR1)[67]. Preliminary support for involvement of EPR1 came from studies that compared the binding characteristics of factor Xa onto phospholipid vesicles and thrombin-activated platelets[68,69]. Others, however, were unable to demonstrate involvement of an EPR1-like protein in the assembly of the prothrombinase complex at the surface of activated platelets[70]. The EPR1 work came into a new perspective with the finding that the so-

called 'EPR1' gene actually encodes for a protein regulating apoptosis, and that the elusive factor Xa receptor protein is not transcribed in cells[71]. The transglutaminase factor XIIIa may also bind to platelets, and functions to mediate the cross-linking of coagulation factors with αIIbβ3 and associated proteins[72].

Of all platelet receptors, especially GPVI has been linked to PS exposure and procoagulant activity[73,74]. However, at the start of the investigations of this thesis, only very little was known of the signaling pathways used by GPVI to evoke this response, nor of the consequences of platelet GPVI stimulation for coagulation factor binding. Thrombin also stimulates procoagulant activity, although its effect is less profound than that of GPVI, but synergizes with the collagen-induced procoagulant response[75]. In the literature it has extensively been discussed which thrombin receptor is responsible for the procoagulant activity. The receptors, PAR1[76,77], PAR4[76], and GPIb-IX-V[78-80], have all been proposed as potential mediators of this thrombin response. Recent results, using selective inhibitors of the various receptors, show that the synergistic effect of thrombin in collagen-induced platelet procoagulant activity is mediated not through GPIb-IX-V or PAR4, but through PAR1[20].

Platelets and coagulation in thrombus formation

So far, the initial events leading to collagen-induced platelet aggregation and thrombus formation under shear have mainly been studied by *in vitro* flow studies using anticoagulated human and murine blood (Figure 3). Under conditions of high, arterial shear rates, platelets initially adhere to immobilized vWF via GPIb-V-IX, and subsequently via integrin αIIbβ3[81,82]. These interactions cause limited platelet activation[83]. Since vWF binds with a high affinity to collagen, the vWF interaction predisposes platelets to collagen-induced activation and facilitates thrombus formation[84,85]. The subsequent order of events differs from platelet to platelet both in man and mouse[86]. Some platelets on collagen become rapidly activated by the collagen receptor, GPVI, which signals to PLC-γ2 and Ca^{2+} mobilization, as described above. Signaling via GPVI leads to activation of a second collagen receptor, integrin α2β1. This event is required for stable adhesion and full activation[87]. However, other platelets, perhaps interacting with different motifs in collagen, follow a different order of events by first stably adhering to the collagen via integrin α2β1 before showing GPVI-induced activation responses. Collagen-adhered platelets release autocoids like ADP and TxA_2, which stimulate passing platelets to activate their αIIbβ3 integrins[88-90]. Instantaneously, these platelets then aggregate via multiple αIIbβ3-fibrinogen interactions, which are

enforced by GPIb-V-IX binding to plasma-derived vWF[81]. Such thrombi are intrinsically unstable structures that require strong adhesion to collagen and continuous platelet activation. This appears from the findings that they disintegrate in the absence of $\alpha 2\beta 1$[91] or when $P2Y_{12}$ signaling is blocked[92,93].

Platelet adhesion to collagen also triggers the so-called procoagulant response[73,94]. In response to prolonged elevation in cytosolic Ca^{2+}, platelets can start to expose PS and form blebs, which later shed as microparticles (Figure 4). Both the blebbing platelets and the shedding of microparticles contribute to the stimulation of coagulation. Thus, in the presence of plasma, this response greatly stimulates thrombin generation[95]. Although the importance of this platelet response is known upon flow over fibrinogen[96], still pertinent questions are how platelet interaction with collagen and vWF can trigger procoagulant activity under venous and arterial flow conditions, and how the various receptors communicate in establishing this platelet response.

In vitro experiments have significantly contributed to our understanding of the mechanisms of platelet activation and thrombus formation. However, *in vivo* thrombosis

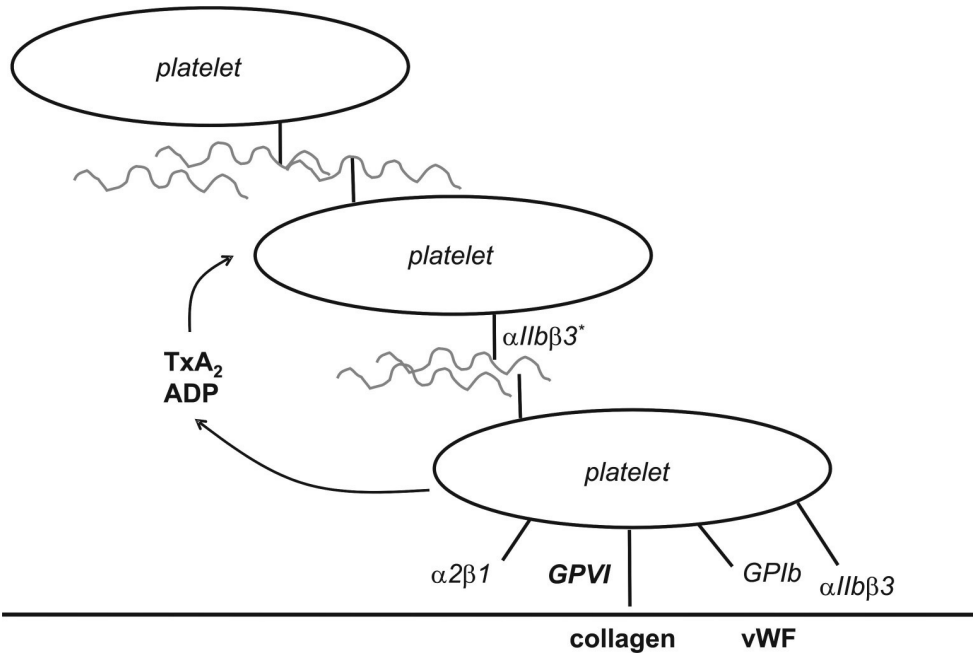

Figure 3 Platelet adhesion and aggregation on collagen under high shear. Platelets bind to collagen fibres containing vWF via multiple receptors and become activated via GPVI. Various signaling pathways mediate activation of integrins and release of paracrine agonists, such as TxA_2 and ADP. Platelet aggregates are formed under shear via interactions of activated αIIbβ3 and GPIb-V-IX with fibrinogen and vWF, respectively.

studies are needed for ultimate verification of the (patho)logical importance of these mechanisms[97]. In the last few years, several animal models have been developed to investigate the role of platelets in the thrombotic process *in vivo*. Here, only murine models that are relevant for the present thesis are introduced. The possibilities and limitations of these models have been discussed more extensively elsewhere[98].

In a well established microvascular model, thrombus formation is induced by topical application of the free radical generating agent, ferric chloride, on mouse mesenteric vessels[99]. Ferric chloride treatment leads to substantial damage of the vessel wall, i.e. denudation of the endothelial cells and exposure of the collagen-containing extracellular matrix to the blood stream. The ensuing thrombotic process is usually followed by intravital microscopic imaging techniques[100]. An other way of injury of these microvessels is by application of high-energy laser light that is focused through microscope optics, which are also used to detect thrombus formation[101]. An advantage of the latter technique is that more precise, local damage can be applied, but a disadvantage is the likelihood of damaging vascular structures by the high energy power of the laser beam. In addition, several macrovascular arterial thrombosis models have been developed. In one of these, vascular injury is induced by ligation of the mouse carotid artery, which also causes loss of the endothelial cell layer[102].

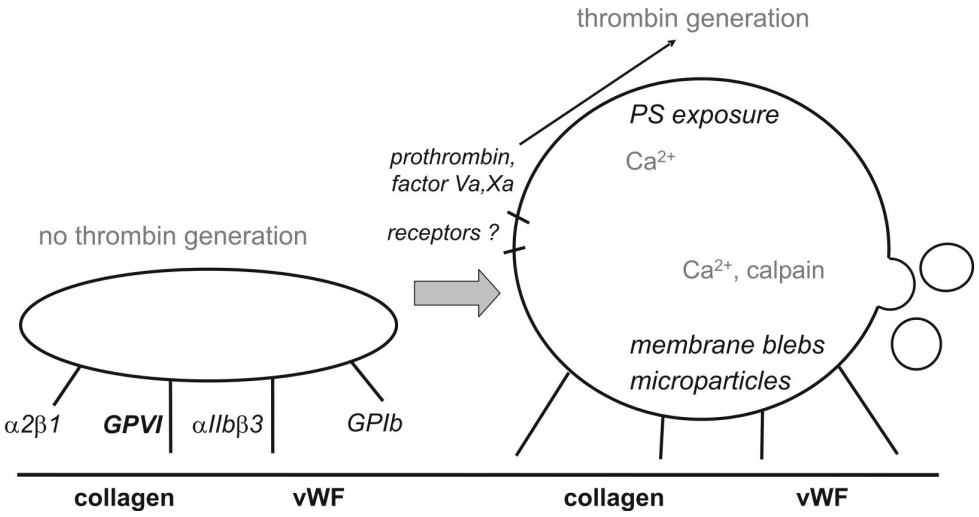

Figure 4 Function of collagen receptors in platelet procoagulant activity. Platelets binding to collagen and activated by GPVI can, depending on their activation state, transform into round structures with membrane blebs and shedding microparticles. These platelets expose PS and are active in thrombin generation and coagulation. Modified after Heemskerk *et al.*[94].

In both these micro- and macrovascular models, collagen-induced platelet activation has been proposed to play a key role in triggering the thrombotic process[102-105]. Alternatively, in both ferric chloride and laser-induced thrombus formation, an important role for tissue factor and thrombin and, hence, for coagulation has been identified[99,105]. In this respect, some authors have even questioned the importance of platelet-collagen interaction[106]. These apparent discrepancies in the literature may be resolved by the possibility that collagen (platelet activation) and thrombin (coagulation) have additive or synergistic roles in arterial thrombosis, and that the relative importance of either of them can vary depending on the local conditions, e.g. the precise way of vascular damage, the local flow and the responsiveness of platelets. Accordingly, in spite of all the *in vitro* and *in vivo* work, it is still unclear whether and how the processes of platelet activation and coagulation integrate in thrombus formation in arteries and veins.

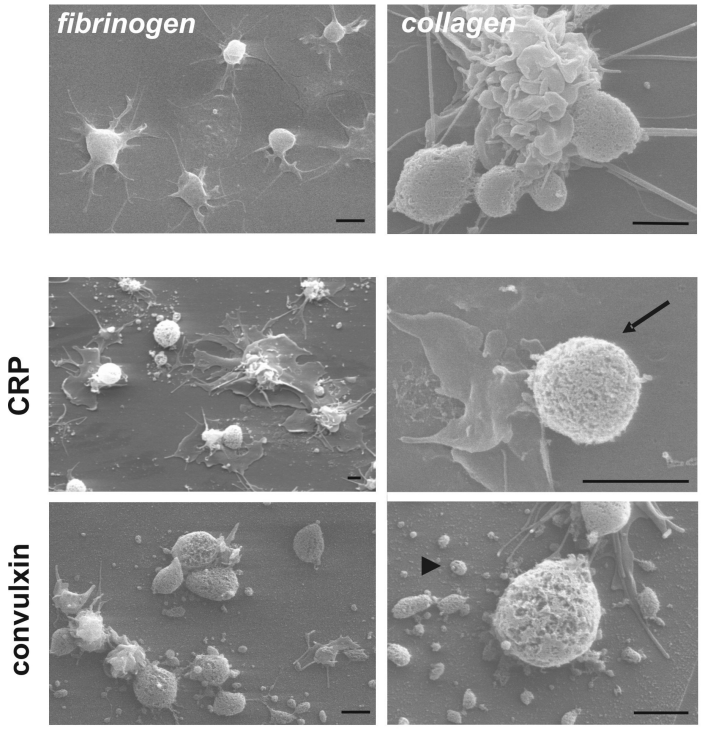

Figure 5 Morphology of procoagulant platelets adhering to collagen under static conditions. Scanning electron microscopic images of human platelets adhered to fibrinogen, collagen, or specific GPVI-binding surfaces. Note sponge-like structures (arrow) and microparticles (arrow head) representing procoagulant structures. Bars represent 2 μm. Modified after Siljander *et al.*[74].

Heterogeneity in platelet responses

Platelets that come into contact with a collagen surface or that are stimulated with GPVI-activating agents can show three different morphologic responses: pseudopod formation followed by spreading, clustering into aggregates, and formation of blebs which shed as microparticles[74]. Some spread platelets may also show membrane contraction and subsequent fragmentation[72]. This heterogeneity in responses even occurs when platelets are adjacent on a collagen surface (Figure 5). A similar heterogeneity is found under flow conditions, when whole blood is perfused over collagen, in which case some platelets integrate into multi-cellular aggregates while other platelets develop into blebbing cells[85,90]. There are some, but incomplete, indications in the literature that may explain these typical differences between platelet responses.

Explanations can be found in inherent, biochemical differences between platelets from an individual as well as in differences in environmental factors. Evidence that not all platelets are structurally similar comes from many flow cytometric studies, where the staining of platelets with antibodies or probes against surface glycoproteins or receptors in many instances shows considerable differences in binding extent among platelets of one subject, regardless of the antigen or probe used. Thus, even independent of their size, some platelets will express more surface receptors than others[107]. Such structural heterogeneity has already been seen at the level of differentiating megakaryocytes. For instance, individual human megakaryocytes express different levels of αIIbβ3 and GPIb-V-IX, while they simultaneously differ in Ca^{2+} signaling properties[108]. Furthermore, some differentiating megakaryocytes appear to keep their immature signaling properties (e.g. prostacyclin-induced Ca^{2+} mobilisation), and can transmit these to a subpopulation of circulating platelets[109]. Thus, platelets in the blood are derived from megakaryocytes with a markedly different expression of adhesive receptor and signaling molecules. It is therefore not a surprise that single platelets from one subject, when placed on a coverslip, also show large differences in Ca^{2+} signal patterns [29]. Such differences in responsiveness may explain why, upon stimulation with collagen and thrombin, only a subpopulation of platelets expresses PS[110], sheds microparticles[72], transforms into coated platelets with serotonin binding sites[111], or preferentially binds coagulation factors[112].

Especially under shear conditions, individual platelets are likely to encounter different activating environments. For instance, during flow over collagen, the first platelets that bind to collagen (designated as vanguard cells) were found to communicate their activation status to subsequent follower platelets, and thus recruiting

the latter into developing aggregates[113]. Vanguard platelets may differ from other platelets by releasing ADP, which implicates that some collagen-bound platelets are more capable in attracting other (follower) platelets than others.

Taken together, it is well conceivable that, during the thrombotic process, biochemical and structural differences between platelets as well as micro-environmental diversity sensed by these platelets determines the different types of platelet responses. However, the present literature has only begun to determine the extent and relevance of this heterogeneity for the thrombus-forming process.

Research outline of this thesis

In this thesis the interaction mechanisms between platelet activation and coagulation are studied on different levels of complexity: using isolated platelets; by way of an *in vitro* model of thrombus formation under flow in the presence and absence of coagulation; and by *in vivo* models of thrombus formation in small and large vessels. Since store-mediated Ca^{2+} entry is essential for the procoagulant response of platelets, we have investigated in **chapter 2** whether the Ca^{2+} entry process is impaired in the Scott syndrome. Measurements are performed of Ca^{2+} fluxes and PS exposure in isolated platelets and in B-cell lymphoblasts from two Scott patients. In **chapter 3**, we describe a study on the role of various PI3K isoforms in the regulation of Ca^{2+} signaling and procoagulant activity using platelets that are stimulated via their collagen or thrombin receptors. In the *in vitro* flow studies reported in **chapter 4**, the interacting roles of the adhesive receptors for vWF (GPIb-V-IX and αIIbβ3) and collagen (GPVI and α2β1) are examined upon thrombin formation and procoagulant activity during high-shear perfusion of whole blood. Novel antibodies and peptides directed against the two collagen receptors are used to specifically unravel the function of each receptor molecule. **Chapter 5** introduces the processes of thrombin generation and coagulation in flow-adhesion experiments. The investigations aim to determine how signaling modules of the GPVI pathway of platelet activation control thrombus formation under coagulant conditions both *in vitro* and *in vivo*. Particular attention is paid to the generation and importance of PS-exposing platelets. To study the interaction mechanisms between platelet activation and thrombin generation *in vivo*, **chapter 6** describes the development and use of a mouse model of arterial and venous thrombosis. Intervention experiments have allowed to study the synergy and interplay of these two processes. In **chapter 7**, this work is extended by demonstrating that temporal changes in intracellular signaling properties determine the 'fate' of a platelet to become either procoagulant or

proaggregatory *in vitro* and *in vivo*. Finally, in **chapter 8** I have discussed the novel findings of this thesis in relation to the current literature.

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Chapter 2

Store-mediated calcium entry in the regulation of phosphatidylserine exposure in blood cells from Scott patients

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Abstract

The Scott syndrome is a bleeding disorder, characterized by impaired surface exposure of procoagulant phosphatidylserine (PS) on platelets and other blood cells, following activation with Ca^{2+}-elevating agents. Since store-mediated Ca^{2+} entry (SMCE) forms an important part of the Ca^{2+} response in various blood cells, it has been proposed that deficiencies in Ca^{2+} entry may relate to the impaired PS exposure in the Scott syndrome. Here, we have tested this hypothesis by investigating the relationship between Ca^{2+} fluxes and PS exposure in platelets as well as B-lymphoblasts derived from the original Scott patient (M.S.), a newly identified Welsh patient (V.W.) with similar bleeding symptoms, and two control subjects. Procoagulant activity of V.W. platelets in suspension, measured after stimulation with collagen/thrombin or Ca-ionophore, ionomycin, resulted in 52% or 17%, respectively, compared to that of correspondingly activated control platelets. Procoagulant activity of V.W. erythrocytes treated with ionomycin resulted in less than 6% of the activity of control erythrocytes. Single-cell Ca^{2+} responses of M.S. and V.W. platelets, adhering to collagen, were similar to those of platelets from control subjects, while PS exposure was reduced to 7% and 15%, respectively, compared to controls. Stimulation of non-apoptotic B-lymphoblasts derived from both patients and controls with ionomycin or agents causing Ca^{2+} mobilization and SMCE, resulted in similar Ca^{2+} responses. However, in lymphoblasts from M.S. and V.W. Ca^{2+}-induced PS exposure was reduced to 7% and 13% of the control lymphoblasts, respectively. We conclude that *i.* patient V.W. is a new case of Scott syndrome, *ii.* Ca^{2+} entry in the platelets and lymphoblasts from both Scott patients is normal, and *iii*. elevated $[Ca^{2+}]_i$ as caused by SMCE is not sufficient to trigger PS exposure.

Introduction

Cell surfaces containing anionic phospholipids, in particular phosphatidylserine (PS), play a pivotal role in blood coagulation. These procoagulant lipid membranes form a catalytic surface for the assembly and proper conformation and juxtaposition of the factor X and prothrombin activating enzyme complexes. Formation of these complexes strongly accelerates the rate of thrombin formation, which is responsible for the production of a stabile fibrin clot. Cellular procoagulant lipid surfaces are mainly provided by platelets. Upon appropriate stimulation, platelets lose their asymmetric transbilayer phospholipid distribution, resulting in exposure of PS at the exterior cell surface.

The Scott syndrome is a rare, hereditary bleeding disorder characterized by impaired surface exposure of PS at the plasma membrane of activated platelets and decreased shedding of procoagulant platelet-derived microvesicles[1]. The Scott phenotype, as apparent in platelets, has also been observed in erythrocytes and Epstein-Bar virus (EBV)-transformed B-lymphocytes[2-4], indicating a cell type-independent trait. Weiss *et al.*, in 1979, were the first to identify a patient (M.S.) with this disorder[1]. One year later, a report was published on ten individuals from three unrelated Welsh families, who also suffered from an unusual bleeding disorder[5]. The latter patients have not been studied extensively since then, but the original data suggest that their bleeding abnormality might be similar or possibly identical to the Scott syndrome. Later, also a French family has been found of which the propositus had platelets deficient in PS exposure and microvesiculation, but not in secretion and aggregation[6-8]. Recently, a colony of German shepherd dogs was discovered with a hereditary bleeding disorder and deficient PS exposure in platelets, characteristic of Scott syndrome[9]. The inability of platelets and other blood cells to scramble phospholipids, and thus expose PS, has been assigned to abnormal activity of a Ca^{2+}-dependent phospholipid scramblase activity in the plasma membrane of these cells.

Cytosolic $[Ca^{2+}]_i$ serves as an intracellular mediator for many extracellular signals in platelets and other cells, regulating processes such as cellular shape, secretion, growth and differentiation[10-12]. In earlier work with platelets, it appeared that a prolonged, high increase in $[Ca^{2+}]_i$ is required for PS exposure, e.g. as achieved by Ca^{2+} ionophore, collagen receptor (glycoprotein VI) stimulation[13-15] or the combination of thrombin and thapsigargin[16]. Release of Ca^{2+} from intracellular stores is insufficient for full platelet activation. However, following depletion of the intracellular Ca^{2+} stores, a mechanism is activated which allows Ca^{2+} entry across the plasma membrane. This store-mediated Ca^{2+} entry (SMCE) from the extracellular medium is a prerequisite to reach the high Ca^{2+} signals required for full platelet activation[17]. In platelets and related cells at least a substantial part of the SMCE is mediated by plasma membrane ion channels of the Trp family[18]. Blocking of this Ca^{2+} entry process abolishes agonist-induced PS exposure[15,19] and, conversely, increased Ca^{2+} influx in platelets is correlated with higher PS exposure[20,21]. Recently, Martinez *et al.* reported of a reduced SMCE in B-lymphoblasts derived from the propositus of the French Scott family, whereas the release of Ca^{2+} from intracellular stores in these cells was normal[4,22]. Together, these observations suggest that defective store-mediated (capacitative) Ca^{2+} entry may be part of the Scott phenotype. However, studies on SMCE have thus far only been performed with the French Scott patient.

In this paper, we examine the relationship between Ca^{2+} fluxes and exposure of procoagulant PS in the plasma membrane of both platelets and immortalized, EBV-transfected B-cells from two control subjects and two different Scott patients. We simultaneously measured Ca^{2+} fluxes and PS exposure in individual platelets from these patients using fluorescence imaging microscopy. In the viable, non-apoptotic population of the B-cell lines, we determined the Ca^{2+} responses evoked by store-depleting agonists, as well as the PS exposure by dual-label flow cytometry.

Materials and methods

Materials

Fibrillar type-1 collagen from equine tendon (Collagen reagent Horm®) was purchased from Nycomed (Munich, Germany). Fluorescein isothiocyanate (FITC)-labeled annexin V (FI-annexin V) was a product from Nexins Research (Hoeven, The Netherlands). Fura-2 and Fluo-3 acetoxymethyl esters, Pluronic F-127 and Alexa Fluor 647-labeled annexin V (AF-annexin V) were purchased from Molecular Probes (Leiden, The Netherlands). Bovine serum albumin (BSA), apyrase (grade V), ionomycin, caffeine and thapsigargin were from Sigma (St. Louis, MO, USA). RPMI-glutamax I was obtained from Bio-Whittaker (Walkersville, MD, USA). Fetal calf serum and antibiotic antimycotic solution were obtained from Life Technologies (Paisly, UK), and Lymphoprep came from Nycomed Pharma AS (Oslo, Norway). Coagulation factors thrombin, prothrombin, factor Xa and factor Va were purified as described before[23]. Thrombin-specific chromogenic substrate, H-D-Phe-Pip-Arg-para-nitroaniline (S2238) was from Chromogenix (Mölndal, Sweden).

Patients

Clinical features of patient M.S. have been described extensively before[24]. Patient V.W. had presented initially at the age of 14 years with a history of excessive bleeding after dental extractions on five occasions. Dental surgery at age 22 again resulted in excessive blood loss, with a fall in haemoglobin concentration from 14.6 to 9.3 g/dl. At 34 years of age she suffered life-threatening post partum haemorrhage after an uncomplicated forceps delivery. Studies at that time revealed an isolated severe abnormality of prothrombin conversion consistent with a defective interaction between plasma and platelets or phospholipid. All other tests of haemostasis were normal[5].

Platelet preparation and culturing of B-lymphoblasts

Freshly obtained citrated blood from patients M.S., V.W. and two control subjects was shipped at 4°C by airmail to Maastricht (The Netherlands). Immediately upon arrival (24-48 hours later), platelets were washed and used for the experiments.

Loading of platelets with Fura-2 and washing procedures have been described elsewhere[25,26]. Washed platelets were resuspeded in Hepes buffer (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% BSA and 0.1% glucose, adjusted at pH 7.45) at a final concentration of 1.0 x 10^8 platelets/ml. Erythrocytes were washed 3 times in the same Hepes buffer (pH 7.45) and resuspended at 1 x 10^8 cells/ml.

Data of platelets from M.S. presented in this study were obtained in 1996 on the last occasion that we received blood from this patient. Since M.S. deceased that same year, there was no opportunity to study the platelets from this patient with those from patient V.W. simultaneously. Experiments with blood cells from patient V.W. were performed in 2002.

EBV-transformed B-cells from M.S. and a control subject were available from previous studies[27]. EBV-transformed B-cells from patient V.W. were prepared by Professor E.G.D. Tuddenham (Imperial College School of Medicine, London). Before use, the EBV-infected cells were expanded in RPMI-glutamax I culture medium containing 10% (v/v) fetal calf serum and antibiotic antimycotic solution. Cells were kept at 37°C in a humidified atmosphere with 5% CO_2, and were sub-cultured in 3 volumes fresh medium every 2 or 3 days. Data of EBV-transformed B-lymphocytes from both patients and two corresponding controls were collected from experiments performed in 2002.

Procoagulant activity of platelets and erythrocytes in suspension

Prothrombinase activity of washed platelets in suspension, either activated for 15 min with collagen (10 μg/ml) plus thrombin (4 nM) or for 5 min with ionomycin (1 μM), was determined by measuring the rate of conversion of prothrombin to thrombin by the enzyme complex factor Xa-factor Va, as described before[26]. The assay conditions were: 3 x 10^6 platelets/ml, 3 mM $CaCl_2$, 3 nM factor Xa, 6 nM factor Va and 4 μM prothrombin (all final concentrations). Chromogenic thrombin substrate S2238 (250 μM) was used to determine the amount of thrombin formed. To measure procoagulant activity of erythrocytes, washed cells at a concentration of 2 x 10^8/ml were incubated with 5 μM ionomycin in the presence of 1 mM $CaCl_2$ for 60 min. Samples from this incubation are

diluted to a cell count of 10^7/ml, and prothrombinase activity was measured using the conditions described above.

Dual measurement of $[Ca^{2+}]_i$ and phosphatidylserine exposure by fluorescence imaging microscopy

Intracellular Ca^{2+} and PS exposure in was determined by a quasi-simultaneous measurement of fluorescence from Fura-2 and FI-annexin V using a combined fluorescence imaging and microphotometric system as described previously[15]. Changes in fluorescence were measured in single platelets after 25 minutes adhering to a collagen-coated coverslip. The incubation chamber contained 5 x 10^7 Fura-2-loaded platelets in 300 µl Hepes buffer with 2 mM $CaCl_2$ and 0.5 µg/ml FI-annexin V. Fluorescence of about 100 cells in three or more microscopic fields was measured. Conversion of Fura-2 fluorescence ratio to levels of $[Ca^{2+}]_i$ was made as described before[17]. During the measurements in 1996 (patient M.S. and control 1) the optical pathway was slightly different from that used in 2002 (patient V.W. and control 2), resulting in slightly different calibration values of $[Ca^{2+}]_i$. Platelets were considered to be elevated in $[Ca^{2+}]_i$ when their Fura-2 ratio was increased with 0.2 compared to basal, corresponding to a rise of about 85 nM; annexin V positivity was defined as all localized fluorescence in the fluorescein channel of >30 pixels.

Dual measurement of $[Ca^{2+}]_i$ and phosphatidylserine exposure by flow cytometry

Cultured EBV-transformed B-lymphocytes derived from patients and control subjects were centrifuged at 700 g for 10 min, and resuspended in Hepes buffer pH 7.45 and the cell count was adjusted to 10^6 cells/ml. Washed B-lymphoblasts were loaded with 3 µM Fluo-3 acetoxymethyl ester in the presence of 1 mg/ml Pluronic F-127 at room temperature for 30 min. Loaded cells were activated with 10 µM ionomycin (5 min), 1 µM thapsigargin (5 min), or 10 mM caffeine (10 min). Activations were carried out in the presence of either $CaCl_2$ (2 mM) or EGTA (2 mM) to study total Ca^{2+} responses and Ca^{2+} mobilization separately. Where indicated, the loaded cells were pre-incubated with AF-annexin V, as described by the manufacturer, to monitor PS exposure. After activation, Fluo-3 and Alexa Fluor 647 fluorescence were simultaneously measured using a FACScan flow cytometer, and Cell-Quest software (Becton-Dickinson, CA, USA). For analysis, viable B-cells were gated based on their forward and side scatter, to exclude apoptotic cells and cellular fragments. Per assay, a minimum of 5000 events was counted. Control measurements were always run with unlabeled/stimulated as well as

labeled/unstimulated cells. Green (Fluo-3) and red (Alexa Fluor 647) fluorescence intensities were expressed as arbitrary units. Dot-plots of fluorescence versus forward scatter of control cells in the absence of fluorescent annexin V were used to define the region containing 99% of the annexin V-negative cells. Events outside this region were considered to be positive. List-mode data were analyzed using WinMDI 2.8 software (http://facs.scripps.edu).

Results

Procoagulant activity of platelets and erythrocytes in suspension

First we established the Scott syndrome phenotype of the Welsh patient, V.W. As shown in Table 1, washed platelets of this patient in suspension activated by collagen plus thrombin resulted in a prothrombinase activity of 52% of that achieved with similarly activated platelets from the control subject, whereas stimulation with ionomycin induced a procoagulant response of only 17% of that of ionomycin-treated control platelets. Considering the normal levels of coagulation factors and normal platelet aggregation[5], these findings confirm the phenotype of a Scott syndrome of patient V.W. This was further substantiated by the observation that washed erythrocytes of V.W., treated with ionomycin and Ca^{2+}, were deficient in forming a procoagulant surface: prothrombinase activity was 5.6 ± 1% (mean ± SD, n=5) of that of control erythrocytes. When the prothrombinase activity of completely lysed cell preparations (obtained by sonication) of either platelets or erythrocytes was measured, there was no appreciable difference between patients and controls. This excludes that the decreased procoagulant activity in the patient's platelets and erythrocytes is due to alterations in the overall phospholipid

	Prothrombinase (nM thrombin/min)			
Activation	**Control 1**	**Patient M.S.**	**Control 2**	**Patient V.W.**
None	136 ± 11	54 ± 3 (40%)	130 ± 22	84 ± 16 (65%)
Collagen + thrombin	358 ± 37	119 ± 44 (33%)	336 ± 10	176 ± 30 (52%)
Ionomycin	1159 ±247	247 ± 71 (18%)	1172 ± 6	200 ± 6 (17%)

Table 1 Prothrombinase activity of suspensions of platelets from control subjects and Scott patients. Values presented are means ± SD of three independent measurements performed in 1996 for patient M.S and in 2002 for patient V.W., each with their corresponding control. In brackets is given the activity expressed as a percentage of the correspondingly treated control platelets. It should be emphasized that due to the time between collection of the blood and the prothrombinase measurement (24-48 hrs), the prothrombinase activity of the non-stimulated platelets is slightly increased compared to that of freshly prepared platelet suspensions.

composition, i.e. a reduced content of PS.

Finally, similar to previous findings on the morphology of the erythrocytes of propositus M.S.[2], the red cells of V.W. did not change into echinocytes after treatment with ionomycin and were unable to produce microvesicles.

Relation between calcium responses and phosphatidylserine exposure of single platelets

Fluorescence video microscopic imaging was used to examine whether the Ca^{2+} fluxes and PS exposure of the platelets from the patients differ from those of control subjects. In one set of experiments, Fura-2-loaded platelets from M.S. were compared with the simultaneously collected platelets from control 1. In a later set of experiments,

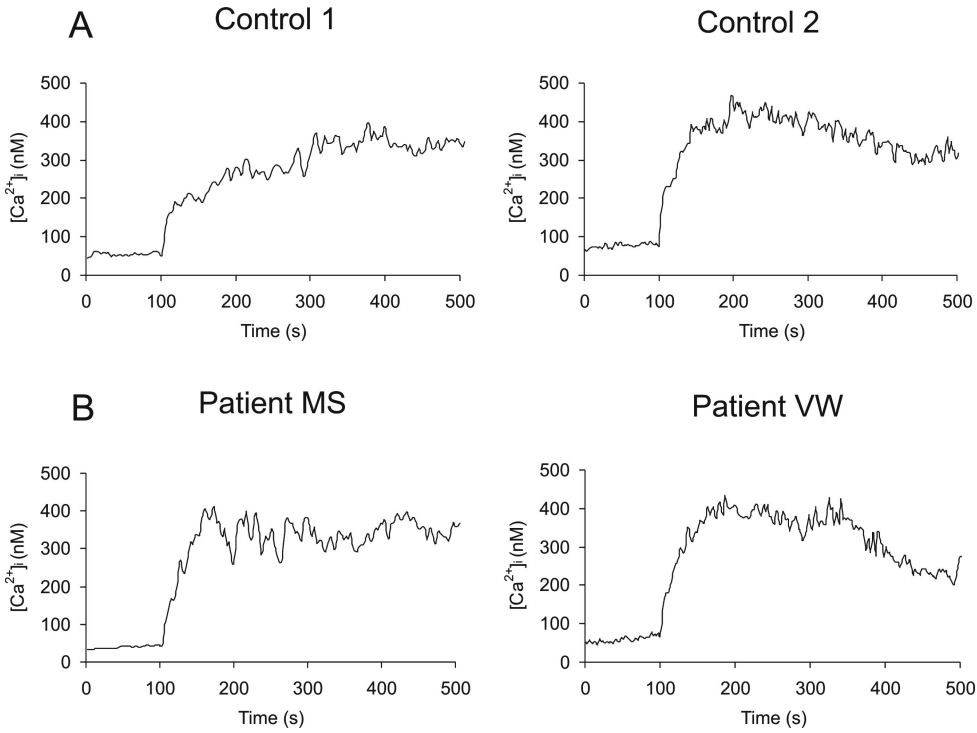

Figure 1 Collagen-induced Ca^{2+} responses of single platelets from control subjects and Scott patients. Fura-2-loaded platelets were allowed to adhere to collagen fibers in the presence of $CaCl_2$ (2 mM). Changes in Fura-2 fluorescence ratio in single platelets were measured by fluorescence video imaging microscopy. (A) Experiments were performed in 1996 with platelets from control 1 and patient M.S. (B) Experiments with platelets from control 2 and patient V.W. were carried out in 2002. Changes in Fura-2 fluorescence ratio (340/380 nm excitation) were recorded during adhesion and activation, as described in Materials and Methods. Traces are averaged overlays of single traces obtained from at least twenty platelets per subject (n≥3 incubations/subject).

platelets from V.W. were compared with those of control 2. The platelets were allowed to interact with collagen fibers, during which glycoprotein VI-induced changes in $[Ca^{2+}]_i$ were measured at a single-cell level by capturing fluorescence ratio images. Patient and control platelets showed prominent increases in $[Ca^{2+}]_i$ shortly after adhesion to collagen (Figure 1). It appeared that the collagen-induced rises in $[Ca^{2+}]_i$ in the platelets from either patient were similar in magnitude to those of the platelets from the two controls.

As the glycoprotein VI-induced increase in $[Ca^{2+}]_i$ is a major signal in the procoagulant response, i.e. PS exposure[19], we determined in the same experiment also the binding to platelets of FI-labeled annexin V, which binds to surface-exposed PS[28,29]. After 25 min adhesion to collagen, 40% of the activated platelets of control 1 were annexin V positive, in comparison to less than 3% of the platelets of Scott patient M.S. (Figure 2A). Similarly, the collagen-activated platelets from patient V.W. showed a strongly reduced PS exposure in comparison to those of control 2, although still 8% of the patient platelets did bind FI-annexin V, i.e. slightly more than observed for patient M.S. (Figure 2B). These observations confirm the Scott syndrome phenotype in patient V.W.

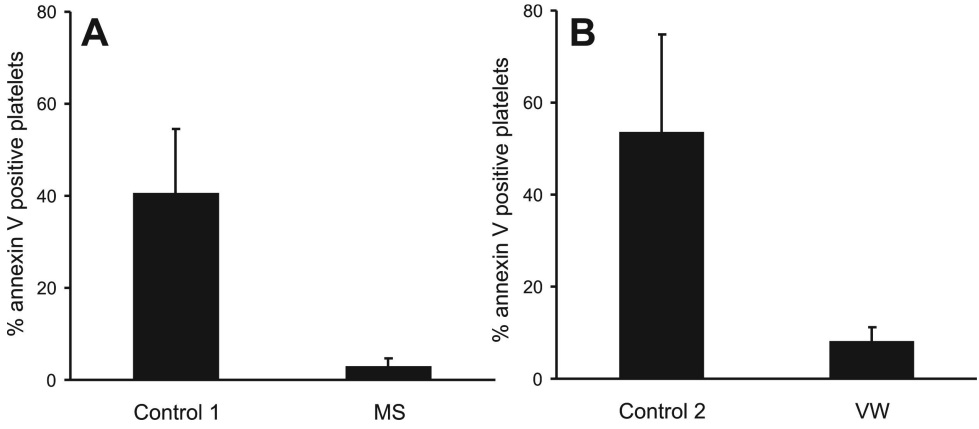

Figure 2 Phosphatidylserine exposure of activated, collagen-adherent single platelets from control subjects and Scott patients. Fura-2-loaded platelets were allowed to adhere to collagen fibers in the presence of FI-annexin V and $CaCl_2$, as described for Figure 1. Results given are percentages of annexin V-positive platelets which exhibit an increased Fura-2 fluorescence ratio, i.e. elevated $[Ca^{2+}]_i$. Values are means ± SD from at least three independent incubations. (A) Experiments were performed in 1996 with platelets from control 1 and patient M.S. (B) Experiments with platelets from control 2 and patient V.W. were carried out in 2002.

Calcium responses of activated B-lymphoblasts

Immortalized B-cells from patient M.S. and control subject 1 and from patient V.W. and control subject 2 were compared simultaneously. To analyze the Ca^{2+} responses of these four lymphoblast cell lines, the cells were loaded with the fluorescent probe Fluo-3 and then stimulated with agonists, reported to cause reduced SMCE in B-lymphoblasts derived from the French Scott patient[22]. Activation was carried out in the presence of EGTA to measure Ca^{2+} mobilization from intracellular stores only, and in the presence of $CaCl_2$ to allow complementary SMCE. Agonist-evoked changes in Fluo-3 fluorescence were measured in single cells by flow cytometry, using a gating profile so that only fluorescence from non-apoptotic, viable cells was analyzed. Treatment of lymphoblasts from patients and control subjects with a high dose of ionomycin (10 μM) in the presence of EGTA caused the same increase in Fluo-3 fluorescence, indicative for a similar, maximal degree of Ca^{2+} store depletion (Table 2). Activation with the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (1 μM) in the presence of EGTA to deplete SERCA-related store compartments, induced similar $[Ca^{2+}]_i$ rises in control and patient cell lines, but the levels were slightly lower than obtained with ionomycin (Table 2). Addition of caffeine (10 mM) in the presence of EGTA, which causes depletion of the ryanodine-sensitive Ca^{2+} stores (in which Ca^{2+} is released through ryanodine- rather than inositol-trisphosphate-activated receptors)[22], had an effect on all cell lines comparable to that of thapsigargin. Together, these data indicate that the Ca^{2+} release from the intracellular store compartments is similar for the various control and patient B-cell lines.

	Fluo-3 fluorescence (fold increase)			
Condition	**Control 1**	**Control 2**	**Patient M.S.**	**Patient V.W.**
Unstimulated	1.00	1.00	1.00	1.00
Ionomycin, EGTA	1.4 ± 0.2	1.5 ± 0.1	1.3 ± 0.1	1.4 ± 0.2
Thapsigargin, EGTA	1.3 ± 0.2	1.2 ± 0.1	1.2 ± 0.1	1.4 ± 0.1
Caffeine, EGTA	1.4 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.5 ± 0.3
Ionomycin, $CaCl_2$	7.5 ± 1.4	6.6 ± 0.6	6.6 ± 1.1	6.3 ± 2.2
Thapsigargin, $CaCl_2$	4.8 ± 0.8	5.0 ± 0.8	4.9 ± 0.7	4.2 ± 1.6
Caffeine, $CaCl_2$	2.9 ± 1.1	3.1 ± 1.2	2.3 ± 0.9	3.9 ± 1.1

Table 2 Total Ca^{2+} responses and Ca^{2+} mobilization in activated EVB-B lymphoblasts from two controls and two Scott patients. Flow cytometric results are expressed as fold increase in fluorescence intensity (arbitrary units) relative to the control (1.00). Under unstimulated conditions, averaged fluorescence intensities of the populations of Fluo-3-loaded cells were similar for all four cell lines. Values shown are means ± SD from at least four independent experiments.

To allow capacitative Ca^{2+} entry, the Fluo-3-loaded B-lymphoblasts were activated using the same agonists with extracellular $CaCl_2$ present. Ionomycin/$CaCl_2$ caused a high increase in $[Ca^{2+}]_{i,}$ which was similar for the control and patient B-lymphoblasts (Table 2). Thapsigargin/$CaCl_2$ resulted in a smaller increase in $[Ca^{2+}]_i$ due to SMCE. This response was somewhat lower in the V.W. cells in comparison to the control, but the difference was not statistically significant (Table 2). When capacitative Ca^{2+} entry was induced with caffeine/$CaCl_2$, the Ca^{2+} responses were lower than with thapsigargin/$CaCl_2$, but no differences in responsiveness were observed between the various cell lines. Combined treatment with thapsigargin/caffeine/$CaCl_2$ did not further increase the thapsigargin-induced Ca^{2+} response (fold fluorescence increase: 4.6 ± 0.2 for control 1, 4.7 ± 0.4 for M.S. (mean ± SD, n=3)), indicating efficient coupling of the thapsigargin-sensitive stores to the SMCE Ca^{2+} influx channels. These results typically differ from those obtained with EBV-transfected B-cells from the French Scott patient, in which case both Ca^{2+}-ionophore/$CaCl_2$ and thapsigargin/$CaCl_2$ induced substantially lower Ca^{2+} responses[22]. Thus, the present observations show that Ca^{2+} release from intracellular stores and Ca^{2+} entry are not significantly altered in viable, non-apoptotic B-lymphoblasts cells from these two different Scott patients.

Relation between calcium responses and phosphatidylserine exposure in B-lymphoblasts from patients

To address the question whether the B-lymphoblasts from the patients have retained the Scott-type characteristics, we used a double-labelling procedure to directly compare Ca^{2+} responses (Fluo-3 loading) and PS exposure (AF-annexin V addition) by flow cytometric analysis. Knowing that apoptosis of B-lymphoblasts, even from Scott patients, is accompanied by PS exposure[27], all analysis were performed exclusively on

	% AF-annexin V binding cells			
Condition	**Control 1**	**Control 2**	**Patient M.S.**	**Patient V.W.**
Unstimulated	4.0 ± 1.8	2.0 ± 0.5	1.0 ± 0.1*	3.0 ± 2.0
Ionomycin, $CaCl_2$	90.0 ± 2.7	85.0 ± 18.0	6.0 ± 1.4*	11.0 ± 2.1*
Thapsigargin, $CaCl_2$	5.0 ± 2.5	5.0 ± 2.9	2.0 ± 0.7	4.0 ± 1.9
Caffeine, $CaCl_2$	3.0 ± 0.1	2.0 ± 1.4	1.0 ± 0.1	1.0 ± 1.3

Table 3 Phosphatidylserine exposure in non-apoptotic Fluo-3-loaded B-lymphoblasts from two controls and two Scott patients. Data represent percentages of AF-annexin V positive cells (means ± SD, n = 4-6). *P<0.05 compared to value of corresponding control cells.

the non-apoptotic cell population. In the absence of stimulation, both the control B-cells and the cells derived from patients M.S. and V.W. exhibited comparably low PS exposure (Table 3). When treated with ionomycin (10 μM) in the presence of $CaCl_2$, 85-90% of the B-lymphoblasts from the controls became PS-positive, versus only 6% and 11% of the B-lymphoblasts of patient M.S. and V.W., respectively.

When SMCE was induced by $CaCl_2$ after emptying the Ca^{2+} stores with thapsigargin, no PS exposure was observed in either control or patient B-lymphoblasts (Table 3), despite the fact that the thapsigargin-induced Ca^{2+} responses were high (see Table 2). Similarly, addition of caffeine – either alone or in combination with thapsigargin – did not lead to significant PS exposure in viable cells for any of the cell lines.

