Platelet receptors and procoagulant activity in thrombus formation

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**Platelet receptors
and procoagulant activity
in thrombus formation**

Platelet receptors and procoagulant activity in thrombus formation

Marijke Kuijpers

Thesis Universiteit Maastricht – with summary in Dutch

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Platelet receptors and procoagulant activity in thrombus formation

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Contents

Chapter 1	General introduction	9
Chapter 2	Platelet collagen receptors and coagulation – A characteristic platelet response as possible target of antithrombotic treatment	37
Chapter 3	Complementary roles of glycoprotein VI and $\alpha 2\beta 1$ integrin in collagen-induced thrombus formation in flowing whole blood ex vivo	49
Chapter 4	Role of integrin $\alpha 2\beta 1$ in thrombus stabilization and embolization	69
Chapter 5	Facilitating roles of murine platelet glycoprotein Ib and $\alpha 1\text{Ib}\beta 3$ in phosphatidylserine exposure during vWF-collagen-induced thrombus formation	87
Chapter 6	Regulation of tissue factor-induced coagulation and platelet aggregation in flowing whole blood	107
Chapter 7	Role of platelet procoagulant response in collagen- and tissue factor-induced thrombosis in arterioles and venules	127
Chapter 8	General discussion	151
	Summary / Samenvatting	169
	Dankwoord	180
	Curriculum vitae	184
	Publications	186
	Awards	188

Chapter 1

General introduction

Hemostasis is the physiological process responsible for cessation of bleeding from an injured vessel, while thrombosis is pathophysiological thrombus formation that may develop into vascular occlusion, causing venous or arterial thrombosis, resulting in heart attack or stroke. In hemostasis and thrombosis interactions of platelets with components from the vessel wall play a very important role. Also, the coagulation process is activated upon vascular injury, generating fibrin and consolidating thrombus formation. The processes of platelet activation and coagulation are closely entwined and both are essential for effective thrombus formation. Furthermore, hemostasis is the result of a balance between anti- and prothrombotic factors. When this balance is disturbed, this may lead to bleeding or thrombosis. Nowadays, mice are frequently used as models to study hemostasis and thrombosis, which leads to important knowledge regarding the factors involved in these processes and subsequently to improved treatment of patients.

1.1. Discovery of platelets

Platelets have been recognized as separate entities in blood in the middle of the nineteenth century. At that time a number of scientists claimed to be the first to describe blood platelets (1). The first drawings of platelets were made independently by George Gulliver and William Addison around 1840. However, the roles of platelets in hemostasis and experimental thrombosis were not discovered until the studies of Giulio Bizzozero in 1881 and 1882 (2,3). As a medical doctor, he studied mesenteric venules in guinea pigs using intravital microscopy and observed that platelets circulate as isolated disk-shaped cells, but adhere to the vessel wall after vascular injury and then aggregate with each other. Ironically, Bizzozero was also the first to identify bone marrow megakaryocytes (4), but he never recognized them as the precursors of platelets. This discovery was made by James Homer Wright in 1906 (5). Around 1880 William Osler established the role of blood platelets (which he called blood "plaques") in human thrombotic disease, identifying them in white thrombi that were formed on "atheromatous ulcers" and on vegetations on heart valves and in aortic aneurisms (6). At that time, the development of thrombosis (thrombogenesis) had already been described by Rudolph Virchow in 1856 (7). He described the nowadays classical triad of factors that are associated with the development of venous thrombosis: endothelial injury/damage, changes in blood flow and blood hypercoagulability.

Why were platelets discovered so late, while erythrocytes were known already in the seventeenth century and leukocytes in the eighteenth century? Antoni van Leeuwenhoek first observed the erythrocyte (and many other minute objects) with a simple home-made microscope (1670), which magnified up to 250 times (8). Earlier, the compound microscope had been developed, which, in its simplest form, consisted of two convex lenses aligned in series and achieved a two-step magnification. Compound microscopes developed during the

seventeenth and eighteenth centuries were hampered by optical aberration, a flaw that was worsened by the use of multiple lenses. These microscopes were actually inferior to single lens microscopes of that time because of these artifacts. The images they produced were often blurred and had colorful halos associated with chromatic aberrations that not only degraded image quality, but also hampered resolution. Hence, it is not surprising that before 1830 nobody was able to identify a translucent object of only 3 μm in diameter. A significant optimization of the compound microscope has been made by Joseph Jackson Lister in 1826. One year later, Thomas Hodgkin and Joseph Lister wrote a paper on 'Microscopical observations of the blood and animal tissues' (9), which marks the beginning of real animal histology; with Lister's microscope it was now possible to resolve objects about 1 μm apart.

Since Bizzozero's early description of platelets in 1881, our understanding of hemostasis and thrombosis and the important roles of platelets in these processes has been greatly increased. Hemostasis is the physiological process which results in stopping the bleeding from a damaged vessel, while thrombosis is an acute complication that develops on chronic atherosclerotic lesions and causes heart attacks and stroke (10,11). Nowadays, atherothrombosis, i.e. thrombus formation at the surface of atherosclerotic plaques in arteries (12) is the main cause of morbidity and mortality in the western world (13). As an important tool in the fight against these diseases, better understanding of the mechanisms involved in platelet adhesion to the vessel wall and subsequent events leading to thrombus formation is needed. This chapter reviews current literature on platelet morphology, platelet adhesion to the vessel wall and subsequent platelet activation and thrombus formation. Since the majority of the experiments in this thesis have been performed with (genetically modified) mice, attention is also paid to similarities and differences between human and mouse platelets.

1.2 Platelet morphology

Platelets are the smallest anuclear cellular elements present in human blood in concentrations of $150\text{-}350 \times 10^9/\text{L}$, where they circulate mostly in close proximity to the endothelial lining of the vessel wall (14). They are shed from megakaryocytes in bone marrow and lung. Once released, platelets circulate in the human body for 7-10 days, after which they are cleared by spleen, liver and bone marrow. In general, resting platelets have a discoid shape, with a longitudinal diameter of 3 μm , a thickness of 0.5 μm and a volume of 7.5-10 fL in humans (15,16). In mice, the species studied in the present thesis, platelets are smaller (mean platelet volume 4.7 ± 0.3 fL) and have a life span of 4.5-5.5 days, whereas the platelet count is generally higher than in humans ($450\text{-}1690 \times 10^9/\text{L}$) (16). Apart from differences in size and number, human and mouse platelets have similar morphologic structures and react in similar ways to environmental stimuli.

Figure 1.1. Schematic drawing of platelet morphology. This cartoon represents a cross-section through a platelet. The plasma membrane and the open canicular system (OCS) are shown in grey; in the plasma membrane several types of receptors (Rec) are anchored. ER = endoplasmatic reticulum; mtch = mitochondrion; lys = lysosome; α = α -granule; δ = dense granule. The microtubule coil is represented by the dotted line and the actin microfilaments and intermediate filaments are depicted as straight lines.