Further analysis of the double label flow-cytometric data clearly indicate that the Ca^{2+} signals caused by thapsigargin/$CaCl_2$ were lower but in the same range as those evoked by ionomycin/$CaCl_2$, whereas only the latter agonist was capable to induce PS exposure in control B-lymphoblasts (Figure 3). Thus, in the control cells, ionomycin appears to be a much better stimulus for PS exposure than thapsigargin or caffeine,

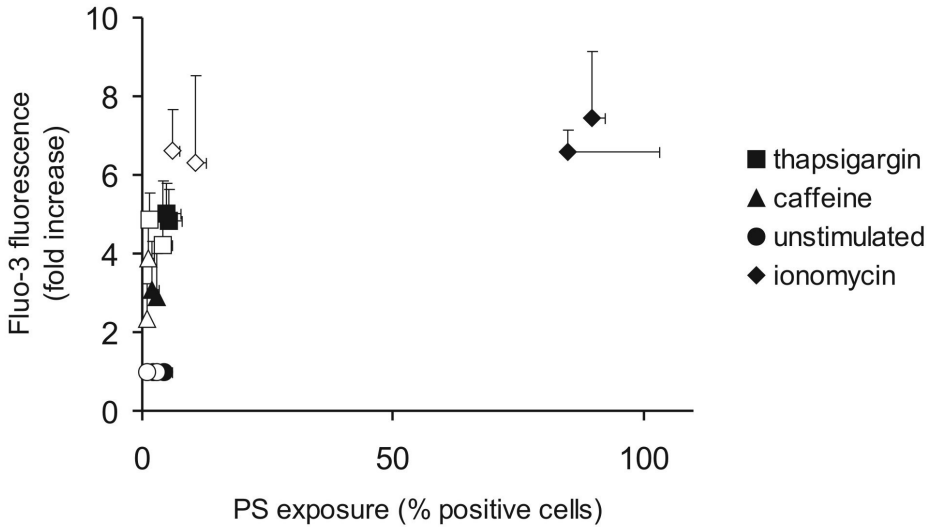

Figure 1 Collagen-induced Ca^{2+} responses of single platelets from control subjects and Scott patients. Fura-2-loaded platelets were allowed to adhere to collagen fibers in the presence of $CaCl_2$ (2 mM). Changes in Fura-2 fluorescence ratio in single platelets were measured by fluorescence video imaging microscopy. (A) Experiments were performed in 1996 with platelets from control 1 and patient M.S. (B) Experiments with platelets from control 2 and patient V.W. were carried out in 2002. Changes in Fura-2 fluorescence ratio (340/380 nm excitation) were recorded during adhesion and activation, as described in Materials and Methods. Traces are averaged overlays of single traces obtained from at least twenty platelets per subject (n≥3 incubations/subject).

despite the fact that the latter agents cause substantial Ca^{2+} influx, suggesting a particular action of ionomycin/Ca^{2+} on the phospholipid scrambling process.

Discussion

The transmembrane phospholipid asymmetry, characteristic of the plasma membrane of most mammalian cells, is maintained by two distinct plasma membrane proteins: the aminophospholipid translocase, which causes a rapid inward transport of aminophospholipids, and –as demonstrated for erythrocytes- the multidrug resistance protein MRP1, which facilitates a slow outward movement of membrane phospholipids, irrespective the composition of the polar headgroup. These proteins assure that procoagulant anionic phospholipids, such as PS, remain localized in the cytoplasmic leaflet of the plasma membrane (reviewed in[30]). A third protein is responsible for a rapid bidirectional transbilayer movement of the phospholipids in the plasma membrane. Activation of this protein causes a collaps of the asymmetric lipid distribution in the plasma membrane; hence this protein has been termed 'phospholipid scramblase'[30]. Activation of the scramblase causes exposure of PS at the cell surface, which serves an important function in the process of blood coagulation. Patients with an impaired scramblase activity suffer from a bleeding disorder, referred to as Scott syndrome, illustrating the importance of this membrane remodelling process. Scrambling activity has been demonstrated in most haematological cells; Sims and coworkers have cloned a protein from erythrocytes with Ca-dependent scrambling activity, a protein which is now referred to as human phospholipid scramblase (hPLSCR1, reviewed in[31]). However, there is increasing doubt about the true function of this protein. As was found recently, adult PLSCR1(-/-) mice showed no obvious haematologic or haemostatic abnormality, and blood cells from these animals normally mobilized phosphatidylserine to the cell surface upon stimulation[32]. Thus, the identity of the scramblase remains to be elucidated. The finding that the phenotype of defective PS exposure in the Scott syndrome affects all cells of the haematological lineage as well as the hereditary transmission of this disorder strongly suggest the involvement of a protein in the scrambling process. Although it has been demonstrated that lipid scrambling does not require hydrolysis of ATP and that activity of the scramblase requires a persistent elevation of intracellular Ca^{2+}, the mode of action and the regulation of the scramblase remain to be elucidated.

In the present study, we introduce a new case with characteristics of the Scott syndrome. Patient V.W. has been described in an early study published in 1980 as the propositus of a family with a platelet-related bleeding disorder despite a normal

coagulation profile[5], but has not been recognised as having Scott syndrome. The data obtained in this study clearly demonstrate an impaired Ca^{2+}-induced PS exposure in the patient's erythrocytes, platelets and lymphoblasts as judged from decreased prothrombinase activities and annexin V binding, confirming the Scott phenotype. Note that for patient V.W. the procoagulant response of platelets treated with ionophore seems to be more impaired than that of platelets activated with collagen plus thrombin. This has been a consistent finding over several years with the platelets of patient M.S. In both the French propositus with Scott syndrome and the canine model of Scott syndrome, platelet prothrombinase activity was virtually absent, independent of whether the cells were activated with ionophore or with collagen plus thrombin[6,9]. The reason for this difference in phenotype is as yet unclear. Whether the partially impaired procoagulant response after collagen plus thrombin stimulation of platelets of patients V.W. and M.S. illustrates different pathways of activation or the existence of more than one mechanism for phospholipid scrambling in platelets remains to be investigated. In this respect, it is of interest to mention that the defect in ionomycin-induced PS exposure in the EBV-transformed B cells of the two patients is not observed for the apoptotic cells from these patients (data not shown), in agreement with the suggestion that phospholipid scrambling in apoptotic cells is differently regulated from the scrambling caused by Ca^{2+}-elevating agents[27]).

SMCE following Ca^{2+} mobilization from intracellular stores presents the most important Ca^{2+} entry pathway in platelets and lymphocytes. Recently, Martinez and co-workers have described that B-lymphoblasts from a French Scott patient exhibit normal Ca^{2+} release from the thapsigargin- and caffeine-depletable Ca^{2+} stores, but show a considerably reduced subsequent Ca^{2+} entry, along with a reduced PS exposure[4,22]. Interestingly, Ca^{2+} entry following depletion of both the thapsigargin and caffeine-sensitive stores became normalized in these B-lymphoblasts. In the present study, we also measured thapsigargin- and caffeine-mediated release of Ca^{2+} from stores as well as subsequent Ca^{2+} entry. In agreement with their observations, we found no indications for disturbances in the release from stores. However, in contrast to the French Scott B-lymphoblasts, the B-cells from both patients M.S. and V.W. appeared to have normal Ca^{2+} influx via SMCE with thapsigargin or caffeine as well as Ca^{2+} ionophore. Furthermore, the combination of thapsigargin and caffeine did not further increase the Ca^{2+} signal or the PS exposure. The reason for this discrepancy is not known, but may reflect a variant of the Scott syndrome.

The finding that Ca^{2+} responses in both the platelets and B-cells from patients M.S. and V.W. are unaltered indicates that aberrant Ca^{2+} signaling -at least in these two

patients- is not part of the Scott phenotype. The double-labelling techniques applied with both platelets and non-apoptotic B-cells show that Scott cells of either type are greatly diminished in PS exposure although being high in $[Ca^{2+}]_i$. Even for the control B-cells, there is no simple relationship between elevated $[Ca^{2+}]_i$ and PS exposure (Figure 3), suggesting that the scrambling process is not dependent on high $[Ca^{2+}]_i$ only, but relies on other intracellular processes as well, in agreement with previous findings[16]. This suggestion is supported by recent evidence that also in stimulated human T-lymphocytes the phospholipid scrambling with Ca^{2+} ionophore is mediated by factors other than elevated $[Ca^{2+}]_i$ only[33].

In summary, the present study shows that patient V.W. can be considered as having a Scott syndrome phenotype. The impaired lipid scrambling in platelets and B-cells from both Scott patients presented here, cannot be ascribed to alterations in Ca^{2+} release from intracellular stores or subsequent Ca^{2+} entry.

Acknowledgement

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Chapter 3

Specific roles of PI3K isoforms α and β in glycoprotein VI-induced platelet signaling to procoagulant activity

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Abstract

Platelets contain various forms of phosphoinositide 3-kinase (PI3K), which contribute to platelet activation in a non-clarified way. Here, we investigated the roles of platelet PI3K isoforms in glycoprotein VI (GPVI)-induced Ca^{2+} signaling and procoagulant activity during thrombus formation. We used mouse platelets deficient in the p85α regulatory PI3K subunit or the p110γ catalytic subunit, as well as high-affinity PI3K inhibitors, specifically blocking the p110 α, β, γ or δ catalytic subunits. Under high-shear flow conditions, absence of murine p85α or inhibition of PI3Kα or β lowered GPVI-induced Ca^{2+} responses and subsequent procoagulant activity (phosphatidylserine exposure). In contrast, absence of p110γ only delayed the GPVI-induced Ca^{2+} signal and did not influence procoagulant activity. Experiments with human platelets showed that the contribution of PI3K to GPVI-induced Ca^{2+} signaling and procoagulant activity was independent of autocrine released ADP. Furthermore, blockage of either PI3Kα or β catalytic activity was sufficient to completely suppress the enhancing effect of PI3K on mobilisation of Ca^{2+} from internal stores. Combined inhibition of PI3Kα and β was without extra effect, while inhibition of PI3Kδ was only partly effective. Taken together, the present results indicate that the principle mechanism of action of PI3K on GPVI-induced Ca^{2+} signaling and PS exposure is enhancement of phospholipase Cγ2-mediated stimulation of Ca^{2+} depletion from internal stores, and subsequent store-mediated Ca^{2+} entry. Furthermore, particularly the PI3Kα and β isoforms, acting via p85α, appear to be involved in this activation pathway, with PKI3δ playing a minor role.

Introduction

Cytosolic $[Ca^{2+}]_i$ elevation is a key regulating signal for most platelet activation processes, such as shape change, release of autocoid mediators, aggregation and, particularly, development of procoagulant activity[1,2]. The signal transduction processes to the procoagulant platelet response are not well investigated, although it is clear that these require a high, threshold $[Ca^{2+}]_i$ elevation, which mediates scrambling of plasma membrane phospholipids, exposure of the phosphatidylserine (PS) at the platelet surface, and shedding of microparticles[3]. The PS-exposing platelets and microparticles avidly assemble coagulation factors, and thus mediate massive formation of factor Xa and thrombin during the propagation phase of the coagulation process[4].

In both human and mouse platelets, the G_q protein-coupled receptor agonists thrombin, ADP and thromboxane A_2 activate phospholipase C (PLC)β and form inositol

1,4,5-trisphosphate (IP_3)[5,6]. By themselves, these agonists are unable to trigger the platelet procoagulant response, likely because of the relative short duration of the Ca^{2+} signal. In contrast, platelet stimulation of the immunoglobulin family receptor, glycoprotein VI (GPVI) – using agonists as collagen, collagen peptide and convulxin – results in a more prolonged elevation in $[Ca^{2+}]_i$[4,7,8]. The GPVI receptor signals via Src and Syk tyrosine kinases to activate PLCγ2 in a pathway that is supported by several other protein kinases, particularly of the Btk/Tec and phosphoinositide 3-kinase (PI3K) families[9,10].

In lymphocytes and mast cells, triggering of immunoglobulin receptors also leads to activation of PLCγ isoforms, in a way promoted by PI3K activation[11,12]. In these cells, plasma membrane accumulation of the PI3K product, phosphatidylinositol 3,4,5-trisphosphate (PIP_3), is assumed to function as an anchoring site of the activated PLCγ via its *N*-terminal pleckstrin homology (PH) domain. Accordingly, the PIP_3 ensures sustained formation of IP_3 and Ca^{2+} signals[13].

Human and murine platelets contain various PI3K isoforms, which are distinguished according to their catalytic subunits. These are the class IA isoforms PI3Kα, β and δ (p110α, p110β and p110δ), and the class IB isoform PI3Kγ (p110γ)[14-16]. Platelets further contain the class IA PI3K regulatory subunits, p85α, p85β and p55γ, and a unique class IB regulatory subunit, p101γ.

In general, the class IA isoforms bind to SH2 domain-containing regulatory subunits, particularly p85α and, therefore, are considered to be regulated by tyrosine kinases. In contrast, class IB isoforms are supposed to be activated by G-protein coupled receptors[15]. Whether this is also the case for platelets, has not yet been investigated. It is also not completely sure which regulatory subunit can bind to which catalytic subunit. As far as known, the inter-SH2 domains of p85α, p55α, p50α, p85β and p55γ constitutively interact with the N-terminal domain of p110α, β and δ. Binding of the dual SH2 domains to tyrosine-phosphorylated adaptor proteins activates the kinase function of the p110 subunits. However, for platelets only little is known of the contribution of the individual PI3K isoforms in signal transduction. In mouse and human platelets, PI3K p85α was found to play a role in GPVI/FcR γ-chain signaling[8,17,18]. This corroborates with the finding that PI3K inhibition reduces the Ca^{2+} signal evoked by collagen peptides[8], although the mechanism of this is still unclear. Furthermore, both PI3Kβ and PI3Kγ have been implicated in ADP-induced platelet aggregation and aggregate stabilization[16,19,20], which deviates from the paradigm of class 1A and 1B isoforms being triggered by distinct signaling processes. To date, the role of individual

PI3K catalytic isoform activities in regulating GPVI-induced Ca^{2+} signaling and procoagulant activity is still unknown.

There are several pieces of evidence that platelet PI3K contributes to Ca^{2+} signaling in response to collagen peptides in yet another way. The PI3K product PIP_3 was found to mediate activation of Ca^{2+} entry either directly by influencing the Ca^{2+} channels themselves[21], or via the Tec-family protein kinase, Btk, independently of PLCγ[22]. This Ca^{2+} influx pathway was suggested to be responsible for collagen-evoked PS exposure[22]. However, the mechanism of inhibition has not further been studied.

In this paper, we studied the contribution of PI3K in GPVI-induced platelet Ca^{2+} responses, using mice deficient in distinct PI3K subunits, p85α or p110γ, and using a panel of isoform-specific PI3K inhibitors in combination with human platelets. Hereby, we focussed on monitoring of Ca^{2+} signaling and PS exposure under physiologically relevant flow conditions.

Materials and methods

Animals

Animal experiments were approved by the local animal experimental committees. Mice deficient in the p85α regulatory PI3K subunit on C57BL/6 genetic background were generated, as described[23]. Mice deficient in the p110γ catalytic subunit (PI3Kγ) were bred on a 129Sv background as described[24]. Wildtype mice of the same genetic background and same breeding programs were used as control animals.

Materials

Fibrillar type-1 collagen (Horm) from equine tendon was purchased from Nycomed. Collagen-related peptide (CRP), containing triple-helical polymers of Gly-Pro-Hyp, was prepared and cross-linked, as described before[25]. The GPVI agonist, convulxin[26], was purified to homogeneity from the venom of *Crotalus durissus terrificus* (Latoxan), as described[27]. Fura-2 and Fluo-3 acetoxymethyl esters and Pluronic F-127 were purchased from Molecular Probes. Oregon green 488-labelled annexin A5 (OG488-annexin A5) was from Nexins Research. Bovine serum albumin (BSA), apyrase (grade V), aspirin, thrombin, wortmannin and MRS2179, a $P2Y_1$ antagonist, were from Sigma. LY294002 and H-Phe-Pro-Arg chloromethyl ketone (PPACK) were from Calbiochem. Isoform-selective PI3K inhibitors were a kind gift from the Baker Heart Research

Institute, and synthesized as described[16,28]. These were (with cell-free IC_{50} concentrations): PI103 and YM024, selective for p110α (0.008 and 0.3 μM, respectively); TGX221, selective for the p110β isoform (0.05 μM); AS252424, selective for p110γ (0.035 μM); and IC87114, selective for p110δ (0.5 μM). Cangrelor (AR-C69931MX), an antagonist of the $P2Y_{12}$ receptor, was kindly provided by The Medicines Company. Acetylsalicylic acid (aspirin) was purchased from Lorex Synthelabo. Other reagents were of analytical grade and from sources as described[29].

Blood collection and platelet isolation

For flow experiments, human blood was collected into 40 μM PPACK, and mouse blood was collected into 40 μM PPACK and 5 U/ml heparin, as described previously[29,30]. For measurements of cytosolic $[Ca^{2+}]_i$, human or mouse blood was collected on one-sixth volume of acid-citrate-dextrose anticoagulant, composed of 85 mM sodium citrate, 78 mM citric acid and 11 mM D-glucose. Platelet-rich plasma and washed platelets were obtained by centrifugation, as described before for human[30] and mouse[29] platelets. Mouse platelets were finally resuspended in Hepes buffer I (5 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 2 mM $MgCl_2$, 0.1% glucose and 0.1% BSA, pH 7.45). Cells were counted with a Coulter counter.

Measurement of thrombus formation and procoagulant response under flow

Whole blood perfusion experiments were performed, as described previously for human[30] and mouse[29] blood. Briefly, coverslips were coated with collagen fibres (12.5 μg/cm^2) and blocked with Hepes buffer at pH 7.45 containing 1% BSA. Blood anticoagulated with PPACK was perfused over the coverslip through a transparent 50-μm deep flow chamber by using a 1-ml syringe and a pulse-free pump, at a shear rate of 1000 s^{-1} for 4 min. The blood was incubated for 10 min prior to perfusion with various inhibitors, as indicated. During and after perfusion, microscopic phase-contrast and fluorescence images were recorded in real-time using a Visitech imaging system, equipped with two intensified CCD cameras. After 4 min of perfusion, flow chambers were rinsed with Hepes buffer I, supplemented with 1 U/ml heparin and 2 mM $CaCl_2$ at the same shear rate for 4 min. Exposure of PS was detected with OG488-annexin A5 (1 μg/ml) in $CaCl_2$ Hepes buffer I. Recorded images were analysed on surface area coverage, as described before[31].

Measurement of Ca^{2+} responses under flow

Human platelet-rich plasma was incubated with 7 μM Fluo-3 acetoxymethyl ester for 45 min at 20°C, gentle rotating. Washed mouse platelets were incubated with 5 μM Fluo-3 acetoxymethyl ester and 0.2 mg/ml Pluronic F-127 under the same conditions. After labelling, the platelets were added back to whole blood from the same donor or mouse strain to give 10% labelled platelets. Changes in fluorescence in Fluo3-loaded, collagen-adhered platelets were obtained using the Visitech imaging system by high-speed (5 Hz) recording of fluorescence images, and off-line analysis of regions-of-interest representing single platelets[32]. Calibration to $[Ca^{2+}]_i$ was by pseudo-ratioing to give F/F_o values and fixed calibration values[33]. For quantitative data, traces from individual cells were superimposed, so that $[Ca^{2+}]_i$ initially increased after 3.0 s.

Measurements of Ca^{2+} responses in platelet suspension

Human Fura-2-loaded platelets were prepared as described previously[34]. ACD-anticoagulated platelet-rich plasma was incubated with aspirin (100 μM) and apyrase (0.2 U ADPase/ml) at 37°C with 2.5 μM Fura-2 acetoxymethyl ester for 45 min. Platelets were collected by centrifugation at 350 *g* for 20 min. The cells were resuspended in Hepes buffer II, containing 145 mM NaCl, 10 mM Hepes, 10 mM D-glucose, 5 mM KCl, 1 mM $MgSO_4$ and 0.1% BSA, which was supplemented with 40 μg/ml apyrase. Concentrations of vehicle Me_2SO did not exceed 0.2 vol%, and equivalent volumes of Me_2SO vehicle were present in control samples.

Fluorescence was recorded from 1.5 ml aliquots of magnetically stirred platelet suspension (10^8 cells/ml) at 37°C using a Cairn Research spectrofluorometer[35] or a SLM-Aminco spectrofluorometer[1], at excitation wavelengths of 340 and 380 nm and emission at 500 nm. Changes in $[Ca^{2+}]_i$ were determined using the calibrated 340/380 fluorescence ratio method[36]. When coloured antagonists were used interfering with the Fura-2 fluorescence signal, calibrations were performed in the presence of these antagonists. Time plots were constructed of agonist-evoked increases in $[Ca^{2+}]_i$ relative to basal level (i.e. the level before addition of agonist). Mobilisation and total Ca^{2+} influx induced by specific agonists were measured by comparing 2.5 min-integrals of the agonist-evoked rises in $[Ca^{2+}]_i$[37]. Therefore, Ca^{2+} response curves were determined in the presence of either 1 mM EGTA (allowing only intracellular Ca^{2+} mobilisation) or 1 mM $CaCl_2$ (allowing Ca^{2+} mobilisation and Ca^{2+} influx).

Statistics

Differences between experimental groups were statistically tested with the non-parametric Mann-Whitney U test. Effects of inhibitors were tested in paired incubations using the Student's paired *t* test. The statistical package for social sciences was used (SPSS 11.0). Data are presented as means ± SEM.

Results

Absence of murine p85α or blocking of PI3Kβ reduces GPVI-evoked PS exposure under flow

With mice lacking the p85α regulatory PI3K subunit (which links to class IA catalytic subunits) and mice deficient in the class IB catalytic subunit p110γ, we studied the contribution of the PI3K isoform classes to GPVI-induced platelet responses. These mice had normal levels of platelets and, furthermore, their platelets expressed normal levels of adhesive receptors (data not shown, but see Refs.[18,24]). Whole blood of these mice, anticoagulated with PPACK-heparin, was used to measure collagen-induced thrombus formation and platelet procoagulant activity under high-shear perfusion. Earlier, we have shown for murine and human blood that, under these conditions, platelet activation, thrombus build-up and procoagulant activity are strictly GPVI-dependent processes[29-31,38]. Perfusion of wildtype blood at a high shear rate of 1000 s^{-1} resulted in platelet aggregate formation on collagen, while staining with OG488-labeled annexin A5 indicated that many of the collagen-bound platelets expressed PS (Figure 1A). With blood from p85α -/- or p110γ -/- mice, platelet aggregates were still formed, although in case of the p110γ -/- platelet aggregate size was relatively small, such in agreement with earlier observations that this PI3K isoform is implicated in aggregate stability[20]. Typically, for p85α -/- blood, platelet deposition on the collagen surface was unaltered, while the number of PS-exposing platelets was significantly reduced (Figure 1B, C) With blood from PI3Kγ -/- mice the number of PS-exposing platelets was unchanged in spite of reduced aggregate formation.

It has been shown, using the specific PI3Kβ inhibitor, TGX221, that this isoform is involved in shear- and ADP-dependent platelet adhesion to vWF[16]. We used this compound at a dose of 1 μM, shown to be selective in whole blood[16], to study the contribution of p110β (PI3Kβ) to thrombus formation and platelet procoagulant activity in wildtype and knockout blood. Addition of TGX221 to p85α -/- blood reduced platelet

deposition but not PS exposure (Figure 1B, C). In contrast, with p110γ -/- blood, TGX221 lowered platelet PS exposure. These results thus demonstrate that both p85α and p110β subunits, but not p110γ, are involved in collagen- and GPVI-induced PS exposure under flow.

Absence of murine p85α and blocking of PI3Kβ reduces and delays GPVI-evoked Ca^{2+} signaling under flow

Knowing the relation between GPVI-evoked rises in $[Ca^{2+}]_i$ and extent of PS exposure under these flow conditions[29,30], we measured the Ca^{2+} responses of platelets

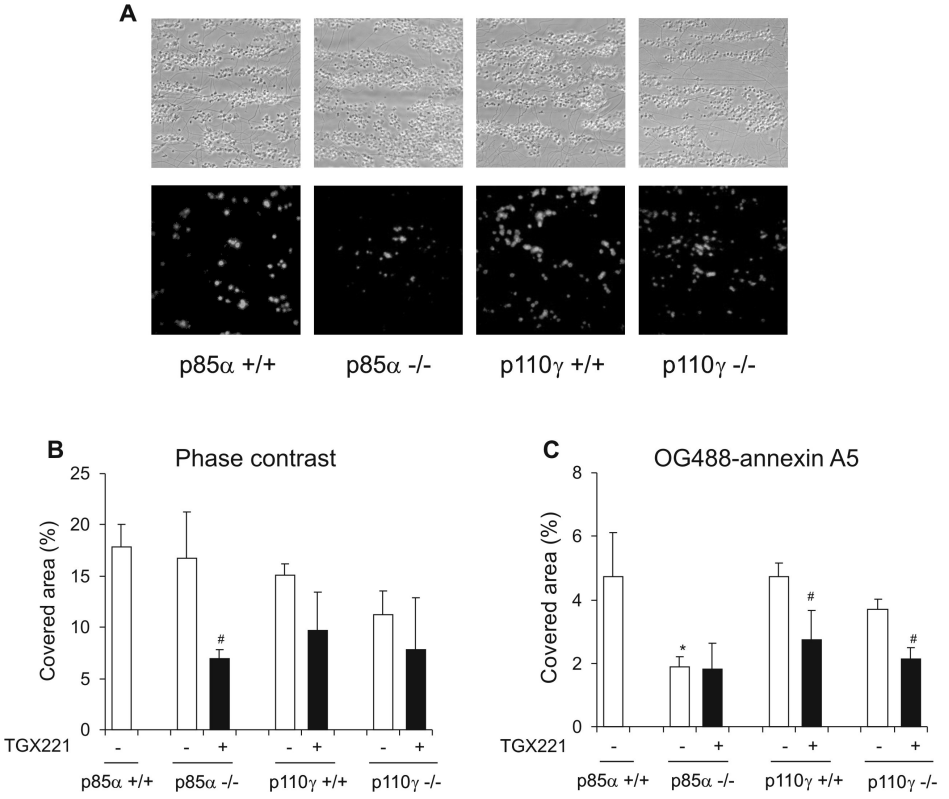

Figure 1 Absence of murine platelet p85α but not p110γ restricts collagen-evoked procoagulant activity under flow. PPACK/heparin-anticoagulated blood from indicated mice was perfused over a collagen surface for 4 min at 1000 s^{-1}, followed by a 4-min wash with buffer in the presence of fluorescently labelled annexin A5 (0.5 μg/ml). Blood was pre-incubated for 10 min with Me_2SO vehicle or PI3Kβ inhibitor TGX221 (1 μM), as indicated. (A) *Upper panels*: representative phase-contrast microscope images of platelet thrombi formed after 4-min perfusion (120×120 μm). *Lower panels*: representative fluorescence images after staining with OG488-labeled annexin A5, detecting PS-exposing platelets (150×150 μm). Surface area coverage of all platelets (B) and PS-exposing platelets (C) with untreated blood (white bars) or blood treated with TGX221 (black bars). Data are mean±SEM (n=4). *p<0.05 compared to wildtype; #p<0.05 compared to vehicle control.

adhering to and activated by collagen under flow[33]. Whole blood was spiked with Fluo-3-loaded platelets derived from the same mouse strain to reach a concentration of 10% labelled platelets. Upon perfusion of blood over collagen, wildtype platelets showed a potent and prolonged Ca^{2+} response (Figure 2A), as is required for PS exposure. Traces from single platelets indicated a rapid rise in $[Ca^{2+}]_i$ that was followed by a persistent, elevated plateau level. With blood from p85α null mice, platelets on average showed lower Ca^{2+} responses compared to the wildtype cells (Figure 2A, B). Interestingly, averaged traces with platelets from p110γ -/- mice showed that initial but not later increases in $[Ca^{2+}]_i$ were reduced, thus pointing to a delayed response. Indeed, in single cell traces, often a long delay time between stable adhesion and start of the Ca^{2+} response was observed (Figure 2A).

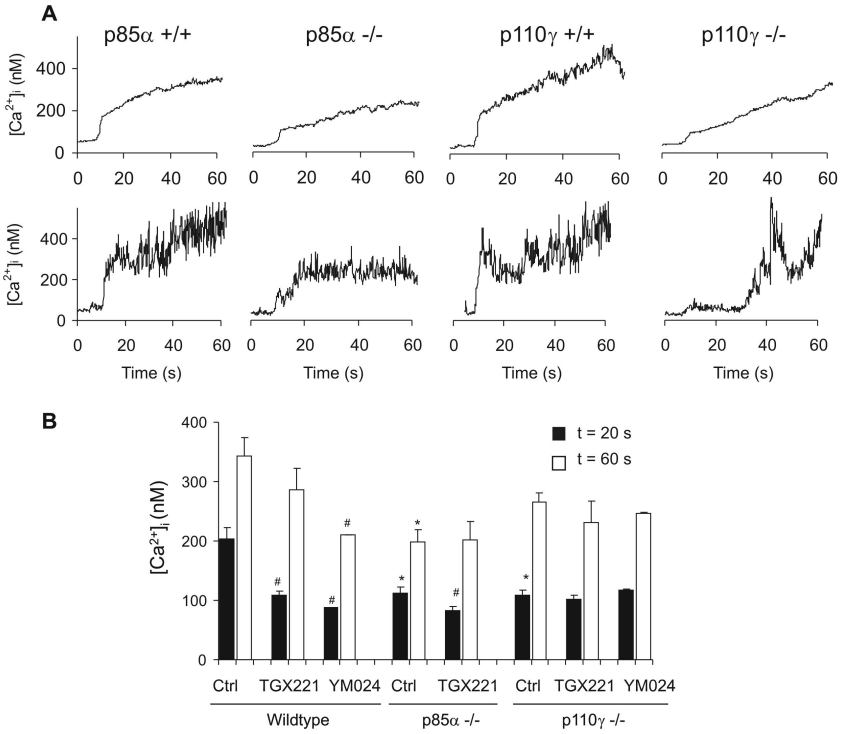

Figure 2 Absence of murine platelet p85α or blocking of PI3Kα or β suppresses collagen-evoked Ca^{2+} responses under flow. Whole murine blood, spiked with 10% Fluo-3-loaded platelets from the same mouse strain, was perfused over collagen at 1000 s^{-1}. Changes in fluorescence were measured during flow in single, adhered platelets. (A) Upper traces show averaged changes in $[Ca^{2+}]_i$, of 15-20 platelets during 60 s (≥3 mice per condition). Lower traces show Ca^{2+} responses of representative, single platelets. (B) Blood was pre-incubated with vehicle (Ctrl), PI3Kα inhibitor YM024 (1 μM) or PI3Kβ inhibitor TGX221 (1 μM), as indicated. Quantification is given of changes in $[Ca^{2+}]_i$, at 20 s (black bars) and 60 s (white bars) after initial increase. Data are mean±SEM (n=3-5). *p<0.05 compared to wildtype; #p<0.05 compared to vehicle control.

To study the additional contribution of PI3Kβ and other class IA isoforms in the GPVI-induced Ca^{2+} signal generation under flow conditions, the PI3Kβ inhibitor TGX221 and the PI3Kα inhibitor YM024 were used. In wildtype mice, TGX221 only reduced the initial phase of the Ca^{2+} response, while YM024 significantly suppressed the full response to the level observed in platelets from p85α -/- mice. In these cells, TGX221 still further reduced the initial Ca^{2+} signal. In platelets from p110γ -/- mice, no additional effect of TGX221 or YM024 was observed. Together, these data suggest that p85α and p110α (PI3Kα) markedly contribute to the GPVI-evoked Ca^{2+} responses of platelets on collagen under flow, while the β and γ PI3K isoforms only contribute to the initial phase of this response.

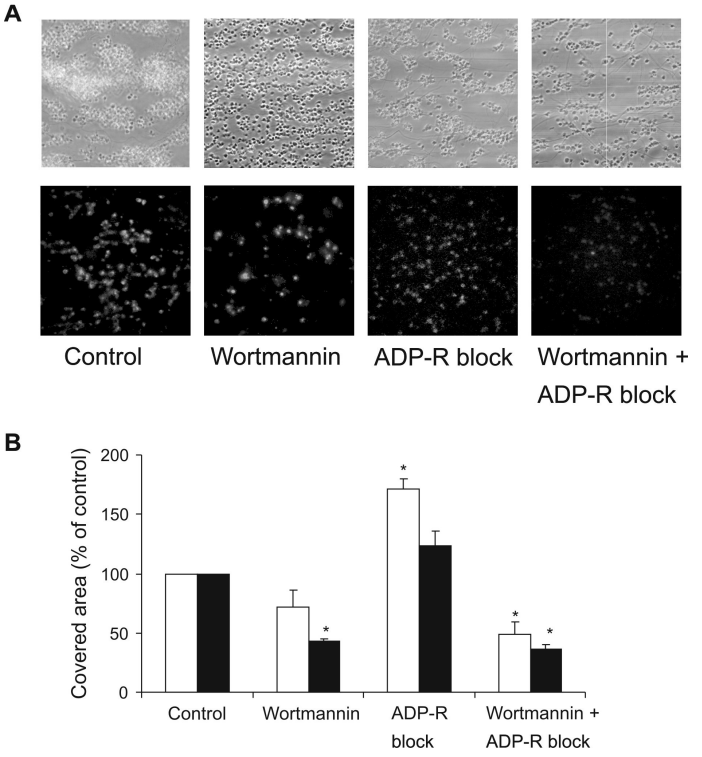

Figure 3 Blocking of human platelet PI3K suppresses collagen-evoked aggregate formation and procoagulant activity under flow. PPACK-anticoagulated human blood was perfused over a collagen surface at 1000 s^{-1}, as indicated for Figure 1. Blood was pre-incubated for 10 min with vehicle (control), wortmannin (1 μM) and/or ADP receptor blockers (40 μM MRS2179, 20 μM cangrelor and 1 unit/ml apyrase), where indicated. (A) *Upper panels*: representative phase contrast images after 4-min of perfusion (120×120 μm). *Lower panels*: representative fluorescence images after staining with OG488-annexin A5, detecting PS-exposing platelets (150×150 μm). (B) Surface area coverage of all platelets (white bars) and PS-exposing platelets (black bars), expressed as percentages of control condition (mean±SEM, n=4). *p<0.05 compared to control.

Human platelet PI3K contributes to GPVI-evoked aggregate formation, Ca^{2+} signaling and PS exposure under flow

To investigate the importance of PI3K for the human system, we performed similar flow experiments with PPACK-anticoagulated human blood at a shear rate of 1000 s^{-1}. Under control conditions, platelets rapidly adhered to the collagen fibres and assembled into aggregates, covering much of the collagen surface (Figure 3A). Post-staining with OG488-annexin A5 again revealed many PS-exposing platelets.

Pre-incubation of blood with the high-affinity general PI3K inhibitor wortmannin (1 μM) profoundly inhibited the GPVI-evoked aggregation (Figure 3A), but did not affect platelet adhesion to the collagen surface, as surface area coverage of platelets was still about 75% of the control condition (Figure 3B). In contrast, wortmannin treatment significantly reduced the presence of PS-exposing platelets to 65% of the control. Similar effects were obtained with another general PI3K inhibitor, LY294002 (not shown).

Because paracrine ADP release contributes to thrombus formation via the ADP-

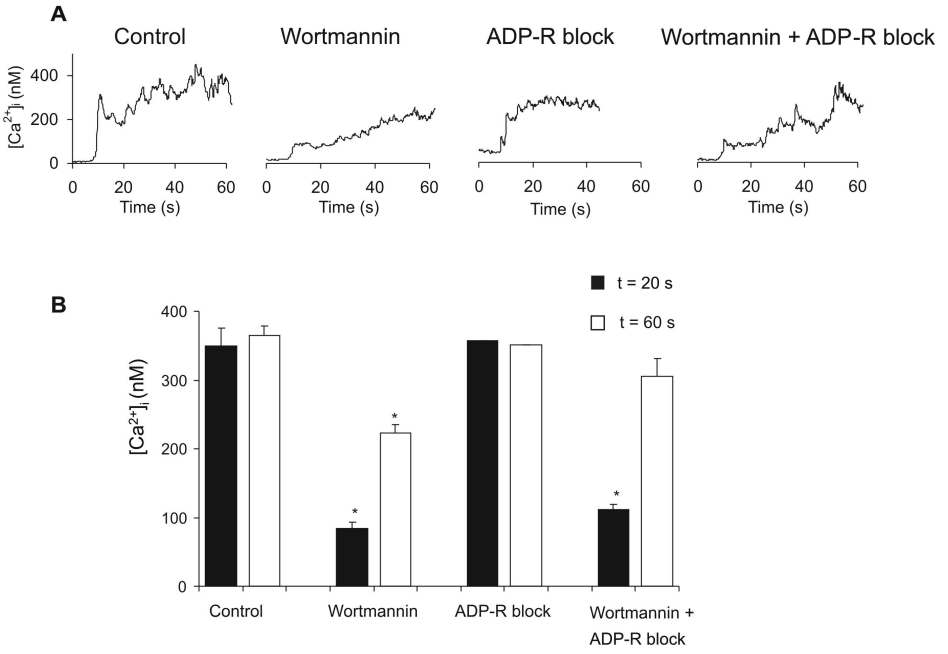

Figure 4 Blocking of human platelet PI3K inhibits collagen-evoked Ca^{2+} responses under flow. Human whole blood was spiked with autologous Fluo-3 loaded platelets, and perfused over collagen, as indicated for Figure 2. Blood was treated with vehicle (control), wortmannin (1 μM) and/or ADP receptor blockers (40 μM MRS2179, 20 μM cangrelor and 1 U/ml apyrase), as indicated. (A) Traces of $[Ca^{2+}]_i$ changes shown are averaged curves of single-cell responses from 15-25 platelets. (B) Quantification of changes in $[Ca^{2+}]_i$, at 20 s (black bars) and 60 s (white bars) after initial increase. Data are mean±SEM (n=3). *p<0.05 compared to control.

receptors $P2Y_{12}$ and $P2Y_1$[38,39], and because PI3K isoforms play a role in $P2Y_{12}$-mediated aggregation under flow[20], we investigated whether ADP release and signaling interfered in the wortmannin effects. Therefore, blood was pre-incubated with saturating doses of ADP-degrading apyrase and the $P2Y_1$ and $P2Y_{12}$ receptor blockers, MRS2179 and cangrelor, respectively. Blockade of the ADP receptors resulted in high deposition of mostly single platelets and small aggregates at the collagen surface (Figure 3A), with as a result a higher surface area coverage with platelets (Figure 3B). However, the ADP-receptor blockade failed to reduce the number of PS-exposing platelets. Interestingly, treatment of wortmannin in combination with ADP receptor blockers reduced platelet deposition, and lowered PS exposure to the level obtained with wortmannin alone. Accordingly, the PI3K contribution to platelet procoagulant activity seems to be independent of indirect, ADP-mediated effects.

Using Fluo-3-loaded platelets, which were added to the human blood, effects of wortmannin and ADP receptor blockade were also investigated on the collagen-evoked Ca^{2+} responses. As indicated in Figure 4, treatment with wortmannin, but not with ADP receptor blockers, greatly reduces the increases in $[Ca^{2+}]_i$. Taken together, these data indicate that under flow blocking of PI3K with wortmannin suppresses collagen-evoked Ca^{2+} signaling and procoagulant activity, independently of secondary ADP effects.

Human platelet PI3K α and β isoforms contribute to GPVI- but not thrombin-evoked Ca^{2+} mobilisation and entry in platelet suspension

To more directly investigate the involvement of PI3K in agonist-induced Ca^{2+} signal generation, suspension measurements were performed with washed, Fura-2-loaded platelets, which were stimulated by near-maximal doses of the GPVI agonist, CRP (5 µg/ml) or the G_q protein-coupled receptor agonist, thrombin (10 nM). Platelets were pre-treated with aspirin and contained apyrase to abolish secondary effects due to released thromboxane and ADP, respectively. Activations were in the presence of EGTA to measure only Ca^{2+} mobilisation from internal stores, while others were with $CaCl_2$, where the Ca^{2+} signal consists of both internal mobilisation and Ca^{2+} entry. Dose-response curves indicated that the general PI3K inhibitors reduced the Ca^{2+} signal evoked by CRP to about 50%, at saturating concentrations of 100 nM wortmannin and 10 µM LY294002 (Figure 5A). Furthermore, with either compound a similar dose-dependency of inhibition was seen for experiments in the presence of EGTA or $CaCl_2$, thus indicating that internal Ca^{2+} mobilisation and Ca^{2+} influx were similarly influenced. Control experiments, performed in the presence of extra MRS2179 (40 µM) plus

cangrelor (20 μM) to block P2Y receptors, gave a similar, maximal inhibition with wortmannin and LY294002 of 45±1.4% and 52±2.8% of control, respectively. Similar results were obtained when platelets were stimulated with the GPVI agonist, convulxin instead of CRP: wortmannin (10 nM) and LY294002 (1 μM) inhibited the Ca^{2+} response to 37±0.9 and 58±10.4% of control, respectively. However, in contrast, platelet treatment with wortmannin or LY294002 no more than slightly lowered the Ca^{2+} responses evoked by thrombin (Figure 5B).

A panel of currently available high-affinity isoform-specific PI3K inhibitors was then used to established the isoforms contributing to the GPVI-induced Ca^{2+} response with convulxin in the presence of external $CaCl_2$. The compounds were used at concentrations around their IC_{50} and higher, as was established *in vitro* using cell-free

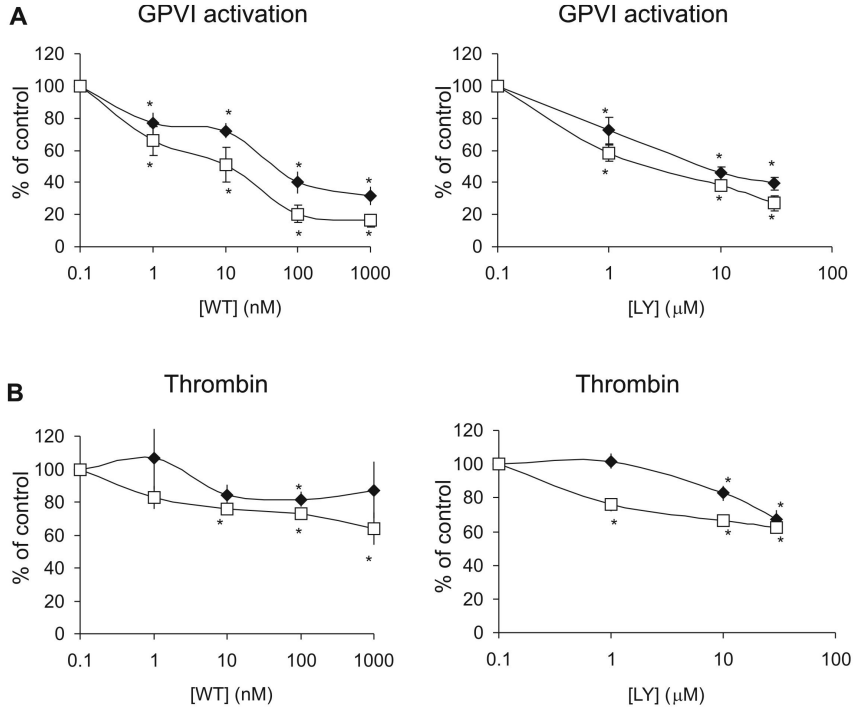

Figure 5 Inhibition of human platelet PI3K suppresses GPVI-, but not thrombin-evoked Ca^{2+} mobilisation and influx. Suspensions of human, Fura-2-loaded platelets were incubated for 10 min at 37°C with Me_2SO vehicle, wortmannin (0.1-1000 nM) or LY294002 (0.1-30 μM). Platelets were then stimulated with (A) GPVI-stimulating CRP (5 μg/ml), or (B) thrombin (10 nM) in buffer containing 1 mM Ca^{2+} or 1 mM EGTA. The platelets were pre-treated with aspirin while loading with Fura-2, and suspensions contained apyrase. Elevations in $[Ca^{2+}]_i$ were measured as time-$[Ca^{2+}]_i$ integrals. Black diamonds represent total Ca^{2+} response with $CaCl_2$, and open squares Ca^{2+} mobilization with EGTA. Results are expressed as percentages of the vehicle control condition (mean±SEM, n=5). *p<0.05 compared to control.

systems[28]. Platelet treatment with the PI3Kα inhibitor PI103 (0.02-0.5 μM), but not with the analogous control substance, PIK112, resulted in a dose-dependent inhibition of the Ca^{2+} response up to 50% of control (Figure 6A). A quite similar effect was obtained with the PI3Kα inhibitor, YM024 (0.1-2.5 μM).

Also, PI3Kβ inhibition with TGX221 (0.1-2.5 μM) gave a 50% reduction in Ca^{2+} response. Interestingly, combined inhibition of PI3Kα and β (PI103 + TGX221) did not have an additional effect, when compared to either inhibitor alone. Platelet treatment with the PI3Kγ inhibitor AS252424 (0.1-1 μM) failed to have an effect on the Ca^{2+} response, but interfered with the Fura-2 fluorescence at high doses. The compound IC87114 (0.2-5

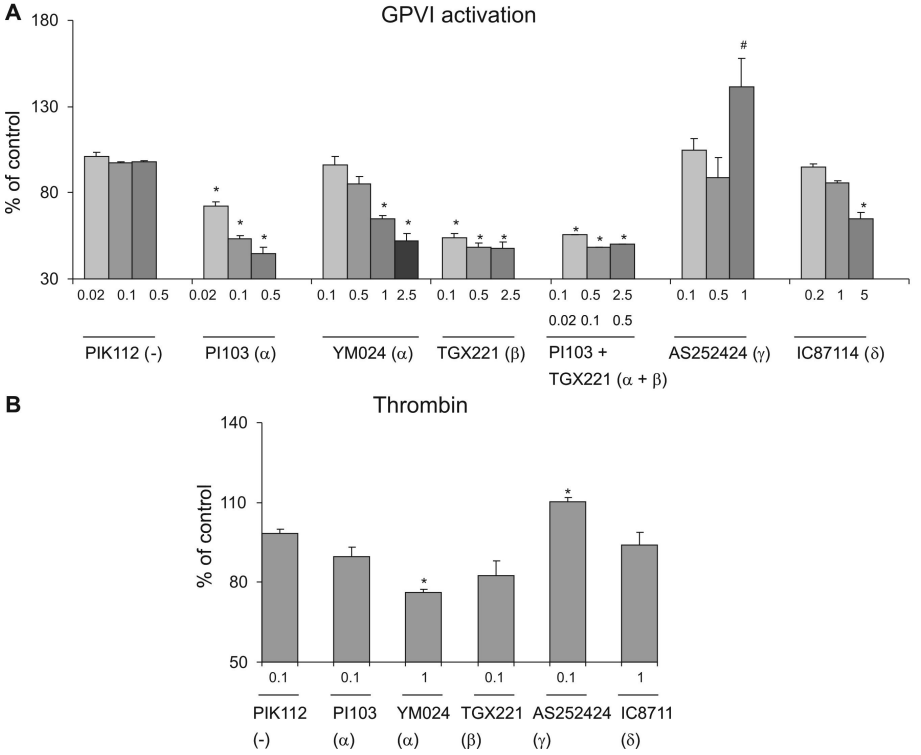

Figure 6 Inhibition of human platelet PI3Kα or β suppresses GPVI-, but not thrombin-evoked Ca^{2+} signaling. Suspensions of human Fura-2-loaded platelets were incubated for 10 min at 37°C in the presence of vehicle or one or more of the following inhibitory substances (0.02-5 μM): PIK112 (negative control), PI103 (PI3Kα), YM024 (PI3Kα), TGX221 (PI3Kβ), AS252424 (PI3Kγ) or IC87114 (PI3Kδ) to block indicated PI3K isoforms. Numbers below bars indicate concentrations of inhibitors. Cells were then stimulated with (A) convulxin (70 ng/ml) or (B) thrombin (10 nM) in Hepes buffer containing 1 mM $CaCl_2$. The platelets were pre-treated with aspirin, while loading with Fura-2, and suspensions contained apyrase, MRS2179 and cangrelor. Elevations in $[Ca^{2+}]_i$ were determined as described for Figure 5. Histograms indicate percentage changes in $[Ca^{2+}]_i$ rises relative to control condition. Values are mean±SEM from three independent experiments. *p<0.05 *versus* vehicle control, # interference with Fura-2 fluorescence.