Platelets react, adhere, spread, secrete, and also interact with each other instantly upon changes in their environment, particularly after vessel wall injury. These responses require platelets to undergo rapid morphological changes, as they convert from their resting discoid shape into an activated shape. To enable these swift morphological changes, platelets are equipped with several features, such as an extensive surface-connected membrane system (Fig. 1.1). The bilamellar plasma membrane extends into the cytoplasm through an open canicular system, which greatly enlarges the surface area of the platelet. In close contact to the open canicular system lies the dense tubular system or endoplasmatic reticulum (ER), in which Ca^{2+} ions are sequestered; Ca^{2+} is an important intracellular messenger during platelet activation. The ER membrane also contains enzymes, including those needed for thromboxane A_2 (TxA_2) synthesis, i.e. the cyclooxygenase (COX-1) and TxA_2 -synthase complex. In the plasma membrane numerous receptor proteins are embedded, which are required for interaction of the platelet with proteins present in plasma, on other blood cells and vascular tissue.

The platelet's shape is determined by the cytoskeleton, which consists of microtubules, microfilaments and intermediate filaments. A thick bundle of microtubules in the periphery of the platelet (microtubule coil) forms a solid, egg-like structure that is responsible for the discoid form of the platelet (15), whereas the actin microfilaments and intermediate filaments (mainly desmin

and vimentin) form an integrated network that coats the inner surface of the plasma membrane and that is involved in platelet shape change (15).

Platelets also disperse several organelles and secretory granules within their cytoplasm (Fig. 1.1). The α -granules are most abundant and contain proteins such as fibrinogen, von Willebrand factor (vWF), P-selectin, platelet factor 4, coagulation factor V, growth arrest specific gene 6 (Gas6) and thrombospondin. The dense granules are rich in serotonin, adenosine diphosphate (ADP), adenosine triphosphate (ATP) and the divalent cations Ca^{2+} and Mg^{2+} . The contents of these granules are released upon platelet activation and strongly support the subsequent aggregation of platelets and formation of a thrombus.

1.3 Platelet adhesion, aggregation and thrombus formation

Following damage or alterations of endothelial cells (the inner lining of blood vessels), the subendothelial extracellular matrix (ECM) may be exposed, after which platelets can come into contact with thrombogenic components in the ECM. The ECM contains different macromolecules that are suitable ligands for platelet adhesion molecules, including various types of fibrillar collagen, laminin, fibronectin, proteoglycans, vitronectin, thrombospondin and von Willebrand factor (vWF). The hemostatic response to vascular injury is dependent on the extent of damage, the specific matrix proteins exposed and on the local blood flow conditions. Collagen plays an important role as it not only supports platelet adhesion, but also strongly activates the cells (17). Under conditions of elevated shear, as in arteries, platelet adhesion to collagen is strictly dependent on the interaction of platelets with collagen-bound vWF (18). Together, collagen and vWF are key components of the extracellular matrix, initiating platelet adhesion and activation.

There are genes for more than 20 forms of collagen in the human genome, 9 of which are expressed in the vessel wall: types I, III, IV, V, VI, VIII, XII, XIII and XIV. Collagens represent up to 40% of the total protein of the vessel wall and fibrillar types I and III are the major constituents of the extracellular matrix. Three polypeptide chains (α -chains) form the fundamental structure of the collagen molecule, which is characterized by the presence of one or more triple-helical domains. Mature vWF is a large multimeric glycoprotein consisting of a variable number of subunits (minimal 2, up to 50-100). VWF is synthesized in endothelial cells and megakaryocytes, and is composed of 4 types of domains (A-D), arranged in the following order: D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2 (19). In endothelial cells, vWF is stored in the Weibel-Palade bodies and can be released, either constitutively or via secretion, in two directions: luminal (into the circulating blood) and abluminal (into the extracellular matrix), where it binds directly to collagen types I and III via its A3 domain.

Figure 1.2. Model of platelet-collagen interaction leading to thrombus formation. After endothelial cell denudation, platelets tether to subendothelial collagen fibers by interaction of their GPIb-V-IX receptors with von Willebrand factor (vWF), which leads to initial rolling of the platelets over the surface. Subsequent stable adhesion to collagen is mediated through direct binding of glycoprotein VI (GPIIb) and integrin $\alpha 2\beta 1$ and indirect binding of integrin $\alpha IIb\beta 3$ to vWF. GPIIb binding to collagen leads to activation of the integrins $\alpha 2\beta 1$ and $\alpha IIb\beta 3$ (indicated by black arrows inside platelet). Activation of integrins involves a conformational change of the receptor from an inactive to an active state. Platelet-collagen interaction also induces exposure of phosphatidylserine on the outer membrane of the platelet, which forms a catalytic surface for the assembly and activation of coagulation factors, leading to the formation of factor Xa and thrombin. Activated platelets form the aggregate via cross-linking of fibrinogen bound to integrin $\alpha IIb\beta 3$. Further cross-linking is established by binding of vWF and fibronectin to $\alpha IIb\beta 3$ and GPIb-V-IX. Also, activated platelets will release TxA_2 and ADP. These autocrine agents, together with thrombin, activate other platelets via their specific receptors, leading to growth of the aggregate. The platelet aggregate is stabilized by fibrin, which is formed out of fibrinogen by thrombin, through the formation of a network.

When collagen is exposed, binding of plasma vWF is a crucial step in the response to vascular injury. Soluble vWF does not bind to resting platelets, but when immobilized on a surface it forms a reactive surface capable of capturing platelets in flowing blood (19). Several residues in the A3 domain display multiple conformations, a flexibility which suggests that binding of A3 to collagen involves an induced-fit mechanism (17). High shear stress induces conformational changes of vWF, resulting in a fast, reversible interaction between platelet glycoprotein (GP)Ib-V-IX and the A1 domain of vWF (20). Moreover, vWF also contains a unique Arg-Gly-Asp-Ser (RGDS) sequence in its C1 domain, the essential binding site for GPIIb/IIIa (integrin $\alpha IIb\beta 3$). Hence, vWF can act as a bridging molecule in platelet-collagen interactions, since it can bind to both collagen and the platelet receptors GPIb-V-IX and $\alpha IIb\beta 3$. The interaction between vWF and GPIb-V-IX reduces the velocity of platelets flowing over (collagen-bound) vWF and results in transient attachment ('rolling') to a collagen surface (Fig. 1.2) (21).

Following platelet rolling, direct interactions between collagen and platelets lead to adhesion (Fig. 1.2) (11,22). Platelets adhere to collagen via two receptors, GPIIb and GPIa-IIa (integrin $\alpha 2\beta 1$) (22,23). This direct binding to collagen induces platelet activation, which is predominantly mediated by GPIIb (22). The activated platelets change shape and expose phosphatidylserine (PS) on their outer membrane (Fig. 1.2). The availability of PS greatly

accelerates the conversion of prothrombin into coagulant thrombin and, thus, enhances clot formation (24-26). Chapter 2 addresses the typical morphological changes accompanying PS exposure in these procoagulant platelets.