μM) slightly, but significantly reduced the GPVI-evoked Ca^{2+} response only at the highest concentration of 5 μM, which is still specific for PI3Kδ inhibition. In marked contrast to the profound effects of PI3Kα and β inhibitors with GPVI agonist, YM024 (1 μM) only slightly lowered the Ca^{2+} response with thrombin (Figure 6B). Thus, inhibition of PI3Kα and/or β, and only weakly of δ, with fair specificity suppressed the Ca^{2+} response induced by GPVI agonists for up to 50%.

Discussion

The present results with mouse platelets indicate that absence of the p85α regulatory PI3K subunit as well as blocking of the p110α or p110β activity significantly suppresses Ca^{2+} signaling and PS exposure elicited by GPVI-induced platelet activation under flow. For platelets from these p85α -/- mice, it has been established that selective disruption of the p85α protein does not results in significant up-regulation of the isoform p55α, p50α and p85β proteins to compensate for the lack of p85α . However, in these knock-out platelets expression of the p110α catalytic subunit (PI3Kα) is almost undetectable, along with a reduced p110β and δ expression, resulting in only 5% kinase activity[18,23,40]. That the class IA isoforms PI3Kα and β play a role in GPVI-dependent Ca^{2+} signaling was confirmed by the suppressive effects of PI103/YM024 and TGX221 which block the p110α and p110β subunits with preference, respectively. However, it should be noted that the PI3Kα inhibitors, PI103 and YM024, also have limited inhibitory activity towards PI3Kβ (S. Jackson, unpublished results).The PI3Kβ inhibitor TGX221 was unable to further suppress the lower Ca^{2+} signal in p85α -/- platelets. Importantly, these compounds showed the same degree of inhibition on GPVI-induced (with convulxin) Ca^{2+} responses in human platelets, thus indicating that the isoform-specific regulation of this signaling pathway is conserved in mouse and human platelets. Earlier work has demonstrated that collagen activation results in association of p85α with the LAT adaptor protein[41], and that absence of p85α results in defective collagen-induced platelet aggregation and degranulation[18]. The current findings significantly extend this, and indicate that p85α acts by activating p110α/β, which potentiate GPVI-induced Ca^{2+} signaling and PS exposure.

Previous studies have shown that GPVI agonist like collagen and CRP stimulate the formation of PIP_3 and $PI(3,4)P_3$ in human platelets, and that this stimulation is antagonized by wortmannin and LY294002, two structurally unrelated inhibitors of all platelet PI3K isoforms. Indeed, it has been shown that PI3K activity and PIP_3 formation

are required for full activation of PLCγ2 in response to GPVI stimulation[8,42,43]. Interestingly, the present data with mouse and human platelets indicate that combined inhibition of PI3Kα and β gives a similar reduction in Ca^{2+} response, and hence in PLCγ2 activity, as inhibition of either isoform alone. While this finding agrees with the concept that the PI3K product PIP_3 acts as a membrane anchoring site of PLCγ2, it argues against a redundancy in function of the two PI3K isoforms. One explanation is that threshold levels of PIP_3 need to be formed – i.e. by simultaneous activity of either isoform – in order to anchor and stimulate PLCγ2, perhaps because of its competition with other PH-domain containing signaling proteins. Thus, the activity of both PI3K isoforms is needed to reach sufficient lipid kinase activity.

In contrast to PI3Kα and β, the class IA PI3Kδ and the class IB PI3Kγ appeared to have a less prominent role in GPVI-induced Ca^{2+} responses. In human platelets, the PI3Kδ inhibitor IC87114 provoked a small reduction of the Ca^{2+} signal, but not the PI3Kγ inhibitor AS252424. This is in agreement with recent data with platelets from PI3Kδ deficient mice, which point to a small but detectable role for the δ isoform in mediating platelet activation by collagen[44]. Typically, under flow conditions, absence of murine p110γ resulted in a collagen-induced Ca^{2+} signal, which was only initially reduced in comparison to that of wildtype platelets. This appeared to be due to an often prolonged delay time between adhesion and start of the Ca^{2+} signal, thus pointing to a delayed onset in GPVI signaling under flow rather than to a reduction in GPVI signaling per se. This conclusion is also supported by the observation that GPVI-induced and Ca^{2+}-mediated PS exposure of platelets is not reduced in the absence of p110γ.

We and others have shown that both the PI3Kβ and γ isoforms play an enhancing role in ADP-induced integrin αIIbβ3 activation, stable adhesion and thrombus stabilization under static and flow conditions[16,19,20]. This was also apparent in the present experiments, i.e. from the reducing effects of wortmannin and ADP receptor blockage on human platelet aggregate formation under flow. However, these ADP-mediated effects of PI3K are independent of the GPVI-mediated effects, because wortmannin still suppresses collagen-induced Ca^{2+} responses and PS exposure under conditions of full blockade of the ADP receptor blockers (MRS2179, cangrelor and apyrase). Similarly, in platelet suspensions, the general PI3K inhibitors, wortmannin and LY294002, and the isoform-specific inhibitors, PI103, YM024 and TGX221, suppress GPVI-induced Ca^{2+} signaling up to 50%, again under conditions where ADP receptors are blocked. This high involvement of PI3K in Ca^{2+} rises was specific for GPVI signaling, as the same inhibitors had only a small, reducing effect on thrombin-evoked Ca^{2+} rises. This agrees well with

the earlier observation that thrombin mobilises Ca^{2+} nearly entirely via PLCβ isoforms with only little contribution of PLCγ1[5,45].

Dose-response curves with either wortmannin or LY294002 showed a similar degree of inhibition of GPVI-mediated Ca^{2+} responses in the presence of EGTA (measuring only internal Ca^{2+} mobilisation) and $CaCl_2$ (recording both Ca^{2+} mobilisation and Ca^{2+} influx from the external medium). This argues against a specific stimulation of platelet PI3Ks on Ca^{2+} entry processes. Moreover, wortmannin or LY294002 also failed to give extra inhibition of thrombin-induced Ca^{2+} responses, when Ca^{2+} entry was allowed in the presence of $CaCl_2$. Accordingly, our results do not support the earlier suggestion by others that PI3K phosphoinositides, e.g. as generated by stimulation of GPVI or thrombin receptors, stimulate a specific pathway of Ca^{2+} entry[21,22]. This discrepancy may come from the higher doses of LY294002 or wortmannin used by Lu *et al.*, at which also PI4-K activity and, thus, e.g. PIP_2 formation is affected[46]. Further, it is possible that increased Ca^{2+} influx, noted in the absence of the PIP_3-degrading SH2 domain-containing inositol 5-phosphatase (SHIP)[22], is caused by a distinct mechanism than that influenced by PI3Ks, e.g. related to the higher basal Ca^{2+} levels in SHIP -/- mice.

Taken together, the present results indicate that the principle mechanism of action of PI3K on GPVI-induced Ca^{2+} signaling and PS exposure is by enhancement of PLCγ2-mediated stimulation of Ca^{2+} depletion from internal stores, and subsequent store-mediated Ca^{2+} entry. Furthermore, particularly the PI3Kα and β isoforms, acting via p85α, appear to be involved in this activation pathway, with PKI3δ playing a minor role.

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Chapter 4

Platelet receptor interplay regulates collagen-induced thrombus formation in flowing human blood

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Abstract

The platelet glycoproteins (GPs) Ib, integrin α2β1, and GPVI are considered central to thrombus formation. Recently, their relative importance has been re-evaluated based on data from murine knockout models. To examine their relationship during human thrombus formation on collagen type I fibers at high shear (1000 s^{-1}), we tested a novel antibody against GPVI, an immunoglobulin single-chain variable fragment, 10B12, together with specific antagonists for GPIbα (12G1 Fab_2) and α2β1 (6F1 mAb or GFOGER-GPP peptide). GPVI was found to be crucial for aggregate formation, Ca^{2+} signaling, and phosphatidylserine (PS) exposure, but not for primary adhesion, even with more than 97% receptor blockade. Inhibiting α2β1 revealed its involvement in regulating Ca^{2+} signaling, PS exposure, and aggregate size. Both GPIbα and α2β1 contributed to primary adhesion, showing overlapping function. The coinhibition of receptors revealed synergism in thrombus formation: the coinhibition of adenosine diphosphate (ADP) receptors with collagen receptors further decreased adhesion and aggregation, and, crucially, the complete eradication of thrombus formation required the coinhibition of GPVI with either GPIbα or α2β1. In summary, human platelet deposition on collagen depends on the concerted interplay of several receptors: GPIb in synergy with α2β1 mediating primary adhesion, reinforced by activation through GPVI, which further regulates the thrombus formation.

Introduction

The platelet response to exposed subendothelial matrix is fundamental to thrombosis and hemostasis. Uniquely, collagen, the most abundant vessel wall protein, mediates platelet adhesion and activation, localizing and regulating the hemostatic response at sites of injury. Discovering the molecular mechanisms that control platelet-collagen interaction is crucial for understanding the pathogenesis of arteriothrombotic diseases such as stroke and myocardial infarction. Under high shear rate conditions, the glycoprotein (GP) Ib/V/IX complex allows initial platelet rolling over von Willebrand factor (VWF) bound to subendothelial collagen fibers, and subsequently collagen receptors come into contact with their specific binding sequences in the collagen. For the next step, platelet arrest and activation, firm evidence exists of a role for only 2 receptors, integrin α2β1 and immunoglobulin superfamily member GPVI, despite the apparent redundancy in collagen receptors (for a review, see Siljander PRM and Farndale RW[1]).

According to the 2-site, 2-step model, high-affinity interaction through $\alpha 2\beta 1$ stops the platelet, allowing low-affinity binding of GPVI, which generates signaling required for the subsequent thrombus formation. Platelet deposition under flow was found to be dependent on GPIb/V/IX and $\alpha 2\beta 1$[2-4],whereas no platelet deposition occurred on the GPVI-specific substrate collagen-related peptide (CRP), even under low shear rates[5]. The limited number of studies with human platelets deficient in either GPVI or $\alpha 2\beta 1$ support the 2-site, 2-step model. Blood depleted of functional GPVI showed only severe impairment of the collagen-induced "second-wave" aggregation response under flow[6]. Although thrombus volume was reduced, the initial adhesive layer of platelets was fully preserved. The few patients with defective $\alpha 2\beta 1$ displayed bleeding problems, suggesting a central role for the integrin in hemostasis[7-10]. Genetic diversity of $\alpha 2\beta 1$ expression also correlates with predisposition to thrombotic events (for a review, see Kunicki TJ[11]). Recently, several lines of evidence strongly encouraged revision of the step-wise model, promoting the idea that GPVI is the primary collagen receptor. Integrin $\alpha 2\beta 1$ was found to change its affinity for collagen when stimulated by noncollagenous agonists such as adenosine diphosphate (ADP) or thrombin, or through GPVI[12], implying that high-affinity interaction through $\alpha 2\beta 1$ requires the involvement of platelet receptors that induce secretion or inside-out signaling. Moreover, mice with Cre/loxP-mediated loss of $\alpha 2\beta 1$ from the platelets displayed undisturbed bleeding times, and their platelets exhibited normal collagen adhesion and signaling[13]. Similarly, $\alpha 2(-/-)$ mice showed no changes in platelet aggregation and adhesion to fibrillar collagen[14], whereas Fcγ (–/–) mice, generating a deficiency in the coexpressed GPVI, completely lacked a response to collagen under flow, as did wild-type mouse blood incubated with an anti-GPVI monoclonal antibody (mAb), indicating a key role for GPVI[13].

Following the deposition of platelets, their procoagulant response is essential for thrombus formation. Platelet aggregates are stabilized through fibrin formation, and newly generated thrombin recruits further platelets and activates other cells. At an advanced stage of activation, collagen-adherent platelets undergo a procoagulant transformation that includes the exposure of phosphatidylserine (PS), the secretion of coagulation factors, and the distinctive morphology of blebbing cells and microvesiculation[15]. We have previously shown that GPVI is essential for triggering this response in collagen-adherent platelets, which is preceded by extensive intracellular Ca^{2+}-signaling[16]. Supporting this, prothrombinase activity was shown to correlate with GPVI density[17] and genotype[18].

This study was undertaken to clarify the role of the human collagen receptors GPVI and $\alpha 2\beta 1$ in thrombus formation under flow. Studies with GPVI have been hindered by the lack of antagonists of human GPVI. Recently, 10B12, an anti-GPVI, single-chain, variable domain antibody fragment (scFv) was cloned by phage display from a library of human immunoglobulin variable domains, using its capacity to block the recombinant GPVI-CRP interaction[19]. Because it blocks CRP- and fibrillar collagen-induced platelet activation, 10B12 provides a small and efficient tool for human studies. To inhibit $\alpha 2\beta 1$ function, a collagen-derived triple-helical peptide, GFOGER-GPP[20], was used along with well-defined inhibitory mAbs[21,22]. Simultaneous ADP and collagen receptor antagonism were also studied. Under high shear rate conditions, specific parameters of thrombus formation—surface coverage of platelets, aggregate size distribution, and PS exposure—were monitored. Further, the intracellular Ca^{2+} response, which regulates these platelet processes and secretion and thromboxane A_2 (TxA_2) production, was measured from adherent platelets.

Our results show that GPVI is crucial to collagen-induced Ca^{2+} responses, aggregate formation, and PS expression but that $\alpha 2\beta 1$ also contributes to these processes. Although GPVI is not directly involved in primary platelet adhesion to collagen, the inhibition of GPVI, along with GPIb and $\alpha 2\beta 1$, was mandatory to eradicate thrombus formation.

Materials and methods

Materials

Fibrillar type I collagen (Horm) from equine tendon was obtained from Nycomed (Munich, Germany). Apyrase (grade 7), heparin, and MRS2179, a $P2Y_1$ antagonist, were from Sigma (St Louis, MO), H-Phe-Pro-Arg chloromethyl ketone (PPACK) was from Calbiochem (La Jolla, CA), and Fluo-3 and calcein acetoxymethyl esters were from Molecular Probes (Leiden, The Netherlands). Annexin V (Apoptest) labeled with Oregon Green 488 (OG488) was obtained from Nexins Research (Hoeven, The Netherlands). AR-C69931MX, an antagonist of the $P2Y_{12}$ receptor, was from Astra-Zeneca (Charnwood, United Kingdom). Acetylsalicylic acid (ASA) was from Lorex Synthelabo (Maarssen, The Netherlands), and low-molecular–weight heparin (Fragmin) was from Pharmacia N.V. (Puurs, Belgium). The GFOGER-GPP and CRP peptides were synthesized as described[20,23]. ScFv antibodies 10B12 and 1C3[19] and mAb 12G1 against GPIbα has been previously described[24,25]: 12G1 inhibits platelet deposition to type 1

collagen by 90% at 5 µg/mL, ristocetin-induced platelet aggregation with an IC_{50} of 1.25 µg/mL, ristocetin-induced VWF binding to GPIb by 35%[24], and 35% of thrombin generation under intermediate shear[25]. Fab_2 fragments were generated by standard methodology. The mAb 6F1 against α2 was a generous gift from Prof B. Coller (Mount Sinai Hospital, New York, NY)[21]. The anti-β1 mAb 4B4 was from Beckman Coulter (High Wycombe, United Kingdom). Saratin was kindly provided by Dr M. Hoylaerts (KU, Leuven, Belgium)[26].

Blood collection

Blood from 9 healthy volunteers was collected in 40 µM PPACK in 0.1 vol saline, supplemented hourly with 10 µM PPACK. Donors had not taken medication for 2 weeks. Platelet counts were determined with a Coulter counter (Coulter Electronics, Hialeah, FL). Donors were genotyped for receptor polymorphisms: GPVI (a/b), α2β1 (α2 807 C/T), and GPIb (5-C/T) using TaqMan at the National Blood Service (Cambridge, United Kingdom). All donors expressed high GPVI levels (7 aa, 2 ab)[18]. Five donors were CT for C807T α2 polymorphism, implying an average density of α2β1 on the platelet surface. Three donors were low expressors of α2β1 (CC), and one was a high expressor (TT). In this small population, C807T α2 polymorphism had no apparent effect on any of the platelet responses. Two donors were CT for GPIb C5T.

Static platelet adhesion

Static platelet adhesion to CRP and fibrillar collagen was performed exactly as previously described[27], using cation-free conditions to examine the adhesive role of GPVI in the absence of α2β1.

Intracellular Ca^{2+} and thrombus volume measurements

Acid citrate dextrose–platelet-rich plasma (ACD-PRP) was incubated with 7 µM Fluo-3 acetoxymethyl ester or calcein for 30 minutes at 37°C. Platelets were centrifuged with 0.1 vol ACD, washed once with HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer containing 10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose, 0.1% bovine serum albumin (BSA), pH 6.6, centrifuged again with 0.1 vol ACD, and suspended in the original volume of PRP with HEPES buffer, pH 7.45. PPACK-anticoagulated blood was spiked with Fluo-3–labeled platelets at 10% of the original platelet count and with 30 µM PPACK.

In control perfusions over collagen, 30% of 7 µM calcein-prelabeled autologous platelets were added. In real-time, *Z*-stacks of *x*-*y* scans were taken during the perfusion (box volume of 171.8 x 171.8 x 50 µm) at a 2-photon excitation wavelength of 800 nm and an emission of 485 to 515 nm (8% of full power) using a Radiance 2000 multiphoton laser scanning fluorescence microscope system (BioRad, Hemel Hempstead, United Kingdom), equipped with a pulsed sub-picosecond Tsunami Ti:sapphire laser (Spectra-Physics, Mountain View, CA). Two-photon images were analyzed with Laserpix software (Bio-Rad).

Flow experiments and image recording

Whole blood perfusion experiments were performed essentially as described for mouse blood[28]. Briefly, glass coverslips were coated with collagen fibers (12.5 µg/cm^2) and blocked with HEPES buffer, pH 7.45, containing 1% BSA and 1% glucose. The blood was placed in a syringe and perfused over the coverslip through a transparent 50 µm deep chamber using a pulse-free pump[29], at a shear rate of 1000 s^{-1} for 4 minutes. Blood was incubated for 15 minutes before perfusion with various concentrations of scFv 10B12 or 1C3, 10 to 20 µg/mL 6F1, 500 µg/mL GFOGER-GPP, 10 µg/mL 4B4, 20 µg/mL Fab_2 fragment 12G1, ASA (100 µM), MRS2179 (40 µM), and AR-C69931MX (20 µM) or 1 U/mL apyrase, as described. Where indicated, autologous Fluo-3–labeled platelets were added to the blood before antagonists.

Microscopic phase-contrast and fluorescent images from Fluo-3–labeled platelets were recorded in real-time using a Visitech digital imaging system (Sunderland, United Kingdom) equipped with 2 intensified, charge-coupled device (CCD) cameras[30]. After perfusion, flow chambers were rinsed at the same flow rate for 4 minutes with HEPES buffer, pH 7.45, supplemented with 1 U/mL heparin and 2 mM $CaCl_2$. Phase-contrast and fluorescent images were collected with a 40x UV-transparent objective and 15x to 20x image magnification. Exposure of PS was detected after incubation of the slide with 100 µL HEPES/$CaCl_2$ buffer, pH 7.45, containing OG488-labeled annexin V (1 µg/mL) for 5 minutes. Antibodies and antagonists were also added to the rinsing buffer and the annexin V incubation.

Image analysis

Surface coverage from phase-contrast images was analyzed using Image-Pro (Silver Spring, MD) software version 4.1, and it was analyzed from platelets stained with OG488-annexin V using Quanticell software (Visitech). Distribution of aggregate sizes in

phase-contrast images was measured using Leica QWin image analysis software (Leica Imaging Systems, Cambridge, United Kingdom). Changes in Fluo-3 fluorescence from individual platelets were converted to $[Ca^{2+}]_i$ as described[31]. To provide a measure of the proportion of procoagulant cells independent of platelet deposition, the ratio of annexin V–binding surface coverage to phase-contrast surface coverage was calculated and was termed procoagulant index (Pi). Although the procoagulant area was overestimated through fluorescent glare in the optics, Pi provided a means of distinguishing the effects of treatments on procoagulant expression from those on platelet deposition.

Experimental design and statistics

Each experimental condition was tested on at least 3 occasions using blood from different donors, and each donor provided blood for at least 10 perfusion experiments. Control conditions were included for each blood sample, together with other permutations of conditions. For each perfusion surface, images from 9 random microscopic fields were collected, and the average percentage area covered by adherent platelets was measured either by phase-contrast or by OG-labeled annexin V fluorescence. Phase-contrast, fluorescent surface coverage (mean ± SE) and Pi were compared among all experimental groups using analysis of variance (ANOVA), with Newman-Keuls or Dunnett posttesting. Data derived from the same donors were compared by paired sample *t* tests or ANOVA. The effect of ADP blockade under different conditions was tested by 2-way ANOVA. Image analysis provided estimates (area and roundness of separate features, such as platelets and aggregates) of thrombus size and shape. Features were segmented digitally and measured with minimal operator intervention. From an average of 9 images, using 3 different donors, at least 200 different features were measured for each treatment, the effects of which were determined by χ^2 analysis. Data are plotted as frequency distributions with mean values per field (± SE). Prism (GraphPad Software, San Diego, CA) was used throughout for multiple comparisons and linear regression.

Results

Blocking of GPVI abolishes aggregate formation, Ca^{2+} signaling, and PS exposure without eradicating primary platelet adhesion

PPACK-anticoagulated whole blood was perfused at high shear rate (1000 s^{-1}) over a surface covered with native collagen type I fibrils. Phase-contrast and fluorescence microscopic images were captured to monitor 2 independent parameters of thrombus formation measured as the surface coverage of deposited platelets and of procoagulant, PS-exposing platelets that stained with OG488-conjugated annexin V. In control perfusions, platelets rapidly adhered to the collagen fibers, forming aggregates that later coalesced (Figure 1A). The deposition of platelets measured by surface coverage was linear in time, indicating regular and continuous scavenging of platelets (Figure 2A). To confirm that the measuring surface area represents a linear progression of thrombus build-up, the thrombus volume was measured by using calcein-labeled platelets from the same donor and real-time scanning of the 2-photon fluorescence signal, which even at more than 50 µm penetration was typically not distorted by the

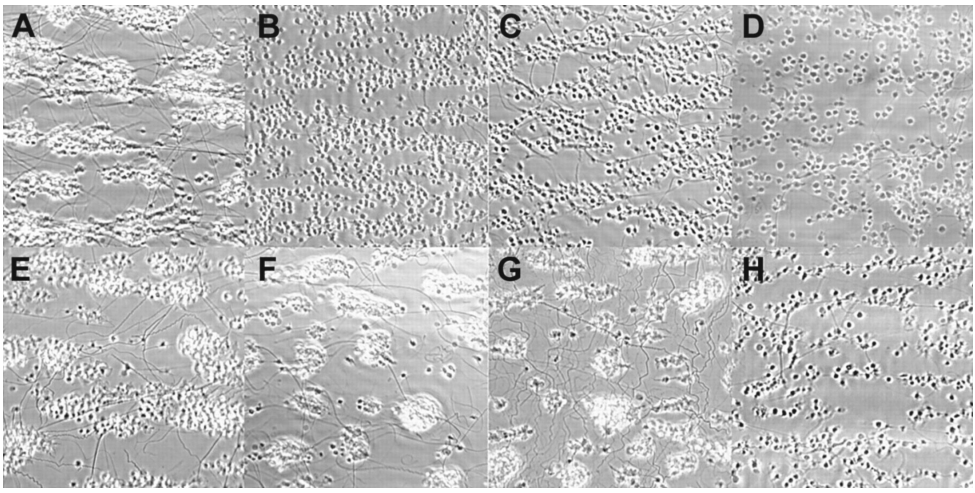

Figure 1 Effects of inhibitory antibodies and receptor antagonists on thrombus formation. Images (120 x 120 µm) were recorded using a phase-contrast microscope after the perfusion of PPACK-anticoagulated whole blood over a collagen-coated surface for 4 minutes at a shear rate of 1000 s^{-1} Antibodies or inhibitors were added 15 minutes before perfusion. (A) Control. (B-D) 10B12 at 50, 100, or 300 µg/mL, respectively. (E) scFv 1C3 at 100 µg/mL. (F) 6F1 at 20 µg/mL. (G) GFOGER-GPP at 500 µg/mL. (H) ADP-receptor and TxA_2 blockade by 40 µM MRS2179, 20 µM AR-C69931MX, 1 U/mL apyrase, and 100 µM ASA. Images shown are representative of 3 to 9 independent experiments. Collagen fibers are visible in some of the panels. Original magnification, x 60.

flowing blood. Again, thrombus volume increased linearly in time (Figure 2B). No differences in platelet deposition were observed within the donor population, relating to genetic polymorphisms of GPVI, $\alpha 2\beta 1$, or GPIbα. By the 4-minute end point, 17.9% ± 0.8% of the surface was covered by platelets. Most platelets were deposited as interconnected islets originating from individual aggregates. The mean feature size was 176 ± 16 μm^2, an area corresponding to approximately 40 platelets. Single platelets made up 22% of features, whereas 10% were large aggregates corresponding to 130 platelets or more (Figure 3A). Large numbers of blebbing platelets were observed in direct contact with collagen fibers, and staining with OG488-annexin V revealed 8.3% ± 0.7% coverage of PS-exposing platelets. To provide a measure of procoagulant platelets independent of the total number of adherent cells, the ratio of the 2 surface parameters was expressed as Pi, giving a value of 0.43 ± 0.03. Thrombin generation was absent during and after perfusion because no fibrin or D-dimer was observed. Including Fragmin (0.5 U/mL) as an additional anticoagulant did not change platelet deposition or annexin V binding from PPACK-only controls (data not shown).

Blocking GPVI by 10B12 inhibited CRP- and collagen-induced platelet aggregation *in vitro*[19]. Static platelet adhesion to CRP was abolished at 5 µg/mL and decreased by 80% ± 4% (n = 4) to collagen fibers at 25 µg/mL. However, under flow, a concentration curve for 10B12 (Figure 4) showed that, up to 300 µg/mL, surface coverage with platelets did not significantly decrease (r^2 = 0.22; P = .42), though,

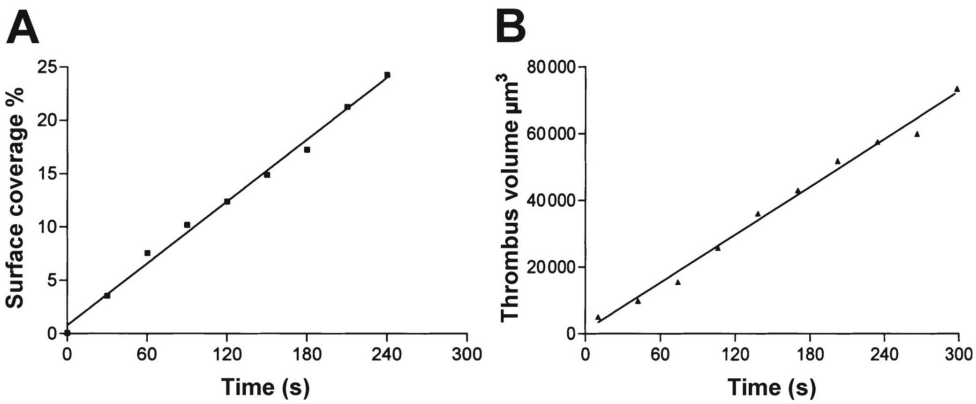

Figure 2 Thrombus formation measured by surface area coverage or thrombus volume is linear in time. (A) Phase-contrast video images captured during the flow of PPACK-anticoagulated whole blood over collagen at 1000 s^{-1} were analyzed for platelet surface area coverage, which was plotted against perfusion time. Linear regression showed R^2 = 0.97-0.99 (n = 3). (B) Thrombus volume was measured using blood supplemented with 30% of calcein-labeled autologous platelets. *Z*-stacks of *x-y* scans were measured in real-time by 2-photon laser scanning microscopy. Fluorescence image stacks were reconstructed 3 dimensionally and analyzed. Plots of thrombus volume compared with time were linear up to 300 s (R^2 = 0.99). Representative plots are shown in panels A and B.

dramatically, platelet aggregates were absent (Figure 1B-D).

Biosensor data indicated that at 300 µg/mL 10B12, occupancy of recombinant GPVI had reached more than 97% (data not shown). The predominance of single platelets with increasing 10B12 concentrations, obvious to the eye, was quantified by image analysis as almost complete elimination of larger aggregates ($P < .0001$) at 50 µg/mL 10B12, and the mean area was reduced to 37 ± 2 μm^2 from 176 ± 16 μm^2 ($P < .001$) (Figure 3B). In contrast to platelet adhesion, surface coverage of PS-exposing platelets decreased progressively with the 10B12 concentration with an IC_{50} value of 23 µg/mL (r2 = 0.94; P < .01) (Figure 4), and blebbing platelets were no longer visible. At the highest concentration of 10B12 tested, the Pi was reduced to 0.02 ± 0.005 ($P < .001$), indicating strong blockade of GPVI function.

ScFvs 1C3 and 10B12 recognizes distinct epitopes, and 1C3 is incapable of blocking static platelet adhesion to CRP or collagen-induced platelet aggregation[19]. As expected, 1C3 (100 µg/mL) did not alter either the surface coverage (Figure 1E) or the size and morphology (Figure 3C) of the formed thrombi. However, 1C3 halved the PS expressing surface coverage (Figure 4), suggesting that the 1C3-binding site is relevant for GPVI function, possibly by influencing receptor clustering or dimerization. The Pi obtained with 1C3 was reduced to 0.24 ± 0.07 ($P < .05$, relative to control), a significantly higher Pi than with 100 µg/mL 10B12 ($P < .001$). Coinhibition with 10B12 and 1C3 did not change surface coverage from that of 10B12 alone (data not shown).

To study the dynamics of platelet activation, Ca^{2+} signal generation was measured in real-time under flow from single, Fluo-3-labeled platelets coming in contact with collagen. Control platelets exhibited a strong Ca^{2+} response, sometimes preceded by an initial Ca^{2+} spike (Figure 5A). A submaximal dose (50 µg/mL) of 10B12 decreased the Ca^{2+} signal amplitude (Figure 5B). Although blocking GPVI did not abolish primary adhesion, the increased translocation of platelets across the collagen surface was observed during Ca^{2+} measurements. Increasing the 10B12 concentration to 300 µg/mL reduced the Ca^{2+} signal slightly further (Figure 5C). No difference from controls was observed in the averaged Ca^{2+} response with 1C3 (100 µg/mL), though Ca^{2+}-spiking occurred in individual platelets (Figure 5D). Together, these data indicate that blocking of GPVI with 10B12 potently suppressed collagen-dependent platelet activation pathways without eradicating primary adhesion.

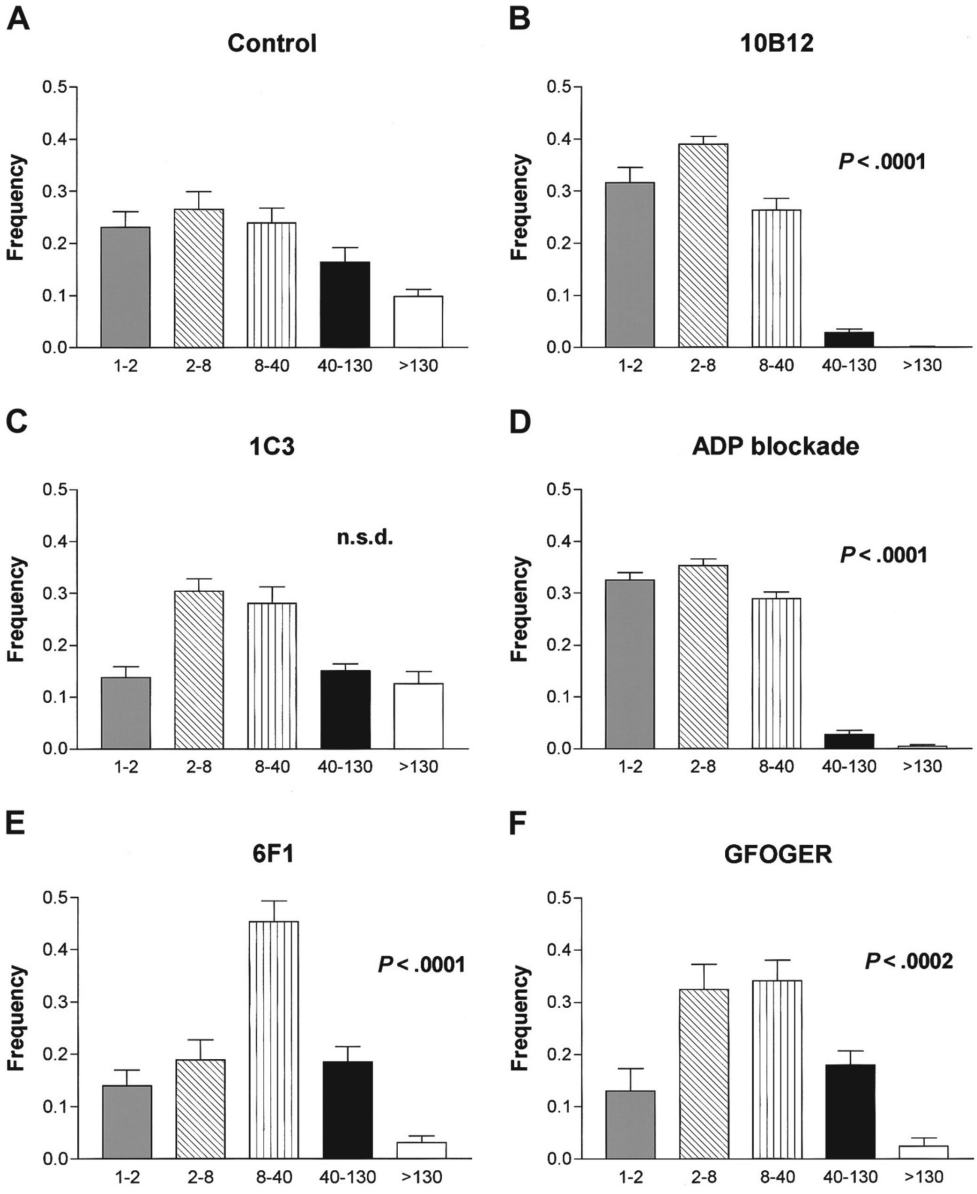

Figure 3 Effects of inhibitory antibodies and receptor antagonists on size distribution of platelet aggregates during thrombus formation. Histograms were obtained by image analysis of phase-contrast micrographs obtained from perfused collagen surfaces. Estimated numbers of platelets per feature were 1 to 2 ( ), 2 to 8 ( ), 8 to 40 ( ), 40 to 130 ( ), and more than 130 platelets ( ). (A) Control. (B) 10B12 at 50 µg/mL. (C) 1C3 at 100 µg/mL. (D) ADP receptor and TxA_2 blockade. (E) 6F1 at 20 µg/mL. (F) GFOGER-GPP at 500 µg/mL. Data (A-F) are mean ± SE from 3 donors. Statistical significance compared with the control was established from contingency tables (χ^2 analysis).

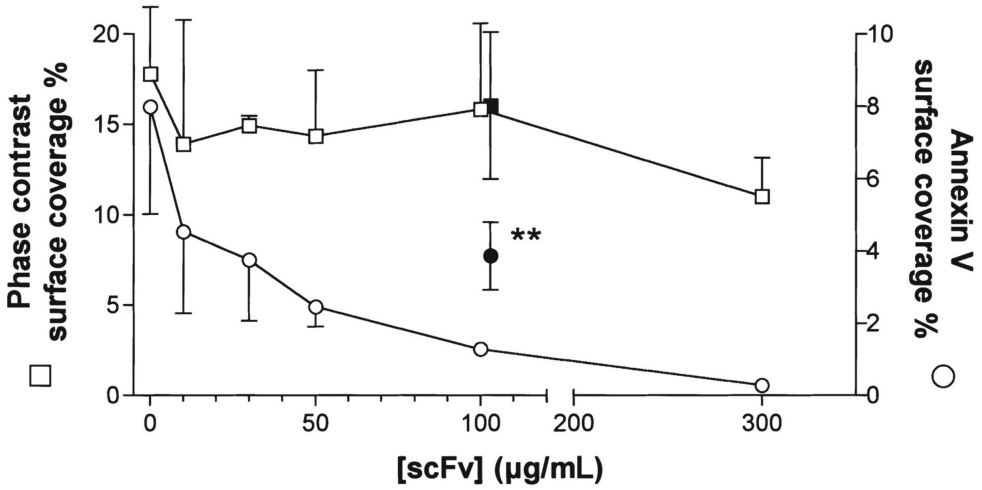

Figure 4 Inhibition of GPVI causes dose-dependent reduction in platelet PS exposure but not in total surface coverage. Blood was treated with 10B12 at 10-300 µg/mL (white symbols) or 1C3 at 100 µg/mL (black symbols). Total surface coverage of platelets (squares) and surface coverage of OG488-labeled annexin V binding platelets (circles) were measured after 4 minutes of perfusion. Data represent mean ± SE from 3 to 17 experiments performed on the blood of 3 donors. **Significant difference from 10B12 treatment ($P < .001$) and from control ($P < .05$).

Blocking of GPIb or α2β1 reduces primary adhesion and aggregate formation, but blocking only α2β1 inhibits PS exposure

High levels of VWF and high α2β1 density have been correlated with increased platelet deposition on collagen[32,33], whereas patients with VWF concomitant with low α2β1 expression bleed more than patients with either condition alone[34]. The essential nature of GPIb and α2β1 in human platelet deposition on collagen under flow has been emphasized[23,32,35], though other studies failed to detect consistent involvement of α2β1[4]. This prompted us to revisit the topic in the context of molecular interplay with GPVI using the following antagonists: 12G1 Fab_2 fragment, recognizing amino acids 1 to 59 of GPIbα, hinders platelet adhesion to collagen by inhibiting VWF-GPIb interaction at high-shear[24,25]; anti-α2 mAb 6F1, whose epitope within the I-domain is separate from the MIDAS motif and lies between residues 173 and 259[36]; and the GFOGER-GPP peptide, which inhibits collagen binding through the α2 MIDAS[37].

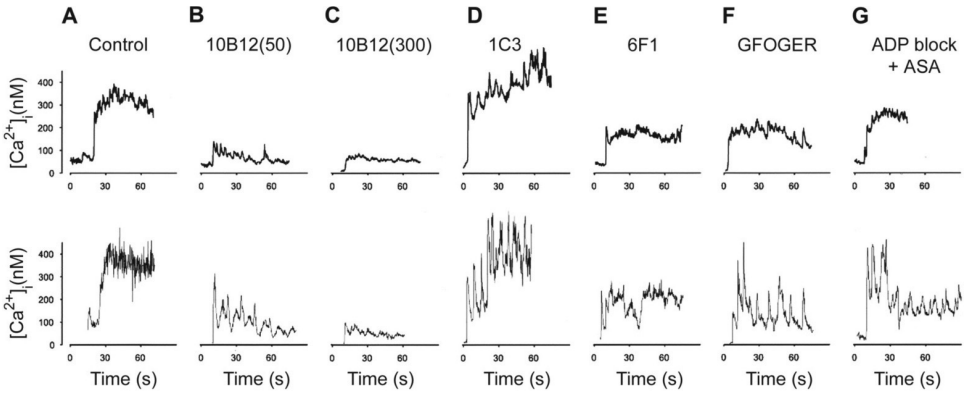

Figure 5 Effects of inhibitory antibodies and receptor antagonists on Ca^{2+} responses of platelets interacting with collagen under flow. Blood was spiked with 10% Fluo-3–loaded autologous platelets, incubated with antagonists, and perfused over collagen, as indicated for Figure 1. Changes in $[Ca^{2+}]_i$ were recorded during perfusion in single collagen-adherent platelets. Traces above are averaged curves from 15 to 25 platelets (3 donors); traces below are representative curves from single platelets. (A) Control. (B) 10B12 at 50 µg/mL. (C) 10B12 at 300 µg/mL. (D) 1C3 at 100 µg/mL. (E) 6F1 at 20 µg/mL. (F) GFOGER-GPP at 500 µg/mL. (G) ADP-receptor and TxA2 blockade, as in Figure 1.

The importance of GPIb in primary adhesion[4] was reconfirmed under the shear rate conditions used here. Using 12G1 Fab_2 at a maximally effective dose of 40 µg/mL, the overall surface coverage was reduced by 50% ± 14% (Figure 6), which confirms that at (arterial) shear rates of approximately 1000 s^{-1}, platelet adhesion to collagen is only partially dependent on VWF-GPIb interaction[38,39]. Although in 2 donors surface coverage was strongly inhibited (63% and 67%), 2 gave a weaker reduction in thrombus formation (38% and 45%), but the differential response did not correlate with –5C/T polymorphism. For all donors, blocking of GPIb strongly influenced platelet tethering. GPIbα-blocked platelets moved swiftly over the collagen surface, but, once adherent, they became fully activated. Adding 12G1 Fab_2 reduced the PS exposure of platelets, but this simply reflected the decrease in surface coverage (Figure 6A) without a change in Pi. Like 12G1, saratin, which blocks the binding of VWF to collagen[40], caused only a partial reduction of platelet surface coverage (68% ± 14.5%) and annexin V staining (27% ± 0.3%; n = 2).

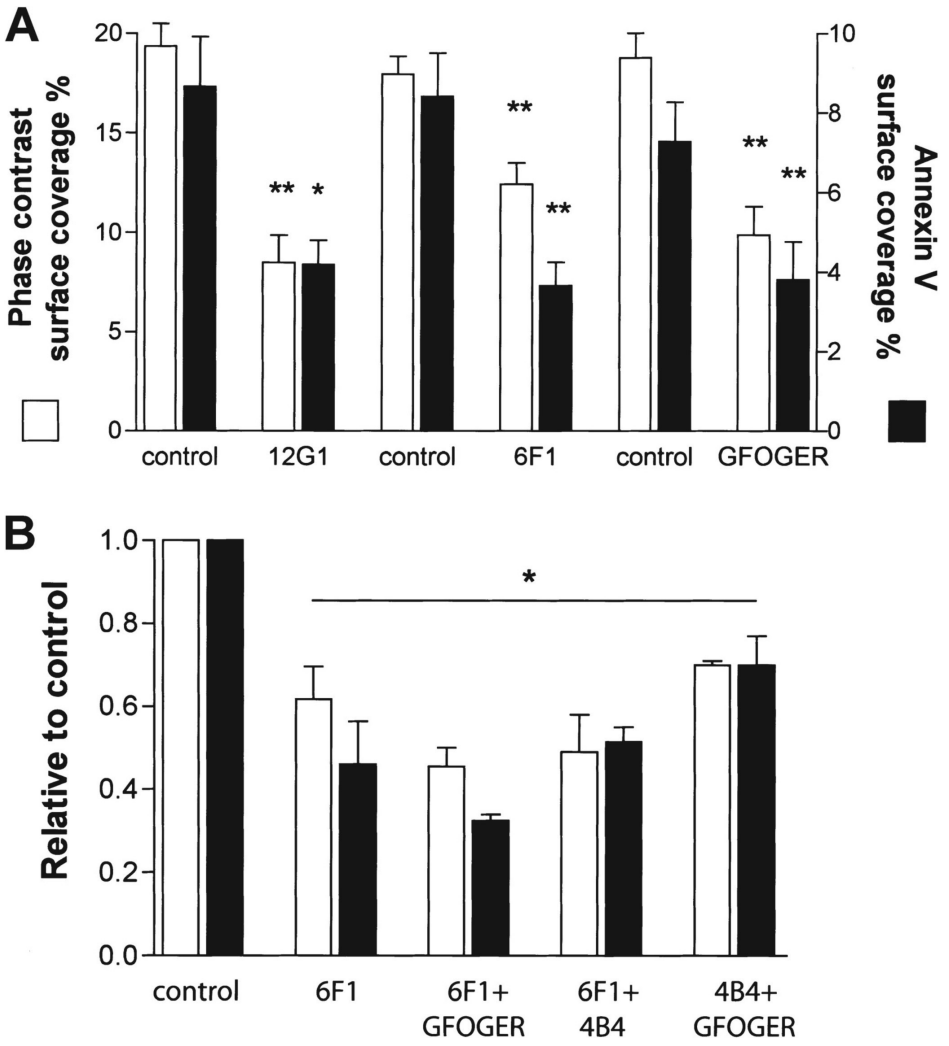

Figure 6 Blocking of GPIbα or α2β1 partially reduces surface coverage on collagen, but blocking only of α2β1 affects PS exposure. (A) Blood was treated with 12G1 Fab_2 at 40 μg/mL and 6F1 at 20 μg/mL or GFOGER-GPP at 500 μg/mL and was perfused over collagen, as described for Figure 1. Data present surface coverage of platelet deposition or OG488-labeled annexin V binding (mean ± SE, from 4-6 experiments). ***P* < .01, and **P* < .05 compared with matched controls. (B) Blood was treated with combinations of anti-α2 6F1 or GFOGER and anti-β1 4B4 (10 μg/mL). Data (mean ± SE) present the proportion of phase- or annexin V–positive surface coverage relative to matched controls. All combinations significantly inhibited platelet responses compared with respective controls (**P* < .05; n = 2) but not with each other.

Inhibition of α2β1 integrin by mAb 6F1, at a saturating concentration of 10 to 20 μg/mL, reduced surface coverage in all donors by approximately 30% ± 11% (Figure 6A). The tethering platelets reluctantly anchored to collagen fibers and, on arrest, formed only small, dense aggregates (Figure 1F). Image analysis indicated a smaller mean

aggregate size of 117 ± 9 μm^2 with a shift toward round structures ($P < .001$). Aggregate sizes showed a kurtotic distribution ($P < .0001$), with a 50% increase in mid-sized features (Figure 3E). Similarly, GFOGER-GPP significantly reduced surface coverage by approximately 45% ± 13% at a maximally effective dose of 500 μg/mL (Figure 6A). Changes in aggregate morphology closely matched those obtained with 6F1 mAb—that is, aggregates appeared as round structures (Figure 1G), of smaller, average size (Figure 3F).

Inhibition of $\alpha 2\beta 1$ with either mAb 6F1 or GFOGER-GPP had a small but significant effect ($P < .01$) on the exposure of PS (Figure 6A). The Pi was reduced from 0.43 ± 0.03 to 0.30 ± 0.04 ($P < .05$) and 0.37 ± 0.08 (not significant), respectively. To ensure maximal blocking of $\alpha 2\beta 1$, we tested combinations of antagonists binding different epitopes. The most effective of 3 anti-$\beta 1$ mAbs tested, 4B4—against the activatory hinge region 207 to 21822—caused less inhibition than GFOGER or 6F1 (data not shown). Slight further inhibition was seen when 6F1 was combined with 4B4 or GFOGER, but these reductions were not significant (Figure 6B). A role of $\alpha 2\beta 1$ in platelet signaling was also supported by the decreased Ca^{2+} response of the collagen-adherent platelets. With both 6F1 (Figure 5E) and GFOGER-GPP (Figure 5F), the average Ca^{2+} response was decreased, and the responses of most single platelets showed transient or spiking increases in intracellular Ca^{2+}. These findings reconfirm the contribution of GPIb and $\alpha 2\beta 1$ to collagen-induced thrombus formation but underscore their different roles. Blocking GPIb interfered with platelet tethering and initial anchorage and dispersed aggregates with little effect on PS exposure, but $\alpha 2\beta 1$ blockade reduced primary adhesion (partially by prolonging firm anchorage) and restricted thrombus size, platelet activation, Ca^{2+} signaling, and PS exposure.

Combined inhibition of GPVI with either GPIb or α2β1 integrin completely abolishes thrombus formation

Coinhibition of GPVI with 10B12 (100 μg/mL) and of GPIb with 12G1 Fab_2 (40 μg/mL) resulted in almost complete abolition of platelet adhesion, with remaining single platelets covering not more than 2% of the surface; PS-exposing platelets were hardly detected (Figure 7A-B). Similarly, coinhibition of GPVI and $\alpha 2\beta 1$ with 6F1 (20 μg/mL) or GFOGER-GPP (500 μg/mL) completely inhibited stable platelet adhesion and all subsequent activation events, eradicating thrombus formation (Figure 7A-B). Calcium responses, measured in platelets adhering in the presence of 6F1 and 10B12, resembled the responses measured in the presence of 10B12 alone (data not shown).

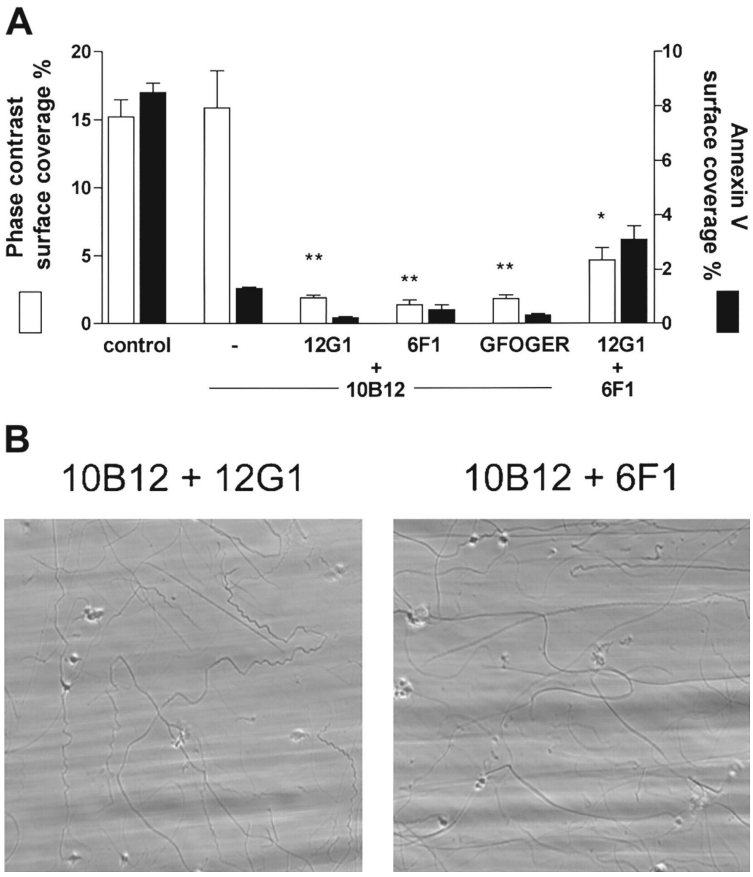

Figure 7 Combined inhibition of GPVI with GPIb or α2β1 abolishes collagen-induced thrombus formation. Blood was treated with the indicated combination of antagonists before perfusion over collagen: 10B12 at 100 µg/mL and 6F1 at 10 to 20 µg/mL, GFOGER-GPP at 500 µg/mL or 12G1 Fab_2 at 40 µg/mL perfused over collagen as in Figure 1. (A) Surface coverage of platelet deposition or OG488-labeled annexin V binding (mean ± SE, from 4-6 donors). ***P* < .001, and **P* < .05 compared with single antibody controls. (B) Representative phase-contrast images after perfusion, also showing visible collagen fibers. Original magnification, x 60.

In contrast, combined blocking of GPIb and α2β1 had a less dramatic effect on platelet deposition and aggregation because significant numbers of small aggregates could still be observed. Surface coverage after combined blockade of GPIb with α2β1 was reduced by approximately 70% (Figure 7A), a greater effect than achieved by blocking either receptor individually (*P* < .05). The remaining platelets were in an activated state, judged by the relatively high surface coverage of PS-exposing platelets and the apparent increase in Pi to 0.72. In conclusion, our data show that GPIb and α2β1 have partially overlapping functions in mediating human platelet adhesion and GPVI-dependent thrombus formation.

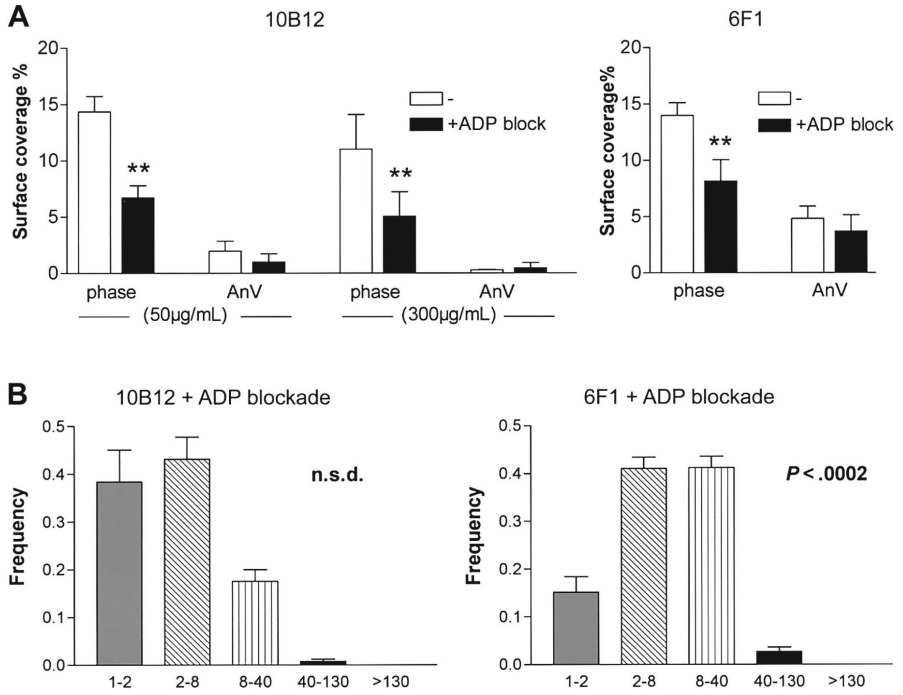

Figure 8 ADP antagonism further reduces thrombus formation after the inhibition of GPVI or α2β1. Blood was treated with 10B12 at 50 or 300 µg/mL, 6F1 at 20 µg/mL with or without ADP receptor antagonism (40 µM MRS2179, 20 µM AR-C69 931MX, and 1 U/mL apyrase). (A) Total surface coverage of platelets and of annexin V–positive platelets was measured (mean ± SE from 4-6 donors). ***P* < .01 compared with control condition. (B) Histograms of feature sizes from phase-contrast micrographs, determined as in Figure 2, where individual antibody effects are shown. Estimated numbers of platelets per feature were 1 to 2 (▤) , 2 to 8 (▨), 8 to 40 (▥) , 40 to 130 (■), and more than 130 platelets (□). Data are means ± SE from 3 donors.

Blocking of ADP receptors further inhibits thrombus formation restricted by GPVI or α2β1 blockade

ADP regulation of thrombus size has been attributed to the platelet $P2Y_{12}$ purinergic receptor; $P2Y_1$ regulates the onset of thrombus formation[41]. To obliterate any effects of ADP, we used apyrase in conjunction with specific $P2Y_{12}$ and $P2Y_1$ inhibitors. Full inhibition of ADP- or TxA_2-mediated (inhibited by ASA) effects reduced the surface coverage by 43% (*P* < .001). The morphology of platelet deposition was similar to that under GPVI blockade: a layer of single platelets remained on the collagen (Figure 1H), and aggregates were eradicated (Figure 3E). However, combined blockade of ADP and TxA2 actions had no effect on Pi (0.53 ± 0.15 [not significant]) or platelet blebbing, though it slightly reduced the Ca^{2+} response (Figure 5G).

Inhibiting the ADP pathway further decreased surface coverage when combined with GPVI blockade. Irrespective of the 10B12 concentration (50 or 300 µg/mL), ADP receptor antagonism caused an additional reduction in platelet surface coverage (55%; *P* < .001) (Figure 8A), suggesting that the presence of ADP was not solely derived from GPVI-dependent secretion. Because GPVI inhibition itself abolished aggregate formation, the additional blockade of ADP receptors had no further effect on the mean feature size: 24 ± 2.6 µm2 (ADP + GPVI blockage) and 23 ± 1.8 µm2 (GPVI blockade alone) (Figure 8B). When applied together with 10B12, ADP receptor blockade reduced the number of PS-exposing platelets by attenuating platelet deposition because the Pi did not significantly decrease.

When the ADP receptors were coinhibited with $\alpha 2\beta 1$ (20 µg/mL 6F1), surface coverage decreased by 41% (Figure 8A), and the mean aggregate size decreased from 121 ± 8 to 44 ± 4 µm2 (P < .0002) (Figure 8B). Again, this was accompanied by a small decrease in PS-exposing platelets and an unaltered Pi value. These results let us conclude that ADP (from various sources) stimulates GPVI- and $\alpha 2\beta 1$-dependent adhesion and contributes to aggregate formation but is insufficient to promote PS exposure. Thus, platelets respond to ADP by inducing one of the thrombus end points, aggregation, in contrast to full activation through GPVI.

Discussion

The present study shows that, in humans, collagen-induced thrombus formation under high shear rate requires the orchestrated interplay of several platelet receptors. Using antibodies and peptides allowed clarification of the complementary and overlapping roles of the GPIb/V/IX complex, $\alpha 2\beta 1$ integrin, GPVI, and the platelet purinergic receptors in the processes of platelet adhesion, aggregation, and procoagulant activity. Our results, though formally in line with the original 2-step adhesion-activation model, also revealed its oversimplification. Although the main role of GPIb is to provide initial contact with collagen-bound VWF, it stabilizes the anchorage of thrombi on collagen together with $\alpha 2\beta 1$. GPVI is involved in GPIb- and $\alpha 2\beta 1$-mediated adhesion through activation, and it is the main signaling receptor during thrombus formation. Conversely, $\alpha 2\beta 1$ assists GPVI in signaling. Complete abrogation of adhesion and subsequent activation thus requires simultaneous inhibition of GPVI together with either GPIb or with $\alpha 2\beta 1$.

We find that GPVI has a crucial role in regulating human thrombus growth. Inhibiting GPVI resulted in the complete loss of platelet aggregates and the full inhibition

of collagen-induced Ca^{2+} mobilization and PS exposure. In contrast, the adhesion of single, nonaggregated platelets was not significantly diminished unless ADP was also antagonized, probably eliminating the contribution of $\alpha 2\beta 1$. These results corroborate findings from earlier flow studies with GPVI-deficient platelets in which the surface coverage equaled that obtained with $\alpha IIb\beta 3$ antagonists[6]. A recent study using the blood of patients with GPVI deficiency also showed the obliteration of collagen-induced thrombus formation though a layer of single platelets was still visible in the PDF-format micrographs[42], supporting our conclusions. In contrast, these results clearly deviate from those obtained in the first flow study using GPVI-deficient mice[13], which showed the total abolition of platelet-collagen adhesion. In mice, GPVI function was severely impaired when more than 90% of receptors are blocked. At the highest level of the scFv 10B12 used here (300 μg/mL), calculated receptor occupancy was 97%, supporting the idea that unaltered platelet adhesion was not caused by the inadequate inhibition of GPVI. Although it is possible that antibody dissociation or displacement by ligand may allow a proportion of GPVI to remain functional during multireceptor interaction under flow, 10B12 caused the near-complete, concentration-dependent abolition of platelet activation processes such as PS exposure, Ca^{2+} response, and aggregate formation. While this paper was in preparation, a true GPVI knockout model was generated in mice in which thrombus formation was similar to that observed in our study[43]. The reasons for the discrepancies regarding the different mouse data remain to be elucidated.

Both GPIb and $\alpha 2\beta 1$ were implicated in the primary adhesive step at high shear rate, as others suggested after using recombinant receptors in liposomes[44]. Inhibiting GPIb or $\alpha 2\beta 1$ on its own, but not GPVI, dramatically reduced the number of collagen-adherent platelets, though those remaining became activated. The interplay of GPVI with both receptors is implicit in the near-complete suppression of adhesion using the combined blockade of GPIb and GPVI or $\alpha 2\beta 1$ and GPVI. This indicates that all 3 receptors are required for optimal adhesion. As a first step, GPIb tethers the platelet, allowing other receptors to interact, which perhaps further regulates GPIb function, as recently reported[42,45]. Adhesion through $\alpha 2\beta 1$ follows closely, with GPVI and $\alpha 2\beta 1$ acting interdependently. Initial $\alpha 2\beta 1$ binding to high-affinity collagen sequences, not requiring activation, supports the weak GPVI-collagen interaction, which, in turn, mediates the switch of the integrin to the high affinity required for stable adhesion. In line with this, blocking GPVI with 10B12 caused a more dramatic (but not full) reduction in surface coverage on collagen type III containing only low-affinity xxxGER sequences, but not on collagen type I containing the high-affinity GFOGER sequence used in this study (P.A.S.,

unpublished data, 2002). Collagen-dependent differences in GPVI inhibition were also observed elsewhere[6]. The failure of α2β1 blockade to cause further reduction in Ca^{2+} response and PS exposure in the presence of 10B12 implies that α2β1 signaling occurs downstream from GPVI. This conclusion was reinforced by almost complete loss of binding to adherent platelets, in the presence of 300 μg/mL 10B12, of an antibody that recognizes only the fully activated state of α2-integrin, IAC-1 (7F6)[46]. However, the signaling end points of this study (Ca^{2+}, PS exposure, and aggregate size) were measured to identify the contribution of specific receptors rather than to study the detail of the signaling processes, which will be subject to further study.

The present model of human thrombus formation deviates in certain respects from that derived from murine data. Although murine thrombus formation seems to rely more on GPVI, it was recently shown that the interplay of α2β1 with GPVI is required for full GPVI-induced platelet activation and procoagulant activity, using β1-deficient mouse platelets[28]. In addition, in another α2(–/–) mouse flow model, platelet-collagen interaction was severely impaired[47]. Thus, the main deviation between human and mouse concerns the role of primary receptors in platelet adhesion to collagen under flow. There may be several nonexclusive reasons for discrepancy. First, human α2β1 may be competent to bind GFOGER sequences in type I collagen but not those of lower affinity, such as GASGER[48], before GPVI involvement. Second, human α2β1 may become activated independently of GPVI, perhaps caused by GPIb activity. It has been proposed that GPIb uses the same Fcγ-mediated signaling pathway as GPVI[49], and it reportedly induces rapid Ca^{2+} signaling under flow[50]. Similarly, the GPVI-independent presence of ADP in the blood may activate α2β1 at an early stage. Third, the threshold for α2β1 activation of human platelets may be low, that is, small signals from the slight percentage of GPVI not blocked by 10B12 may still be sufficient to trigger α2β1 for adhesion. Fourth, the human integrin-collagen contact—though dependent on GPIb and GPVI—may be more prone to autocrine signaling than that in mouse platelets. Finally, the considerable differences in human and murine GPVI structures may influence their contribution to the platelet-collagen interaction. Murine GPVI shares only 64% homology with the human receptor, and its cytoplasmic domain, half the length of the human 51-amino acid tail, lacks signaling motifs[51,52]. A human-to-mouse mutation, K59E, was shown to decrease GPVI binding to CRP, indicating important differences in the function of human and murine receptors[19].

After the primary platelet-collagen contact, platelets form aggregates or become procoagulant; both processes contribute to full-blown thrombus formation. We defined 2

conditions in which the formation of thrombi was inhibited: blocking GPVI and blocking ADP- and TxA_2-mediated events. Inhibiting GPVI suppressed aggregate formation and signaling processes, detected as the greatly reduced Ca^{2+} and procoagulant response. However, ADP/TxA_2 antagonism reduced Ca^{2+} signaling only slightly, though no aggregates were formed, with no effect on Pi. GPVI regulates aggregate formation through autocrine ADP/TxA_2 secretion, in part through synergism with $G_{i/q}$-coupled receptors[53]. We found that ADP contributed to aggregation and primary adhesion but that its influence was not eliminated by GPVI inhibition. This implies that ADP was derived from sources other than GPVI-stimulated platelets.

Platelet aggregation was mostly accompanied by high Ca^{2+} signaling and PS exposure, even when blocking shear-dependent adhesion through GPIb. The platelets that escaped inhibition were activated, as demonstrated by an unchanged Pi. A notable exception occurred with the blocking of $\alpha2\beta1$ function. Ca^{2+} signaling, PS exposure, and aggregate formation were all affected. This implies that $\alpha2\beta1$ has an activatory effect on these processes, in synergy with GPVI. Most, if not all, integrins exhibit inside-out and outside-in signaling[54], suggesting that outside-in signaling from activated $\alpha2\beta1$ can participate in platelet activation. We have previously shown that integrin-mediated adhesion to fibrinogen or collagen increases the responsiveness of platelets toward GPVI agonists[55]. Similar cross-talk between GPVI and $\alpha2\beta1$ also likely controls platelet activation in the present experiments. Previously, others have linked $\alpha2\beta1$ activation with $\alpha IIb\beta3$ up-regulation[56,57] and established cross-talk between $\alpha2\beta1$ and $\alpha IIb\beta3$[58].

Together, the results presented here advance the idea that human thrombus formation occurs through the concerted action of several receptors: the interplay of GPIb/V/IX, integrin $\alpha2\beta1$ and GPVI enables platelet deposition, and GPVI in cross-talk with $\alpha2\beta1$ mediates subsequent thrombus formation, additionally supported by ADP. Yet these receptors may not be the only determinants of human platelet collagen interaction because other collagen receptors[35,57,59,60] may participate in the early stages of platelet contact with collagen, and new receptors are being discovered[61,62]. Ultimately, these other receptors may participate in the interaction and may be shown to allow further fine-tuning of the receptor cross-talk identified in this study.

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Chapter 5

The glycoprotein VI-phospholipase Cγ2 signaling pathway controls thrombus formation induced by collagen and tissue factor in vitro and in vivo

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Abstract

Both collagen and tissue factor can be initiating factors in thrombus formation. We investigated the signaling pathway of collagen-induced platelet activation in interaction with tissue factor-triggered coagulation during the thrombus-forming process. In murine blood flowing over collagen, platelet exposure of phosphatidylserine and procoagulant activity, but not adhesion, completely relied on each of the following signaling modules: glycoprotein VI (GPVI), FcR γ-chain, Src kinases, adaptor protein LAT, and phospholipase Cγ2 (PLCγ2). Upon flow in the presence of tissue factor, these signaling components were essential for platelet aggregation and greatly enhanced fibrin clot formation. Collagen-stimulated thrombin generation relied on the presence and activity of GPVI, FcR γ-chain, Src kinase, LAT and PLCγ2. The physiological importance of this GPVI pathway was shown in a $FeCl_3$-induced *in vivo* murine thrombosis model. In both venules and arterioles, signaling through GPVI, FcR γ-chain and Src kinases enhanced the formation of phosphatidylserine-exposing and fibrin-rich thrombi. In conclusion, the GPVI-PLCγ2 activation pathway regulates collagen-dependent coagulation in venous and arterial thrombus formation.

Introduction

Thrombus formation can be initiated by both platelet- and coagulation-activating factors. Collagens in the extracellular matrix and other vascular layers are though to act as principal platelet-activating components of the damaged vessel wall; they also provide a surface for von Willebrand factor (vWf) adhesion[1]. Tissue factor, also exposed in damaged vessels, is a key trigger of the coagulation process[2]. Because of the proposed major role of platelets in arterial thrombosis and the importance of coagulation in venous thrombosis, current understanding is that collagen/vWf-mediated events are more important in arteries, while tissue factor plays a more prominent role in venous thrombus formation.

Flow studies with human and mouse blood have established that the signaling receptor, glycoprotein VI (GPVI), exclusively mediates collagen-induced platelet procoagulant activity, thus linking the processes of platelet activation and coagulation[3-5]. This procoagulant platelet response is mediated by a prolonged and potent rise in cytosolic $[Ca^{2+}]_i$, which results in exposure of procoagulant phosphatidylserine (PS) at the platelet outer surface[6]. PS exposure is a key regulating factor in the coagulation process. For instance, PS-containing membrane surfaces dramatically increase the

formation of factor Xa and thrombin[7]. However, several authors have argued that this platelet response has only an assistant role in coagulation and that, *in vivo*, other platelet reactions may play important roles as well[2,8]. Thus, while there is no doubt that platelets enhance thrombin generation (coagulation) in plasma or whole blood, the precise mechanism is still a matter of debate.

The platelet immunoreceptor GPVI is co-expressed with the Fc receptor (FcR) γ-chain, although the latter also interacts with other platelet receptors, e.g. GPIb[9]. In human and mouse platelets, activation of GPVI by collagen or other ligands results in a complex cascade of signaling events[10-12]. The initial step is tyrosine phosphorylation of the FcR γ-chain by the Src family kinases, Fyn and Lyn. Subsequent activation of the tyrosine kinase Syk leads to phosphorylation of multiple signaling proteins, including the adaptor proteins LAT, SLP-76 and SLAP-130, and further the G-protein regulator Vav and phospholipase C-γ2 (PLCγ2)[13]. GPVI stimulation activates various other protein kinases, including Btk, Tec, phosphoinositide 3-kinase and, further downstream, protein kinase C, Erk1/2 and focal adhesion kinase, as a result of which platelets respond by integrin activation, Ca^{2+} increase, aggregation, shape change, secretion and procoagulant activity. Although earlier work has shown that GPVI plays a key role in the collagen-induced platelet procoagulant activity in both stasis and flow[14], it is still unclear which signaling elements downstream of GPVI contribute to this platelet reaction.

Evidence that GPVI plays a key role in arterial thrombosis comes from recent *in vivo* studies with mice, where the platelet aggregation *in vivo* was followed after ligation of arteries, causing exposure of vascular collagen[1,15]. Absence of GPVI (as in FcR γ-chain null mice) or down-regulation of GPVI (as in mice treated with JAQ1 antibody) appeared to suppress intravascular formation of platelet aggregates. Thus, knowing the *in vitro* evidence for a procoagulant effect of GPVI, we hypothesized that GPVI can also drive the coagulation process during thrombus formation.

In the present study, we used mice deficient in GPVI or in one of the signaling proteins downstream of GPVI to investigate the signal transduction route leading to GPVI-induced PS exposure, coagulation stimulation and fibrin formation both *in vitro* and *in vivo*. We performed whole-blood flow studies partly in the presence of tissue factor-triggered coagulation to determine the functional effects of PS exposure. Furthermore, *in vivo* experiments where thrombus formation was induced with free radical-forming $FeCl_3$, allowed us to study the importance of this process in a thrombosis model known to rely on thrombin generation and coagulation[16]. The data showed that also *in vivo* the

signaling cascade from GPVI to PLCγ2 led to coagulant activity and enhanced thrombus formation.

Materials and Methods

Materials

Fibrillar type-I collagen (Horm) was purchased from Nycomed (Munich, Germany). MRS2179, a $P2Y_1$ antagonist, was from Sigma (St. Louis, Mo, USA). Annexin A5 labeled with Oregon green 488 (OG488) came from Nexins Research (Hoeven, The Netherlands). OG488-conjugated fibrinogen and annexin A5 labeled with Alexa fluor 647 (AF647) were from Molecular Probes (Leiden, The Netherlands). PD173952 was a gift from Pfizer (Ann Arbor, MI, and Sandwich, UK).Lotrafiban was a gift from GlaxoSmithKline (Middlesex, UK). Anti-GPVI antibody JAQ1 was produced and modified as described[9]; Fab fragments were generated and in part used for fluorescein labeling[3]. Blocking anti-murine GPIb monoclonal antibody (mAb) Xia.B2 was from Emfret Analytics (Würzburg, Germany). ARC69931MX, an antagonist of the $P2Y_{12}$ receptor, was from AstraZeneca R&D (Charnwood, UK). Aspirin was from Lorex Synthélabo (Maarssen, The Netherlands). Recombinant tissue factor came from Dade (Düdingen, Switzerland); Z-Gly-Gly-Arg aminomethyl coumarin (Z-GGR-AMC) from Bachem (Bubendorf, Switzerland); H-Phe-Pro-Arg chloromethyl ketone (PPACK) from Calbiochem (La Jolla, CA); $FeCl_3$ from Merck (Darmstadt, Germany). Other materials were obtained from sources described before[3].

Animals and blood collection

Healthy 4-5 weeks old (11-21 g) or 12 week old C57Bl/6 (20-25 g) (wildtype) mice of either sex were obtained from Charles River (Maastricht, The Netherlands). C57Bl/6 mice deficient in the FcR γ-chain (-/-) were obtained from Taconics (Germantown, NY). Mice homozygously deficient in LAT or PLCγ2 were generated as described previously[17,18], and bred from heterozygotes on a B6 background. Wildtype littermates were used as controls. Where indicated, wildtype mice were injected under anesthesia with 100 µg anti-GPVI JAQ1 antibody (in 100 µL saline) at 5 days before start of the experiment. Other anesthetized animals were injected with 100 µL of 1 mM Src kinase inhibitor, PD173952 (diluted in saline), at 10 min before experimentation. Efficacy of these treatments was verified with collected blood by flow cytometry and platelet aggregation measurements. For experiments in the absence of coagulation, blood was

collected under anesthesia by orbital puncture in 40 µM PPACK and 5 U/mL heparin, as described[3]. For experiments involving coagulation, blood was collected into 10% 0.129 M citrate. Studies were approved by the local animal care and use committees.

Thrombus formation on collagen under flow

Thrombus formation was measured ex vivo with blood from 12 week old mice which was perfused over a collagen surface as described before[4]. Briefly, an area of 20×5 mm in the centre of a glass coverslip was coated with fibrillar Horm type-I collagen (50 µg/mL for 20 min at 37°C), blocked with Hepes buffer pH 7.45 (5 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4 and 1% bovine serum albumin (BSA), and then placed in a parallel plate flow chamber (slit depth of 50 µm). Blood was incubated for 15 min with various inhibitors and/or fluorescent probes, and then perfused over the coverslip at a shear rate of 150-1000 s^{-1} for 4 min. Thereafter, the flow chamber was perfused with Hepes buffer pH 7.45 with 2 mM $CaCl_2$ and 1 U/mL heparin and containing fluorescent-labeled annexin A5 (0.5 µg/mL) for 4 min. Bright-field phase contrast as well as non-confocal fluorescence images of adherent platelets were recorded using a Visitech microscope system equipped with two cameras[5] or an inverted stage video microscope system (DM-IRB, Leica, UK)[19]. To inhibit GPIb activity we used the blocking anti-murine GPIbα mAb Xia.B2 (40 μg/ml), which was demonstrated to be equally effective as the earlier used blocking anti-GPIb antibody p0p/B (B. Nieswandt, unpublished results).

For flow experiments without coagulation, blood collected in PPACK/heparin was used. For coagulation experiments, citrated whole blood was used, which was during perfusion exposed to 10% volume of 20 pM tissue factor in 200 mM $CaCl_2$ upon entry into the flow chamber, as described for rat and human blood[5]. Platelet surface coverage of images was analyzed using ImagePro software (Media Cybernetics, Silver Spring, MD) for phase contrast images and Quanticell software (Visitech, Sunderland, UK) for fluorescent annexin A5 images. At least 10 different fields of view were averaged per experiment (no image processing).

Two-photon laser scanning microscopy (TPLSM)

For two-photon laser scanning microscopy (TPLSM), coverslips with thrombi were observed with a Bio-Rad 2100 multiphoton system. Excitation was by a Spectra-Physics Tsunami Ti:Sapphire laser, tuned and mode-locked at 800 nm, producing pulses of 100 fs wide (repetition rate 82 MHz). Excitation at 647 nm was by a parallel-placed red

diode laser. Fluorescence was detected at 508 to 523 nm and above 660 nm. Thrombi in flow chambers, double labeled with OG-488 fibrinogen (green) and AF647-annexin A5 (red), were scanned at the end of perfusions. Optical sections were recorded in Kalman filtering mode; no further image processing was performed. Mouse mesenteric vessels were imaged in situ using TPLSM, taking advantage of the high penetration depth and intrinsically high optical sectioning of this technique[20].

Thrombin generation measurement

Murine platelet-rich plasma (PRP) and platelet-poor plasma (PPP) collected on citrate were used to measure thrombin generation with the thrombogram method[21], adapted for murine plasma. Briefly, normalized PRP (1.5×10^8 platelets/mL) was preincubated with an antagonist for 15 min and then with collagen for 10 min. Samples of 40 µL PRP or PPP were pipetted into wells of a 96-well plate (Immulon 2HB; Dynex Technologies, Chantilly, VA), containing 10 µL tissue factor (6 pM) in buffer A (20 mM Hepes, 140 mM NaCl, 5 mg/mL BSA, pH 7.35). Coagulation was started by addition of 10 µL Z-GGR-AMC (2.5 mM), dissolved in buffer B (20 mM Hepes, 100 mM $CaCl_2$, 60 mg/mL BSA, pH 7.35). Plates were immediately inserted into a fluorescence well-plate reader (Thermolab Systems, Helsinki, Finland or Molecular Devices, Spectra Max Gemini XS) and processed at 37°C[22]. Fluorescence accumulation from cleaved AMC was measured, and first derivative curves of accumulation of fluorescence were generated; calibrations were performed with human thrombin.

Animal preparation and intravital microscopy

Four to 5-week old mice were anesthetized by subcutaneous injection of 0.1 mg/g body weight ketamine and 0.02 mg/g xylazine. Anesthesia was maintained by continuous infusion of ketamine (0.07 mg/g/h) through a subcutaneous polyethylene (PE)-10 catheter in the neck. Pharmacological agents were administered intravenously through a PE-10 catheter in the tail vein. Body temperature was kept at 37°C using an infrared heating lamp, controlled by a thermoanalyzer system connected to a rectal probe.

A segment of the ileum was exteriorized through a right side abdominal incision. The mesentery was spread over a siliconized glass plate mounted on an electrically heated table (37°C) of a Leitz (Jena, Germany) intravital microscope, and continuously superfused with a Tyrode solution (130 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 0.56 mM $MgCl_2$, 11 mM glucose, 13 mM sucrose, 25 mM $NaHCO_3$ and 1.2 mM NaH_2PO_4; pH 7.35

at 37°C), supplemented with 1 µg/mL isoprenaline and saturated with 95% N_2 and 5% CO_2. The exteriorized ileum was kept moist with overlying wet gauze. Where indicated, fluorescently labeled compounds were infused (bolus, 100 µL) via the tail vein catheter either before or after $FeCl_3$ application.

Thrombus formation in vivo

For *in vivo* experiments, fat-free mesenteric arterioles and proximate venules were selected. Before start of $FeCl_3$ application, superfusion with Tyrode solution was stopped, and remaining fluid was carefully removed from the selected mesenteric tissue. Vessel wall damage was induced by topical application of 30 µL $FeCl_3$ (500 mM), which remained on the tissue during experimentation. The intravascular reactions were observed by trans-illumination and fluorescence microscopy using a LL25x objective (NA 0.35) and a CCD camera (Hamamatsu) for 600 s^{23}. For each vessel, the following parameters were quantified off-line: lagtime to first observation of thrombus formation, thrombus height perpendicular to the vessel wall (percentage of the vessel diameter) at 2, 5 and 10 min after $FeCl_3$ application. Damaged mesenteric vessels were observed *in situ*, as described above, while specimens for histology were fixed in formaldehyde (10% in PBS), routinely processed and embedded in paraffin. Sections of 4 µm were cut and stained with hematoxylin and eosin.

To determine wall shear rates in representative mesenteric arterioles and venules, several mice were injected with rhodamin 6G (30 µL of 1 mg/mL, intravenous) for labeling of circulating leucocytes. Fluorescence microscopy was applied to quantify centerline blood flow velocities of passing leucocytes, as described. Mean wall shear rates were 1183 s^{-1} in arterioles and 344 s^{-1} in venules.

Statistical analysis

Data from *in vivo* experiments displayed non-symmetrical distribution and are presented as median values with interquartile ranges. Differences between experimental groups were tested on significance with a non-parametric Mann-Whitney U test using the statistical package for social sciences (SPSS 11.0, Chicago, IL).

Results

Components of the GPVI-PLCγ2 signaling pathway required for collagen-induced procoagulant activity of platelets in flowing blood

In mouse and human platelets, functional activity of GPVI is required for collagen-induced aggregation and surface exposure of procoagulant PS[4,5]. To investigate which signaling proteins downstream of GPVI are involved in the procoagulant platelet response, we used genetically modified mice and specific blockers

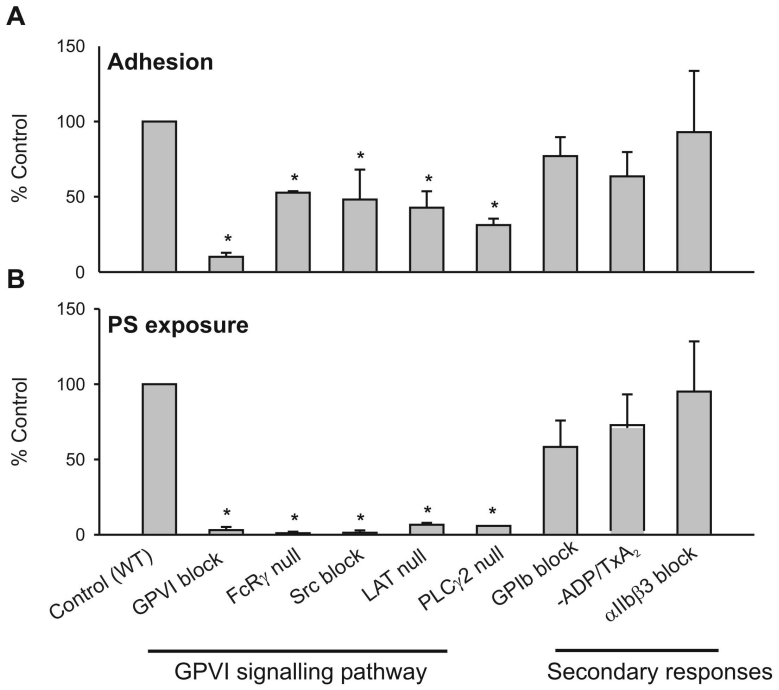

Figure 1 GPVI-PLCγ2 signaling pathway is required for collagen-induced PS exposure in flowing blood. PPACK-anticoagulated blood was perfused over collagen in the absence of coagulation. Perfusion was at 1000 s^{-1} for 4 min, followed by staining with fluorescent annexin A5 (0.5 µg/mL). Wildtype blood was pre-incubated for 15 min with vehicle (control), 40 µg/mL JAQ1 Fab (blocking GPVI) or 25 µM PD173952 (blocking Src kinases). Blood from mice lacking FcR γ-chain, LAT or PLCγ2 was compared to blood from genetically matched wildtype mice. Otherwise, wildtype blood was pre-treated with vehicle or 40 µg/mL Xia.B2 mAb (blocking GPIbα), 10 µM lotrafiban (blocking integrin αIIbβ3) or 20 µM MRS2179, 50 µM AR-C69931MX and 100 µM aspirin (blocking effects of autocrine ADP/thromboxane A_2). Data represent surface area coverage of all platelets (A) and of PS-exposing platelets (B), expressed as percentage of corresponding controls. Surface area coverage with wildtype platelets under control conditions was 17.5±5.5%, and coverage with fluorescent annexin A5 was 7.2±4.1%. Mean±SD (n=5-7); *P≤0.05 compared to control.

of key proteins in the PLCγ2 activation pathway. We used Fab fragments of JAQ1 mAb to block GPVI receptors and, in comparison, GPVI-null mice lacking the FcR γ-chain. Src kinase activity was abolished with the compound PD173952, which has recently been identified as a selective and specific inhibitor of Src-family kinases that is still active in blood. It essentially abolishes all collagen- but not thrombin-induced tyrosine phosphorylation events in platelets[19]. Furthermore, mice deficient in LAT[18] or PLCγ2[17] were used to study the contribution of these downstream signaling entities.

In the first experiments, PPACK-anticoagulated whole blood from wildtype or genetically modified mice was perfused over a collagen surface at a shear rate of 1000 s^{-1}, i.e. representative of that found in murine arterioles. Using wildtype blood, platelets rapidly adhered to the collagen surface and partly assembled into aggregates. As shown before[4,24], platelet deposition and aggregate growth on collagen increased about linearly with time. Blocking of GPVI with JAQ1 Fab fragments abolished platelet adhesion and aggregation (Figure 1A), also confirming earlier results[4,15]. Absence of FcRγ or treatment with Src kinase inhibitor resulted in a moderately reduced deposition of platelets on collagen; these platelets remained single and showed little tendency to aggregate. Similar results were obtained with blood from LAT-null or PLCγ2-null mice (Figure 1A).

Post-staining of wildtype/control thrombi with OG488-annexin A5 revealed many single PS-exposing platelets (Figure 1B). Strikingly, virtually no PS-exposing platelets were detected when: GPVI was blocked (with JAQ1 Fab), FcR γ-chain was absent, Src kinases were blocked (with PD173952), or LAT or PLCγ2 was absent (Figure 1B). In sharp contrast, platelet deposition was only little affected by either blocking of GPIb-vWf interactions (with Xia.B2 mAb), secondary platelet responses due to release of ADP (with receptor antagonists MRS2179 and AR-C69931MX), thromboxane A_2 (aspirin), or by blocking integrin αIIbβ3 (with lotrafiban) (Figure 1A). Also, these blocking conditions did not significantly influence PS exposure compared to the control situation (Figure 1B).

A typical observation under control conditions was that wildtype platelets on the collagen surface only showed PS exposure when not assembled into aggregates. Conversely, aggregated platelets did not expose PS. We used the high resolution of TPLSM, allowing simultaneous detection of two fluorescent probes, to better characterize the populations of PS-exposing and aggregated platelets. With OG488-fibrinogen and AF647-annexin A5 added to wildtype blood, fibrinogen label exclusively incorporated into platelet aggregates, whereas labeled annexin A5 was differently localized, only staining single platelets around the aggregates. After perfusion, platelets from FcR γ-chain null mice and also wildtype platelets treated with PD173952 failed to

bind fibrinogen, pointing to the requirement of the FcR γ-chain and Src kinases to the aggregate-forming process[19]. Together, these results indicate that the signaling components of the GPVI-PLCγ2 pathway (involving Src kinases, FcRγ, Syk and LAT) are indispensable for PS exposure on collagen under flow. The PS-exposing platelets typically represent a different population than the platelets assembled into aggregates and binding fibrinogen.

Components of the GPVI signaling pathway determining platelet procoagulant activity and thrombus formation in flowing blood triggered with tissue factor

To induce coagulation under flow conditions, citrated blood was mixed with tissue factor and $CaCl_2$ just before entering the flow chamber. The perfusion protocol was such that coagulation occurred at physiological, millimolar Mg^{2+} and Ca^{2+} concentrations[25]. With wildtype blood, perfusion in the presence of tissue factor resulted in PS exposure and the formation of platelet aggregates, which gradually transformed to

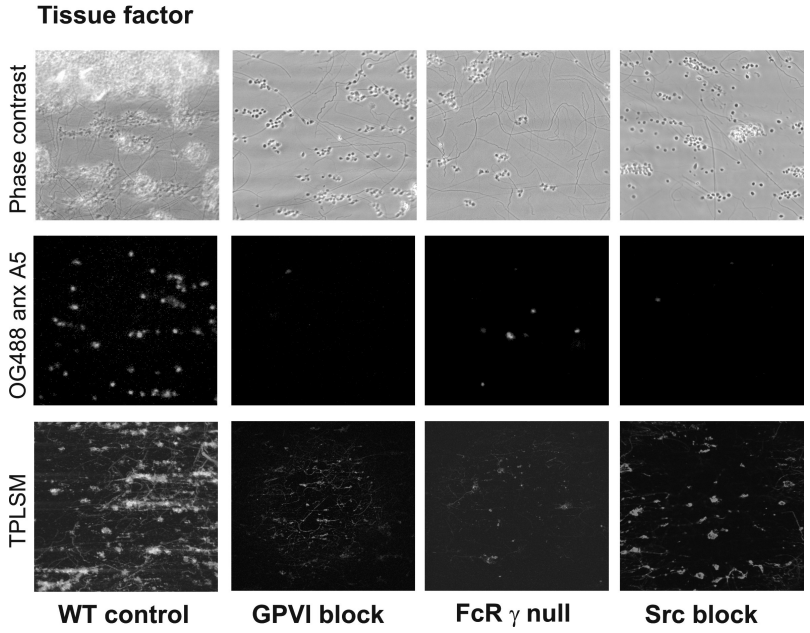

Figure 2 GPVI/FcR γ-chain signaling pathway controls tissue factor-induced fibrin formation and platelet activation in flowing blood. Citrate blood from wildtype mice was incubated for 15 min with vehicle, GPVI-blocking JAQ1 Fab, or Src kinase inhibitor PD173952 (see Figure 1). Blood was then perfused over collagen together with tissue factor (2 pM, f.c.) and $CaCl_2$ (2 mM free Ca^{2+}, f.c.) to allow coagulation. Standard perfusion time was 4 min at shear rate of 1000 s^{-1}. Post-labeling was with OG488-annexin A5 (0.5 µg/mL). *Upper panels*: phase-contrast images after perfusion (120×120 µm); *middle panels:* images of OG488-annexin A5 fluorescence (different fields, 150×150 µm); *lower panels:* TPLSM images of OG488-fibrinogen and AF647-annexin A5 fluorescence (77×77 µm). Images are representative of 5-10 experiments.

clots trapping erythrocytes (Figure 2). TPLSM analysis indicated that the thrombi contained extensive networks of fibrin and aggregated platelets, which were surrounded by single PS-exposing platelets. Perfusion of JAQ1-treated blood (with blocked GPVI) or blood from FcRγ-null mice in the presence of tissue factor gave only limited platelet adhesion without aggregate formation. Fibrin formation was greatly suppressed, and only few PS-exposing platelets were observed (Figure 2). PD173952 was somewhat less inhibitory in the presence of tissue factor, with small fibrin(ogen)-binding aggregates still being formed (Figure 3). This reflects the presence of Src kinase-independent pathways of platelet aggregation. Pre-treatment of blood from FcR γ-chain null mice with PD173952 did not further reduce platelet adhesion.