Following adhesion to a collagen surface, activated platelets can recruit platelets from flowing blood to form a platelet aggregate. They activate nearby platelets via secretion of autocrine agents such as ADP and TxA_2 , which bind to specific receptors on the platelet membrane. TxA_2 is formed from arachidonic acid, that is released from membrane phospholipids by phospholipase A_2 upon platelet activation. Once formed, TxA_2 diffuses through the platelet membrane and activates other platelets by binding to the thromboxane-prostanoid receptors, $\text{TP}\alpha$ and $\text{TP}\beta$ (27). ADP, secreted from dense granules by exocytosis, activates platelets via two receptors, P2Y_1 and P2Y_{12} ; the first receptor mediates shape change and initiates aggregation, while the latter is required for complete aggregation (28). Platelets also contain P2X_1 receptors, which enhance collagen-induced platelet aggregation through a rapid ATP-induced Ca^{2+} influx (29).

Formation of a platelet aggregate is strongly dependent on the presence of integrin $\alpha\text{IIb}\beta_3$, which upon platelet activation binds several adhesive molecules to form cross-links between platelets (Fig. 1.2) (30). Fibrinogen is the major platelet cross-linker, present both in plasma and platelet α -granules (15). It is an extended dimeric molecule with unique RGDS binding sites for $\alpha\text{IIb}\beta_3$ on both ends. Integrin $\alpha\text{IIb}\beta_3$ can also cross-link platelets via vWF and fibronectin (31,32). Moreover, platelet cross-linking by interaction of vWF and GPIb-V-IX supports aggregation, particularly under high shear conditions (33).

Thrombin is a crucial protein linking coagulation, platelet activation and thrombus formation. It is formed during coagulation (Fig. 1.3) and cleaves fibrinogen into fibrin, which forms networks and stabilizes the platelet aggregate and, hence, consolidates the formation of the thrombus (Fig. 1.2). Thrombin is also a potent platelet agonist. Three thrombin receptors have been identified on human platelets, namely the GPIb-V-IX complex and the protease activated receptors, PAR-1 and PAR-4 (34). Activation of PARs occurs when thrombin cleaves the N-terminus of the receptors, exposing a new N-terminus, which serves as a ligand for the receptor. Current data indicate that the vast majority of thrombin responses is due to PAR-1 (35). Interestingly, mouse platelets do not express PAR-1, and in these cells low concentrations of thrombin can bind to PAR-3, enabling PAR-3 to function as a co-factor for PAR-4 (36). However, at high thrombin concentrations, PAR-4 can signal independently of PAR-3 (37). In humans, PAR-1 and PAR-4 signal independently in response to thrombin, with PAR-1 responding at low concentrations of thrombin, whereas PAR-4 responds only at higher thrombin concentrations (38,39).

1.4 The platelet procoagulant response

The plasma membrane of resting platelets has an asymmetric distribution of phospholipids. The outer leaflet is composed predominantly of the choline phospholipids sphingomyelin and phosphatidylcholine, whereas the inner leaflet mostly contains the amino-phospholipids phosphatidyl-ethanolamine (PE) and phosphatidylserine (PS). At least two transporter proteins are involved in maintaining this phospholipid asymmetry: the aminophospholipid translocase (40), which transports PS and PE from outer to inner leaflet, and floppase, which transports all phospholipids outward with a halftime about 10 times longer than translocase. Upon platelet activation, intracellular calcium levels rise and inhibit translocase and floppase activity. In addition, the high calcium levels activate a non-selective phospholipid scramblase, which allows phospholipids to move randomly between the two leaflets and results in the appearance of PS and PE on the outer surface of the platelet (40,41). Until now, the identities of translocase and scramblase are still unknown (26). The prolonged rise in intracellular calcium required for the procoagulant response can be mediated by collagen or high doses of thrombin; secreted ADP is known to potentiate this response (as discussed below) (26,42).

Figure 1.3. Tissue factor induced coagulation. Upon vascular injury, tissue factor (TF) is exposed at surfaces of smooth muscle cells and fibroblasts in the vessel wall, or inflammatory cells at the site of injury. Circulating factor VIIa or VII binds to TF and VII is consequently activated. The membrane-bound TF/FVIIa complex converts factors IX and X into their active forms (initiation phase). Factor IX and X bind to exposed PS (dark circles) on procoagulant platelets and assemble into the tenase and prothrombinase complexes, which respectively convert the zymogens Factor X and prothrombin (Factor II) into their active forms factor Xa and thrombin. During the propagation phase of coagulation, factor IXa stimulates the tenase reaction, after which factor Xa stimulates prothrombinase. Thrombin subsequently converts soluble fibrinogen into fibrin and stimulates the formation of factors XIIIa, Va, VIIIa and XIa.

The exposure of PS on the platelet surface strongly promotes two important reactions of the coagulation cascade, the tenase and prothrombinase reactions (Fig. 1.3). This involves the binding of the vitamin K-dependent coagulation factors II (prothrombin), VII, IX, X, V and VIII to PS. Factors II, VII, IX and X contain glutamate residues which are carboxylated by vitamin K-dependent carboxylase to gamma-carboxyglutamate (Gla)-residues. These Gla-residues can bind to PS in a calcium-dependent way. The cofactors Factor V and VIII can bind to platelets, independently of exposed PS. The mechanism by which this occurs is presently unknown.

1.5 Main intracellular signaling pathways

Many of the functional platelet responses, including secretion, TxA_2 formation and the PS exposure are mediated by a rise in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). $[\text{Ca}^{2+}]_i$ elevation is to a large extent due to the activation of phosphoinositide-specific phospholipase C (PLC) β and γ isoforms, which hydrolyze phosphatidylinositol 4,5-bisphosphate to generate the second messengers inositol 1,4,5-trisphosphate (InsP_3) and sn-1,2-diacylglycerol (DAG). InsP_3 binds to Ca^{2+} -channels in the endoplasmatic reticulum, which release stored Ca^{2+} into the cytosol (43). This depletion of the Ca^{2+} stores triggers the influx of extracellular Ca^{2+} into the cell through store-regulated Ca^{2+} influx channels. Together, this results in a series of transient $[\text{Ca}^{2+}]_i$ increases (spikes) with irregular frequency and amplitude (44). However, upon platelet stimulation by collagen or high concentrations of thrombin, a non-oscillating prolonged rise in $[\text{Ca}^{2+}]_i$ is seen (43). The other second messenger DAG activates protein kinase C, which in turn phosphorylates many effector molecules. Activated protein kinase C in combination with elevated $[\text{Ca}^{2+}]_i$ causes secretion of autocrine agents and contributes to integrin activation and, hence, platelet aggregation. Rises in $[\text{Ca}^{2+}]_i$ are also implicated activation of phospholipase A_2 , in actin rearrangements and shape change (43). In addition, $[\text{Ca}^{2+}]_i$ is necessary to activate the alleged enzyme phospholipid scramblase, a membrane activity that is assumed to regulate the procoagulant response (26,41).