Quantitative image analysis indicated that platelet deposition and PS exposure were significantly reduced by the various treatments (Figure 3). Pre-treatment of wildtype blood with the anti-GPVI Fab JAQ1 reduced total surface area coverage of platelets from 25.8% (control) to 6.3% and PS exposure from 7.9% (control) to 0.1% (Figure 3). This was also the case when using FcR γ-chain null blood or PD173952-treated blood, where platelet deposition was decreased to 3.8% and 4.9%, and annexin A5 binding was even more reduced to 0.5% and 0.7%, respectively (Figure 3). Reduced thrombus formation and coagulant activity under all above conditions was also seen at a lower shear rate of

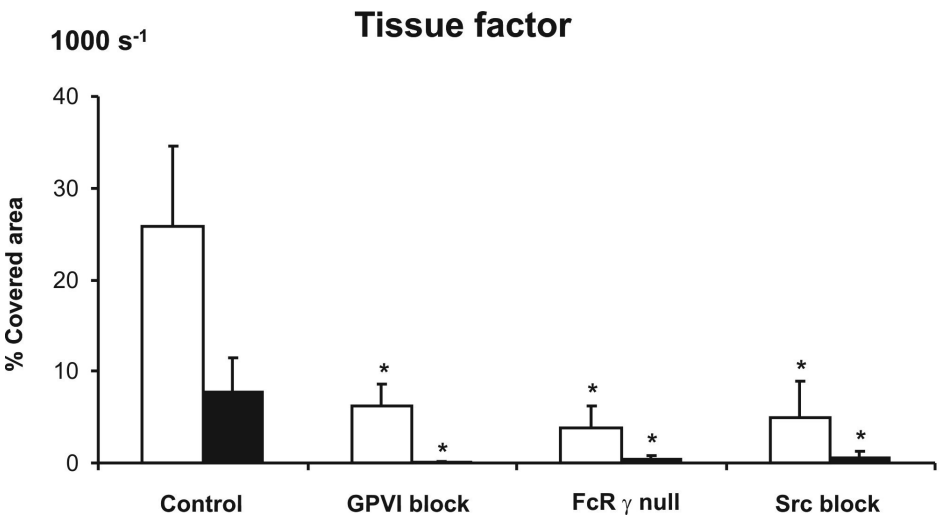

Figure 3 GPVI/FcR γ-chain signaling pathway controls platelet deposition and procoagulant activity in blood triggered with tissue factor under flow. Citrate blood was perfused over collagen in the presence of tissue factor and $CaCl_2$, as described for Figure 2. Post-perfusion was with AF647-labeled annexin A5. Data are percentages of area coverage of all platelets (white bars) or PS-exposing platelets (black bars). Mean±SD (n=5); *$P \leq 0.05$ as compared to control.

150 s^{-1} (data not shown), which is representative of that in venules.

Together, these results stress the importance of the GPVI pathway in aggregate formation on collagen under flow. They also significantly extend earlier work by showing that, independently of the shear rate, GPVI signaling plays a key role in the coagulation process, i.e. in thrombin and fibrin formation triggered by tissue factor. Apparently, collagen-induced PS exposure via the FcR γ-chain is a key regulatory factor in coagulation.

Components of the GPVI-PLCγ2 signaling pathway involved in collagen-enhanced thrombin generation

To investigate more directly how coagulation is controlled by collagen-GPVI interaction and subsequent signaling, we examined the effect of collagen on thrombin generation in mouse PRP triggered with tissue factor under static conditions. Addition of tissue factor/$CaCl_2$ to wildtype PRP resulted in high thrombin generation, a process which was greatly enhanced with a submaximal dose of collagen (peak level increased by 97%, (Figure 4). Control experiments demonstrated that essentially no thrombin was formed during 60 min, when platelets or tissue factor were absent (not shown). Collagen did not alter the thrombin generation curve with PRP from FcR γ-chain null mice (Figure 4). In agreement with this, in PRP from wildtype mice, blocking of GPVI (with JAQ1 Fab)

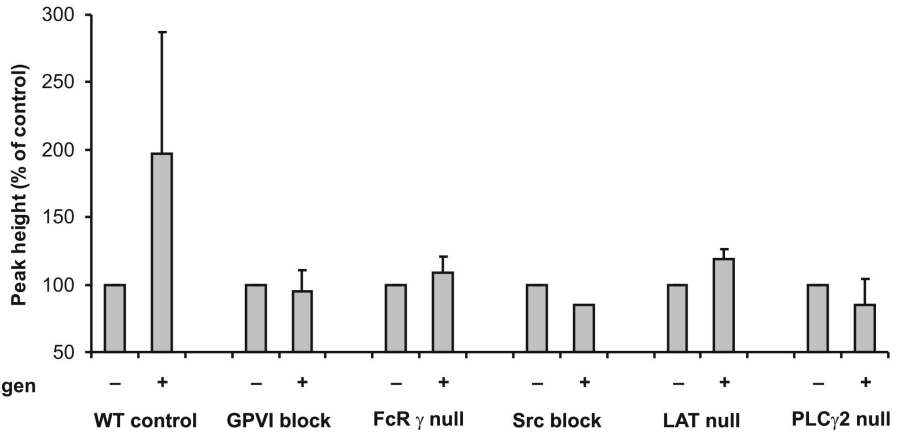

Figure 4 GPVI-PLCγ2 signaling pathway mediates collagen-enhanced thrombin generation. Mouse PRP (1×10^8 platelets/mL, f.c.) was pre-incubated with vehicle (control), JAQ1 Fab (40 µg/mL) or Src kinase inhibitor, PD173952 (25 µM). PRP was activated with vehicle (control) or 5 µg/mL collagen, after which coagulation was initiated with tissue factor (1 pM) and $CaCl_2$. PRP (1×10^8 platelets/mL, f.c.) from mice deficient in FcR γ-chain, LAT or PLCγ2, or from matched wildtype mice was similarly activated. Enhancing effect of collagen on thrombin peak height; peak height normalized to control condition or wildtype PRP (mean±SD, n=4).

or Src kinases (with PD173952) completely abolished the enhancing effect of collagen on thrombin generation. These interventions did not affect thrombin generation in PRP from FcR γ-chain null mice. In the absence of collagen, PRP from mice deficient in LAT or PLCγ2 showed normal thrombin generation curves compared to wildtypes. Collagen addition did not increase the thrombin-generating activity in this PRP (Figure 4). Accordingly, the enhancing effect of collagen on thrombin generation was lost in case one of these components of the GPVI-PLCγ2 signaling pathway was missing.

Absence of GPVI signaling diminishes thrombus formation in venules and arterioles on exposed extracellular matrix

To determine the physiological relevance of the collagen-induced activation pathway, we used an *in vivo* mouse model of microvascular thrombus formation that relies on exposure of the extracellular matrix and ensuing thrombin generation[26]. Injury of the mesenteric vessels of anesthetized mice was induced by topical application of $FeCl_3$, which caused local, but complete denudation of the endothelium in venules and nearby arterioles. Intravital microscopy was used for real-time imaging of thrombus formation in the damaged vessels (Figure 5).

To study the involvement of GPVI, wildtype mice were injected with JAQ1 antibody, which causes biphasic, long-term *in vivo* depletion of GPVI on platelets, without affecting other platelet glycoproteins[15,27]. After 10 min, this injection resulted in a

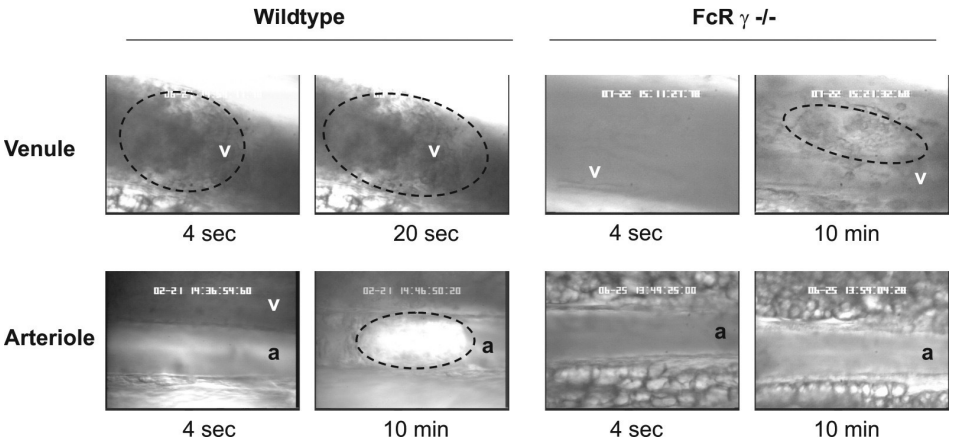

Figure 5 In vivo thrombus formation in venules and arterioles of wildtype and FcR γ-chain null mice. Thrombi were raised *in vivo* by application of $FeCl_3$ on selected mesenterial venules (v) and arterioles (a) from wildtype or FcR γ-chain null mice. Shown are representative trans-illumination images (280×350 µm) from damaged venules and arterioles, captured at indicated times after $FeCl_3$ application; thrombus area is indicated by dotted lines.

greatly reduced platelet concentration (20% of normal platelet count) and a specific disappearance of GPVI on the platelet surface with other platelet glycoproteins remaining unchanged (not shown)[15]. At 5 days after injection, however, platelet count was normalized, while GPVI expression on platelets was still completely absent.

In venules from wildtype mice, thrombus formation started after seconds, while in arterioles there was a lagtime of several minutes. Thrombus size, quantified as thrombus height perpendicular to the vessel wall, increased with time in the vessels from wildtype mice. After 10 min, 39% and 31% of the venules and arterioles were occluded, respectively. When $FeCl_3$ was applied to vessels from mice that were injected with JAQ1 antibody (5 days before), thrombus formation in both venules and arterioles was greatly delayed and reduced (Figure 6). Similarly, in FcR γ-chain null mice, thrombus formation induced by $FeCl_3$ was markedly delayed in both venules and arterioles. Thrombi in this case remained small in size in venules, and were not formed at all in arterioles ($P \leq 0.05$). No occlusion was observed within 10 min. Furthermore, pre-injection of mice with PD173952 at 10 min before $FeCl_3$ application (estimated final plasma concentration of 50 µM), resulted in a significant decrease in arterial thrombus size ($P \leq 0.05$), but not in venular thrombus size (Figure 6). PD173952 injection caused full blockage of collagen-induced aggregation *in vitro*. We conclude that, in this microvascular model of extracellular matrix exposure, the presence of GPVI, FcRγ and Src kinase signaling are critical for venous and arterial thrombosis.

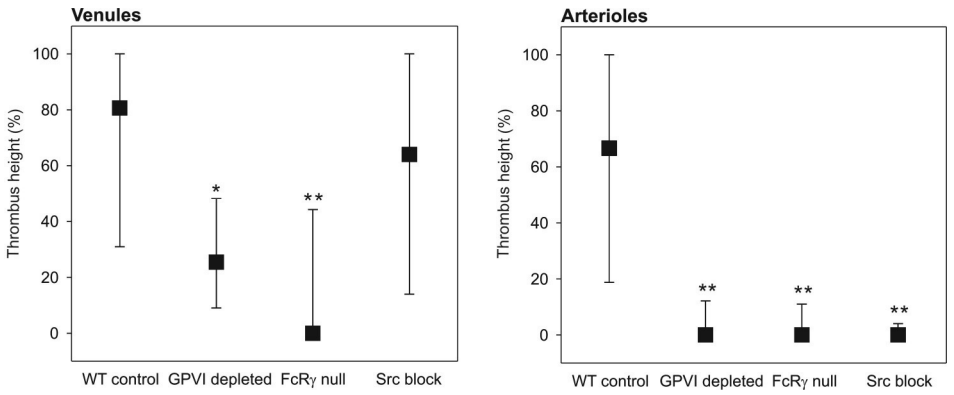

Figure 6 Reduced thrombus formation in arterioles and venules in the absence of GPVI signaling. Wildtype mice were pre-infused with saline vehicle (WT control) or Src kinase inhibitor PD173952 (100 µL of 1 mM in saline) at 10 min before start. Other mice were injected with anti-GPVI JAQ1 mAb (100 µg in saline) at 5 days before experimentation. Thrombi were raised by topical application of $FeCl_3$ of adjacent mesenteric arterioles and venules. Thrombus size was measured at 10 min after $FeCl_3$ application, and expressed as percentage of the local vessel diameter. Data are median values with interquartile ranges (n=6). *$P \leq 0.1$; **$P \leq 0.05$ compared to control.

Reduced fibrin formation and PS exposure in arterial thrombi after GPVI depletion

TPLSM was used for better visualization of platelet activation and coagulation in arterioles after $FeCl_3$ application. Wildtype mice were pre-injected with OG488-labeled fibrinogen or annexin A5, and fluorescent thrombi were subsequently scanned. In $FeCl_3$-treated arterioles from control mice, extensive deposition of OG488-fibrin(ogen) was detected and smaller spots of labeled annexin A5 (Figure 7). In contrast, in arterioles from mice pretreated with JAQ1 antibody (5 days) to down-regulate GPVI, $FeCl_3$ application led to formation of only small domains of fibrin labeling and no annexin A5 labeling. Thus, both fibrin formation and PS exposure were reduced after down-regulation of platelet GPVI.

Discussion

The present results indicate that the GPVI receptor, acting via a straightforward signaling pathway, acts as principal mediator of PS exposure, platelet-dependent thrombin generation and fibrin formation/coagulation in the presence of tissue factor. The signaling modules involved in the procoagulant platelet response are the FcRγ, Src

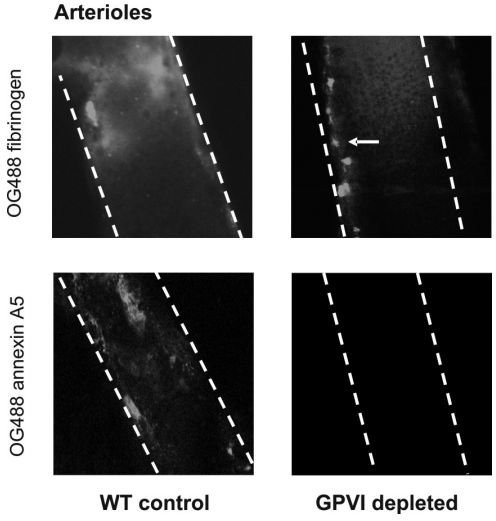

Figure 7 Decreased accumulation of fluorescent fibrin(ogen) and annexin A5 in arteriolar thrombi from GPVI-depleted mice. Wildtype mice were untreated (control) or depleted in GPVI by injection of 100 μg JAQ1 mAb at 5 days before start of experimentation. Prior to thrombus induction, mice were infused with either 200 μg OG488-labeled fibrinogen or 200 μg OG488-labeled annexin A5 in 100 μl saline. Mesenteric arterioles were exposed to $FeCl_3$ for 10 min, after which fluorescence was observed *in situ* by TPLSM. Small fibrin clots were formed after GPVI depletion (arrow). Images (206×206 μm) are from representative damaged arterioles.

kinases (likely Fyn/Lyn), the adaptor protein LAT and PLCγ2, the latter of which is responsible for Ca^{2+} mobilization. In addition, we find that under flow this signaling pathway mediates the build-up of a platelet-fibrin thrombus. Evidence comes from a number of approaches to eliminate GPVI or subsequent activation steps. Blocking anti-GPVI JAQ1 Fab fragments were used. Further, the Src kinase inhibitor, PD173952, which completely inhibits collagen-induced aggregation and tyrosine phosphorylation of mouse platelets in plasma, but leaves platelet responses to G protein-coupled receptor agonists unchanged[19]. Abolished PS exposure and thrombus formation was also seen in blood from mice lacking either the FcR γ-chain, the adaptor protein LAT or the effector protein PLCγ2. Especially the recognition that LAT is required for the procoagulant response is important, because earlier work suggested that LAT is dispensable for collagen- and convulxin-induced platelet aggregation[28].

Interestingly, addition of JAQ1 antibody fragments resulted in lower platelet adhesion than with FcR γ-chain deficient blood. This can be explained by the recent observation that GPVI and GPIb-IX-V associate on platelets[29]; and the possibility that the JAQ1 antibody can thereby interfere with GPIb-mediated effects, while GPIb remains functionally active under flow in the absence of FcRγ[30]. We have shown before that GPIb plays a more prominent role in platelet-collagen interaction at reduced levels of GPVI and FcRγ, which argues against a significant role of the FcR γ-chain in thrombus formation independently of GPVI, such as others have proposed[31].

Earlier perfusion studies have indicated that platelet-collagen interaction via GPVI is the principal trigger of platelet activation and aggregation in mouse and human blood[5,15]. Here, we significantly extend this observation by showing that GPVI signaling also controls thrombus formation on collagen in the presence of coagulation. We found that not only platelet aggregate formation, but also PS exposure and fibrin formation were greatly suppressed in blood from FcR γ-chain null mice, both at high (arteriolar) and low (venular) shear rates. This was also the case when GPVI or Src kinases were blocked with JAQ1 Fab or PD173952, respectively. During flow in the absence of GPVI activity, tissue factor was still active in triggering thrombin formation, as deduced from the traces of fibrin that still were formed, but apparently propagation of the coagulation process and fibrin clot formation were prevented. Accordingly, platelet activation by GPVI, most likely via PS exposure, is required for full coagulation activity.

In a macrovascular thrombosis model triggered by mechanical damage of the carotid artery, it has been demonstrated that GPVI is required for platelet adhesion to the vessel wall[1]. Whether or not coagulation contributes to the thrombotic process in that

model is unknown. Here, we used a microvascular model, relying on free radical formation with $FeCl_3$, known to be driven by thrombin formation and coagulation[26]. The application of $FeCl_3$ resulted in almost complete disappearance of the endothelium in both arterioles and venules, causing exposure of the collagen-containing extracellular matrix (M. Kuijpers, unpublished data 2004). In either vessel type GPVI activity appeared to control the thrombus-forming process. For instance, in venules and arterioles from FcR γ-chain null mice, thrombi remained small in size (venules) or were not formed at all (arterioles). Long-term injection with anti-GPVI JAQ1 mAb, which completely down-regulates GPVI on the platelet surface[15], delayed and reduced the thrombus formation in venules and arterioles to a similar extent. Furthermore, observation with TPLSM indicated that in vessels from mice pre-injected with JAQ1 mAb only small spots of labeled fibrin were present, while labeled annexin A5 was not detectable at all. Finally, injection of PD173952 into mice to block (GPVI-activated) Src kinases suppressed the thrombotic process in arterioles.

Together, these data provide first *in vivo* evidence that GPVI, acting via a relatively simple signaling pathway (Src kinase, FcRγ, Syk, LAT, PLCγ2) has a key role

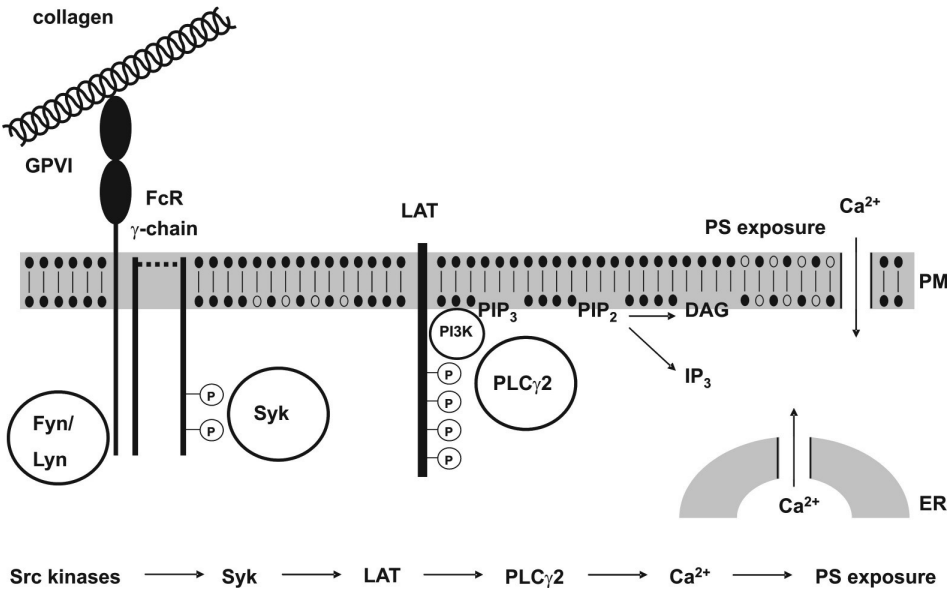

Figure 8 GPVI signaling cascade. Crosslinking of GPVI induces tyrosine phosphorylation of the FcR γ-chain ITAM by the Src kinases, Fyn and Lyn. This initiates a Syk-dependent signaling cascade that leads to formation of a LAT complex and activation of PLCγ2. PLCγ2 associates directly with LAT, and with the membrane via binding of its PH domain to PIP3. PLCγ2 activation causes Ca^{2+} mobilization as well as Ca^{2+} entry via plasma membrane channels, which subsequently leads to phosphatidylserine (PS) exposure.

in platelet procoagulant activity and subsequent thrombin and fibrin formation. The procoagulant function of GPVI appears to contribute to arterial thrombus formation and, interestingly, also to venous thrombus formation. These findings thus point to a dual role of GPVI, both procoagulant and aggregatory, in collagen-induced thrombus formation *in vivo*.

Acknowledgements

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Chapter 6

Key role of platelet procoagulant activity in tissue-factor- and collagen-induced thrombus formation in arteries and veins in vivo

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Submitted for publication

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Abstract

Blood coagulation and platelet activation are mutually dependent processes, but contribute with different extent to venous and arterial thrombosis. The processes of platelet activation and thrombin generation have been extensively studied *in vitro*, but the precise interactions between these processes are unclear *in vivo*. We investigated the interplay of platelet activation and coagulation in a mouse model of both arterial and venous thrombosis. Thrombus formation was studied *in vivo* by topical application of $FeCl_3$ on mouse mesenteric vessels. Both in arteries and veins, the thrombotic process relied on tissue factor-factor VII(a) interaction and collagen exposure, followed by glycoprotein VI-mediated platelet activation. Mild thrombin inhibition or platelet inhibition suppressed arterial thrombus formation, while strong thrombin inhibition or mild thrombin inhibition in combination with platelet inhibition was necessary to suppress venous thrombosis. Thrombus formation in both vessel types was characterized by the presence of phosphatidylserine (PS)-exposing platelets, detected with fluorescently labeled annexin A5. Shielding of exposed PS by injected annexin A5 abolished formation of both arterial and venous thrombi, while mutant M1234-annexin A5 was ineffective. Decreased anticoagulant activity by using mice carrying the factor V Leiden mutation had only little effect. In this microvascular model of combined tissue factor/collagen-induced thrombosis, propagation of thrombin generation by PS-exposing platelets is a key regulatory process in the formation of thrombi. Platelet-dependent thrombin generation is most active in venous thrombus formation and may be a clinically relevant target to reduce thrombosis.

Introduction

Cardiovascular disease is still the leading cause of death in Western countries. Current understanding is that pathological thrombosis in veins and arteries is caused by the interplay between activated platelets and the coagulation system. However, these two processes contribute differently to either arterial or venous thrombus formation. Thrombi formed in the venous system are mostly rich in fibrin and trapped red cells, though relatively poor in platelets. This is in paradox with the fact the thrombin is one of the most potent platelet agonists[1], which implicates that platelets must become activated by thrombin in thrombotic veins. On the other hand, arterial thrombosis is associated with undesired platelet activation. 'White' thrombi formed in atherothrombotic arteries are rich in aggregated platelets, although fibrin is detectable here as well. This is in agreement with the fact that -in clinical practice- anticoagulant drugs such as heparins and active-

site thrombin inhibitors are preferentially used to reduce venous thromboembolism, while platelet inhibition is preferred in case of arterial thrombosis. Intriguingly, recent *in vivo* experiments, using several animal thrombosis models, have shown that thrombin is also a key regulator of thrombus formation in arteries[1-5]. How this finding relates to the assumed more predominant role of thrombin in venous thrombosis is unclear.

In vitro studies on the regulation of thrombin generation in platelet-rich plasma (PRP) have clearly shown that platelet inhibition, e.g. with cAMP-elevating agents or integrin αIIbβ3 antagonists, have an anticoagulant action by slowing down the formation of thrombin and fibrin[6,7]. Although there is evidence that (activated) platelets stimulate the coagulation process[8,9], the mechanism behind this effect is disputed. Studies *in vitro* have pointed to specific binding of coagulation factors (prothrombin and factors V, X and XI) to integrins and other receptor proteins on platelets[10,11]. The binding of activated factor V is of interest, as platelets are supposed to play a role in the inactivation of factor Va by activated protein C[12]. Also, tissue factor can bind to platelet aggregates and thrombi, and then stimulate the coagulation process[2]. Another proposed mechanism concerns the exposure of procoagulant phosphatidylserine (PS) at the outer surface of activated platelets. At least *in vitro*, PS serves as a site for the assembly of coagulation factor complexes that greatly enhance thrombin generation[13]. A strong trigger of PS exposure is platelet interaction with immobilized collagen, mediated by glycoprotein VI (GPVI)[14,15], while platelet stimulation via thrombin receptors is less effective[16]. Whether and how these platelet-dependent processes indeed contribute to pathological thrombus formation in arteries and veins *in vivo* is poorly understood.

In the present study the importance and the mechanism of platelet-dependent coagulation was investigated in arterial and venous thrombosis. We used an established murine thrombosis model of $FeCl_3$-induced vascular damage, known to be sensitive to both platelet and thrombin inhibition[1,4,17-19]. The data show that thrombin and PS-exposing platelets play prominent, but different roles in the thrombotic process in arteries and veins. This platelet procoagulant response may constitute an attractive novel target for antithrombotic therapies.

Materials and Methods

Animals

C57Bl/6 mice of 4-5 weeks old (11-21 g) or 12 weeks old (20-25 g) of either sex were obtained from Charles River (Maastricht, the Netherlands). Mice deficient in FcRγ-

chain of the same age and background were from Taconics (Germantown, NY, USA)[20]. Mice carrying the Factor V Leiden mutation (Arg 504 to Gln) were described previously[21]. Factor V R504Q mice were backcrossed to C57BL/6J mice (Jackson Labs, Bar Harbor, ME) for at least 8 generations (N8), and N8 heterozygous mice were intercrossed to produce homozygous ($FV^{Q/Q}$), heterozygous ($FV^{Q/+}$), and wildtype offspring ($FV^{+/+}$). The $FV^{Q/Q}$ and $FV^{+/+}$ were intercrossed to produce the $FV^{Q/Q}$ and $FV^{+/+}$ littermates used in the present study. Genotyping for Factor V Leiden was performed by PCR analysis of tail DNA with the primers previously described[21]. Local animal care and use committees approved the experiments.

Materials

Fibrillar Horm collagen (type-I) was purchased from Nycomed Pharma (Munich, Germany). Unfractionated heparin (ISH 1), prostaglandin E_1 and rhodamin 6G were from Sigma (St. Louis, MO, USA). Annexin A5, unlabeled and labeled with fluorescein isothiocyanate (FITC), and M1234-annexin A5 were from Nexins Research (Hoeven, the Netherlands); acridine red came from Chroma (Stuttgart, Germany). Recombinant human tissue factor was from Dade (Düdingen, Switzerland); Z-Gly-Gly-Arg aminomethyl coumarin (Z-GGR-AMC) and Gly-Pro-Arg-Pro (GPRP, Pefablock) were from Bachem (Bubendorf, Switzerland); $FeCl_3$ was from Merck (Darmstadt, Germany). Hematoxylin and eosin came from Klinipath (Duiven, the Netherlands). Ketamine and xylazine were from Eurovet (Bladel, the Netherlands). Transfected cells expressing cDNA encoding for the annexin A5 mutant M1234 were provided by dr. F. Russo-Marie (Bionexins-Pharmaceuticals, France). Melagatran was kindly provided by dr. M. Elg (AstraZeneca, Mölndal, Sweden). Mouse tissue factor, anti-mouse tissue factor antibody and mouse factor VIIai (active site-inhibited factor seven, ASIS)[22] were kindly provided by dr. P. Tijburg (Novo Nordisk, Bagsværd, Denmark).

Perfusion of whole blood under coagulant conditions

Collagen- and tissue factor-induced thrombus formation under coagulant and flow conditions was measured as described[23]. Briefly, mice were bled retro-orbitally, and blood (9 volumes) was collected in 0.129 M citrate (1 volume). The blood was supplemented with FITC-labeled annexin A5 (0.5 µg/ml) and perfused through a parallel-plate transparent flow chamber that contained a coverslip coated with collagen. Coagulation was introduced by co-perfusion with 0.1 volume of $CaCl_2$ (200 mM) and

tissue factor (20 pM). Final wall shear rate was 1000 s^{-1}. Recording of bright-field and fluorescent images was performed with a non-confocal microscopic imaging system.

Thrombus formation in vivo

Four- to 5-week-old mice were subjected to $FeCl_3$-induced thrombus formation in mesenteric blood vessels as described[23]. Topical application of $FeCl_3$ (30 µL, 500 mM) damaged the vessel wall of selected arteries and veins. At baseline, the wildtype arteries had a diameter of 68 ± 4 µm (n=30), while the venous diameter was 107 ± 8 µm (n=20). Diameters of the vessels monitored in genetically modified animals were not different (not shown). An intravital microscopic imaging system allowed continuous recording of thrombus formation in arteries and veins for at least 10 minutes (min). For each vessel, the following parameters were quantified from video recordings off-line: lag-time to first observation of thrombus formation, thrombus height perpendicular to the vessel wall (expressed relative to the local vessel diameter) at 2, 5 and 10 min after $FeCl_3$ application, and time to occlusion.

Where indicated, inhibitors were injected intravenously (bolus, 100 µl) at 5-10 min before application of $FeCl_3$. Control mice received the same volume of saline (vehicle). Injection of FITC-labeled annexin A5 (0.16 µg/g body weight (bw)) shortly after $FeCl_3$ application, visualized PS exposure *in vivo*. Epi-illumination with a Xenon lamp using a sensitive, high-resolution fluorescence imaging system and an intensified CCD camera (Photonic Sciences), enabled capture of high-resolution fluorescence images as described[24]. Centerline blood flow velocities (v) of labeled leukocytes were measured in 8 arteries and 8 veins after injection of rhodamin 6G (30 µl, 1 mg/ml; i.v.). This was converted to wall shear rates (r) according to the Newtonian equation, $r = 8 \times (v / 1.6) /$ vessel diameter.

Thrombin generation measurements

Murine platelet-poor plasma (PPP) or PRP was prepared from blood from 12 week old mice[25]. Thrombin generation was measured using the thrombogram method[26], adapted for murine plasma. Normalized PRP (1.5×10^8 platelets/ml) or PPP supplemented with 4.0 µM phospholipid vesicles of phosphatidylserine : phosphatidylcholine : phosphatidylethanolamine (1:3:1, mol/mol) was used. Plasmas were preincubated with inhibitors for 10 min at room temperature, and subsequently added to a 96-well plate, containing tissue factor (1 pM, final concentration) in Hepes buffer (20 mM Hepes, 140 mM NaCl, 5 mg/ml bovine serum albumin, pH 7.35).

Thrombin formation was started by addition of $CaCl_2$ (16.6 mM) and Z-GGR-AMC (2.5 mM) in Hepes buffer. Fluorescence was continuously measured in time at 37°C with a Fluoroscan Ascent well-plate reader (Thermolab Systems). First-derivative curves of accumulation of fluorescence were generated as described[27]. Assays were run in duplicates or triplicates; calibrations were performed with human thrombin. Effects on peak height of thrombin generation curves (maximal levels) were measured, which parameter is proportional to the maximal rate of thrombin generation.

Statistical analysis

Data are presented as means ± SEM. Differences between experimental groups were tested with the Mann-Whitney U test, using the statistical package for social sciences (SPSS 11.0).

Results

Interaction between tissue factor-induced thrombin formation and platelet phosphatidylserine exposure in blood flowing over collagen

The importance of interactions between platelet activation and coagulation in thrombus formation under flow was evaluated with mouse blood. Thrombin generation was triggered by co-infusion of citrate-anticoagulated blood with a low dose of tissue factor (2 pM) in the presence of $CaCl_2$, directly before entering a collagen-containing flow chamber. At an arterial shear rate of 1,000 s^{-1}, this resulted in early platelet deposition, subsequent aggregate formation and appearance of fibrin fibers after 2-3 min. At a more advanced stage (7-8 min), the chamber occluded with large fibrin-platelet thrombi and trapped red cells (Figure 1A). Labeling of the blood with FITC-annexin A5 (0.5 µg/ml, a low dose not influencing coagulation) indicated that many of the platelets on collagen and on aggregates exposed PS, covering about 20% of the surface area (Figure 1B). In the presence of melagatran (10 µM), a high affinity, active-site thrombin inhibitor[28], aggregate formation still occurred, but fibrin fibers and occlusive thrombi did not form (Figure 1C). In this case, only collagen-bound platelets exposed PS, covering 5% of the surface area (Figure 1D). Quantitative analysis indicated that this melagatran concentration markedly but incompletely delayed and reduced the appearance of PS-exposing platelets during the flow experiment (Figure 1E), while at the same time it suppressed thrombin generation in PRP by 95 ± 2% (mean ± SEM, n=3). Thus, under these high shear flow conditions, melagatran suppressed the formation of PS-expressing

platelets in parallel with the formation of platelet-fibrin clots. Previously, using anticoagulated murine blood, we have shown that the (remaining) platelet aggregate formation and PS exposure results from collagen-induced GPVI activation[25]. Together with the present results, this indicates that the processes of GPVI-induced platelet activation and coagulation activation cooperate in generating procoagulant platelets.

Tissue factor and collagen trigger thrombus formation in arteries and veins

To investigate the role of procoagulant platelets in arterial and venous thrombus formation *in vivo*, we used a $FeCl_3$ model of thrombosis induction in wildtype mouse mesenteric vessels, as described earlier[23]. Wall shear rates in the mesenteric arteries and veins were 1183 ± 187 (n=8) and 344 ± 77 s^{-1} (n=8), respectively. Topical application of $FeCl_3$ resulted in damage of adjacent mesenteric arteries and veins. Histochemical staining indicated that in both vessel types the endothelial layer completely disappeared after $FeCl_3$ treatment, while the vascular structure remained intact (not shown). Real-time visualization by intravital microscopy showed that $FeCl_3$

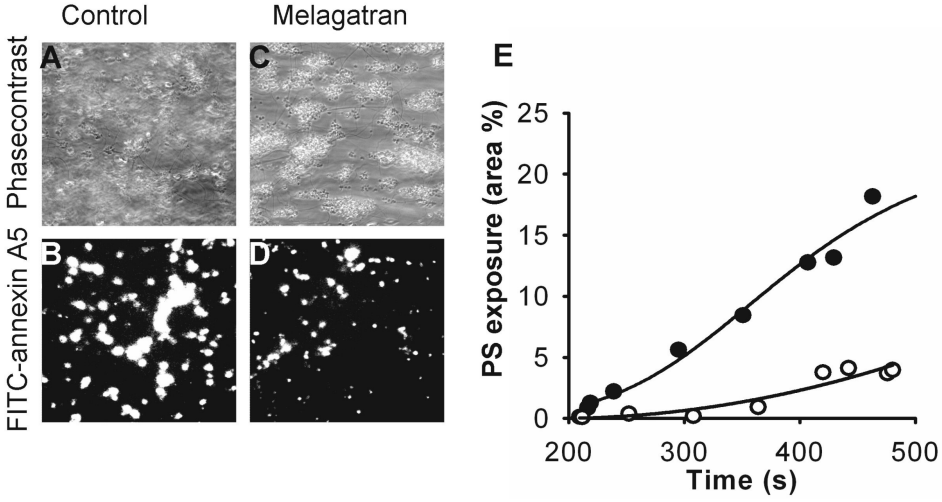

Figure 1 Role of procoagulant platelets in collagen- and tissue factor-induced thrombus formation under shear. Citrated mouse blood was perfused for 4 min over a collagen surface at a shear rate of 1,000 s^{-1}. During perfusion, coagulation was triggered with 2 pM tissue factor and 20 mM $CaCl_2$ (resulting in about 2 mM free Ca^{2+}) in the absence (A, B) or presence (C, D) of 10 µM melagatran. Shown are representative phase-contrast images (A, C) and fluorescence images of PS-exposing platelets (B, D), taken at 4 min after perfusion of blood labeled with 0.5 µg/ml FITC-annexin A5 (n=3). (E) Inhibitory effect of melagatran on PS exposure under flow. Surface area coverage with fluorescence is given during perfusion with blood labeled with 0.5 µg/ml FITC-annexin A5 and triggered with tissue factor/$CaCl_2$. Control condition (•), melagatran condition (o) (n=3).

caused rapid adhesion of platelets in veins, followed by formation of thrombi, while these processes started only after several minutes in arteries (Figure 2A). Under these control conditions, thrombus formation was progressive in both vessel types (Figure 2B). At 10 min after $FeCl_3$ application, 33% of the arteries and 42% of the veins were fully occluded.

To establish the involvement of tissue factor in this thrombosis model, we inhibited tissue factor activity or, alternatively, its ligand factor VII(a) in wildtype mice. Intravenous administration of anti-murine tissue factor antibody (3 µg/g bw) significantly delayed (Figure 2A) and reduced (Figure 2B) the thrombotic process in both arteries and veins. Injection of mouse active site-inhibited factor VII (factor VIIai, 10 µg/g bw) had similar inhibitory effects (Figure 2). *Ex vivo* experiments, using plasma samples from mice injected with factor VIIai, showed that tissue factor-induced (1 pM) thrombin generation was severely delayed and reduced to 6.8 ± 2.4% of control level (n=3). *In*

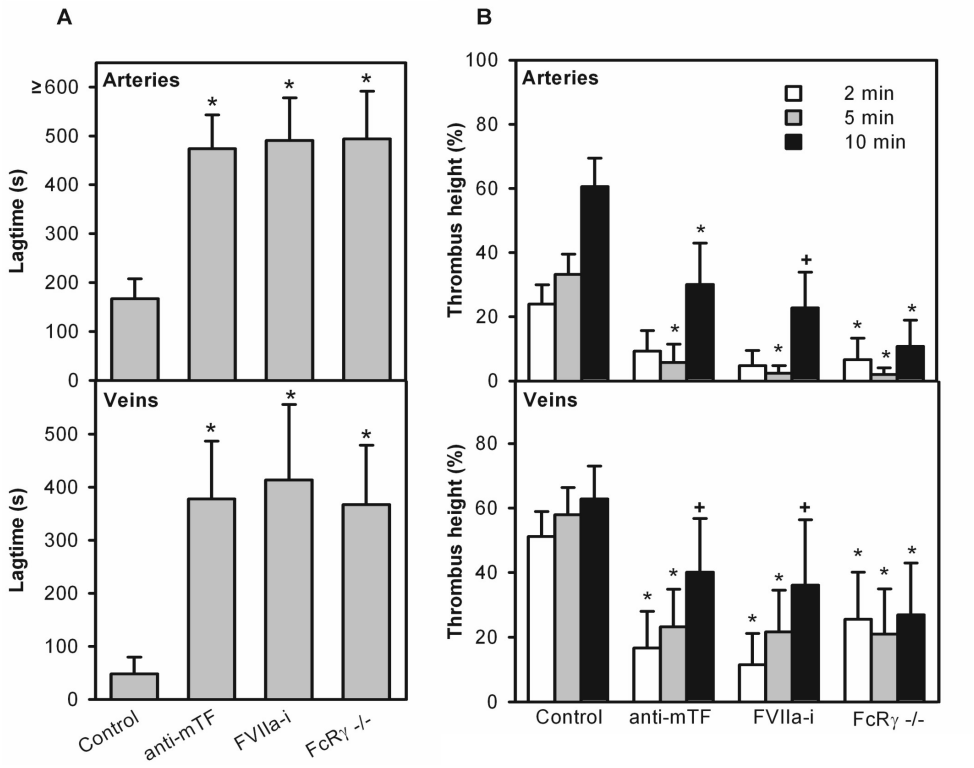

Figure 2 Complementary contribution of tissue factor and collagen in $FeCl_3$-induced thrombus formation in mesenteric arteries and veins in vivo. Wildtype mice were injected intravenously with vehicle (control), 3 µg/g bw anti-mouse tissue factor antibody (anti-mTF) or 10 µg/g bw factor VIIai (FVIIai). Mesenteric vessels of wildtype and FcRγ-chain -/- mice were damaged by topical application of $FeCl_3$. (A) Lag-time to thrombus formation after $FeCl_3$ application. (B) Thrombus height measured at indicated times after $FeCl_3$ application (% of vessel diameter). Data are means ± SEM (n=6-12). *p<0.05; $^{+}$p< 0.1 compared to control.

vitro experiments confirmed that anti-tissue factor antibody or factor VIIai (each 20 µg/ml) blocked thrombin generation in mouse plasma by >85%. To demonstrate the involvement of platelet-collagen interaction in this model, we used mice deficient in the FcRγ-chain, whose platelets do not express the GPVI collagen receptor[29]. In comparison to wildtypes, application of $FeCl_3$ resulted in a greatly delayed and reduced thrombus buildup in both arteries and veins (Figure 2). Earlier results show that injection of wildtype mice with inhibitory anti-GPVI antibody JAQ1 also suppresses thrombus formation in these arteries[23]. We therefore conclude that, in this model, the thrombotic process is regulated by both coagulation activation by exposed tissue factor/factor VII(a) and interaction of collagen with platelets. Interestingly, the role of tissue factor in this process was similar in arterial and venous thrombus formation.

Stronger inhibition of thrombin is required to affect thrombus formation in veins

To investigate how thrombin generation and coagulation drive the thrombotic process in arteries and veins, wildtype mice were injected with a moderate or higher dose of melagatran (0.175 or 0.5 µg/g bw, respectively). *Ex vivo* analysis indicated that these doses gave 3-fold and higher prolongation in clotting times (see also below). In comparison to control mice, injected with saline vehicle, injection of 0.175 µg/g bw melagatran did not influence the lag-time to thrombus formation in arteries (Figure 3A), but thrombus height was significantly reduced (Figure 3B). After administration of the higher melagatran dose (0.5 µg/g bw), the arterial lag-time to thrombus formation was prolonged to 514 ± 86 s (mean ± SEM, n=7, Figure 3A), while thrombus build-up was again greatly reduced (Figure 3B). Strikingly, venous thrombus formation was inhibited only by the higher dose of melagatran (Figure 3A, B). None of the veins occluded within 10 min with the moderate or high dose of melagatran. Similar observations were made after injection of 0.05 U/g bw unfractionated heparin, an indirect thrombin inhibitor. This only inhibited thrombus formation in arteries ($p<0.05$), but not significantly in veins (not shown).

Plasma samples were obtained from blood collected after 30 min of melagatran administration. The thrombogram method was used to determine the activity of melagatran in *ex vivo* mouse plasma and indicated that thrombin peak height was diminished to 18% and 4% of control, respectively (Figure 3C).

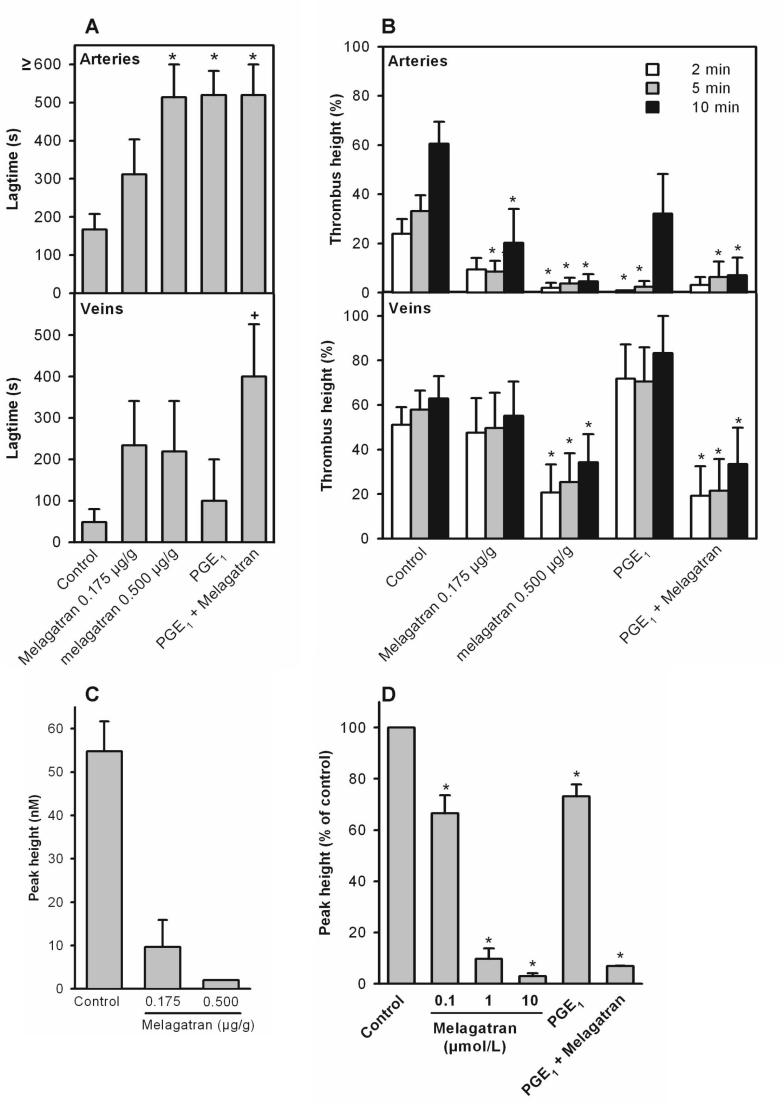

Figure 3 Complementary roles of thrombin and platelet activation in $FeCl_3$-induced thrombus formation in arteries and veins. Mice were injected with saline vehicle (control), melagatran (0.175-0.500 µg/g bw) and/or prostaglandin E_1 (PGE_1, 0.4 mg/g bw). Mesenteric vessels were damaged by topical application of $FeCl_3$ as in Figure 1. (A) Lag-time to thrombus formation after $FeCl_3$ application. (B) Thrombus height measured at indicated times after $FeCl_3$ application (% of vessel diameter). Data are means ± SEM (n=4-9). *p<0.05; $^{+}$ p<0.07 compared to control. (C) Effect of *in vivo* injection of melagatran (0.175-0.5 µg/g bw) on thrombin generation. After *in vivo* experimentation, blood was collected, and PPP was prepared and supplemented with 4.0 µM phospholipid vesicles. Thrombin generation was triggered with tissue factor/$CaCl_2$. (D) Effect of *in vitro* added inhibitors on thrombin generation. Mouse PRP (1.5×10^8 platelets/ml) was pre-incubated with melagatran (0.1-10 µM) and/or PGE_1 (10 µM), and thrombin generation was triggered with tissue factor/$CaCl_2$. Data show effects on peak height relative to control condition; means ± SEM (n=3-5); *p<0.05 compared to control.