The $\text{PLC}\gamma_2/\text{Ca}^{2+}$ signaling cascade is triggered by collagen via GPVI (22). GPVI recognizes the specific sequence Gly-Pro-Hyp (GPO) within collagen, and activation is achieved by dimerization of two GPVI receptors (17). GPVI is a member of the immunoglobulin receptor superfamily (45) and is non-covalently associated with the signal transducing $\text{FcR}\gamma$ -chain (46,47). Cross-linking of GPVI induces tyrosine phosphorylation on the $\text{FcR}\gamma$ -chain immunoreceptor tyrosine-based activation motive (ITAM) (46) by the Src kinases Fyn and Lyn (48). This is followed by the binding and activation of another tyrosine kinase Syk, which initiates downstream signaling events that lead to the activation of a number of effector enzymes, including $\text{PLC}\gamma_2$, small G-proteins and phosphoinositide-3 kinase (PI-3 kinase). The many adaptor proteins, including LAT and SLP-76 play critical roles in the further signaling cascade (reviewed in (22,49)). In vitro, platelet GPVI can be selectively stimulated by a

synthetic, triple-helical GPO-containing peptide, termed collagen-related peptide (CRP) or by convulxin, a toxin isolated from venom of the tropical rattlesnake.

The integrin $\alpha 2\beta 1$ is a heterodimeric type I transmembrane receptor, which is present in a low-affinity state on the membrane of resting platelets, like the other main platelet integrin $\alpha IIb\beta 3$. Ligand binding upon platelet activation requires a conformational switch mechanism, which shifts the integrin to a high-affinity state, and probably also induces receptor clustering (23,50-52). This so-called inside-out signaling can be achieved by several agonists, most importantly collagen (via GPVI), but also thrombin, TxA_2 , ADP (53) and vWF (via GPIb-V-IX) (54). Once in its active state, integrin $\alpha 2\beta 1$ recognizes its own specific sequence within collagen, Gly-Phe-Hyp-Gly-Glu-Arg (GFOGER), through a particular extracellular domain located on $\alpha 2$, the so-called inserted (or I-) domain (55). Like GPVI, the integrin $\alpha 2\beta 1$ has been proposed to couple to the intracellular signaling proteins Syk, SLP-76 and $PLC\gamma 2$, but not to the $FcR\gamma$ -chain (56). The relative roles of GPVI and integrin $\alpha 2\beta 1$ in platelet activation on collagen have been a subject of considerable debate in recent years, and this topic is investigated in chapter 3 of this thesis.

The $PLC\beta/Ca^{2+}$ pathway is triggered by several members of the G-protein coupled receptor family. Several heterotrimeric GTP-binding proteins (G-proteins) are involved in the signal transduction process in platelets (57). G_q couples to $PLC\beta$ following activation of the thrombin receptors PAR-1 and PAR-4, the ADP receptor $P2Y_1$ or the TxA_2 receptor $TP\alpha$ (43). Since G_q can interact with several types of receptors, it is involved in platelet responses to several agonists. As a result, G_q -deficient platelets show severely impaired responses to thrombin, ADP and the TxA_2 analog U46619 (58). Typically, these platelets are still capable of shape change in response to thrombin and U46619, in a way independently of elevated $[Ca^{2+}]_i$. Recent studies show that this shape change is mediated by other G-proteins, G_{12} and G_{13} , which activate a Rho/Rho-kinase (ROCK) mediated pathway after platelet activation by thrombin or TxA_2 (59). Experiments with G_{12} - and G_{13} -deficient mice showed that the $G_{12/13}$ -mediated effects are mainly caused by G_{13} (60). Another important G-protein is G_i , which couples to PAR-1, $P2Y_{12}$ and possibly $TP\beta$, and inhibits the enzyme adenylate cyclase (61). This results in reduced levels of the platelet-inhibitory second messenger cyclic adenosine monophosphate (cAMP). However, G_i likely also mediates another signaling pathway, leading to activation of phosphoinositide (PI) 3-kinase, which is involved in the activation of $\alpha IIb\beta 3$ and potentiation of secretion (62,63). It has been shown that full platelet activation via G-protein coupled receptors needs stimulation of two out of the three G-protein coupled pathways (64,65). However, the three G-protein pathways are tightly entwined and all play an important role in platelet responses in humans (66,67) and mice (58,60).

It is known that certain combinations of agonists lead to high levels of $[Ca^{2+}]_i$ and PS exposition. More than twenty years ago, Bevers and colleagues demonstrated that thrombin

and collagen only moderately affect the platelet procoagulant response individually, while dual stimulation with both agonists appeared to be the most potent inducer of procoagulant activity (68). Collagen induces PS exposure mainly via GPVI (as described above), while thrombin mediates procoagulant activity mainly via PAR-1 (35,69). Moreover, it has been shown that ADP is also involved in the expression of PS, mediated via P2Y₁₂ (70,71).

Signaling towards aggregation is regulated in a manner that differs from signaling towards procoagulant activity. In fact, signaling which leads to aggregation, i.e. activation of integrin α IIb β 3, has not been clearly defined (50). Briefly, most agonists cause integrin α IIb β 3 activation via the ADP-P2Y₁₂ pathway (72). This makes P2Y₁₂ receptor antagonists (such as clopidogrel) interesting pharmacological agents for the treatment of thrombotic patients. Further, aggregation is inhibited when intracellular cAMP levels are elevated, which is mediated via protein kinase A. Protein kinase A is activated by prostacyclin or prostaglandin E₁. As P2Y₁₂ antagonists and prostaglandin E₁ can also suppress procoagulant activity and thrombin generation (70,73,74), it is possible that their effects on thrombin generation are via regulation of integrin activation. This subject is the topic of chapter 6.

At arterial shear conditions, the first platelet-vessel wall contact is mediated via GPIb-V-IX interaction with vWF. The importance of this interaction in supporting platelet-vessel wall and platelet-platelet interactions is clearly delineated, but the contribution of this interaction to platelet activation is less clear. Controversy exists with respect to the question whether ligand-bound GPIb-V-IX is signaling. In various reports it is proposed that there are no, weak or strong activating signals generated by vWF binding (reviewed in (49)). Also, the mechanism by which GPIb generates signals is controversial (75), with some studies suggesting direct signals through the cytoplasmic tails of GPIb-V-IX and the accessory protein 14-3-3- ζ (75), while others point to signaling through associated surface molecules like the FcR γ -chain (76,77) or indirect mechanisms via ADP release and TxA₂ generation (49).

Besides the collagen receptors GPVI and integrin α 2 β 1, other proteins have also been proposed as receptors for collagen in platelets (22). One of these is glycoprotein IV (CD36), which has been shown to bind to collagen (78). GPIV is now no longer considered as a functional receptor for collagen, as human and mouse platelets that lack GPIV respond to collagen normally (79,80). Furthermore, GPV is also a serious candidate collagen receptor. GPV $-/-$ mouse platelets show a moderate decrease in collagen-dependent adhesion and aggregation and additional studies demonstrated that GPV specifically binds to collagen (81). However, in vivo studies have yielded conflicting results regarding the significance of GPV deficiency (81,82). Thus, the question whether there is a third, functional collagen receptor remains open.