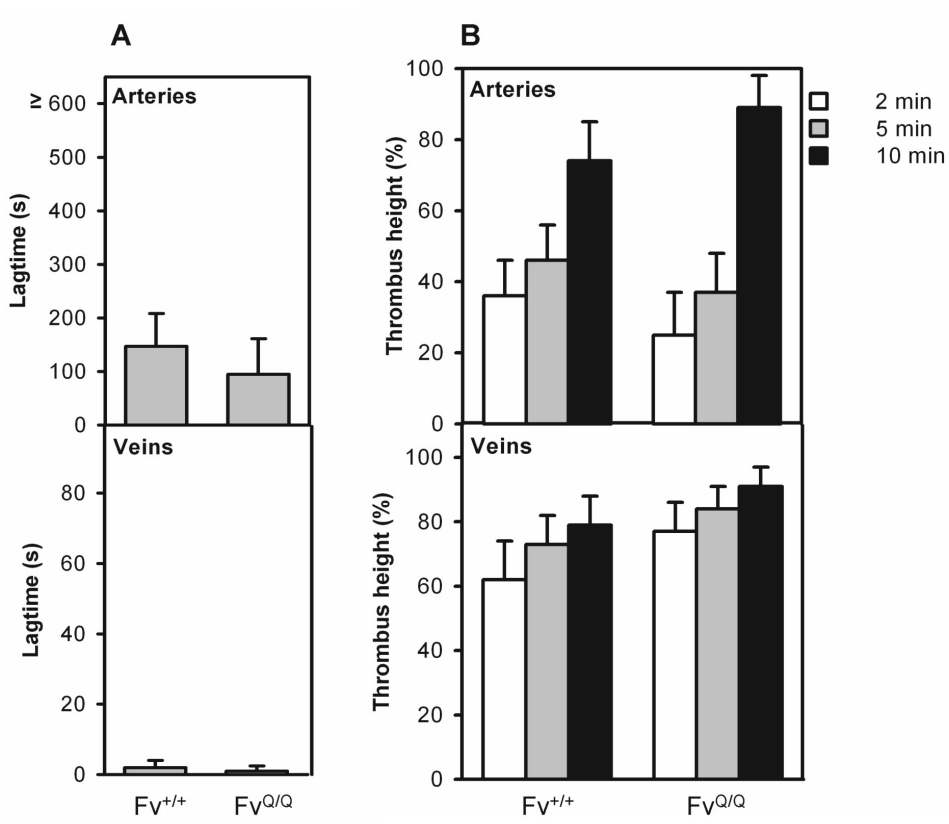

Figure 4 Normal $FeCl_3$-induced thrombus formation in arteries and veins of factor V Leiden mice. Mesenteric vessels of homozygous factor V Leiden ($Fv^{Q/Q}$) and wildtype ($Fv^{+/+}$) mice were subjected to $FeCl_3$-induced thrombus formation. (A) Lag-time to thrombus formation after $FeCl_3$ application. (B) Thrombus height at indicated times after $FeCl_3$ application (% of vessel diameter). Data are means ± SEM (n=8-13).

Similarly, when added to PRP *in vitro*, melagatran (Figure 3D) or heparin (not shown) dose-dependently reduced tissue factor-triggered thrombin generation; the IC_{50} for melagatran was about 0.5 µM. Together, these data stress the importance of thrombin generation and coagulation in this mouse thrombosis model, and, furthermore show that stronger inhibition of the thrombin-generating process is required to affect venous compared to arterial thrombus formation.

Additive effect of platelet and thrombin inhibition on venous thrombus formation

To inhibit platelet activation, the cAMP-elevating agent prostaglandin E_1 (PGE_1) was used. In isolated mouse PRP, 10 µM PGE_1 substantially reduced platelet aggregation (not shown) and platelet-dependent thrombin generation (Figure 3D, peak

height reduced to 73 ± 5%, p=0.003). This corresponds to results obtained with human PRP[6,27]. *In vivo* treatment of mice with 0.4 mg/g bw PGE_1 (giving a plasma concentration of about 10 µM) significantly delayed and reduced thrombus formation in arteries, but not in veins (Figure 3A, B). *Ex vivo* measurements demonstrated that the injected PGE_1 caused a 40% reduction in thrombin formation.

Combined administration of PGE_1 (0.4 mg/g bw) and the lower dose of melagatran (0.175 µg/g bw) resulted in significant retardation and reduction of the thrombotic process, not only in arteries, but also in veins (Figure 3A, B). Thus, in veins, the combined administration was much more effective than that of PGE_1 or moderate melagatran alone. Or stated otherwise, platelet inhibition by PGE_1 or moderate thrombin inhibition by melagatran alone was sufficient to inhibit arterial thrombus formation, but the combined action was needed for reducing venous thrombosis.

Unchanged thrombus formation in arteries and veins of factor V Leiden mice

To investigate the involvement of anticoagulant activity in the thrombus-forming process, we used transgenic mice carrying the factor V Leiden mutation (homozygous $FV^{Q/Q}$), which renders factor Va relatively resistant to inactivation by activated protein C and hence enhances thrombin generation[21,30]. When compared to wildtype controls ($FV^{+/+}$), in $FV^{Q/Q}$ mice neither the lag-time to thrombus formation (Figure 4A) nor the extent of thrombus formation (Figure 4B) was altered in arteries or veins. However, the time to arterial occlusion of $FV^{Q/Q}$ mice was significantly shorter than that of $FV^{+/+}$ mice (462 ± 62 *vs.* 531 ± 41 s, n=8, $p<0.05$). Apparently, in spite of the key role of thrombin in this process, resistance of factor Va to inactivation by activated protein C, as in $FV^{Q/Q}$ mice, did not have a major effect on arterial and venous thrombus formation.

Procoagulant platelets control thrombus formation in arteries and veins

To investigate the role of procoagulant platelets in the thrombus-forming process, $FeCl_3$ was applied to induce thrombus formation, and mice were subsequently injected with a tracer amount of FITC-labeled annexin A5 (0.16 µg/g bw), which detects cell surface-exposed PS. Overlays of transmission and fluorescence images indicated that the thrombi in both arteries and veins incorporated labeled annexin A5 (Figure 5A), pointing to the presence of PS-exposing platelets. During the experiments no staining with FITC-annexin A5 was observed inside the vessel wall or in vessels without thrombi (not shown).

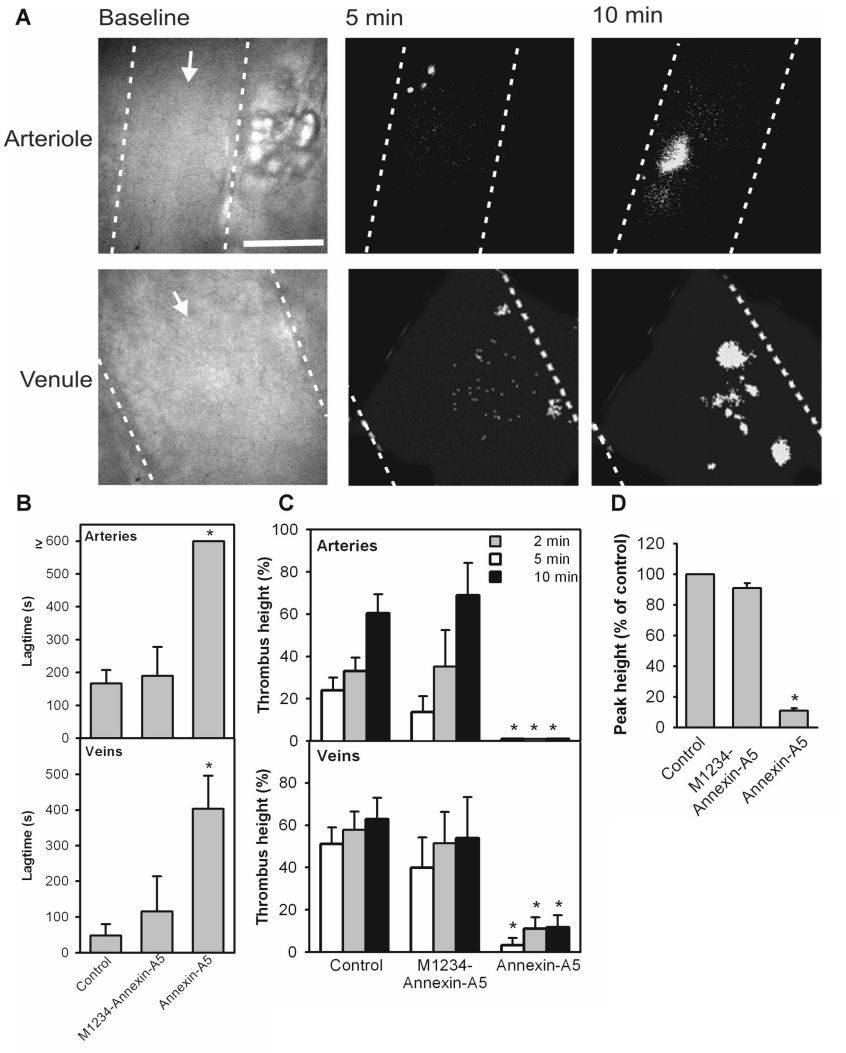

Figure 5 Role of platelet procoagulant activity in arterial and venous thrombus formation in vivo. (A) Vessel wall damage was induced by topical $FeCl_3$ application, directly after which FITC-annexin A5 (0.16 µg/g) was infused intravenously. Trans-illumination images were taken at baseline; fluorescence images were taken at 5 and 10 minutes after $FeCl_3$ application. Dotted lines indicate vessel wall, arrows indicate direction of flow; bar represents 50 µm. (B) Mice were infused with a high dose of 7.7 µg/g bw annexin A5 or mutated M1234-annexin A5, resulting in plasma concentration of 70 µg/ml. Mesenteric vessels were damaged by topical application of $FeCl_3$. (B) Lag-time to thrombus formation after $FeCl_3$ application. (C) Thrombus height measured at indicated time points after $FeCl_3$ application (% of vessel diameter). Data are means ± SEM (n=6-7); *p<0.05 compared to control. Note that M1234-annexin A5 infusion led to a similar thrombus build-up in arteries and veins as vehicle (control) infusion (p=0.179). (D) *In vitro* effect of annexin A5 on thrombin generation in PRP. Thrombin generation was measured in PRP, treated with a moderate dose (10 µg/ml) mutated M1234-annexin A5 or wildtype annexin A5. Values are thrombin peak heights as % of control (means ± SEM, n=3-4).

The importance of PS exposure was further investigated by injecting mice with a saturating dose (7.7 µg/g bw) of unlabeled annexin A5. Quadruple-mutated annexin A5 (M1234-annexin A5, 7.7 µg/g bw) was used as a negative control. The M1234-annexin A5 has single amino acid mutations in each of the four Ca^{2+} binding sites that greatly reduces its affinity for binding to PS[31]. Whereas injection of M1234-annexin did not influence thrombus formation as compared to vehicle control ($p \geq 0.18$), wildtype annexin A5 almost completely inhibited this process in both arteries and veins (Figure 5B, C). In fact, in none of the 7 damaged arterioles more than little platelet deposition was detected within the 10-min time frame, while in 3 out of 6 venules only very small thrombi assembled.

Thrombin generation experiments *in vitro* showed that addition of 10 µg/ml annexin A5 to PRP was sufficient to reduce the thrombin peak to 11 ± 1.6% (n=3) of control, while M1234-annexin A5 was without effect (Figure 5D). In plasma collected from mice injected with annexin A5 (7.7 µg/g bw), thrombin generation was reduced to 17 ± 4% (n=4) of control plasma. Thus, in either vessel type, thrombus formation appears to rely on PS exposure and ensuing thrombin generation by (activated) platelets. Similarly, in *in vitro* flow experiments (see Figure 1), addition of annexin A5 reduced PS exposure by platelets and clot formation (not shown).

Discussion

In this paper, we investigated the interaction mechanisms of platelets and coagulation in thrombus formation under *in vivo* conditions, using an established murine thrombosis model of vascular damage induced by $FeCl_3$. In both arteries and veins, we detected substantial externalized PS on platelets trapped into thrombi, using fluorescently labeled annexin A5. This probe has previously also been used for *in vivo* monitoring of PS-exposing, apoptotic cardiomyocytes in a mouse model of ischemia/reperfusion of the heart[32]. A major finding was that injection of a high dose of annexin A5, sufficient to suppress PS-dependent thrombin generation in plasma, results in a profound delay in initiation and in an almost complete suppression of arterial and venous thrombus formation. In contrast, a mutated non-binding annexin A5, was without any effect. These *in vivo* data are in good agreement with results from *in vitro* flow experiments, where PS-exposing platelets were found to accumulate under conditions of high thrombin generation and clot formation. Together, this is first evidence for a key role of PS exposure and platelet-dependent coagulation in the thrombotic process *in vivo*, in both arteries and veins. This work thereby significantly extends knowledge from

experiments in test tubes that PS-exposing platelets promote prothrombin cleavage[9,13]. Indirect evidence for the clinical importance of this process comes from the finding that Scott patients, whose platelets are deficient in PS exposure upon activation, are characterized by a mild bleeding defect[33].

Strikingly, in this $FeCl_3$-induced microvascular model the same initiating factors appear to be involved in the thrombus-forming process in arteries and veins. Accordingly, in both vessel types blocking of tissue factor with a monoclonal antibody or injection of inactivated factor VIIa (both of which interventions block the tissue factor pathway of coagulation) delayed and suppressed thrombus formation. Others have shown that tissue factor, platelets and fibrin gradually accumulate into developing murine thrombi, produced in microvascular arterioles[2]. For the current model, we cannot answer the question whether[34] or not[35] blood-borne tissue factor is involved in the thrombotic process. However, the data do support the proposal that tissue factor is a promising target for new antithrombotic treatment[36].

Another similarity between arteries and veins we found in this model, is that the absence of FcRγ-chain and GPVI delays and reduces thrombus formation in both vessel types. The arterial results agree with those of others, who found diminished arterial thrombus formation in mice lacking the FcRγ-chain, using a similar model[3,37]. This also holds for high shear flow studies showing impaired thrombus formation with blocked or absent GPVI signaling *in vitro*, regardless of the presence of coagulation[23,25]. In contrast, laser-induced thrombosis is not diminished in arteries of FcRγ-chain deficient mice, likely to due a high generation of thrombin after this type of injury[3,4].

Furthermore, our results show that, in arteries as well as veins, thrombin is an important mediator of the thrombotic process. In both vessel types, thrombus formation is abrogated by infusion of the thrombin inhibitor melagatran at an established fully effective concentration. Interestingly, however, while in arteries also a lower, partly active melagatran concentration is inhibiting, in veins this concentration is without effect, except when given in combination with platelet-inhibiting PGE_1. For arterial thrombus formation induced by $FeCl_3$, laser or mechanical damage, significant contribution of thrombin is also noted by others[1,4,5,18].

The importance of anticoagulation was investigated using $FV^{Q/Q}$ mice carrying the factor V Leiden mutation, rendering factor Va relatively resistant to inactivation by activated protein C[21]. It has been proposed that factor Va inactivation is controlled by binding to the platelet surface[12], implicating that thrombus formation is potentially regulated by factor Va-platelet binding. Here, we found that $FV^{Q/Q}$ vessels had no more than a small increase in arterial or venous thrombus formation; significant effects, i.e. on

arterial occlusion, were only present at a late stage of thrombus formation. Thus, although in the present model arterial and venous thrombosis is controlled by coagulation factor activation (thrombin), factor Va inactivation only appears to play a significant role at later stages of the process. Therefore, our results do not support the observation that the factor V Leiden mutation enhances arterial thrombosis in mice[38].

Taken together, the present data indicate that the thrombotic process in arteries as well as veins involves: *(i)* platelet-collagen interaction via GPVI/FcRγ-chain, *(ii)* tissue factor-induced initiation of thrombin generation, *(iii)* platelet activation and aggregation, and *(iv)* development of platelet procoagulant activity, i.e. PS exposure, to potentiate thrombin generation and clot formation. Apparently, in both arteries and veins the thrombotic process is driven by sequential loops of thrombin generation, platelet activation and PS exposure.

While the mechanisms of arterial and venous thrombus formation are strikingly similar, the present data also point to typical quantitative differences. Thrombus formation in veins was faster in onset than in arteries. Such differences between arteries and veins were also found in other, non-$FeCl_3$ thrombosis models[39]. In the present model, one of the reasons of the faster onset of venous thrombosis may be a quicker penetration of $FeCl_3$ through the relatively thin venous vessel wall. Another possible explanation is the slower dilution in veins of locally formed prothrombotic substances (factor VII, ADP, thrombin). Support for the latter comes from the observation that stronger inhibition of thrombin, or combined inhibition of thrombin and platelets, is required to suppress thrombus formation in veins in comparison to arteries. This points to a relatively high accumulation of thrombin in the slowly flowing venous blood. A third possibility is that more thrombin is formed in damaged veins, for instance because they may express more tissue factor, which is known to be distributed through the vascular system in a non-uniform way[40].

In conclusion, the present study provides new insights into similarities and differences in the dynamic process of arterial and venous thrombus formation. In this murine model, platelet PS exposure appears to be an important regulatory process in either vessel type, integrating platelet activation and thrombin generation. This *in vivo* study thus indicates that platelet procoagulant activity may be a clinically relevant target for antithrombotic therapy.

Acknowledgments

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Chapter 7

Signaling-induced transient integrin activation controls segregation of procoagulant and aggregatory microdomains in thrombus formation

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Abstract

Platelets play a dual role in thrombosis by forming aggregates and stimulating coagulation. We investigated the commitment of platelets to these separate functions during thrombus formation *in vitro* and *in vivo*. Flow of human or mouse blood over collagen under coagulant conditions *in vitro* results in fibrin-rich thrombi with platelets exposing phosphatidylserine (PS). Two-photon microscopy reveals these thrombi to be heterogeneous in composition. They partly consist of microdomains of aggregated, fibrin(ogen)-binding platelets with activated αIIbβ3 integrins. These are segregated from strings of PS-exposing platelets, low in αIIbβ3 activation but displaying increased binding of all components of the prothrombinase complex (factor Va, factor Xa and prothrombin). Typically, "coated" platelets, binding factor Va, overlap both types of microdomains. *In vivo*, damage of the murine carotid artery leads to production of thrombi with a similar, patch-like composition. A key regulatory event in the appearance of PS-exposing platelets is the secondary down-regulation of active αIIbβ3, which is mediated by increased tyrosine kinase activity. Together, these results point to a local segregation within thrombi of aggregated and coagulation-active platelets, whereby tyrosine kinases trigger to integrin inactivation, thus reducing the aggregatory properties of procoagulant platelets.

Introduction

Activated platelets have a dual role in hemostasis and thrombosis. They form the building blocks of a thrombus and provide the membrane surface for coagulation factor activation, which results in thrombin and fibrin formation[1,2]. Once formed, thrombin greatly enhances the activation and aggregation of platelets. Given this strong interdependency of thrombin generation and platelet activation, it is intuitively assumed that the platelets that participate in aggregate formation are also involved in coagulation.

The mechanism(s) by which platelets contribute to the coagulation process are still incompletely identified. Kinetic evidence shows that collagen/thrombin-activated platelets expose phosphatidylserine (PS) at their outer surface and then bind Gla domain-containing coagulation factors, mediating factor Xa and thrombin generation[3]. However, even after activation with strong Ca^{2+}-mobilizing agonists, not all platelets expose PS[4-6]. Conversely, there is evidence that PS exposure alone is insufficient to fully explain the procoagulant effect of platelets[7,8]. Another point of debate is whether subfractions of activated platelets have specific functions in the coagulation process. Several reports indicate that only part of the PS-exposing platelets are capable to bind

one or more of the coagulation factors Va, VIIIa, IXa and Xa[9-11]. One study identifies a subpopulation of activated platelets with so-called SCIP morphology (for **s**ustained **c**alcium-**i**nduced **p**latelet), which are targets for factor XIIIa or other transglutaminases and partly express PS[12]. Some authors describe a subpopulation of **co**llagen- **a**nd **t**hrombin-activated (COAT or 'coated') platelets, which express serotonin binding sites at their outer surface that function in the assembly of sheets of fibrinogen, von Willebrand factor (vWF) and factor Va[13-15]. However, others conclude that factor XIIIa is not required for generation of the coated platelet phenotype[16]. Thus, there is substantial evidence that individual platelets respond in a different way to collagen/thrombin activation by binding to coagulation factors. However, the mechanism of origin and the function of these differences are still unclear. Moreover, the relevance of this for the thrombus forming process is completely unknown.

Recently, we and others have shown that platelets which adhere to collagen under high shear flow conditions are heterogeneous with respect to Ca^{2+} signaling and PS exposure[12,17]. Here, we aimed to extend this work by hypothesizing that differences in intracellular signaling properties determine the 'fate' of a platelet to become either procoagulant or proaggregatory. Our results show that, both in flow chambers *in vitro* and in arterial thrombosis models *in vivo*, platelets in a thrombus appear to segregate into distinct microdomains with aggregatory or procoagulant functions. A key discriminatory event in the segregation process is the activation and subsequent inactivation of integrin αIIbβ3.

Materials and methods

Materials

Convulxin was purified to homogeneity from the venom of *Crotalus durissus terrificus* (Latoxan, France)[4]. Annexin A5 labeled with Oregon Green (OG)-488, Alexa Fluor-568 (AF568) or AF647 was from Molecular Probes (Leiden, The Netherlands), as were OG488-conjugated human fibrinogen, Syto-44 and AF532-labeled streptavidin. Fluorescein isothiocyanate (FITC)-labeled monoclonal antibody against human P-selectin (α-CD62 mAb) was from WAK Chemie (Steinbach, Germany); FITC-labeled PAC1 antibody from BD Biosciences (San Jose, CA); (FITC-labeled) anti-phosphotyrosine mAb 4G10 from Upstate Biotechnology (Dundee, UK); anti-actin mAb AC-40 from Sigma (St. Louis, MO). Lotrafiban was a gift from GlaxoSmithKline (Middlesex, UK). Biotin-pentylamine-succinylated bovine serum albumin (BPA-sBSA),

prepared by reacting succinylated BSA with biotin-pentylamine, was kindly provided by dr. G. Dale (Dept. of Medicine, Health Science Center, Oklahoma). BPA-sBSA recognizes serotonin binding sites on coated platelets and is less sensitive to oxidation than the parent compound, biotin-BSA-$(5HT)_n$[14]. Biotin was detected with AF488- or AF532-labeled streptavidin. Other materials were from sources described earlier[17].

Fluorescent coagulation factors

Active site OG-labeled, human factor Xa was prepared by inactivation of native factor Xa with N^{α}-[(acetylthio)acetyl]-(*D*-Phe)-Pro-Arg-CH_2Cl, and covalent modification with OG488-iodoacetamide following mild NH_2OH treatment, as described[18]. OG-prothrombin was labeled at the active site by a similar method, following formation of the catalytic site on the prothrombin zymogen by the use of a staphylocoagulase fragment[19]. Stoichiometries of OG incorporation into factor Xa and prothrombin were 0.8 and 0.9 mol probe per mol protein, respectively. OG-prothrombin had 0.1% residual native prothrombin, determined as described[19]. Bovine factor V, purified as described[20], was suspended in phosphate-buffered saline and labeled with AF488 protein labeling kit using a PD-10 column (Molecular Probes). The AF-488 labeled factor V contained 15 mol probe per mol protein. After activation with thrombin, the labeled factor Va had retained its cofactor function in enhancing factor Xa activity. SDS-gelelectrophoretic analysis under reducing conditions showed that both the heavy and light chains of factor Va were labeled. Concentrations of labeled coagulation factors were confirmed by protein assay.

Collection of human and mouse blood

Blood was obtained from healthy volunteers with full informed consent. For experiments in the absence of coagulation, blood was collected into 40 μM *D*-Phe-Pro-Arg-chloromethyl ketone (PPACK) in 10% saline, and supplemented hourly with additional 20 μM PPACK. For experiments involving coagulation, blood was collected in 10% 0.129 M trisodium citrate.

Wildtype 12-week-old C57Bl/6 mice of either sex (20-25 g) were obtained from Charles River (Maastricht, The Netherlands). Blood was collected under anesthesia with ketamine and xylazine (Eurovet, Bladel, The Netherlands) by orbital puncture. For experiments in the absence of coagulation, blood was collected in PPACK/heparin[21]; for experiments involving coagulation, blood was collected into 0.129 M trisodium citrate. Experiments were approved by the local animal care and use committees.

Two-photon laser scanning microscopy (TPLSM) and confocal microscopy

TPLSM was performed with a BioRad 2100 multiphoton system. Excitation was with a Spectra Physics Tsunami Ti:Sapphire laser, tuned and mode-locked at 800 nm, producing pulses of 100 fs wide (repetition rate 82 MHz), which was connected to an upright Nikon E600FN fluorescence microscope[22]. Two photomultipliers with separate pinholes detected fluorescence, selected by optical filters, at 508-523 nm and 570-620 nm. A third photomultiplier detected fluorescence above 660 nm from a parallel-placed red diode laser, exciting at 647 nm. This system was used for confocal scanning of thrombi in flow chambers during or after perfusion, and for deep scanning of intact carotid arteries, mounted in a home-build perfusion chamber. Thrombi were double or triple labeled to detect fluorescence from the probes OG488 (color-coded green), AF568 or Syto-44 (color-coded blue), and AF647 (color-coded red). Optical sections were scanned in Kalman filtering mode without further image processing. Single-photon, two-color confocal laser scanning microscopy, using a Leica confocal microscope (DM, IRE2; Leica, Milton Keynes, UK), was performed as described[23]. Analysis of all confocal images (gray level bit maps) and 3D reconstruction of images were with ImagePro/LaserPix software (Media Cybernetics, Silver Spring, MD). Degree of co-localization of two fluorophores was evaluated using the Pearson's correlation coefficient (R_r), which describes the overlap between two colored patterns, and is independent upon pixel intensity values. The overlap coefficient (R) and the sub-coefficients k_1 and k_2, which vary with differences in color intensities, were also calculated.

Thrombus formation on collagen under flow

Human or murine blood was perfused at shear rate of 150-1000 s^{-1} over a collagen surface, as described[24]. Briefly, glass coverslips were coated with fibrillar Horm type-I collagen, blocked with Hepes buffer pH 7.45 (5 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4 and 1% BSA), and then placed in a parallel plate flow chamber (slit depth of 50 µm). Blood pre-incubated for 15 minutes with inhibitors and/or probes, was perfused for up to 4 minutes over the collagen. Thereafter, the flow chamber was post-perfused for a further 4 minutes with Hepes buffer, pH 7.45 containing 2 mM $CaCl_2$ and 1 U/mL heparin. For coagulation experiments, citrate-anticoagulated blood was co-perfused with 10 vol% of Hepes buffer, pH 7.45 containing 20 pM tissue factor and 200 mM $CaCl_2$ upon entry into the flow chamber[24].

Bright-field phase-contrast and fluorescence images of adherent platelets were also recorded using a non-confocal two camera system[25]. Surface coverage with

platelets was analyzed using ImagePro software. Data from >10 different fields of view were averaged per experiment without image processing.

Thrombus formation in vivo

Twelve-week-old mice were anesthetized by subcutaneous injection of ketamine and xylazine, followed by continuous infusion of ketamine. Fluorescently labeled compounds were administered intravenously through a PE-10 catheter in the tail vein[26]. Carotid arteries were dissected free and ligated vigorously for 5 minutes to induce vascular injury. In other animals, the adventitial surface of the carotid artery was damaged by local application of a filter paper (0.5×1.0 mm) with 1 M $FeCl_3$ for 5 minutes. With either method, thrombus formation was allowed to proceed for 10 minutes, after which fluorescence in the still intact vessel was monitored by TPLSM. For *ex vivo* evaluation, 5 mm of the artery was carefully removed, mounted between two micropipettes in a perfusion chamber, and subjected to 1.0 atmosphere pressure[22]. Mounted arteries were post-labeled for 30 minutes with Syto-44 (2 μM in phosphate-buffered saline), to stain for nuclei.

Flow cytometry

Diluted PRP or washed platelets (1×10^8 platelets/mL) were activated with 50 ng/mL convulxin and 4 nM thrombin in the presence of 2 mM $CaCl_2$ for 5-30 minutes. Samples were incubated with fluorescent labels and/or antibodies, as described in the text. For double labeling experiments, probes exciting at 488 and 647 nm were added simultaneously at saturating concentrations. Detection of fluorescence was with a FACScan flow cytometer, equipped with an argon and a red diode laser (Becton-Dickinson, Franklin Lakes, NJ). For analysis, platelets were gated based on their forward scatter/side scatter (FSC/SSC) characteristics. A minimum of 10,000 events was counted per assay. Control measurements were performed with unlabeled/stimulated and labeled/unstimulated platelets. List-mode data were analyzed using WinMDI 2.8 software (http://facs.scripps.edu).

Protein tyrosine phosphorylation in adherent platelets

After flow experiments, adherent platelets on collagen-coated coverslips were stained with AF647-annexin A5, and then fixed for staining with FITC-4G10 anti-phosphotyrosine mAb. Alternatively, platelets on coverslips were carefully lysed with ice-cold NP-40-based lysis buffer pH 7.4 (300 mM NaCl, 20 mM Tris-HCl, 2 mM EGTA, 2

mM EDTA, 2 mM Na_3VO_4, 1 mM 4-(2-aminoethyl)-bezenesulfonylfluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 2% NP-40). Protein was quantified in the lysates with a BioRad DC protein kit. Lysates with calculated equal protein amounts were resolved on 10% SDS-PAGE gels, then transferred to blotting membranes by semi-dry transfer. Membranes were immuno-blotted using an ECL system (Amersham Biosciences, Bucks, UK) with 4G10 mAb followed by stripping of the membranes and re-probing with AC-40 anti-actin mAb to verify equivalent loading[27].

Statistics

Differences were tested on significance with the Mann-Whitney U test using the statistical package for social sciences (SPSS 11.0, Chicago, IL). Results are given as mean values ± SD, unless otherwise indicated.

Results

Microdomains of aggregated and procoagulant platelets in thrombi formed under flow

In the absence of coagulation, flow of human or murine blood over collagen leads to GPVI-dependent formation of thrombi containing PS-exposing platelets[21,25,26]. We studied how the tissue factor coagulation pathway contributed to this thrombus formation, by flowing human blood over collagen under high shear conditions in the presence of different fluorescent labels. Using PPACK-treated blood, OG-labeled fibrinogen (0.2 mg/mL) slowly, but steadily incorporated into platelet aggregates that were formed on collagen (Figure 1A). The probe OG-annexin A5 (0.5 μg/mL), which labels exposed PS[4], only started to accumulate after a delay of 200 seconds (Figure 1B). When coagulation was triggered by co-perfusion of citrate blood together with tissue factor/$CaCl_2$, the formation of platelet aggregates and fibrin fibers was followed by that of larger clots containing erythrocytes. The incorporation of OG-fibrinogen was increased in this condition, and it further enhanced at the time point (~170 seconds) of clot formation (Figure 1A). The addition of OG-annexin A5, which is an anticoagulant[28], typically delayed the clotting time to 200 seconds. After this time point, annexin A5 labeling greatly increased (Figure 1B, see also below).

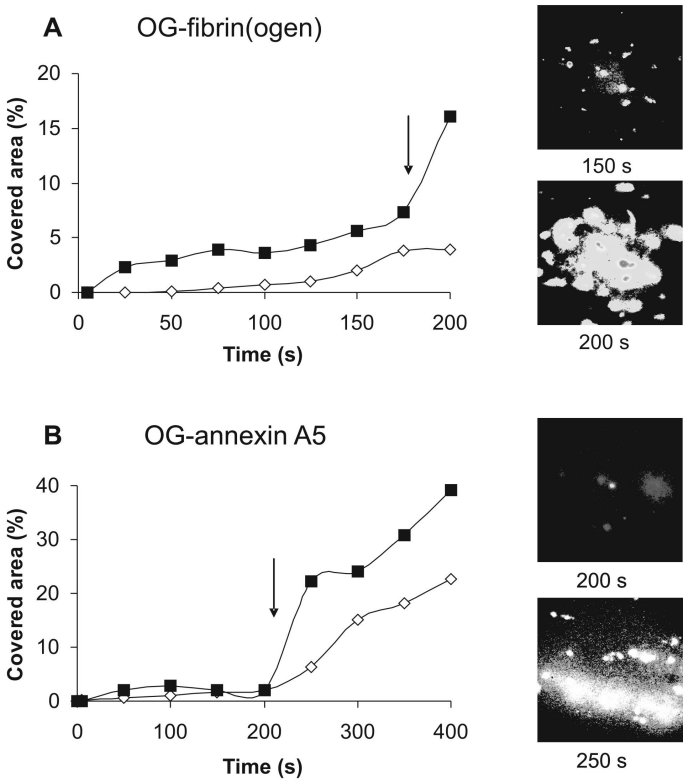

Figure 1 Time-dependent accumulation of labeled fibrin(ogen) and annexin A5 in thrombi formed in the absence or presence of coagulation. Human blood containing 0.2 mg/mL OG-fibrinogen (A) or 0.5 µg/mL OG-annexin A5 (B) was perfused over collagen at a shear rate of 1000 s^{-1}, and fluorescent images were captured from the collagen surface. PPACK-anticoagulated blood was used (open diamonds) or, alternatively, citrate-anticoagulated blood that was co-perfused with 2 pM tissue factor and 2 mM free $CaCl_2$ (final concentrations) to reach physiological Ca^{2+}/Mg^{2+} concentrations (filled squares). Surface area coverage with fluorescence of a representative experiment out of 4 performed is shown; arrows indicate appearance of microscopic clots. Images (150×150 µm) shown are examples of experiments in which coagulation occurred (150-250 s).

Multicolor TPLSM with high optical resolution and high penetration power was used to better localize the bound OG-fibrin(ogen) and AF647-annexin A5 within human and murine thrombi formed on collagen. These labels were simultaneously or sequentially added to the blood, either during or after flow, with essentially the same results (data not shown). Comparison of bright-field contrast images and two-color fluorescent images indicated that OG-fibrinogen was only present on aggregated platelets, whilst AF647-annexin A5 bound a distinct population of single platelets (Figure 2, *upper and middle rows*). Triggering of coagulation with tissue factor/$CaCl_2$ did not influence the labeling of OG-fibrin(ogen), still binding to aggregated platelets. Again, AF647-annexin A5 labeled a different population of platelets around the clots. The same

staining patterns were obtained with both human and murine blood (Figure 2), including when any one of the two labels was omitted (data not shown). Thus, the heterogeneous labeling pattern was not due to competition between labels for the same binding sites on platelets.

'Coated' platelets are recognized by their property to bind multi-amine, serotonin-like compounds such as BPA-sBSA, and may participate in coagulation[13,14]. To detect the formation of coated platelets under flow, we allowed thrombi to form on collagen in the presence of labeled BPA-sBSA. Simultaneously, AF647-annexin A5 was present in this experiment to detect PS-exposing platelets. Surprisingly, the BPA-sBSA labeling pattern was markedly different from that of annexin A5 in both human and

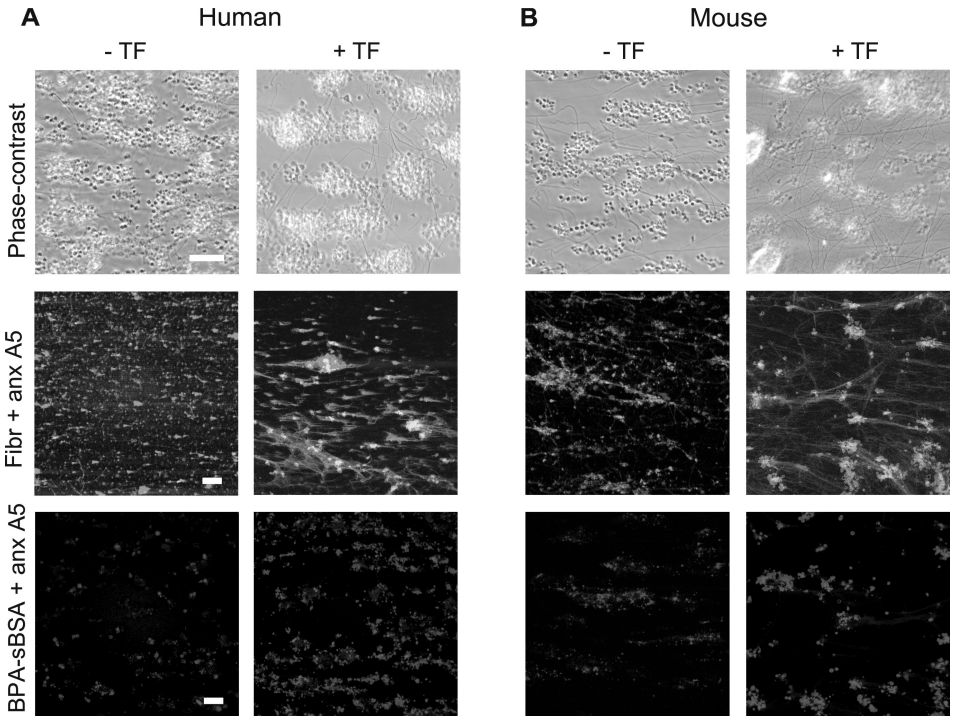

Figure 2 Heterogeneity in human and murine thrombi formed on collagen. Flow experiments were performed with human (A) or murine (B) blood in the absence of tissue factor (-TF) using PPACK-anticoagulated blood (left columns); or with citrate blood that was perfused together with tissue factor (+TF, 2 pM, f.c.) and $CaCl_2$ (2 mM free Ca^{2+}, f.c.) to allow coagulation (right columns). Standard perfusion time was 4 minutes at a shear rate of 1000 s^{-1}. Blood was preincubated with 0.2 mg/mL OG-fibrinogen. Alternatively, preincubation was with 50 µg/mL BPA-sBSA and post-labeling with 1 µg/mL AF532-labeled streptavidin. In both cases, AF647-annexin A5 was also present. *Upper panels:* bright-field phase-contrast images after perfusion. *Middle panels*: TPLSM images of OG-fibrinogen and AF647-annexin A5 fluorescence. *Lower panels:* TPLSM images of BPA-sBSA and AF647-annexin A5 staining. Images are representative of 4-8 experiments; bars indicate 20 µm.

mouse blood, with many of the fluorescent BPS-sBSA corresponding to platelets in aggregates (Figure 2, *lower row*). Clot (thrombin) formation affected the labeling pattern only slightly, although some of the label coincided with annexin A5. Control experiments indicated that BPS-sBSA labeling was not affected by the presence of annexin A5 (not shown). This pointed to a clear difference between coated and PS-exposing platelets.

The biphasic increase in annexin A5 labeling under coagulant conditions suggested that platelets expose PS after interaction with either collagen or clot material (*e.g.* fibrin). This was indeed visible in high magnification images (Figure 3). In the absence of clots, or prior to their formation, only isolated, collagen-bound platelets with a bleb-shaped appearance bound annexin A5. A few minutes after coagulation, strings or patches of annexin A5-positive, bleb-shaped platelets appeared around the clots. Interestingly, the majority of aggregated platelets did not form blebs nor stained positively for annexin A5. Thus, microdomains of PS-exposing platelets are clearly separated from the microdomains of aggregated platelets.

Heterogeneity of integrin activation and PS exposure upon stimulation of collagen and thrombin receptors

To search for the mechanism responsible for the heterogeneity, other dual labeling flow studies were performed under coagulant conditions in the presence of FITC-PAC1 mAb (detecting activated αIIbβ3) and AF647-annexin A5 (for PS exposure).

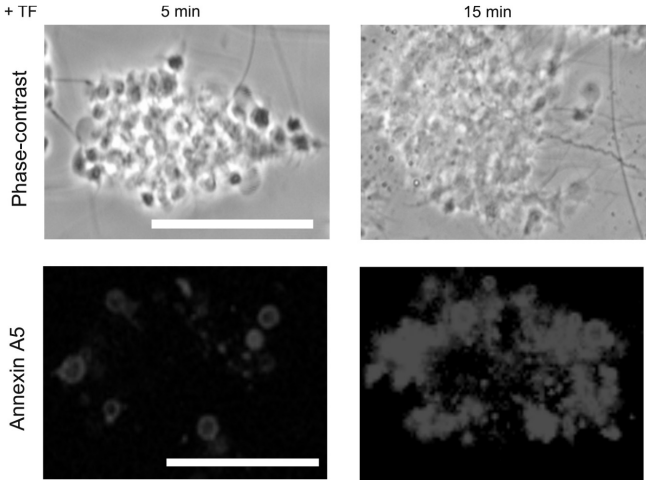

Figure 3 Bleb-shaped annexin A5-binding platelets on fibrin-containing aggregates. Human thrombi were formed on collagen during perfusion with tissue factor, and stained as described for Figure 2. Representative high-resolution phase-contrast and fluorescence images are shown after 5 (*left*) and 15 minutes (*right*) of perfusion. Bars indicate 20 μm.

TPLSM showed that platelets assembled into aggregates and clots expressed activated αIIbβ3 (Figure 4A). These platelets were well separated from the annexin A5-binding platelets (which did not bind PAC1), e.g. around clots. Again the same labeling patterns were obtained, when the experiments were performed with either probe alone (not shown). Control experiments indicated that the aggregated and bleb-shaped platelets both bound mAb, directed against activation-independent epitopes of αIIbβ3, indicating that integrins were still present on the PS-exposing platelets.

To validate these findings, also platelets in suspension were stimulated with a GPVI agonist, convulxin, alone or in combination with thrombin. Two-color flow cytometry showed that populations of FITC-PAC1 positive and negative platelets were well separated in the *FL1* channel, while those of AF647-annexin A5 positive and negative cells were separated in the *FL4* channel (Figure 4B). Time curves indicated that shortly after stimulation, most of the platelets bound FITC-PAC1, but were negative for AF647-annexin A5 (Figure 4B,C). After 10-15 minutes, PAC1 binding was down-regulated in the

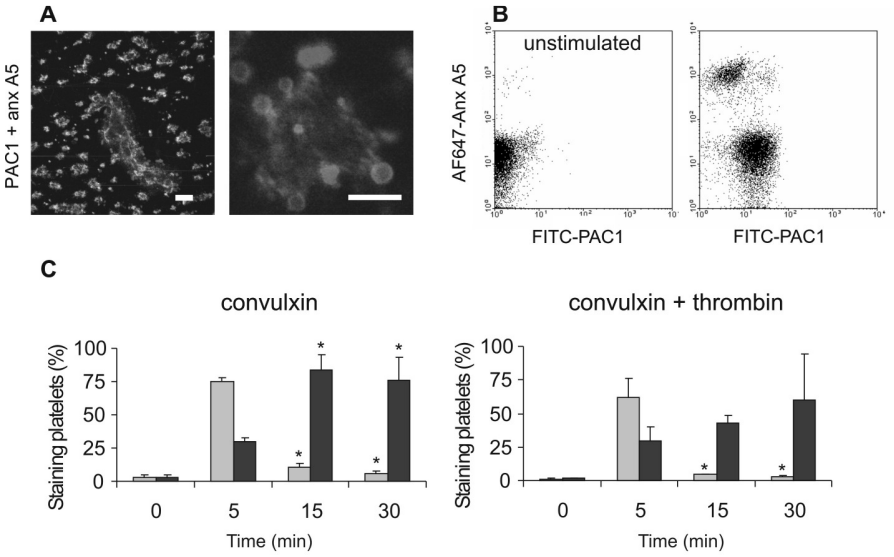

Figure 4 Temporary integrin activation state in procoagulant platelets. (A) Human thrombi were formed on collagen during tissue factor-induced coagulation, as described for Figure 2. Stains were 0.6 µg/mL FITC-PAC1 and 0.5 µg/mL AF647-annexin A5. Representative two-photon images are shown of FITC and AF647 fluorescence at lower and higher magnifications. Bars indicate 20 µm. (B, C) Washed platelets were stimulated in the presence of 2 mM $CaCl_2$ with 50 ng/mL convulxin alone or with 4 nM thrombin, for 5-30 minutes. (B) Representative dot plots are given of FL1 (FITC) versus FL4 (AF647) after 5 minutes of stimulation with convulxin. (C) Two-color flow cytometry after co-staining at indicated times with FITC-PAC1 and AF647-annexin A5, showing transient appearance of PAC1 binding sites and persistent PS exposure. Data are percentages of platelets staining with FITC-PAC1 (grey bars) or OG-annexin A5 (black bars). Mean ± SD (n=4), *p<0.05 compared to t=5 minutes.

majority of the platelets, which now bound annexin A5. Pretreatment of platelets with the transglutaminase inhibitor, mono-dansyl cadaverine (MDC), or the calpain inhibitor, calpeptin, left annexin A5 binding unchanged, but caused a 30% or 3% decrease in PAC1 binding, respectively. Apparently, transglutaminase and calpain are not major factors in regulating the inactivation of integrins and the heterogeneity in PS exposure.

Further characterization of microdomains of PS-exposing platelets in thrombi

It was shown earlier that PS-exposing membranes function as assembly sites for the prothrombinase complex, to form thrombin[29]. To investigate this further, flow studies were performed with blood containing labeled factors of this complex, i.e. AF488-factor Va, OG-factor Xa or OG-prothrombin, always in the simultaneous presence of AF647-annexin A5. Without coagulation, weak staining was visible at the aggregated platelets (not shown). In the presence of tissue factor and coagulation, the labeling of thrombi with these factors increased, e.g. AF488-factor Va fluorescence per pixel increased by 2.3 fold (n=4). High-magnification images showed that particularly patches of (bleb-formed) platelets were double-stained with annexin A5 and the coagulation factors (Figure 5A). The PS-exposing platelets also stained positively for CD62. Further evidence that especially the PS-exposing platelets may have a function in coagulation

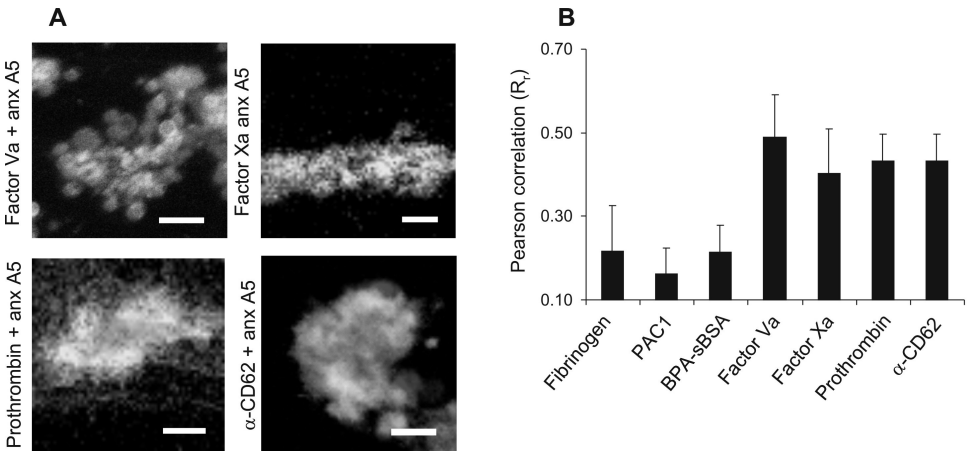

Figure 5 Partial overlap of coagulation factor and annexin A5 binding to stimulated platelets. Human thrombi were generated on collagen under coagulant conditions in the presence of AF647-annexin A5 (see Figure 2) in combination with either 20 nM AF488-factor Va, 200 nM OG-prothrombin, 100 nM OG-factor Xa or 1.25 µg/mL FITC-α-CD62 mAb. (A) Representative TPLSM fluorescence images are shown of AF647-annexin A5 and indicated coagulation factor or antibody. Bars indicate 20 μm. (B) Pattern overlap analysis of TPLSM images with annexin A5 and indicated factor or antibody. Pearson's correlation coefficients (R_r) of corresponding green and red bit maps are given. Data are from at least 4 experiments.

came from quantitative overlap analysis of the various sets of two-colored fluorescence images. The Pearson's correlation coefficient (R_r) was calculated to determine the pattern overlap of complementary images of red annexin A5 and green probe. Strikingly, this coefficient was low for green fibrinogen, PAC1 or BPA-sBSA, but much higher for green factor Va, factor Xa, prothrombin or anti-CD62 mAb (Figure 5B). Quite similar results were obtained when the overlap coefficient (R) was calculated for the same image sets (not shown), although it is sensitive for color intensity variation.

Two-color flow cytometric analysis was performed, following platelet stimulation with convulxin and thrombin, to confirm the separation of platelets into groups with different properties. On average, the population of stimulated, annexin A5-negative platelets relatively weakly bound the fluorescent-labeled factor Va, factor Xa and

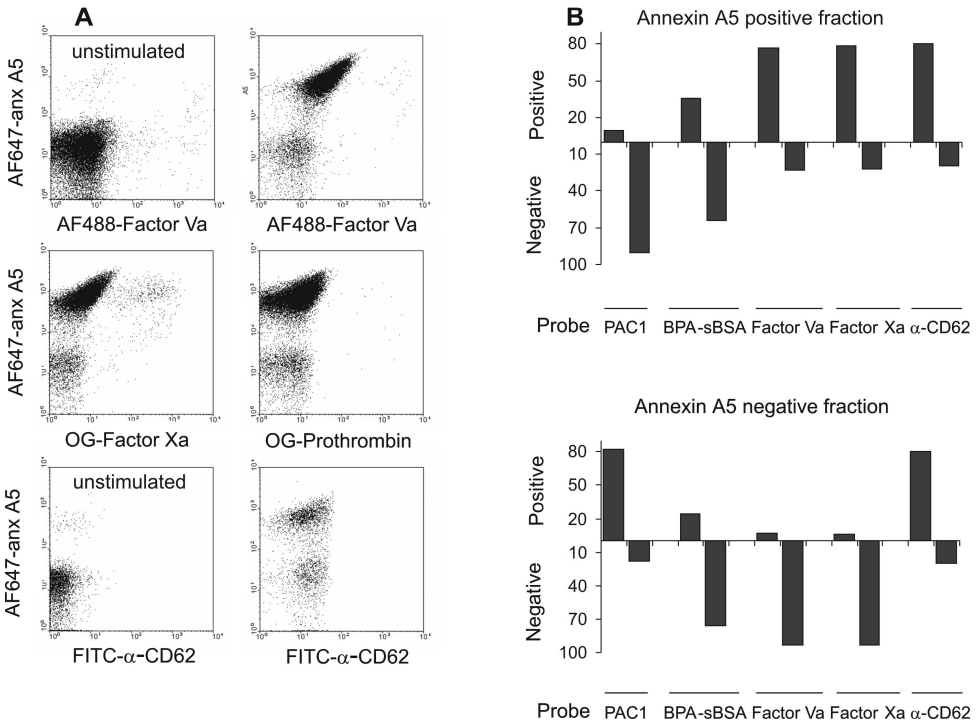

Figure 6 Heterogeneity in binding properties of PS-exposing platelets. Platelets in suspension were either unstimulated or stimulated with 50 ng/mL convulxin, 4 nM thrombin and 2 mM $CaCl_2$ for 10 minutes. Two-color flow cytometry was performed after staining with AF647-annexin A5 in combination with either 20 nM AF488-factor Va, 100 nM OG-factor Xa, 200 nM OG-prothrombin or 1.25 µg/mL FITC-α-CD62 mAb. (A) Shown are dot plots of FL1 (AF488, OG) versus FL4 (AF647), representative of at least 4 experiments. (B) Characterization of the annexin A5 positive and annexin A5 negative fractions of platelets after 10 minutes of activation. Data are percentages of platelets staining positively (upside bars) or negatively (downside bars) with indicated probe: FITC-PAC1, AF532-streptavidin BPA-sBSA, AF488-factor Va, OG-factor Xa, or FITC-α-CD62. Data are representative for 4 independent experiments.

prothrombin, whereas the annexin A5-positive platelets showed increased binding of these coagulation factors by 2.7, 3.1 and 2.0 fold, respectively (Figure 6A). In contrast, both populations of activated platelets were similarly high in CD62 expression.

For further analysis of these populations of AF647-annexin A5 positive and negative platelets, threshold levels of green fluorescence were set to indicate activation-induced binding of labeled antibodies and coagulation factors (Figure 6B). Comparison showed that hardly any of the PS-exposing (annexin A5-positive) platelets bound PAC1, while only a minority bound BPA-sBSA (probing coated platelets). In contrast, a large fraction of the PS-exposing platelets had increased binding of factor Va, factor Xa and anti-CD62 mAb. On the other hand, the PS-negative platelets preferentially bound PAC1, anti-CD62, and to a moderate extent PBA-sBSA, but they were low in factor Va and factor Xa binding. Taken together, these data provide strong indications that platelets in a thrombus are segregated into aggregates that bind fibrin(ogen), PAC1 and BPA-sBSA and, at the other hand, patches of PS-exposing platelets with increased binding of all components of the prothrombinase complex (factor Va, factor Xa, and prothrombin) at the expense of integrin activation.

Tyrosine phosphorylation state regulates platelet heterogeneity

The observation that in platelets stimulated by convulxin and thrombin, αIIbβ3 inactivation is linked to PS exposure may point to the involvement of a common signaling

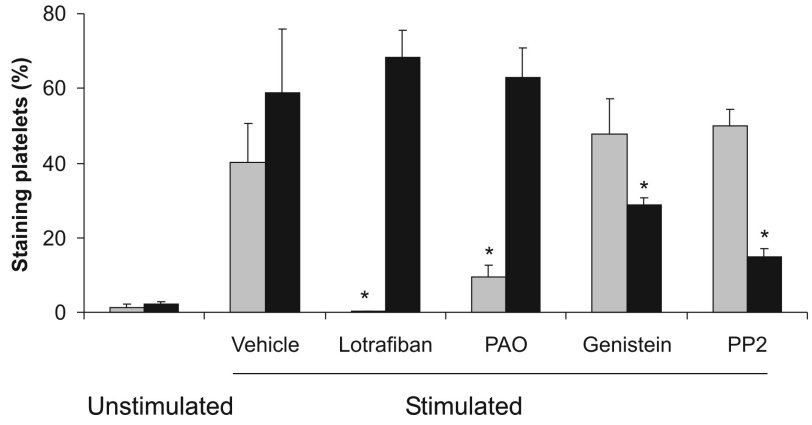

Figure 7 Modulation of protein tyrosine phosphorylation alters integrin activation and PS exposure. Human washed platelets were stimulated with 50 ng/mL convulxin and 4 nM thrombin in the presence of 2 mM $CaCl_2$. The cells were pre-incubated for 10 minutes, as indicated, with vehicle, 200 µM phenylarsine oxide (PAO), 100 µM genistein, 20 µM PP2, or 10 µM lotrafiban. Co-labeling was with a mixture of FITC-PAC1 and AF647-annexin A5. Data are expressed as percentages of platelets staining with FITC-PAC1 (grey bars) or OG-annexin A5 (black bars). Mean ± SD (n=4), *p<0.05 compared to stimulation in the presence of vehicle.

factor.

To investigate this further, platelets in suspension were pretreated with the αIIbβ3 blocker, lotrafiban[17], prior to stimulation with convulxin/thrombin. Lotrafiban treatment abolished PAC1 binding, while annexin A5 binding remained high (Figure 7), suggesting that integrin activation does not regulate PS exposure. Because both collagen and thrombin receptors activate platelet tyrosine kinases, we then investigated the effects of modulation of the tyrosine phosphorylation state. Pretreatment of platelets with the protein tyrosine phosphatase inhibitor, phenylarsine oxide (PAO), resulted in increased tyrosine phosphorylation (not shown), which was accompanied by substantially reduced PAC1 binding and unchanged PS exposure (Figure 7). By contrast, platelet pre-incubation with the general protein tyrosine kinase inhibitor genistein or the Src-kinase inhibitor PP2 left PAC1 binding virtually unchanged, while

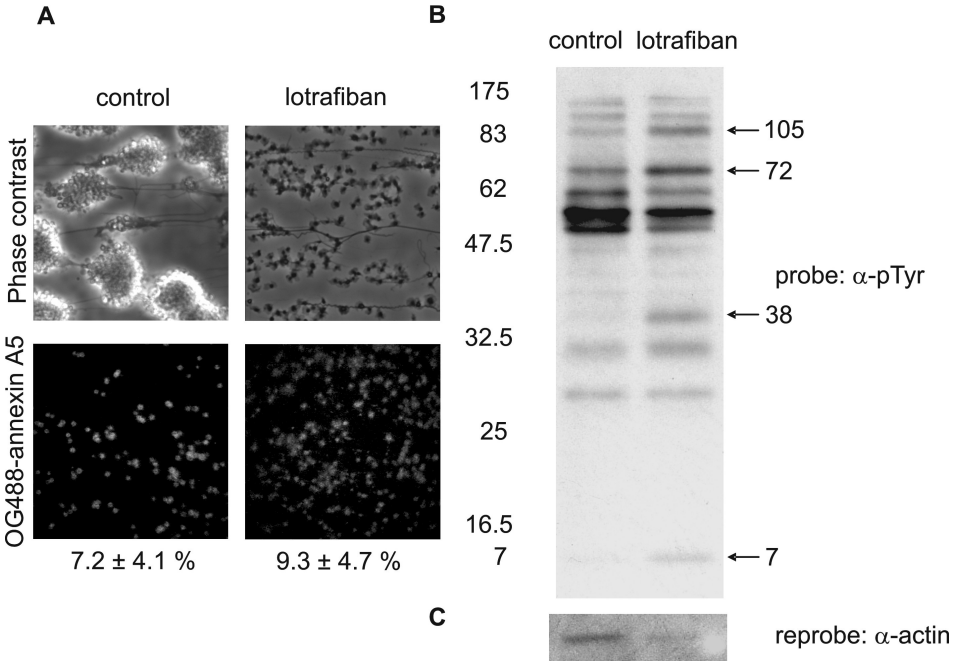

Figure 8 Role of integrin αIIbβ3 in tyrosine phosphorylation of collagen-adherent platelets. Human blood pre-treated with vehicle (control) or 10 μM lotrafiban was flowed over collagen for 4 minutes at a shear rate of 1000 s^{-1}, and stained with OG488-annexin A5. Numbers below images indicate mean surface area coverage ± SD. (A) Representative phase-contrast (120×120 μm) and fluorescence (150×150 μm) images. (B) Flow chambers were perfused with lysis buffer to remove adherent platelets and lysates (equalized as far as possible for protein quantity) were run on a SDS-PAGE gel, transferred to PVDF and blotted for phosphotyrosine using mAb 4G10, (C) then stripped and re-probed for actin using mAb AC-40. Representative data of 2-4 experiments. Note the low actin staining for the lotrafiban lane, indicating the increased phosphorylation of 7, 38, 72 and 105 kDa proteins is underestimated for this gel.

these compounds reduced the number of PS-exposing cells. Yet, in all conditions, the majority of the platelets that bound PAC1 did not bind annexin A5, and vice versa. Together, this indicated that a prolonged high tyrosine phosphorylation state (e.g., by Src kinase activity) reduced integrin activation, but stimulated PS exposure.

The importance of protein tyrosine phosphorylation was further examined in flow studies (in the absence of coagulation). Pre-incubation of blood with lotrafiban increased the number of PS-exposing platelets, but reduced the total number of adherent platelets, because aggregation was abolished (Figure 8). Gel electrophoresis of the platelet proteins, followed by immunoblotting with anti-phosphotyrosine 4G10 mAb,

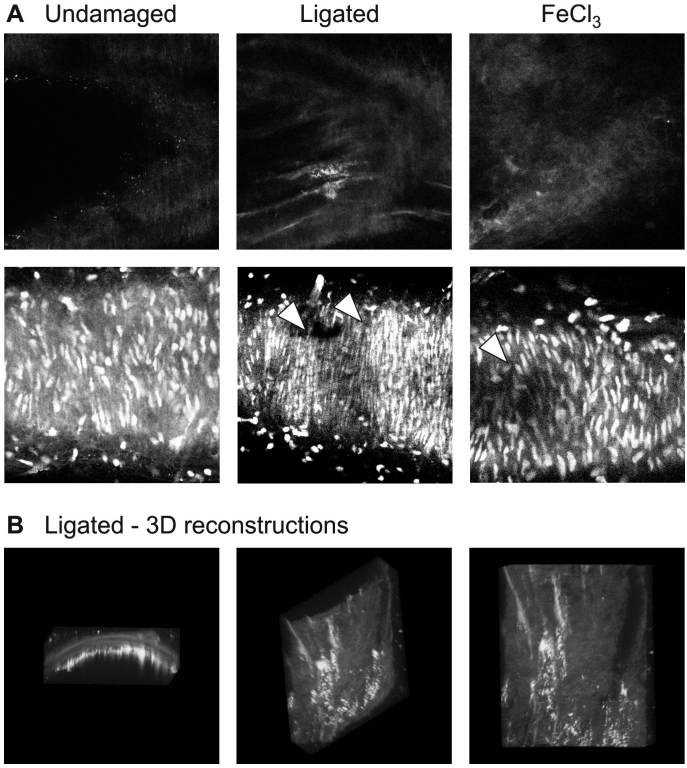

Figure 9 Heterogeneity in arterial thrombi induced by carotid ligation or by $FeCl_3$ application. Mice were infused with OG-fibrinogen and AF568-annexin A5 (200 µg each). *Left panels:* undamaged control carotid artery. *Middle panels:* One carotid artery was damaged by tight ligation at the bifurcation for 5 minutes to induce vascular damage. *Right panels:* in another animal, the carotid artery was damaged by local application of saturated $FeCl_3$. Thrombus formation proceeded for 10 minutes, after which fluorescence inside the arteries was recorded by TPLSM. (A) *Upper row*: images of fibrin(ogen) and annexin A5 fluorescence taken on the luminal side of the vessel wall. Note distinct patches of green and red fluorescence. *Second row:* images of Syto-44 fluorescence at cross section through the vessel wall. Arrow heads indicate absence of cell nuclei at sites of damage. (B) Three-dimensional reconstructions of fibrin(ogen) and annexin A5 fluorescence in the ligated artery: side view through vessel wall, turned view, and view from inside of vessel lumen. Images are 206×206 µm.

indicated that lotrafiban treatment specifically increased the tyrosine phosphorylation levels of proteins of about 7, 38, 72 and 105 kDa (Figure 8). More direct evidence for an increased tyrosine phosphorylation in PS-exposing platelets was obtained by staining the collagen-bound platelets on coverslips with FITC-4G10 mAb. Comparison of brightfield and confocal fluorescent images indicated that, typically, the single (AF647-annexin A5 positive) platelets were more fluorescent than adjacent platelets in aggregates. Quantitative analysis showed that fluorescence levels per pixel of the single cells were 2.4 ± 0.8 (n=7) fold higher, in comparison to pixels with aggregated platelets. Lotrafiban treatment maintained this high FITC-4G10 staining. Accordingly, high tyrosine phosphorylation levels accompany PS exposure, but not integrin activation.

Heterogeneity in fibrin(ogen) binding and PS exposure of thrombi formed in vivo

A key question is how the platelet heterogeneity as observed in thrombi formed *in vitro* relates to the *in vivo* situation. To investigate this, anesthetized mice were co-infused with fluorescently labeled OG-fibrinogen and AF568-annexin A5. Thereafter damage to the carotid arteries was provoked using two different techniques. In the first method, the artery was ligated, which results in mechanical exposure of subendothelial collagen[30]. Alternatively, $FeCl_3$ was applied locally to the vessel, which causes endothelial denudation by free radical formation[26,31]. After *in vivo* thrombus formation for 10 minutes, the injured and control (undamaged) vessels of the animals were scanned using the high penetration power of TPLSM. Typically, fluorescence from both probes was detected only at sites of ligation or $FeCl_3$ application, and was absent in control arteries. Optical cross-sections showed local spots of OG-fibrin(ogen) at the wall-lumen interface, which to a small extent overlapped with spots of AF568-annexin A5 (Figure 9A, *first row*). Three-dimensional reconstruction of the images at thrombotic sites showed distinct patches of fluorescent fibrin(ogen) and annexin A5, again only at the damaged vascular lumen (Figure 9B). After scanning, the still intact arteries were post-stained for vessel wall cells with the nuclear stain Syto-44, and then rescanned. Optical cross-sectioning demonstrated absence of Syto-44 fluorescence at the sites of ligation or $FeCl_3$ application, thus confirming the local damaging effect of the prothrombotic interventions (Figure 9A, *second row*). Together, these data show that fibrin(ogen)-rich and annexin A5-binding domains appear at adjacent but distinct locations during *in vivo* thrombus formation.

Discussion

The present data indicate that during the thrombus-forming process different clusters of platelets contribute to aggregate formation and to coagulant activity, which points to a clear spatial separation of these two key functions of platelets. In shear-dependent thrombus formation triggered by collagen (GPVI) and thrombin receptors, we distinguish at least two, and likely even three microdomains of activated platelets: *(i)* platelets packed in fibrin-containing aggregates (clots) with activated αIIbβ3 integrin at their surface; *(ii)* strings of non-aggregated platelets which are low in activated αIIbβ3, but expose PS and efficiently bind the components of the prothrombinase complex; *(iii)* platelets binding serotonin probes in addition to factor Va, which partly overlap with the aggregated platelets.

The microdomains of aggregated platelets, rich in fibrin(ogen) under condition of thrombin generation, were most readily distinguished by high binding of PAC1 mAb, which stains activated αIIbβ3 integrins. Part of these platelets expressed CD62 at their surface, indicating secretion. Typically, as long as aggregated, these platelets did not bind annexin A5 and, thus, did not expose procoagulant PS. In both confocal microscopy and flow cytometry, the annexin A5 negative platelets showed low binding to coagulation factors Va and Xa and prothrombin.

The second type of microdomain consists of mostly bleb-shaped platelets, which often appear as strings at the edge of clots. Visual inspection and also overlap analysis of two-color fluorescence images pointed to a clear separation of the annexin A5-binding platelets, exposing PS, from the fibrinogen- and PAC1-binding platelets in aggregates or clots. The round morphology and lack of pseudopods is well compatible with the low integrin activation state and thus diminished adhesion. Likely, these platelets play an important role in thrombotic thrombin generation, given the increased binding of factor Va, factor Xa and prothrombin. For these studies, we used fluorescent labeled factor Va, which had fully kept its co-factor function in prothrombinase activity; and active site-labeled factor Xa and prothrombin, with proven unaltered binding characteristics[19].

The appearance of single, bleb-shaped, PS-exposing platelets upon interaction of platelets with collagen via GPVI was already reported earlier[24,25]. However, a novel finding is that this platelet response becomes much more frequent during perfusion of blood in the presence of thrombin generation and coagulation. In this case, strings of platelets often located around clots jointly transform into PS-exposing cells. The result is that thrombi develop into a patched structure with microdomains of platelet/fibrin aggregates, alternated with loose clusters of PS-exposing platelets. It is tempting to

suggest that the collagen-bound platelets in the first phase of PS exposure contribute to initial phases of thrombin generation, while the procoagulant platelets around clots help to propagate thrombin production. A similar patched structure of fibrin(ogen)- and annexin A5-binding cells is observed by TPLSM imaging of the thrombi inside murine arteries, generated following two different models of vascular damage *in vivo*. Thus, we hypothesize that the microdomain organization with separate aggregated/clotted and coagulation-active platelets is functionally relevant to arterial thrombosis in humans.

The experiments distinguish a third subpopulation of platelets, but with less characteristic binding properties. Using BPA-sBSA as a probe that specifically detects serotonin binding sites, we observed appreciable labeling of platelet aggregates and to a smaller extent to single, PS-exposing platelets. In fact, the Pearson correlation coefficient R_r of BPA-sBSA versus fibrinogen staining is 0.54, i.e. higher than the R_r of BPA-sBSA versus annexin A5 labeling of 0.21. The fact that aggregates were labeled with BPA-sBSA is not surprising, because the initial description of 'coated' (or COAT) platelets referred to platelets that are covered with serotonin-derivatized proteins such as fibrin(ogen) and thrombospondin in association with the granular proteins vWF, factor V, and fibronectin[14], all of which are present on platelet aggregates formed on a collagen surface[26,32,33]. On the other hand, current literature is inconsistent in the description of coated platelets - e.g. as formed by convulxin/thrombin stimulation -, which may coincide with PS-exposing platelets[15], or with fibrin(ogen)-binding platelets[16]. The present data can resolve this discrepancy by showing that BPA-sBSA binds aggregates (in thrombi) with a certain preference, but also to some extent PS-exposing platelets (in suspensions).

Recent studies have suggested that platelet-secreted proteases are involved in the formation of subpopulations of coagulation-active platelets. One proposal is that extracellular transglutaminases like factor XIIIa contribute to this platelet response by cross-linking membrane surface proteins with secretion factors, e.g. via serotonin conjugation to form coated platelets[12,13]. Also, extracellular or intracellular calpain activity may contribute to development of the procoagulant platelet phenotype[4,12]. In our hands, inhibition of transglutaminases (MDC) or calpain (calpeptin) had only little effect on PS exposure, while only MDC had a small inhibitory effect on integrin inactivation in convulxin/thrombin-stimulated platelets. This indicated that transglutaminases did not play a major role in the regulation of platelet procoagulant activity, which is in line with a recent report that the binding of secretory proteins and annexin A5 to platelets on collagen requires GPVI/FcR γ-chain activity, but not factor XIIIa[16].

Therefore, we investigated the possibility that an intracellular signaling pathway rather than an extracellular mechanism is responsible for platelet heterogeneity in thrombus formation. Reason for this is the knowledge that GPVI alone or in combination with thrombin receptor stimulation causes a potent Ca^{2+} response, which is a prerequisite for PS exposure in both human and murine platelets[4,12,24,34]. The current data provide evidence that protein tyrosine kinase activity is stimulatory to PS exposure (likely via stimulation of the GPVI - phospholipase C-γ2 pathway), but inhibitory to persistent integrin αIIbβ3 activation. This is concluded from the relatively high tyrosine phosphorylation state of PS-exposing, PAC1-negative platelets on collagen as probed with fluorescent 4G10 mAb, and also from the reducing effect of tyrosine phosphatase inhibition on PAC1 binding with unchanged PS exposure. Further support came from perfusion experiments in the presence of an αIIbβ3 antagonist, which increased the tyrosine phosphorylation of several platelet proteins along with the number of PS-exposing platelets on collagen.

In summary, we conclude that in collagen- and thrombin-induced thrombus formation, differences in intracellular signaling state controlled by αIIbβ3 activation accomplish the separation of microdomains of aggregated and PS-exposing platelets. Typically, in PS-exposing platelets, down-regulation of integrin binding is accompanied by up-regulation of coagulation factor binding. These results point to a balance, in which coagulant activity suppresses integrin activation, while conversely, aggregate formation reduces the procoagulant activity of platelets.

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Chapter 8

General discussion

The majority of cardiovascular diseases involve undesired thrombus formation and embolisation following vascular damage or plaque rupture. The thromboembolic process is known to be driven by platelet activation and the coagulation system. As these processes are of major importance in both arterial and venous thrombosis, clear understanding of the interactions between these two processes will be important for a successful fight of cardiovascular diseases. The overall goal of the studies presented in this thesis is to identify and study the interaction between platelet activation and coagulation at different levels of complexity. Part of the investigations considers sub-cellular and cellular aspects of the signaling pathways of platelets that contribute to the generation of their procoagulant activity. Other experiments focus on the intercellular processes defining the formation of multi-platelet thrombi formed *in vitro* under conditions of blood flow and coagulation. Furthermore, *in vivo* mouse models of experimental intravascular thrombosis have been developed and used to assess the role of procoagulant platelets in coagulation and the differences among platelets in a thrombus. The signaling receptor for collagen, glycoprotein VI, appears to play a key role in this platelet response.

Signaling pathways of glycoprotein VI to procoagulant activity

Knowing that platelet-collagen interaction via glycoprotein VI (GPVI) can induce procoagulant activity, we were interested to determine which processes operating alongside and downstream of GPVI contribute to the procoagulant response under physiologically relevant conditions. Chapter 4 discusses the important question how the other collagen receptor, integrin $\alpha 2\beta 1$, contributes to the procoagulant function of GPVI in human blood flowing at arterial shear rate. Using a newly generated single-chain phage antibody fragment, 10B12, we were able to demonstrate that GPVI is the exclusive receptor implicated in all collagen-induced activation responses, such as Ca^{2+} signaling, aggregate formation and phosphatidylserine (PS) exposure. Inhibition of $\alpha 2\beta 1$ with specific peptides or antibodies reveals that this integrin does contribute to the regulation of Ca^{2+} signaling, platelet aggregation and PS exposure, but only by enforcement of the responses evoked by GPVI. Inhibitory experiments also show that blocking of another adhesive receptor, the GPIb-V-IX complex which binds von Willebrand factor (vWF), has only little effect on PS exposure. These results thus indicate that only two of the three human platelet receptors for vWF/collagen are involved in the procoagulant response. However, in mice GPIb interaction with vWF was found to enhance GPVI-induced PS exposure, along with stimulation of the rise in

$[Ca^{2+}]_i$[1]. The reason for this discrepancy is unclear. However, in the human system we have not investigated the contribution of GPIb to procoagulant activity at shear rates higher than 1000 s^{-1}, at which more prominent GPIb effects are expected[1].

During the progress of this work, several other studies with human and mouse blood with different anti-GPVI antibodies and GPVI-deficient mouse models indicated that GPVI plays a key role in collagen-induced thrombus formation under flow; GPVI thus appears to be the principal collagen receptor causing Ca^{2+} signaling, secretion, integrin activation and procoagulant activity[2-4]. In the present context it is of interest that the data of chapter 4, and also those of other papers, demonstrate a direct relation between the GPVI-evoked rises in intracellular Ca^{2+} levels and the extent of phosphatidylserine (PS) exposure by platelets. Together, this supports the early suggestion that the PS-exposing mechanism ('scramblase') is directly or indirectly regulated by a strong elevation in intracellular Ca^{2+} concentration[5].

The murine studies described in chapter 5, are of particular interest for the signal transduction route by which GPVI is capable to induce PS exposure in the process of thrombus formation. Using mice deficient in FcR γ-chain, the adaptor protein LAT or phospholipase Cγ2 (PLCγ2), we show that these signaling proteins, which are downstream of GPVI, are all required for PS exposure. In addition, the essential role of Src-family kinases, likely Lyn, in this process was examined by using the specific inhibitor PD173952. It is known that the GPVI/Lyn/LAT signaling pathway is the most important mechanism for tyrosine phosphorylation and activation of PLCγ2 via protein assembly in the so-called LAT signallosome[6,7]. The same research indicated that Syk is the principal tyrosine kinase mediating phosphorylation of both LAT and PLCγ2[6,7]. It was also known that genistein, which is an inhibitor of Syk kinase, suppresses collagen-induced PS exposure[8], which thus makes it quite likely that also Syk is an essential component of the signaling route from GPVI to PS exposure. However, as Syk deficient mice are not viable[9], we have not been able to demonstrate this unequivocally. Interestingly, recent data using vital Syk -/- chimeras pointed to a novel signaling pathway of GPVI in supporting thrombin-induced activation, which operates independently of Src and Syk kinases[10]. In view of the current results, it is unlikely that this GPVI pathway is involved in procoagulant activity.

A number of other members of the LAT signallosome have been identified, which all act downstream of GPVI and Syk kinase, including the tyrosine kinases Btk and Tec, the G-protein-stimulating factors Vav1/3, and the adaptor proteins SLP-76 and Gads[7]. Although we have not investigated involvement of these components, we predict that they will only play a role in PS exposure as far as operating upstream of PLCγ2.

Together, our data show that the role of GPVI in PS exposure is mediated via a relatively simple signaling pathway, consisting of Src kinase (Lyn), Syk and LAT, and resulting in activation of PLCγ2 as a key intermediate step (Figure 1). This work thereby emphasizes the importance of PLCγ2 in platelet activation[7].

Activation of PLCγ2 leads to production of inositol 1,4,5-trisphosphate ($InsP_3$) and subsequent Ca^{2+} release from intracellular stores. In cell types other than platelets, the PH domain-containing PLCγ isoforms bind to and are activated by phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which is formed in the plasma membrane by phosphoinositide 3-kinase (PI3K) activity[11]. Therefore, the membrane-bound PLCγ has easier access to its substrate, PIP_2. Especially members of the class 1A/B PI3K isoforms appear to be responsible for PIP_3 formation[12]. Earlier work with platelets showed that GPVI activation is accompanied by incorporation of the class 1A regulatory subunit p85α into the LAT signallosome[13]. We therefore investigated whether the GPVI pathway to PLCγ2 activation, Ca^{2+} signaling and PS exposure involves activity of one or

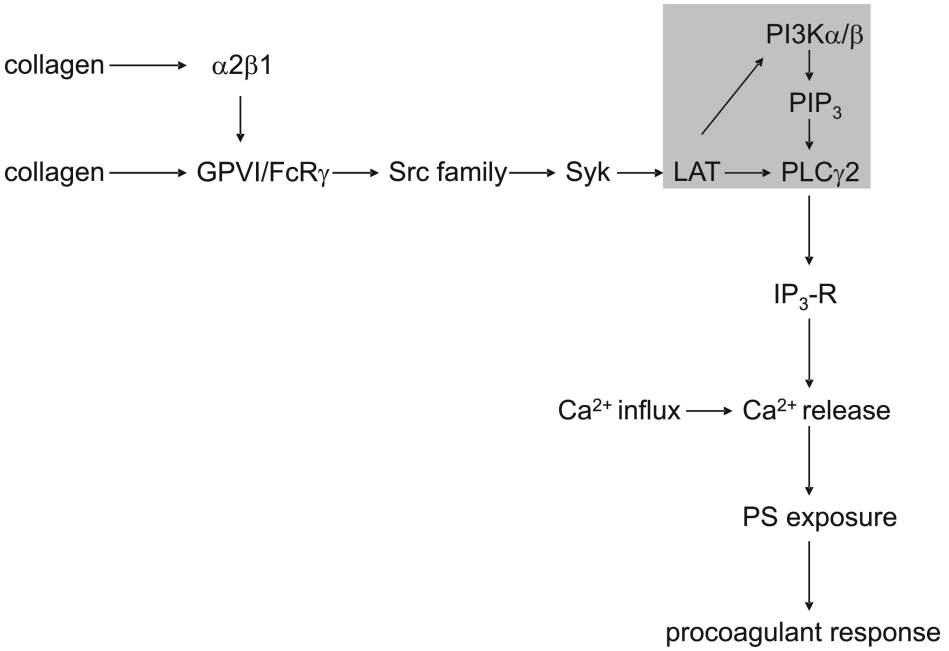

Figure 1 Overview of intracellular platelet signaling leading to procoagulant activity. The indicated GPVI pathway to PLCγ2 activation is recognized as the key signaling mechanism for Ca^{2+}-dependent PS exposure and procoagulant activity. Several signaling proteins cluster together in the LAT signallosome (grey box). The PI3Kα and β isoforms support in this platelet response, by enhancing Ca^{2+} mobilisation and subsequent store-mediated Ca^{2+} entry.

more PI3K isozymes. Platelets contain various class IA (α, β and δ) and class IB (γ) PI3K isoforms[14,15]. However, some authors report that the expression of the p110δ catalytic subunit is only limited[16-18]. For the study presented in chapter 3, we used several newly developed inhibitors interacting with the catalytic subunits of the various PI3Ks, as well as mice lacking the p85α regulatory subunit (for class 1A PI3Ks) and animals lacking the p110γ catalytic subunit (class 1B PI3Kγ). The overall results indicate that both the p85α regulatory subunit and the catalytic p110β activity contribute significantly to Ca^{2+} signaling and PS exposure elicited by GPVI ligands. This contribution occurs via the classical Ca^{2+} pathway, i.e. by enhancing Ca^{2+} mobilization and subsequent store-mediated Ca^{2+} entry (Figure 1). In our hands, the class 1A PI3Kδ and the class 1B PI3Kγ do not seem to have a prominent role in this process.

For platelets it is known that deletion of the murine p85α regulatory subunit does not influence the expression of other type IA regulatory subunits (p55α, p50α and p85β); however, it leads to a major reduction in the level of the p110α catalytic subunit, along with greatly reduced p110β and δ expression[16,19,20]. Recent experiments with PI3Kδ-deficient mice pointed to only a minor but detectable role for the δ isoform in mediating platelet activation by collagen[21]. Furthermore, both PI3Kβ and γ isoforms were found to control ADP-dependent platelet activation processes[12,17,22]. Together with the present results, this indicates that in mouse as well as human platelets particularly the PI3Kα and β isoforms, acting via p85α, are involved in GPVI-induced activation, while the β and γ isoforms rather act downstream of ADP receptors.

GPVI-induced PLCγ2 activation produces $InsP_3$, which opens the $InsP_3$ receptor Ca^{2+} channels to release Ca^{2+} from intracellular stores. It is recognised that intracellular Ca^{2+} mobilisation, by itself, is insufficient for full platelet activation. The process of store-operated Ca^{2+} entry, which causes Ca^{2+} influx from the extracellular medium, is required to reach the high cytosolic Ca^{2+} concentration needed for PS exposure[5,8,23]. One research group provided evidence that the store-operated Ca^{2+} entry is impaired in a French Scott patient and, thereby, suppresses surface exposure of PS on activated platelets and other blood cells[24,25]. Chapter 2 describes the unexpected observation that the Ca^{2+} responses in both platelets and immortalized B-cells from two different Scott patients (including the propositus patient) are unaltered in comparison to cells from control subjects. On the other hand, PS exposure in response to GPVI is strongly reduced in cells from these patients. These results indicate that the impaired lipid scrambling in the Scott cells can not be ascribed to alterations in Ca^{2+} release from intracellular stores or subsequent Ca^{2+} entry. Unfortunately, we have not been able to

make a direct comparison with cells derived from the French Scott patient. Although the reason for the discrepancy with the literature is unclear, it is noteworthy that the diminished Ca^{2+} influx in the cells from the French patient was only detected with specific time-dependent protocols to measure Ca^{2+} influx[24] and, therefore, could be due to subtle signaling changes distantly from the Ca^{2+} influx channels. Alternatively, the differences might reflect variants of the Scott syndrome, which is still not genetically characterised.

Platelets from (our) Scott patients are interesting, because these are so far the only platelet types, in which high Ca^{2+} does not automatically lead to PS exposure. This implies that the defect in these patients should lay downstream of Ca^{2+} signal generation and, moreover, that Ca^{2+} entry channels themselves are not determinants for PS exposure. This was confirmed by the finding that the expression pattern of Trpc channels, which are strong candidates of the channels responsible for store-regulated Ca^{2+} entry, is similar in the cells from Scott patients and control subjects (I. Munnix, unpublished results). A major drawback in the field is that the molecular identity of the components (enzymes, channels?) that are responsible for the lipid scrambling implicated in PS exposure is unknown. After many efforts, a phospholipid scramblase has been identified[26], but this enzyme does not mediate PS exposure in platelets[27]. It is shown that in addition to Ca^{2+} channels, also Gardos-type of K^+ channels play a role in PS exposure[28]. Other, newly identified proteins involved in store-regulated Ca^{2+} entry are the ORAI channels[29] and STIM1, which is a calcium sensor for Ca^{2+} channels and couples Ca^{2+} stores to the plasma membrane[30]. STIM1 is also expressed on platelets[31]. However, it is unlikely that ORAI or STIM1 is changed in the Scott syndrome, since platelets from at least two patients show normal Ca^{2+} responses.

Multiple contributions of glycoprotein VI to thrombus formation under flow

Whereas the function of platelet GPVI in collagen-induced intracellular Ca^{2+} rises and PS exposure under flow has not been questioned, the precise role of GPVI in platelet adhesion and thrombus formation is less clear. Concerning adhesion, the relative contribution of the two collagen receptors, GPVI and $\alpha 2\beta 1$, was the subject of considerable discussion in the past decades. According to the original two-site two-step adhesion model, high affinity interaction with collagen through $\alpha 2\beta 1$ would slow down a platelet, and allow low affinity binding of GPVI[32,33]. However, studies with GPVI- or $\alpha 2\beta 1$-deficient mice demonstrated that GPVI had a critical role in initial platelet adhesion and subsequent aggregation on collagen[34]. The same work showed that $\alpha 2\beta 1$ was not

required for these events. However, several later studies re-established that mouse α2β1 can facilitate the role of GPVI in platelet adhesion to collagen[2,35].

Since for human platelets, the contribution of the two collagen receptors had remained unclear, we have studied this in chapter 4. We observed that under high-shear flow conditions, GPVI does stimulate, but is not essential for primary platelet adhesion to collagen. Furthermore, we detected redundancy in the roles of α2β1 and GPIb-V-IX for platelet adhesion. Inhibiting GPIb or α2β1 on its own, but not GPVI, only partially reduced the number of collagen-adherent platelets. Combined inhibition of GPIb or α2β1 had a greater effect on platelet deposition, but still a significant number of aggregates were observed. That GPVI is in interplay with both other receptors is concluded from the observation that adhesion was completely abolished using a combined blockade of GPIb and GPVI or α2β1 and GPVI. This indicates that all three receptors play a role in adhesion, in contrast to procoagulant activity where only two receptors are involved. These results clearly deviate from those obtained using GPVI-deficient mice, which showed total abolition of platelet-collagen adhesion[34]. This issue of possible differences between human and mouse platelets was addressed by Auger *et al*.[36], who directly compared adhesion, activation and aggregation in either species. Two mechanisms of stable platelet adhesion and activation on collagen were shown to co-exist. In some platelets, adhesion involving α2β1 appears to be followed by GPVI-mediated increases in Ca^{2+} concentration, while in other platelets it precedes the Ca^{2+} response. This heterogeneity in responses is preserved in mouse and human platelets, and can explain apparent differences in the literature.

A number of studies have demonstrated that even minute amounts of GPVI signaling are sufficient to bring integrin α2β1 in a higher-affinity state, which permits this receptor to play a prominent role in adhesion[37-39]. Together with the present work, this has led to the recognition that platelet collagen responses are in a game of molecular ping-pong between different collagen receptors, in which GPVI is the first player to serve[40]. With respect to platelet aggregation under flow, our data in chapter 4 and 5 are in full agreement with other studies with mouse and human blood that GPVI plays a key role in collagen-induced thrombus formation[2,34,36,41]. The GPVI signaling pathway underlying shear-dependent platelet aggregation involves the FcR γ-chain, Src-family kinases, Syk, LAT and PLCγ2 (chapter 5). These are the same signaling components that have been shown to be involved in the GPVI-dependent aggregation response of suspensions of human and mouse platelets[6,42,43]. Our results with LAT, however, are remarkable, since with suspended LAT-deficient platelets aggregation was only reduced

in the presence of low doses of collagen or collagen-related peptide, and became normalized at higher agonist doses[44]. This suggests that, the LAT pathway can be bypassed in collagen-induced suspension measurements, but not in (more physiological) flow measurements on collagen.

As shown by others, aggregate formation on a collagen surface is the result of several platelet responses, including activation of the fibrinogen-binding αIIbβ3 integrin and release of the paracrine agents thromboxane A_2 and ADP (Figure 2)[38,45,46]. Particularly the release and the formation of thromboxane A_2 and the secretion of ADP are Ca^{2+}-dependent responses, thus likely to be controlled by PLCγ2 activation. The results presented in chapter 4 show that also human platelet aggregation under flow – similarly as discussed for PS exposure – occurs through the concerted action of GPVI in cross-talk with α2β1. Accordingly, as indicated in Table 1, the same or similar sets of proteins and signaling mechanisms are implicated in aggregation and PS exposure during the thrombus-forming process.

In chapter 5, we further investigated the thrombus-forming process under conditions where the coagulation was triggered with tissue factor and the platelet aggregates accumulated massive amounts of fibrin. Although in the absence of GPVI activity, still trace amounts of fibrin could be detected (indicative of non-zero thrombin

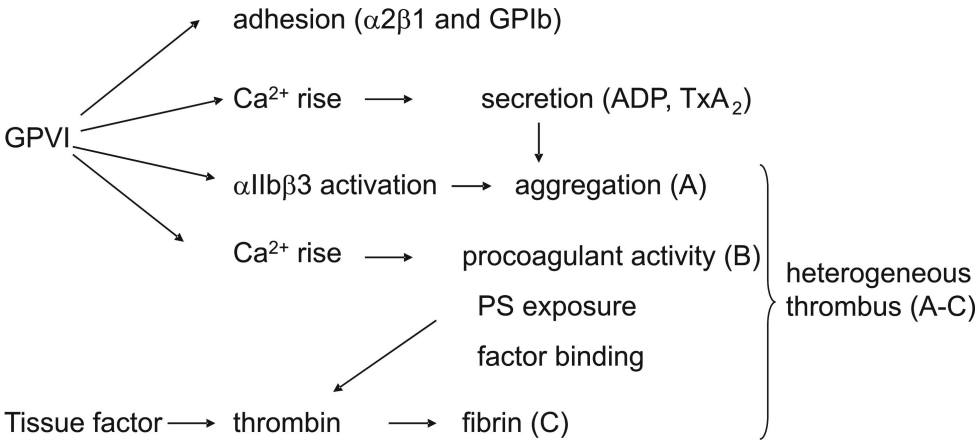

Figure 2 Overview of the roles of glycoprotein VI in thrombus formation. This thesis describes that platelet adhesion to collagen/vWF is mediated by GPVI in combination with α2β1 and GPIb. GPVI subsequently plays a key role in several platelet activation processes, including rise in intracellular Ca^{2+} concentration, secretion, integrin αIIbβ3 activation and PS exposure. In the thrombus-forming process, this results in two populations of platelets either clustered into aggregates or with procoagulant activity. Both populations are embedded in a network of fibrin, formed as a result of the coagulation.

Table 1 Contribution of platelet receptor and signaling proteins to thrombus formation on collagen

	Adhesion	Ca^{2+} signaling	PS exposure	Secretion	Aggregation
GPVI	+*	++	++	++	++
$\alpha 2\beta 1$	+	+	+	+	0
FcRγ	+	++	++	++	++
LAT	+	++	++	++	++
PI3Kα/β	0	+	+	+	+
PI3Kγ	+	0	0	0	+
PLCγ2	+	++	++	++	++

*0, no contribution; +, potentiating; ++, required.

formation), aggregated and PS-exposing platelets were hardly detected under this condition,

indicating that platelet activation by GPVI, most likely via PS exposure, is of key importance for full coagulant activity under the present flow conditions. Thus, GPVI-induced platelet activation can act as a driving force of the coagulation process in these flow chamber experiments. Interestingly, in the presence of GPVI activity, the number of PS-exposing platelets increased under conditions of coagulation (chapter 7). The most obvious explanation is that the processes of collagen-induced PS exposure and thrombin generation are interrelated and influence each other, likely through a positive feed forward loop, in a similar way as was previously suggested for platelet interaction with fibrin[47]. Only few comparable studies with coagulation have been reported, but these particularly concerned *ex vivo* flow chamber experiments where effects of thromboxane A_2 and ADP blockade were investigated instead of GPVI[48].