1.6 Human disorders of platelet receptors or ligands

The process of thrombus formation is a intricately orchestrated response of platelets and coagulation factors to damage of the vessel wall, involving many receptor-ligand interactions and signaling pathways. The importance of a platelet receptor or adhesive protein becomes evident when it is missing, which may have severe consequences for hemostasis in humans. Table 1.1 summarizes some disorders in which (the function of) platelet receptors or their ligands are disturbed and, hence, clearly demonstrates their importance in physiology. These receptors are also very interesting with respect to platelet procoagulant activity, although in most patients this has not been studied.

Deficiency of collagen receptors is very rare in humans. Integrin $\alpha 2\beta 1$ deficiency has been described in only three patients (Table 1.1), and was associated with a bleeding phenotype. One patient had platelets with about 15 to 25% of normal GPIa (integrin $\alpha 2$) expression, and her platelets failed to adhere to collagen, but spread normally on other subendothelial surfaces (83,84). Platelets from this patient showed defective binding to a number of adhesion molecules, raising the possibility that the bleeding effect was caused by defective binding to other ligands besides collagen (85). In another patient, collagen-induced platelet aggregation was markedly reduced, but these platelets were deficient not only in GPIa but also in thrombospondin-1 (86). Platelet aggregation in response to collagen could be restored in this patient by the addition of thrombospondin. Soon after the initial studies, the patient passed through menopause, after which collagen responsiveness recovered. A similar recovery of the platelet functions was seen in the first patient after passing menopause. The third (male) patient had an acquired deficiency of platelet GPIa, due to a myeloproliferative disorder (87). His platelets also completely lacked collagen-induced aggregation and adhesion.

Only seven patients lacking GPVI have been reported so far (Table 1.1). Given the important role of GPVI in platelet adhesion to and activation by collagen (see above), it is surprising that these patients only show a mild bleeding tendency with a slightly prolonged bleeding time. The first patient was reported almost 20 years ago. She appeared to carry antibodies recognizing a 62 kD platelet membrane protein and her platelets did not react to collagen (88). In 2001 and 2004, two other female patient were reported with auto-antibodies against GPVI; their platelets also showed defective collagen-induced aggregation and an impaired adhesion to collagen (89,90). Furthermore, 4 other patients have been discovered, who were (almost) completely lacking the GPVI receptor on their platelets (91-94). All patients were women, three Japanese and one French, and their platelets failed to aggregate in response to collagen, but had an almost normal response to ADP and other agonists. In the French patient GPVI deficiency accompanied gray platelet syndrome (characterized by a low platelet count and absence of α -granules). The investigators suggested that such GPVI

Table 1.1 Human disorders with altered platelet receptor (ligand) expression or function.

Platelet receptor/ Ligand	Copies per platelet	Ligand	Number of known patients	Gene map locus	Inheritance	Phenotype	Refs
$\alpha 2\beta 1$, GPIIb/IIIa	2,000-4,000	collagen	3	5q23-q31	unknown	Bleeding. Decreased platelet adhesion and aggregation in reaction to collagen. Platelet responses to other agonists preserved.	83,84, 86, 87
GPVI	1,000-2,000	collagen	7	19q13.4	unknown	GPVI deficiency (4 patients) or auto-antibodies against GPVI (3 patients). Mild bleeding. Lack of collagen-induced platelet aggregation, decreased adhesion to collagen. Near normal platelet responses to other agonists.	88- 94,140
α IIb β 3 GPIIb/IIIa	80,000	fibrinogen, vWF	1:1x10 ⁶	17q21.33	AR	Glanzmann's thrombasthenia. Greatly prolonged bleeding with normal pc and morphology. Absence of platelet aggregation with most agonists. Classification into 3 types: 1 (less than 5% expression), 2 (10-20%) and 3 (50-100%).	96
GPIIb-V-IX	25,000	vWF	< 1:1x10 ⁵	17p	AR	Bernard-Soulier syndrome. Greatly prolonged bleeding with thrombocytopenia and giant platelets. Mutations in any of 3 genes: GPIIb α (most abundant), GPIIb β or GPIX (not GPV).	96,141
P2Y ₁	500-1,000	ADP	1	3q25.2	unknown	Mild bleeding. Reduced platelet responses to ADP	101
P2Y ₁₂	500-1,000	ADP	5	3q25.1	AR	Bleeding. Blunted platelet aggregation and secretion in reaction to ADP, normal shape change.	97,98- 100
P2X ₁	13-130	ATP	1	17p13.3	dominant negative mutation	Bleeding. Normal pc, but reduced hemoglobin and iron. Partial impairment of activation induced by ADP or ATP.	102
vWF	stored in α - granules	GPIb-V-IX	0.1- 1.6:100	12p13.2	Type 1: AD; Type 2: AD or AR; Type 3: AR	Von Willebrand's disease. Bleeding. Quantitative or qualitative defect in vWF. 3 types: 1 (60-80% of frequency): partial quantitative decrease of vWF and FVIII, mild clinical symptoms; 2 (7-30%): qualitative defects of vWF, mild bleeding; 3 (5-20%): most severe form with marked deficiencies of vWF and FVIII in plasma; absence of vWF in platelets and endothelial cells, severe bleeding.	95,96
Fibrinogen	stored in α - granules	α IIb β 3 GPIIb/IIIa	1:1x10 ⁵	4q2	AR	A(dys)fibrinogenemia. Bleeding, normal pc. Afibrinogenemia or hypofibrinogenemia with decreased level of normal fibrinogen. Dysfibrinogenemia with structurally abnormal fibrinogen (sometimes prothrombotic).	142- 144
PS	stored in cytosolic leaflet pm complexes	tenase and prothrombi- nase	4	unknown	suggested AR	Scott syndrome. Bleeding, normal pc. Strongly decreased phospholipid scrambling, number of factor Va and VIIIa binding sites, procoagulant activity and microparticle formation	103- 106

Refs References; AD autosomal dominant; AR autosomal recessive; PS phosphatidylserine, pc platelet count; pm plasma membrane.

deficiency may be present in more patients with this syndrome, since abnormal collagen-induced aggregation has been reported more often (94).

Deficiencies of integrin $\alpha\text{IIb}\beta 3$ or GPIb-V-IX are more common (about 1:1,000,000), and are designated as Glanzmann's thrombasthenia and Bernard-Soulier syndrome, respectively (Table 1.1). These patients have severe bleeding problems in common. While Glanzmann patients have normal platelet counts and morphology, Bernard-Soulier patients display thrombocytopenia and extremely large ('giant') platelets. A large number of individuals suffer from von Willebrand's disease (vWF deficiency). The latter is the most common disorder of platelet activity occurring in about 1% of the general population (95,96). The severity of symptoms is dependent on the type of von Willebrand's disease (Table 1.1); the majority of patients have only mild clinical symptoms (type 1).