In summary, we have shown that the GPVI receptor, acting via a relatively simple signaling pathway, is a principal mediator of aggregation and PS exposure, as well as platelet-dependent thrombin generation and fibrin formation (Figure 2).

Composition and structure of a thrombus

As shown in Table 1, a quite similar set of signaling proteins downstream of GPVI appears to be involved in two different platelet responses, i.e. PS exposure and aggregate formation. In chapters 5 and 6, we show that these two responses are generated by quite different populations of platelets. In addition, during blood flow over collagen, we find that the aggregated and PS-exposing platelets tend to cluster in separate micro-domains within thrombi. The aggregated platelets bind fibrin(ogen), have

activated αIIbβ3 integrins and express P-selectin at their surface, which is a strong indicator of secretion. Separately, the thrombi contain patches of single PS-exposing platelets, which bind annexin A5 and have a bleb-forming morphology. The latter platelets are reduced in adhesion, do not tend to bind fibrin(ogen), but display increased binding for prothrombin, factor Va and factor Xa, which properties are all compatible with a role in coagulation. While it is clear that the Gla-domain-containing factors Xa and prothrombin bind to PS-exposing membranes, we have not further examined the coagulation factor binding sites on platelets.

Previously, others have characterised a population of so-called coated platelets that express serotonin binding sites and attract factor Va, fibrinogen, vWF, fibronectin and thrombospondin[49]. However, in our studies with labelled fibrinogen and coagulation factors, the platelets that express serotonin binding sites could not be distinguished as a separate population of cells, but partially overlapped with the two other platelet populations. Given these results it can be questioned whether the coated platelets described by others in fact represent a unique platelet population.

An important question is how one and the same signaling pathway, starting from GPVI and leading to PLCγ2 activation, can result in so different platelet responses within a single thrombus. In chapter 7, a first clue observation that can explain the platelet heterogeneity came from flow cytometry experiments. These showed that all GPVI-activated platelets at first express activated forms of integrin αIIbβ3, while at a later stage secondary inactivation of the integrins on PS-exposing platelets occurs. This suggests that initially all platelets are well equipped to incorporate into aggregates, but that later only the PS negative platelets have retained this property. This is in agreement with the observation that, under flow conditions, platelets in aggregates fail to expose PS. It can be speculated that platelets in aggregates are protected from PS exposure, but we do not have evidence to support this hypothesis. Earlier data indicate that platelets with a prolonged and high Ca^{2+} signal tend to expose PS and become procoagulant[50,51]. In combination with the present findings, this suggests that a prolonged Ca^{2+} increase not only causes phospholipid scrambling, but also leads to αIIbβ3 inactivation (Figure 3).

A second novel observation is that blocking of protein tyrosine phosphatases enhances the secondary integrin inactivation in GPVI-stimulated platelets. Since tyrosine kinases can act Ca^{2+}-dependently, it is likely that prolonged elevation in Ca^{2+} leads to both PS exposure and tyrosine kinase-dependent integrin inactivation. Measurement of Ca^{2+} fluctuations in platelets under flow conditions indeed showed considerable heterogeneity between platelets. Considering that the height of the Ca^{2+} response is a

critical determinant of PS exposure and is coupled to this integrin inactivation, there are several possible explanations for the response heterogeneity.

It can be caused by variation in the expression levels of one or more platelet receptors or signaling proteins, e.g. by allelic differences in the GPVI gene[52]. Also the platelet's age may play a role, since older platelets may loose or gain surface expression of functional glycoprotein receptors. For instance, GPIb-V-IX and GPVI have been shown to be sensitive for clustering and protease-mediated cleavage during storage[53-55]. Following this reasoning, GPIb clustering may make older platelets more susceptible for tethering on (collagen-bound) vWF or for aggregation mediated via GPIb-vWF interaction. Another possibility is that micro-environmental diversities upon platelet adhesion cause later response heterogeneity. All together, this leads to the idea that restricted GPVI activity and/or signaling leads to a proaggregatory response, while high stimulation of the GPVI pathway enhances procoagulant activity. This hypothesis is supported by a recent study, in which protein kinase C was identified as a signaling protein to enhance platelet aggregation but suppresses Ca^{2+} and procoagulant responses during thrombus formation on collagen[56].

Some authors have proposed that extracellular transglutaminases like factor XIIIa contribute to the formation of coagulation-active platelets by cross-linking surface-membrane proteins to secretion factors, e.g. via serotonin conjugation to form coated platelets[51,57]. However, we were not able to confirm this result, since in our hands inhibition of transglutaminases had no effect on PS exposure by GPVI-stimulated platelets. Our findings are, however, in line with a recent report in which it was shown

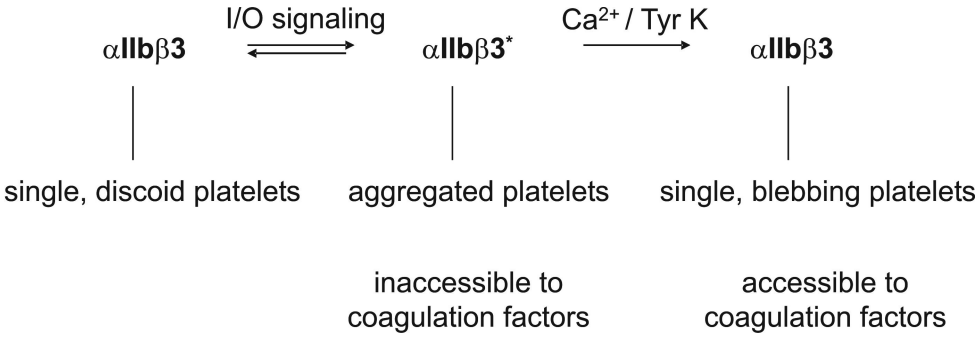

Figure 3 New model of transient integrin αIIbβ3 activation. Inside-out signaling is known to lead to αIIbβ3 activation, exposing fibrinogen-binding epitopes on the integrin. We provided evidence that this activated conformation is a transient state, as prolonged Ca^{2+} elevation and tyrosine phosphorylation events lead to secondary inactivation of the integrins on PS-exposing platelets. Typically, these procoagulant platelets are single, non-adherent with a blebbing morphology. For further explanations, see text.

that the binding of secretory proteins and annexin A5 to platelets on collagen requires GPVI/FcR γ-chain activity, but not factor XIIIa[58]. It was also proposed that intracellular or extracellular calpain activity contributes to the development of procoagulant platelets [51,59]. In agreement with earlier results, however, we find no major role for calpain in the regulation of platelet procoagulant activity, although calpain is involved in gaining the bleb-shaped morphology of the cells[50].

A novel finding in chapter 7 is that, while heterogeneity is maintained during perfusion of blood under conditions of thrombin generation and coagulation, the frequency of procoagulant, PS-exposing platelets becomes higher in this case. Furthermore, it appears that the PS-exposing platelets bind all components of the prothrombinase complex. Taken together, these results indicate that collagen provides an adhesive substrate, at which the processes of platelet aggregation and coagulation are integrated but also separated over different micro-domains.

Roles of collagen and glycoprotein VI in thrombus formation *in vivo*

In the studies presented in chapters 5 and 7, an established $FeCl_3$ microvascular thrombosis model was used to study the importance of collagen and GPVI in the thrombotic process *in vivo*. Upon application to adjacent arteries and veins, the free radical-generating compound $FeCl_3$ caused endothelial denudation and subsequent exposure of the collagen-containing extracellular matrix[60]. We used several approaches to show that arterial thrombus formation relies on collagen-GPVI interaction and subsequent signaling. Thus, in mesenteric arteries from mice lacking the FcR γ-chain, no thrombi were formed after $FeCl_3$ application. Also, in mice where GPVI on the platelet surface was down-regulated by long-term injection with anti-GPVI JAQ1 mAb, thrombus formation in arteries was delayed and reduced. In the damaged arteries of wild-type control mice, we were able to visualize externalized PS on platelets that were trapped in thrombi, using fluorescently labeled annexin A5. However, in vessels from mice pre-injected with JAQ1 mAb, only small spots of labeled fibrin were present and labeled annexin A5 was not detectable at all. Accordingly, both GPVI-induced responses, aggregation and PS exposure, observed *in vitro*, were also present in thrombi formed *in vivo*. That murine GPVI has a function in arterial thrombus formation *in vivo* was also proven in other mouse studies using the same $FeCl_3$ method[61,62]. Yet, with mild $FeCl_3$-induced injury, thrombus formation in FcR γ-chain deficient mice appeared to be normal, probably due to minimal exposure of collagen[62]. Confusing results were obtained when thrombus formation was induced by superficial or severe laser injury. Here, GPVI/PLCγ2

effects were only detected after the superficial laser injury[63,64]. Furthermore, also in large arteries, ligation models demonstrate a role for GPVI in thrombus formation[61].

We used the method of $FeCl_3$-induced injury to study the role of procoagulant platelets and thrombin in arterial thrombosis *in vivo* (chapter 7). Infusion of annexin A5 completely inhibited thrombus formation in the mesenteric arteries. Moderate thrombin inhibition with melagatran or platelet inhibition with prostaglandin E_1 completely blocked arterial thrombus formation. Thus, besides GPVI, procoagulant platelets mediating thrombin generation and coagulation play a key role in this microvascular thrombosis model. In agreement with the present findings, others also describe that thrombin[60] and platelet thrombin receptors[65,66] contribute to $FeCl_3$-induced thrombus formation in small arteries. In this context, it should be noted that a drawback of many *in vivo* thrombosis models is the lack of information on the precise type of vascular damage and the extent of collagen exposure.

Some of the experiments in this thesis are focussed on perfusion of blood at intermediate shear over a glass surface coated with fibrillar collagen. It is important to know whether this flow-chamber system provides a physiological model for the events of thrombus formation *in vivo*. Interestingly, comparison of the *in vitro* flow studies with the *in vivo* mice data shows great similarity between the two methods used (chapters 5-7). Both *in vitro* and *in vivo* we detect a key role of GPVI in the thrombus formation and procoagulant activity, and similarly, in the heterogeneous composition of the formed thrombi. Therefore, the *in vitro* flow model seems to mimic the conditions of *in vivo* thrombus formation, although obviously no vessel is present. There are, however, clearly limitations of the flow-chamber model, one being that type I collagen is used in the present work, while other types of collagen (type IV) are also abundantly present in the vascular extracellular matrix, and can support platelet activation. In addition, the contribution of other matrix proteins, e.g. laminin, to platelet adhesion and thrombus formation can not be ruled out[67]. It is therefore of interest to extend the present model to the study of additional collagen types and other matrix proteins, either in isolation or in combination.

Role of thrombin in arterial and venous thrombosis

An important question is to which extent platelets and coagulation contribute to arterial and venous thrombosis. Using the $FeCl_3$ method, a number of characteristic differences between thrombus formation in mesenteric arteries and veins are described in chapter 6. In this model, platelet inhibition by prostaglandin E_1 or moderate thrombin

inhibition by melagatran was insufficient to affect venous thrombus formation, although arterial thrombosis was greatly inhibited. The combined action of both agents or a higher dose of melagatran was needed to reduce the thrombotic process in the veins. An attractive explanation for this difference is that thrombin generation is locally higher in veins than in arteries, for instance due to a slower flow-mediated dilution of thrombin and other coagulation factors[68]. Alternatively, since endothelial cells of arteries and veins can have different antithrombotic properties[69], increased suppression of the coagulation process in the mesenteric arteries may explain their relatively high responsiveness to thrombin inhibition. More speculative is the hypothesis that $FeCl_3$-induced damage of the mesenteric veins results in the exposure of more coagulation-stimulating vascular material, such as tissue factor. Indeed, the expression of tissue factor does not appear to be uniform throughout the vascular tree[70]. An interesting parallel is seen in a model of laser-induced arterial thrombus formation, where suppressed platelet activation (using FcR γ-chain deficient mice) only affected thrombus formation, when thrombin was simultaneously inhibited[63]. These data suggests that, also in arterial thrombosis, high thrombin formation can overrule the contribution of specific platelet-activating pathways.

In prospective

The studies in this thesis shed new light on the importance of coagulation and platelet activation for thrombus formation under arterial and venous flow conditions. A relevant finding is the observation that absence or inhibition of the collagen receptor, GPVI, can significantly suppress thrombus formation and platelet procoagulant activity both in small and large arteries. This makes GPVI a new interesting target for future antithrombotic treatment. Another relevant finding is the relatively high accumulation of thrombin *in vivo* in veins, and *in vitro* at venous shear rates. It is thus important to realize that the suggested antithrombotic effect of GPVI can be bypassed by high thrombin generation and coagulation under conditions of low shear. This also explains why patients lacking GPVI suffer from no more than a mild bleeding diathesis. Similarly, in mice, GPVI deficiency is not accompanied by prolonged bleeding times. Together, this suggests that anti-GPVI treatment may be most effective in case of arterial thrombosis (low thrombin), rather than in case of venous thrombosis (high thrombin). In addition, it provides more information on the structural organization of such thrombi. Currently, several clinical trials are started to validate the antithrombotic effect of GPVI.

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Summary

Intravascular thrombosis is classified as venous thrombosis (e.g., in deep veins) or arterial thrombosis (as in myocardial infarction and ischemic stroke). Irrespective of other factors (vessel wall, circulation and blood cell composition), venous thrombosis is commonly associated with high coagulation activity, which can cause undesired fibrin clot deposition. On the other hand, arterial thrombosis is often linked to high platelet activation, which leads to the formation of platelet-rich thrombi. Although nowadays there are good examples of favorable combination therapy with anticoagulant and antiplatelet drugs, anticoagulants are still preferred in reducing venous thromboembolism while antiplatelet agents are mostly used in the prevention of arterial thrombosis. Irrespective of this, laboratory investigations indicate that the processes of platelet activation and coagulation are strongly mutually dependent. The crucial coagulation product, thrombin, is one of the most potent platelet agonists known. Conversely, activated platelets are essential in providing the procoagulant surface at which thrombin generation occurs. This is in apparent contrast with the supposed differential roles of thrombin and activated platelets in venous and arterial thrombosis, respectively. Hence, the main goal of this thesis is to investigate the diversity in interaction mechanisms between platelet activation and coagulation in different situations and on different levels of complexity.

Platelets can be activated by complex cascades of signaling pathways. Current knowledge of a number of these pathways is described in **chapter 1**. Emphasised is how the ADP receptor, $P2Y_{12}$, the thrombin receptors, PAR1 and PAR4, and the collagen receptor, glycoprotein (GP)VI, activate the phosphoinositide 3-kinase (PI3K) pathway by producing phosphoinositide 3,4,5-trisphosphate. Particularly, PI3K that is activated downstream of GPVI plays a role in recruiting phospholipase C-γ2 (PLCγ2) to the plasma membrane. Activation of PLCγ2 leads to Ca^{2+} elevation by mobilisation of Ca^{2+} from internal stores, and subsequent secretion and exposure of phosphatidylserine (PS) at the platelet surface. Earlier literature is cited that PS-exposing platelets are active in thrombin generation and coagulation, by providing a surface at which coagulation factors assemble. Separately, $P2Y_{12}$ and PAR receptor stimulation activates the so-called Rap1b, PI3K and Akt pathway, which leads to integrin αIIbβ3 activation and hence platelet aggregation.

The rare Scott syndrome is a bleeding disorder, characterized by impaired surface exposure of procoagulant PS on activated platelets and other blood cells. Since store-mediated Ca^{2+} entry is essential for the procoagulant response of platelets, we investigated in **chapter 2** whether the Ca^{2+} entry process is impaired in blood cells from Scott patients. Measurements of Ca^{2+} fluxes and PS exposure were performed in isolated platelets and in immortalized B-cell lymphoblasts from two different patients. The

responses of platelets and B-cells from the Scott patients appeared to be unaltered in comparison to cells from control subjects. On the other hand, the extent of PS exposure in response to GPVI stimulation was strongly reduced in patient platelets. These results thus indicate that impaired phospholipid scrambling in Scott cells can not be ascribed to alterations in Ca^{2+} signaling, e.g. of diminished Ca^{2+} entry.

Inositol phospholipids, especially those produced by PI3K, play key roles in Ca^{2+} signaling in platelets. **Chapter 3** describes a study on the role of various platelet PI3K isoforms in the regulation of Ca^{2+} signaling, following platelet activation by collagen or thrombin. Mice lacking the p85α regulatory subunit (class 1A PI3K) or the p110γ catalytic subunit (class 1B PI3Kγ) were used, as well as several newly developed inhibitors interacting with the catalytic subunits of the various PI3Ks present in platelets. The results indicate that both the p85α regulatory subunit and the catalytic p110β activity contribute significantly to Ca^{2+} signaling and subsequent PS exposure elicited by collagen receptor activation. This contribution occurs via the classical Ca^{2+} pathway, i.e. by enhancing the PLC-dependent Ca^{2+} mobilisation and subsequent store-mediated Ca^{2+} entry. The class 1A PI3Kδ and the class 1B PI3Kγ isoforms do not seem to have a prominent role in this process.

In the *in vitro* flow studies reported in **chapter 4**, the interacting roles of the platelet adhesive receptors for von Willebrand factor (vWF), i.e. GPIb-V-IX and integrin αIIbβ3, and for collagen, i.e. GPVI and integrin α2β1, were examined. Studies were performed to measure thrombus formation and procoagulant activity during high-shear perfusion of human whole blood over a collagen surface. Novel antibodies and peptides directed against the two collagen receptors were used to specifically unravel the function of each receptor molecule. GPVI was found to be crucial for platelet aggregate formation, Ca^{2+} signaling and PS exposure, but not for primary adhesion to collagen/vWF. Inhibition of α2β1 revealed that this integrin had a modulating role in Ca^{2+} signaling, PS exposure and aggregate size, since it enforced the responses evoked by GPVI. Interestingly, both GPIb-V-IX and α2β1 contributed to primary adhesion, and showed a partly overlapping function. Co-inhibition studies revealed synergism of distinct platelet receptors in thrombus formation. Co-inhibition of ADP with collagen receptors resulted in greatly decreased adhesion and aggregation. Co-inhibition of GPVI with either GPIbα or α2β1 was required to cause complete eradication of thrombus formation. It was concluded that platelet deposition on collagen depends on the concerted interplay of several receptors, GPIb-V-IX in synergy with integrin α2β1 mediating primary

adhesion, reinforced by platelet activation through GPVI, which in turn regulates thrombus formation.

Coagulation activation with tissue factor may contribute to collagen-induced thrombus formation. **Chapter 5** introduces an assay to trigger thrombin generation and coagulation in flow-adhesion experiments on collagen. Investigated was by which signaling pathway collagen-induced platelet activation interacts with tissue factor-triggered coagulation both *in vitro* and *in vivo*. In murine blood flowing over collagen, platelet exposure of PS and procoagulant activity, but not adhesion, completely relied on each of the following signaling modules: GPVI, the FcR γ-chain, Src kinases, the adaptor protein LAT, and PLCγ2. Upon flow in the presence of tissue factor, these signaling components were essential for platelet aggregation and greatly enhanced the formation of fibrin clots. The physiological importance of this GPVI pathway was shown in a $FeCl_3$-induced *in vivo* murine thrombosis model. In both venules and arterioles, the presence and activity of GPVI, FcR γ-chain and Src kinases resulted in an enhanced formation of PS-exposing and fibrin-rich thrombi. These findings demonstrate that the GPVI-PLCγ2 activation pathway can regulate collagen-dependent coagulation in venous and arterial thrombus formation.

In **chapter 6**, the mutually stimulatory processes of platelet activation and thrombin generation in arterial and venous thrombus formation *in vivo* were investigated in more detail. In the murine $FeCl_3$ model, the thrombus-forming process appeared to be triggered by both collagen exposure and complex formation of tissue factor and factor VII(a). Interestingly, mild thrombin inhibition or platelet inhibition suppressed arterial thrombus formation, while strong thrombin inhibition or mild thrombin inhibition in combination with platelet inhibition was necessary to suppress venous thrombosis. Thrombus formation in both vessel types was characterized by the presence of PS-exposing platelets, as detected with fluorescently labeled annexin A5. Shielding of exposed PS by injection of a surplus of unlabelled annexin A5 abolished the formation of both arterial and venous thrombi, while a mutant M1234-annexin A5 was ineffective. Surprisingly, decreased anticoagulant activity in mice carrying the factor V Leiden mutation had no more than little effect. Together, these findings demonstrated that the propagation of thrombin generation by PS-exposing platelets is a key regulatory process in both veins and arteries. Since platelet-dependent thrombin generation is most active in venous thrombus formation, this process may be a clinically relevant target for antithrombotic therapy.

As indicated, platelets play a dual role in thrombus formation by assembling into aggregates and by stimulating the coagulation. In **chapter 7** we investigated the

commitment of platelets to either of these functions both *in vitro* and *in vivo*. High-resolution two-photon fluorescence microscopy revealed that during thrombus formation under flow, fibrin(ogen)-binding platelets assembled into separated aggregates, after which distinct patches formed of non-aggregated platelets, exposing PS. The latter platelet population had inactivated αIIbβ3 integrins and displayed increased binding of coagulation factors. Coated platelets, expressing serotonin binding sites, were not identified as a separate population. Thrombin generation (coagulation) favored the integrin inactivation and the transformation into PS-exposing platelets with reduced adhesion. Prolonged tyrosine phosphorylation *in vitro* resulted in secondary down-regulation of active αIIbβ3. Together, these results lead to a new, spatial model of thrombus formation, in which aggregated platelets ensure thrombus stability, while distinct patches of non-aggregated platelets effectuate procoagulant activity and generate thrombin and fibrin. Herein, the haemostatic activity of a developing thrombus seems to be determined by a balance in formation of proaggregatory and procoagulant platelets. This balance likely influences the efficacy of antiplatelet or anticoagulant medication.

The significance of the findings described in this thesis is discussed in **chapter 8**, particularly in the light of relevant, recent literature. In summary, the data recognize the GPVI pathway to PLCγ2 activation as a key signaling mechanism for Ca^{2+}-dependent PS exposure and procoagulant activity. Signaling proteins that cluster together in the LAT signallosome, including the PI3Kα and β isoforms support this pathway. Yet, only the population of platelets with prolonged Ca^{2+} elevation and tyrosine phosphorylation shows this procoagulant activity. Altogether, the key role of GPVI in the thrombus-forming process makes this platelet receptor as a promising new antithrombotic tool. However, the data also suggest that anti-GPVI treatment may be most effective in case of arterial thrombosis (low thrombin), rather than in case of venous thrombosis (high thrombin), since high thrombin formation can overrule the antithrombotic effect of anti-GPVI treatment. This knowledge may help to develop improved treatment strategies of the still numerous patients who suffer from cardiovascular thrombotic diseases.

Samenvatting

Trombose, dat wil zeggen verstopping van de bloedvaten is nog steeds een van de meest voorkomende aandoeningen in onze samenleving. De medische wereld maakt daarbij onderscheid tussen veneuze trombose (zoals bijvoorbeeld een trombosebeen) en arteriële trombose (zoals bij een hart- of herseninfarct). Veneuze trombose is het gevolg van ongewenste bloedstolling, waarbij een fibrineprop gevormd wordt in de aders of venen. Arteriële trombose daarentegen is veelal het gevolg van schade van de bloedvatwand en daaropvolgende activering van de bloedplaatjes, die daarbij samenklonteren tot een plaatjesprop of plaatjesaggregaat. Veelal wordt veneuze trombose tegengegaan door stollingsremmers ('bloedverdunners'), terwijl arteriële trombose eerder onderdrukt wordt door medicatie die de plaatjesactivering remt (bijvoorbeeld aspirine). De laatste jaren is echter ook nagegaan, in hoeverre combinatietherapie met stollings- en plaatjesremmers effectief is ter preventie van trombose. Onderzoek met geïsoleerd bloed, met name in Nederland uitgevoerd, heeft laten zien dat de processen van stolling en plaatjesactivering heel nauw met elkaar verweven zijn. Trombine, dat een belangrijk product is van de stolling, is tevens een van de meest krachtige activatoren van plaatjes. Anderzijds zorgen geactiveerde plaatjes voor een oppervlak, waarop stollingsreacties kunnen plaatsvinden. Dit lijkt echter tegengesteld aan de verschillende functies van stolling (trombine) en geactiveerde plaatjes bij veneuze en arteriële trombose. Het doel van het onderzoek beschreven in dit proefschrift is daarom na te gaan hoe de interacties tussen trombinevorming en plaatjesactivering afhankelijk zijn van de condities zoals die naar verwachting optreden tijdens veneuze en arteriële trombose.

Bloedplaatjes worden geactiveerd door een aantal ingewikkelde, trapsgewijs werkende signaleringsroutes, waarbij vele eiwitten betrokken zijn. Een algemeen overzicht van de routes, die relevant zijn voor dit proefschrift, is gegeven in **hoofdstuk 1**. Hier wordt het werkingsmechanisme uitgelegd van de receptor voor ADP, $P2Y_{12}$, de trombinereceptoren PAR1 en PAR4, en de collageenreceptor glycoproteïne (GP)VI. Al deze receptoren activeren de zogenaamde fosfoinositide 3-kinase (PI3K) route, die zorgt voor de vorming van het signaleringslipide fosfoinositide-3,4,5-trifosfaat. Activering van PI3K door GPVI speelt een rol bij de rekrutering en activering van fosfolipase C-γ2 (PLCγ2) naar de plasmamembraan. Deze activering van PLCγ2 leidt tot een verhoging van het intracellulaire calcium, dat gemobiliseerd wordt vanuit interne opslagplaatsen. Een daaropvolgende stap is de calcium-afhankelijke expositie van het stollingsactieve fosfatidylserine (PS) op het plaatjesoppervlak. Gememoreerd wordt dat PS-exposerende plaatjes actief zijn in de generatie van trombine en het stimuleren van de stollingsactivieit, doordat zij als bindingsplaats dienen van stollingsfactoren. Stimulering

van de plaatjes via de $P2Y_{12}$ en PAR receptoren leidt daarnaast tot activering van de Rap1b - PI3K - Akt route, die zorgt voor plaatjesaggregatie via activering van het integrine $\alpha IIb\beta 3$.

Het zeer zeldzame syndroom van Scott is een milde bloedingsziekte, die gekenmerkt wordt door een verminderde expositie van stollingsactief PS op geactiveerde plaatjes en andere bloedcellen. De literatuur bevatte aanwijzingen dat de calciuminstroom als gevolg van interne calciummobilisatie verstoord is in plaatjes van Scott-patiënten. Aangezien dit een verklarende factor zou kunnen zijn voor de verminderde procoagulante respons van deze plaatjes, is in **hoofdstuk 2** onderzocht in hoeverre deze calciuminstroom daadwerkelijk veranderd is. In geïsoleerde plaatjes en geïmmortaliseerde B-cel lymfoblasten van twee verschillende Scott-patiënten zijn daarom metingen verricht aan zowel de calciuminstroom als de PS expositie. Er werden echter geen verschillen waargenomen tussen de cellen van Scott-patiënten en overeenkomstige cellen van controlepersonen. Wèl was in Scott plaatjes de mate van PS expositie na GPVI stimulatie sterk gereduceerd. Deze resultaten weerleggen daarmee de veronderstelling dat een deficiëntie in calcium-signaaltransductie ten grondslag ligt aan het Scott syndroom.

Inositol-bevattende fosfolipiden, die voornamelijk geproduceerd worden door PI3K, spelen een rol bij de activering van plaatjes. Welke rol is nog niet helemaal duidelijk. **Hoofdstuk 3** beschrijft onderzoek naar de functie van verschillende PI3K isovormen in plaatjes na activering met collageen of trombine. Voor dit onderzoek werd gebruik gemaakt van muizen, die deficiënt waren in ofwel de $p85\alpha$ regulerende subunit (klasse IA PI3K) ofwel de $p110\gamma$ katalytische subunit (klasse 1B PI3K), en verder van enkele nieuw ontwikkelde remmers van specifieke katalytische subunits. Wij konden aantonen dat zowel de $p85\alpha$ als de katalytische $p110\beta$ subunit bijdraagt aan de calciumsignalering en daaropvolgende PS expositie. In plaatjes gestimuleerd met collageen verloopt deze bijdrage via de klassieke calciumsignaleringsroute, dat wil zeggen door verhoging van PLC-afhankelijke calciummobilisatie en daarop volgende opslag-gemedieerde calciuminstroom. In tegenstelling tot de isovormen $PI3K\alpha$ en $PI3K\beta$ (beide klasse 1A), blijken de isovormen $PI3K\delta$ en $PI3K\gamma$ een minder belangrijke rol te hebben in dit proces.

Middels *in vitro* perfusieproeven, beschreven in **hoofdstuk 4**, is de rol van verschillende plaatjesreceptoren voor adhesieve eiwitten, namelijk het glycoproteïne Ib (GPIb) en integrine $\alpha IIb\beta 3$ als receptoren voor von Willebrand factor (vWF), en GPVI en integrine $\alpha 2\beta 1$ als receptoren voor collageen, onderzocht bij de trombusvorming. Het

trombusvormende proces en de procoagulante activiteit zijn daarbij gemeten tijdens de stroming van humaan bloed over een collageenoppervlak onder een hoge afschuifsnelheid (*shear rate*). Gebruik werd gemaakt van nieuw ontwikkelde antilichamen en peptiden gericht tegen collageenreceptoren, teneinde de specifieke functie van elke receptor te ontrafelen. Gebleken is dat GPVI essentieel is voor de vorming van plaatjesaggregaten, de calciumsignalering en de PS expositie. Anderszins speelt GPVI geen rol in de primaire adhesie van plaatjes aan het collageen/vWF oppervlak. Integrine $\alpha 2\beta 1$ heeft slechts een modulerende rol in de calciumsignalering, plaatjesaggregatie en PS expositie, doordat het de werking van GPVI versterkt. De glycoproteïnen GPIb en $\alpha 2\beta 1$ overlappen elkaar gedeeltelijk in primaire adhesie. Co-inhibitie van ADP- en collageenreceptoren geeft een sterk verminderde adhesie en aggregatie. Om complete remming van de trombusvorming te krijgen moet echter zowel GPVI als GPIb of $\alpha 2\beta 1$ geblokkeerd worden. Op basis van deze resultaten konden wij concluderen, dat de plaatjesadhesie en -aggregatie op collageen bepaald worden door onderlinge samenwerking van de verschillende adhesieve receptoren: GPIb in synergie met $\alpha 2\beta 1$ voor de primaire adhesie, welk in een proces dat versterkt wordt door GPVI leidt tot trombusvorming.

Op grond van de literatuur werd aangenomen dat activering van de stolling middels weefselfactor betrokken is bij de collageen-afhankelijke trombusvorming. **Hoofdstuk 5** beschrijft een nieuwe methode om trombinegeneratie en stolling op te wekken, door bloed onder stromingscondities over collageen te leiden. Deze methode is gebruikt om de vraag te beantwoorden via welk signaleringspad de plaatjesactivering door collageen bijdraagt aan de stolling onder invloed van weefselfactor. In muizenbloed dat geperfundeerd werd over collageen bleek de expositie van PS, maar niet de plaatjesadhesie, volledig afhankelijk van de volgende signaleringmodules: GPVI, de FcR γ-keten, Src kinases, het adaptor eiwit LAT en PLCγ2. In aanwezigheid van weefselfactor waren deze signaleringcomponenten essentieel voor de plaatjesaggregatie en droegen zij bij aan de vorming van fibrinestolsels. De fysiologische betekenis van dit GPVI signaleringspad werd onderzocht in een *in vivo* trombosemodel in muizen. Hierbij werd trombose opgewekt na vasculaire beschadiging met behulp van ijzertrichloride ($FeCl_3$). In de microscopische arteriën en venen van het darmvlies resulteerde de afwezigheid van actief GPVI, FcRγ-keten of Src kinases in een sterk verlaagde vorming van PS-exposerende en fibrinerijke trombi. Deze bevindingen geven aan dat de activeringsroute van GPVI naar PLCγ2 van belang is voor het trombusvormende proces, inclusief stolling en fibrinevorming onder arteriële en veneuze stromingscondities.

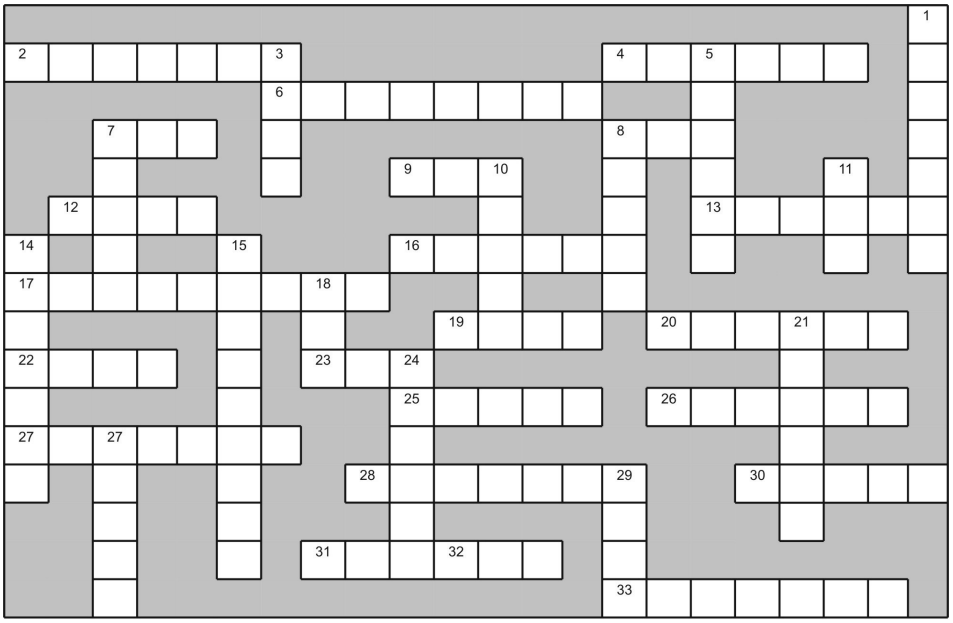
De interactie tussen plaatjesactivering en trombinegeneratie in arteriële en veneuze trombusvorming *in vivo* is onderzocht in **hoofdstuk 6**. In hetzelfde $FeCl_3$ trombosemodel bleek dat de trombusvorming op gang werd gebracht door vasculaire blootstelling van zowel collageen als weefselfactor. De trombusvorming in arteriën kon worden onderdrukt door plaatjesremming of door lage dosering van een trombineremmer. Echter in venen kon de trombusvorming alleen onderdrukt worden door een combinatie van beide remmers, of door een hoge dosis van de trombineremmer. De procoagulante respons van bloedplaatjes verdween na infusie van annexine A5, een eiwit dat bindt aan PS. Wanneer annexine A5 vooraf aan muizen toegediend werd, resulteerde dit in een complete remming van de trombusvorming in beide typen bloedvaten. Dat er inderdaad procoagulante bloedplaatjes aanwezig zijn in de arteriële en veneuze trombi werd bevestigd door deze *in vivo* aan te kleuren met fluorescerend annexine A5. Verrassend genoeg had verlaagde antistollingsactiviteit in muizen met een factor V Leiden-mutatie slechts een klein effect op het tromboseproces. Samen laten deze resultaten zien dat de versterking van trombinegeneratie door PS-exposerende plaatjes van belang is voor het trombotische proces in venen en arteriën. Aangezien PS-afhankelijke trombinegeneratie het meest actief lijkt bij de veneuze trombusvorming, kan deze plaatjesreactie een doelwit zijn voor nieuwe antitrombotische therapie.

Zoals eerder aangegeven hebben plaatjes een tweeledige rol in het proces van trombusvorming, namelijk door vorming van aggregaten en door stimulering van de bloedstolling. In **hoofdstuk 7** onderzochten wij welke van deze twee functies plaatjes vertonen na activering onder *in vitro* of *in vivo* condities. Hoge resolutie tweefoton fluorescentie-microscopie liet zien dat tijdens de trombusvorming een groot aantal fibrinogeen-bindende plaatjes clusterde tot aggregaten, terwijl een andere groep van niet-aggregerende plaatjes PS exposeerde. Deze laatste plaatjespopulatie bleek in versterkte mate stollingsfactoren te binden, terwijl hun αIIbβ3 integrines in een niet-actieve conformatie waren. Zogenaamde 'coated' plaatjes, die gekarakteriseerd zijn door een expressie van serotonine bindingsplaatsen, vormden geen aparte populatie. Interessant is dat de vorming van trombine (stolling) de inactivering van integrines bevorderde en ook zorgde voor een verhoogde transformatie van plaatjes tot losse, PS-exposerende cellen. De secundaire inactivering van αIIbβ3 integrines kon herleid worden tot een verlengde fosforylering van tyrosines middels protein tyrosinekinasen. Samengevat resulteren deze resultaten in een nieuw, ruimtelijk model van trombusvorming, waarbij clusters van geaggregeerde plaatjes zorgen voor trombusstabiliteit, terwijl andere, niet-aggregerende plaatjes zorgen voor de

procoagulante activiteit en de vorming van trombine en fibrine. Verondersteld wordt dat de hemostatische activiteit van een zich ontwikkelende trombus een resultante is van de balans in vorming van proaggregerende en procoagulante plaatjes. Deze balans is waarschijnlijk van belang voor de werkzaamheid van antiplaatjes- en antistollings-medicatie.

Hoofdstuk 8 beschrijft het belang van de bevindingen van dit proefschrift in het licht van relevante, recente literatuur. Aangegeven is dat de GPVI route naar PLCγ2 activering van groot belang is voor calcium-afhankelijke PS expositie en de procoagulante activiteit van plaatjes. Diverse signaleringseiwitten die samenklonteren in het LAT signalosoom zijn betrokken bij het ontstaan van deze procoagulante activiteit, inclusief de PI3Kα en β isovormen. De cruciale rol van het GPVI signaleringpad in het trombusvormende proces maakt de GPVI receptor tot een veelbelovend doeleiwit voor nieuwe antitrombotische middelen. Het is daarbij te verwachten dat anti-GPVI behandeling het meest effectief zal zijn bij de preventie van arteriële trombose (laag trombine) en minder effectief bij de veneuze trombose (hoog trombine). Immers een hoge trombinevorming zal het antitrombotische effect van een anti-GPVI behandeling ongedaan kunnen maken. De in dit proefschrift beschreven kennis zal hopelijk bijdragen tot de ontwikkeling van nieuwe behandelingsmethoden voor de nog zeer vele trombosepatiënten.

Dankpuzzel



15	29	32	25	3	33	12

Horizontaal

2 enthusiastic Birmi-girl **4** de dokter in huis **6** degenen die ik vergeten ben **7** de grote baas **8** deze extroverte Belgische windt er geen doekjes om **9** bezorgde topklusser en onvoorwaardelijke steun **12** degene die de deur naar Biochemie opende….zucht! **13** m'n enige echte zus en reismaatje **16** gezellige, ongedwongen crea-bea **17** master op MTB, bijna master in de kinderfysio: de top is de limit **19** voor iedere foton een dochter **20** vasculaire, oogopende meedenktank **22** mijn liefste huisgenootje ooit **23** de tafeltje-dekster aan het thuisfront en toekomstig kunstenares? **25** m'n onechte tweelingzusje die hard-loopt door het embryonale wereldje van de meerlingen **26** recht voor z'n raap en eerste hulp op reis **27** m'n bed and breakfast adresje **28** alles pluis met de muis? materiaal zorgvuldig in de kluis! **30** expert in superb spicy curries **31** avontuurlijke Belgische met doorzettingsvermogen **33** visolie distributeur en Audi-minded: Deutsche gründlichkeit?

Verticaal

1 oost, west, Posterholt best **3** de eerste zwangere man **5** klein van stuk, speelt graag toneel, maar neemt geen blad voor de mond **7** de gedreven plaatjesexpert, alias de harembewaarder **8** m'n kleine zusje en de benjamin van de groep **10** hippe, altijd jatsende flatmate **11** proud mother and good scientist **14** m'n grote voorbeeld en muizenvanger **15** collegiale groep bloedzuigers die bloedstollend werk verrichten **18** deze reislustige echtgenote kan van chocolade echt genieten **21** kordate, maar krachtige Obbichtse met strakke plannen **24** labmanager en psychologe in èèn **27** wekt spanning op in vaten en tevens in zijn fietsbanden **29** hij heeft mij de promotiebal op hoog niveau toegespeeld

Oplossing

Horizontaal

2 Jocelyn **4** Saskia **6** iedereen **7** Jan **8** Kim **9** pap **12** Theo **13** Nadine **16** Yvonne **17** Annemieke **19** Marc **20** Mirjam **22** Inge **23** mam **25** Aafke **26** Sandra **27** Kirsten **28** Viviane **30** Steve **31** Sandra **33** Kristof

Verticaal

1 Jeffrey **3** Niko **5** Simone **7** Johan **8** Karen **10** Paola **11** Pia **14** Marijke **15** Biochemie **18** Kim **21** Judith **24** Marion **27** Remco **29** Erik

Curriculum vitae

Imke Munnix werd geboren op 16 maart 1979 te Roermond. Na voltooiing van het Voorbereidend Wetenschappelijk Onderwijs (VWO) aan het Bisschoppelijk College Schöndeln te Roermond, startte ze in 1997 met de opleiding Gezondheids-wetenschappen aan de Universiteit Maastricht. Na het behalen van haar propedeuse in 1998 vervolgde zij deze studie in de richting Biologische Gezondheidskunde. Tijdens haar studie slaagde zij voor het examen Stralingshygiëne, deskundigheidsniveau 5^b. Haar afstudeerstage liep zij in 2001 bij de capaciteitsgroepen Biochemie en Moleculaire Genetica onder begeleiding van Dr. J.W.M. Heemskerk en Dr. G.J.J.M. van Eys. Voor een extra buitenlandstage vertrok ze in april 2001 voor 5 maanden naar Cambridge, Engeland. Hier deed zij onderzoek bij the Department of Physiology, University of Cambridge, onder begeleiding van Dr. S.O. Sage. Op 30 september 2001 mocht zij haar universitaire diploma in ontvangst nemen. Per oktober 2001 begon zij als onderzoeksassistent binnen het Cardiovascular Research Institute Maastricht (CARIM) van de Universiteit Maastricht bij de capaciteitsgroep Biochemie van Prof. Dr. J. Rosing. Hier werd zij per november 2002 Assistent in Opleiding (AIO) op een project gesubsidieerd door de Nederlandse Hartstichting. Het onderzoek dat zij daar deed en wat beschreven staat in dit proefschrift vond plaats onder directe begeleiding van Dr. J.W.M. Heemskerk. Na het volgen van verscheidene cursussen werd haar in 2005 het CARIM PhD-Training Certificate uitgereikt. Tevens verwierf ze de status van artikel 9-deskundigheid volgens de Wet op Dierproeven. Zij bezocht verschillende congressen in onder andere Birmingham, Eberbach, Erfurt, Nottingham, Oxford, Reading en Sydney om haar onderzoeksresultaten te presenteren en won hiervoor tweemaal een “young investigator award”. Zij bezocht verscheidene malen het Institute of Molecular Cell Biology, University of Jena (Duitsland) van Dr. R. Heller om daar proeven te doen. Tevens werkte zij in 2006 gedurende 3 maanden bij het Centre for Cardiovascular Sciences Institute of Biomedical Research, University of Birmingham (Engeland), onder leiding van Prof. Dr. S. Watson. Sinds 1 maart 2007 is zij werkzaam als postdoctoraal onderzoeker bij de capaciteitsgroep Biochemie van de Universiteit Maastricht.

Curriculum vitae

Imke Munnix was born on March 16th 1979 in Roermond, the Netherlands. After completing secondary school (VWO) at the Bischoppelijk College Schöndeln in Roermond in 1997, she started to study Biological Health Sciences at Maastricht University. During her study she passed the exam Radiation hygiene, expertise level 5b. In 2001, she followed her graduation internship at the Departments of Biochemistry and Molecular Genetics, under supervision of Dr. J.W.M. Heemskerk and Dr. G.J.J.M. van Eys. In the same year, she spent five months in Cambridge, United Kingdom, for a research internship at the Department of Physiology, University of Cambridge, under the guidance of Dr. S.O. Sage. On September 30st 2001 she received her Master's degree. On October 1st she started as a research assistant at the Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, at the Department of Biochemistry directed by Prof. Dr. J. Rosing. From November on she worked here as a PhD student on a project financed by the Netherlands Heart Foundation. This research, which is described in the present thesis, was supervised by Dr. J.W.M. Heemskerk. After taking several courses, she received the CARIM PhD training certificate and became an Article 9 expert according to the Law on Animal experiments. She visited congresses in Birmingham, Eberbach, Erfurt, Nottingham, Oxford, Reading and Sydney to present research data, which were honoured twice with a "young investigator award". She visited the Institute of Molecular Cell Biology of Dr. R. Heller at the University of Jena (Germany) for several times to perform experiments. In 2006, she worked for 3 months at the Centre for Cardiovascular Sciences Institute of Biomedical Research, University of Birmingham (United Kingdom) under the supervision of Prof. Dr. S. Watson. Since March 1st 2007 she is working as a postdoctoral researcher at the Department of Biochemistry at Maastricht University.

List of publications

Full Papers

- **Munnix ICA**, Harmsma M, Giddings JC, Collins PW, Feijge MAH, Comfurius P, Heemskerk JWM, Bevers EM. Store-mediated calcium entry in the regulation of phosphatidylserine exposure in blood cells from Scott patients. Thromb Haemost. 2003; 89: 687-695.
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Awards

- Travel awards from the Dutch Cancer Foundation (KWF) and the University Foundation Limburg (SWOL) to visit the Department of Physiology from Cambridge University (2001).
- Poster award, Euregional Life Science Conference, Maastricht 2004.
- Young investigator award of the International Society on Thrombosis and Haemostasis, XXth Congress ISTH, Sydney, Australia (August 2005).
- Young investigator award of the Netherlands Foundation for Thrombosis and Haemostasis to visit XXth Congress ISTH, Sydney, Australia (April 2005).
- Travel award from the Netherlands Heart Foundation to visit the Centre for Cardiovascular Sciences Institute of Biomedical Research from Birmingham University (2006).