Two unrelated patients with prolonged bleeding times and P2Y₁₂ receptor deficiency are described, whose platelets change shape normally, but undergo no rapidly reversible aggregation when exposed to ADP (97,98). Furthermore, a family is described in which two sisters have a defect in the P2Y₁₂ receptor. Both show easy bruising, menorrhagia and severe bleeding complications after surgery (99). Recently, another patient was described with a homozygous mutation in the translation initiation codon of the P2Y₁₂ gene (100). This patient had a mild bleeding tendency, impaired platelet aggregation at low agonist concentrations, impaired platelet spreading and small, unstable thrombus formation in flow experiments over collagen. Preliminary reports of congenital defects of platelet P2Y₁ and P2X₁ receptors have also been presented (101,102). In either case, this is associated with a bleeding diathesis and reduced platelet aggregation (Table 1.1).

The Scott syndrome is a rare deficiency in platelet procoagulant activity. It is associated with severe bleeding and platelets from these patients show strongly decreased phospholipid scrambling and a decreased number of factor Va and VIIIa binding sites. Moreover, these platelets lack the ability to promote tenase and prothrombinase activity in response to agonists and show impaired capacity to shed microparticles (103-105). As intracellular calcium responses are normal, the defect should lay downstream of calcium signaling (106).

1.7 Targeted gene disruption of platelet receptor proteins and ligands in mice

The platelet, coagulation and extracellular matrix proteins of the common laboratory mouse show striking similarities to the human systems, with virtually every protein represented in both species and with every cascade appearing to serve similar functions in both species (16). Nonetheless, significant differences exist at the molecular and structural level, with varying

Table 1.2 Deletion or modification of mouse platelet membrane receptors and their ligands.

Platelet receptor/ Ligand	Ligand	Mouse genotype	Homology to human	Gene on chr	Phenotype	Refs
$\alpha 2\beta 1$	collagen	$\alpha 2, GPIa -/-$	13 (64)		Viable and normal, normal bleeding time, reduced platelet interaction with collagen	107, 108
$\alpha 2\beta 1$	collagen	$\beta 1, GPIIa -/-$	8		Embryonic lethal	109
$\alpha 2\beta 1$	collagen	$\beta 1, GPIIa$ null	8		Conditional knock-out. Viable and normal, normal bleeding time	110, 111
GPVI	collagen	FcR γ -chain $-/-$	1 (93.3)		Viable and normal, no severe bleeding, defective platelet activation by collagen	145
GPVI	collagen	GPVI depleted	64.4 %	7	Viable and normal, moderate bleeding. Reduced platelet activation by collagen and protected against collagen-induced TE (depletion induced by JAQ1 100 μ g i.p.)	114
GPVI	collagen	GPVI $-/-$	64.4 %	7	Viable and normal, no severe bleeding, defective platelet activation by collagen	115, 116
$\alpha IIb\beta 3$	fibrinogen	$\beta 3, GPIIa -/-$	11 (68)		<i>Glanzmann's thrombasthenia phenotype</i> . Impaired viability and bleeding, defective platelet aggregation and adhesion to fibrinogen and prothrombin. Reduced thrombus formation in vivo	117-119, 146
$\alpha IIb\beta 3$	fibrinogen	$\beta 3, GPIIa$ (Y747, 759F)	92 %	11 (68)	<i>diYF mouse</i> . Bleeding tendency, but viable. Selective impairment of $\alpha IIb\beta 3$ outside-in signaling, reduced aggregation and clot retraction in vitro.	120, 121
GPIb-V-IX	vWF	GPIb $\alpha -/-$	75 %	11 (42)	<i>Bernard-Soulier syndrome phenotype</i> . Viable, but bleeding. Giant platelets and thrombocytopenia.	122
GPIb-V-IX	vWF	GPIb $\beta -/-$	88 %	11 (42)	<i>Bernard-Soulier syndrome phenotype</i> . Viable, but bleeding. Giant platelets with increased α -granules and thrombocytopenia.	123
GPIb-V-IX	vWF	GPV $-/-$	70 %	16	Viable and normal. Shortened bleeding time, platelets hyperresponsive to thrombin in vivo. Decreased platelet aggregation and thrombus formation in response to collagen in vitro.	81, 82, 124, 125
P2Y ₁	ADP	P2Y ₁ $-/-$	3		Viable, bleeding, decreased ADP-induced platelet responses.	131
P2Y ₁	ADP	P2Y ₁ $-/-$	3		Viable, bleeding. Absent platelet response to ADP, resistant to TE.	132, 133
P2Y ₁₂	ADP	P2Y ₁₂ $-/-$	3		Viable and bleeding. Platelets show reduced adhesion and activation. Reduced thrombus formation, and thrombus instability in vivo.	130
P2X ₁	ATP	P2X ₁ $-/-$	11 (40)		Viable and normal, infertility in males. Impaired platelet aggregation with low agonist doses. In vivo: decreased thrombus formation and TE.	134, 148
P2X ₁	ATP	P2X ₁ over-expression	11 (40)		Viable. Enhanced platelet aggregation and secretion with low agonist concentrations. Increased systemic TE in vivo.	29
Fibrinogen	$\alpha IIb\beta 3$	fibrinogen $-/-$	3 (41-48)		Viable, spontaneous bleeding. Reduced platelet aggregation and clotting. In vivo unstable thrombus formation and increased embolization.	126, 128, 146, 147
vWF	GPIb-V-IX	vWF $-/-$	6 (60.8)		Viable, bleeding. Delayed thrombus formation in vivo. Increased sensitivity to inflammation. Strongly reduced plasma FVIII levels.	127, 147
Fibronectin	$\alpha 5\beta 1$	fibronectin $-/-$	1 (36.1)		Conditional knock-out. Viable and normal. Delayed thrombus formation and occlusion in vivo. Thrombi continuously shed platelets or small platelet clumps, resulting in slow growth; diminished platelet/platelet cohesion.	129

NB. All mice deficient in platelet receptors or their ligand are indicated as $-/-$, regardless of the method used to accomplish this deficiency. Chr chromosome, Refs References; TE thromboembolism.

degrees of homology between corresponding mouse and human proteins (Table 1.2). The introduction of targeted gene disruption in mice has provided unique opportunities to study platelet function, and of every patient described in Table 1.1 a mouse deficient in the same receptor or ligand has been generated (Table 1.2). Comparison of both tables reveals many similarities regarding the regulation of platelet activation, aggregation and procoagulant activity. Undoubtedly, many of the observations in these animal models will have direct relevance for human thrombosis and hemostasis.

Murine integrin $\alpha 2\beta 1$ can be knocked-out from platelets by targeting either the $\alpha 2$ - or the $\beta 1$ -gene. Two research groups almost simultaneously reported the generation of $\alpha 2$ -integrin-deficient mice (Table 1.2) (107,108). General depletion of the $\beta 1$ gene is embryonically lethal (109) and this lethality was overcome by the generation of conditional $\beta 1$ -integrin-deficient mice (110,111). All these mice were found to have normal tail bleeding times, although in vitro their platelets display partial defects in interaction with collagen. These unexpected findings have led to considerable debate in literature. Therefore, we decided to investigate the contribution of integrin $\alpha 2\beta 1$ to platelet activation and thrombus formation in more detail using $\beta 1^{-/-}$ (chapter 3) and $\alpha 2^{-/-}$ (chapter 4) animals.

Mouse GPVI has been depleted by different methods (Table 1.2). Platelets lacking the FcR γ -chain were found to lack expression of GPVI on their surface as well (112). As these platelets are refractory to stimulation by various types of collagen, it was argued that the GPVI/FcR γ -chain complex is a key receptor for platelet-collagen interactions (79). However, some defects of these platelets may also be related to the absence of GPIb, which has also been reported to signal via the FcR γ -chain (76,77). Another strategy to deplete platelets from GPVI was used in studies with mice that were injected with JAQ1, an anti-mouse GPVI monoclonal antibody (113). This treatment leads to a transient GPVI deficiency, while the expression of other platelet receptors is not affected, including that of $\alpha 2\beta 1$, $\alpha IIb\beta 3$, GPIb and GPV (114). Like FcR γ -chain $^{-/-}$ platelets, the GPVI-depleted platelets are refractory to activation by collagen. Only recently the GPVI gene has been deleted in mice (115,116). Platelets from these mice also failed to aggregate in response to collagen, and showed defective platelet activation following normal initial tethering to a collagen surface, as well as complete abolishment of thrombus formation. Similarly to GPVI-deficient patients, the various GPVI-deficient or -depleted mice did not reveal severe bleeding tendencies.

Mice deficient in $\beta 3$ -integrin lack both $\alpha IIb\beta 3$ and $\alpha V\beta 3$ on their platelets. These animals have a bleeding diathesis and platelet function abnormalities similar to those of patients with Glanzmann's thrombasthenia (117). In addition to impaired aggregation, $\beta 3$ -integrin-deficient mouse platelets are unable to adhere to fibrinogen and prothrombin (118). Furthermore, $\beta 3$ -integrin-deficient mice are protected from thrombosis in vivo (119). Upon outside-in signaling, integrin $\alpha IIb\beta 3$ becomes tyrosine phosphorylated at the $\beta 3$ -integrin

cytoplasmic tyrosine (ICY) domain (reviewed in (120)). The critical role of this $\beta 3$ tyrosine phosphorylation in platelet function has been established in diYF mice, which express an $\alpha \text{IIb}\beta 3$ form in which the two $\beta 3$ ICY domain tyrosines (Y) are mutated to phenylalanine (F) (121). These mice are selectively impaired in outside-in $\alpha \text{IIb}\beta 3$ signaling, and show defective aggregation and clot-retraction responses *in vitro*, and also a bleeding phenotype *in vivo* (102).

Genetic deletion of either GPIb α or GPIb β in mice produces a Bernard-Soulier syndrome phenotype (122,123). These mice present all characteristics of the human disease, i.e. prolonged bleeding times, giant platelet size and thrombocytopenia. Moreover, platelets from GPIb β $-/-$ mice have α -granules with increased size (123). In contrast, mice lacking GPV on platelets do not present a Bernard-Soulier syndrome phenotype, but instead appear to have hyperresponsive platelets, manifested in a shorter bleeding time (124). GPV-deficient platelets appear to be more sensitive to activation by low concentrations of thrombin, suggesting that GPV acts as a negative modulator of the thrombin response (125). *In vitro* aggregation studies demonstrated a decreased response of GPV-deficient platelets to collagen (81), while *in vivo* studies showed accelerated platelet adhesion and thrombus formation, as well as compromised thrombus stability, probably due to increased thrombin sensitivity (82).

Also, mice lacking fibrinogen (126) and vWF (127), i.e. the ligands of integrin $\alpha \text{IIb}\beta 3$ and GPIb-V-IX, have been generated (Table 1.2). Surprisingly, thrombi are still formed abundantly in the absence of fibrinogen, but they detached from the subendothelium causing downstream occlusion, as investigated by intravital microscopy of ferric chloride-injured mesenteric vessels (128). Absence of murine vWF significantly delayed platelet deposition and increased the time required for the formation of a first thrombus (127). Importantly, vWF-deficient mice closely mimic the phenotype of severe human von Willebrand's disease. Mice deficient in both fibrinogen and vWF have delayed thrombus formation, but are still capable of forming occlusive thrombi at later time points (128). It was shown that platelets from these mice excessively accumulate fibronectin in their α -granules, suggesting that fibronectin is the ligand supporting platelet aggregation via $\alpha \text{IIb}\beta 3$ in these double knock-out animals. Studies in fibronectin knock-out mice show that initial platelet adhesion is not affected, but thrombus formation is delayed for several minutes. Moreover, thrombi of fibronectin-deficient mice continuously shed platelets or small platelet aggregates, leading to slow thrombus growth (129).

Like platelets from P2Y₁₂-deficient patients, platelets from P2Y₁₂ $-/-$ mice change shape normally and do not undergo rapidly reversible aggregation when exposed to ADP or to low doses of collagen or PAR-4-activating peptide (130). Adhesion of P2Y₁₂ $-/-$ platelets to vWF is impaired, whereas adhesion to collagen and fibrinogen appear normal; thrombi raised *in vitro* are less dense and less activated compared to wildtype counterparts. *In vivo* experiments point to delayed thrombus formation with small unstable thrombi in a mesenteric artery injury model (130). P2Y₁-deficient mice have been generated by two research groups (131,132). Platelets

from these mice are unable to aggregate at low doses of ADP or collagen, but they aggregate without shape change in response to high concentrations of ADP. In vivo, P2Y₁ deficiency increases bleeding time and protects from collagen- and ADP-induced thromboembolism. P2Y₁-deficient mice display significantly less arterial thrombosis in reaction to ferric chloride compared to controls, and in combination with P2Y₁₂ inhibition the P2Y₁ deficiency leads to additive antithrombotic effects (133).

The importance of the P2X₁ cation receptor channel has become clear from the generation of mice with altered expression levels. In vitro, P2X₁-deficient mouse platelets show impaired aggregation in response to collagen, both in the presence and absence of flow, and reduced secretion of autocrine agents. In vivo, P2X₁ ^{-/-} mice have a reduced mortality in a model of collagen-provoked systemic thromboembolism. The size of thrombi formed after laser-induced vessel wall injury is decreased as compared to normal mice (134). These findings are confirmed with transgenic mice over-expressing the human P2X₁ ion channel in the megakaryocytic cell lineage (29). P2X₁ over-expression results in a prothrombotic phenotype, characterized by enhanced platelet secretion and aggregation evoked by low doses of collagen and other agonists, as well as increased aggregate formation under flow conditions.

1.8 Methods to study platelet-matrix interactions: whole blood perfusion chamber versus in vivo studies

Much knowledge about platelets has been generated by in vitro studies under static conditions using blood, platelet-rich plasma, or isolated platelets from healthy subjects, patients or animals. However, shear forces generated by flowing blood have a significant impact on platelet adhesion and thrombus formation. Flow chambers have been devised to mimic these shear conditions in perfusion studies with flowing blood (135,136). In combination with (fluorescence) microscopy and novel real-time imaging facilities, these devices enable data collection under well-controlled conditions of exposure time, thrombogenic surface and reproducible shear stress. In this thesis most studies have been performed using the parallel-plate perfusion chamber shown in Fig. 1.4 (42). This setup allows real-time monitoring of shear-dependent platelet adhesion and aggregate formation, as well as platelet activation and generation of platelet procoagulant activity using suitable fluorescent probes.

Although the parallel-plate flow chamber is an excellent instrument to study (single) platelet activation and thrombus formation under controlled flow conditions, this type of studies is still relatively far away from the in vivo situation. The endothelial cells, lining the vessel wall, with their pro- and antithrombotic properties significantly influence platelet behavior in vivo (reviewed in (137)). Hence, it is also important to study platelet-vessel wall interactions in vivo, where cells are in their natural environment. Therefore, in the present thesis (chapters 4 and 7),

Figure 1.4 Schematic drawing of experimental setup used in chapters 3-7 of this thesis. Thrombus-forming experiments under flow conditions were performed with (mouse) blood anticoagulated with PPACK and heparin or citrate. Rectangular coverslips (24x60 mm) were coated with collagen fibers (chapters 3-5) or fibrinogen (chapter 6). A coverslip was placed in a parallel-plate transparent flow chamber (slit depth of 50 μm), which was provided with polyethylene tubing (diameter 1 mm). Blood was perfused through the flow chamber using a syringe and a pulse-free pump, at low or high wall-shear rate (150-1500 s^{-1}). Phase-contrast microscopy was performed by illumination with a tungsten lamp through an infrared (IR) filter, when required. Fluorescence microscopy was performed under epi-illumination with a xenon light source. The light passed through an excitation band-pass filter and a dichroic mirror, while emitted light from the sample was filtered with an emission filter (em; long-pass). During and after perfusion, high-resolution microscopic transmission and/or fluorescent images were recorded in real-time with a Visitech imaging system (Sunderland, UK). Digital images were captured with two parallel-placed intensified, charge-coupled device (CCD) cameras, recording phase-contrast (infrared) light and epifluorescence light. Fluorescent probes were added to the blood or to the rinsing buffer medium

platelet-vessel wall interactions and ensuing thrombus formation were also studied in an established murine microvascular thrombosis model (127,128). In this model, the thrombotic process was induced by topical application of ferric chloride (FeCl_3) on mouse mesenteric arterioles and venules, which resulted in the formation of intravascular thrombi. FeCl_3 induces local free radical production, which leads to denudation of endothelial cells and exposure of the extracellular matrix (128). The resulting thromboembolic process can be studied by intravital microscopy by bright-field microscopy using trans-illumination and/or by fluorescence microscopy under epi-illumination (chapter 7). Many conclusions on the alleged anti-thrombotic or thrombophilic phenotypes of genetically modified mice (Table 1.2) have been drawn from studying microvascular thrombosis using similar equipment (127,128,138,139). This model allows real-time imaging of platelet-vessel wall interactions, with separate quantification of thrombus formation and embolization. Furthermore, activation processes in participating platelets can be studied with the use of fluorescent probes (chapter 7). This *in vivo* model, as

well as the parallel-plate flow chamber, has many advantages but, of course, also has its limitations, as will be elaborated in the general discussion (chapter 8).

1.9 Aim and outline of this thesis

The main goal of the present thesis is to investigate the involvement of key platelet receptors involved in collagen-induced platelet activation and in the platelet procoagulant response. While most research groups have limited their focus on platelet adhesion and aggregation, the studies in this thesis particularly address the signaling towards procoagulant activity, the regulation of PS exposure and the subsequent generation of thrombin and fibrin. The studies were performed using an *in vitro* model of thrombus formation under controlled conditions of flow and coagulation, as well as in an *in vivo* model of intravascular thrombus formation. The results indicate that platelet aggregation and procoagulant activity are differently regulated processes, which, however, involve similar sets of receptors. In **chapter 2** the present understanding of how platelet interaction with vascular collagen supports the process of thrombin generation and coagulation is addressed. In this chapter the profound morphological changes of procoagulant platelets are also described.

As the role of the integrin $\alpha 2\beta 1$ receptor for collagen in platelet adhesion and activation was controversial, in **chapter 3** the precise function of this integrin in both platelet adhesion and procoagulant activity was investigated using various genetically modified mice with platelets deficient in either GPVI or $\alpha 2\beta 1$. It was discovered that the integrin has a secondary, yet complementary role to GPVI in collagen-dependent platelet activation and PS exposure. The flow experiments in chapter 3 also demonstrated that $\alpha 2\beta 1$ -deficient platelets formed thrombi of a decreased stability with higher tendency of detachment from the collagen surface. These results provide novel insights into the differential roles of these platelet receptors in the hemostatic process. In **chapter 4** this subject was taken further by *in vitro* and *in vivo* studies determining how $\alpha 2\beta 1$ contributes to thrombus growth and stability. Attention was paid to the contribution of autocrine thromboxane A_2 and PS exposure.

Since platelet-collagen interaction under high shear is also dependent on vWF, GPIb and $\alpha IIb\beta 3$, the focus of **chapter 5** was to determine the contribution of these proteins to the generation of the platelet procoagulant surface under flow conditions. It was demonstrated that at high shear rates vWF and GPIb – and $\alpha IIb\beta 3$ further downstream – enhance GPVI-mediated PS exposure, especially under conditions of reduced GPVI activity.

The flow studies described in chapters 3 and 5 have been performed in the absence of thrombin and coagulation. To include the role of thrombin, in chapter 6 the thrombus-forming process is followed in flowing blood upon triggering of coagulation with tissue factor. We concentrated on the antithrombotic potential of platelet-inhibiting agents which can also prevent thrombin generation. The compounds studied (a cyclic AMP-elevating agent and a P2Y₁₂

receptor antagonist) appeared to retain their platelet-inhibiting activity under conditions where the anticoagulant effect had disappeared.

Knowing that only in vitro studies have been performed to establish the mutual interaction of (collagen-induced) platelet activation and thrombin generation, the studies of **chapter 7** were designed to give first evidence for the interaction of these two processes in thrombus formation in vivo. It was found that in an in vivo model, relying on both collagen and tissue factor, the propagation of thrombin generation by PS-exposing platelets is a key regulatory step in thrombus formation not only in arterioles but also in venules. Further, evidence was obtained that in the venous circulation, local thrombin generation may be too high to be suppressed by classical platelet inhibitors. In the general discussion in **chapter 8** the relevance of the present findings in relation to literature are described.

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Chapter 2

Platelet collagen receptors and coagulation

A characteristic platelet response as possible target of antithrombotic treatment

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Collagen is a unique agonist of platelets, because it acts as an immobilized ligand which only causes platelet activation after stable adhesion. This review addresses the present understanding of how platelet interaction with collagen supports the process of thrombin generation and coagulation. Only some of the collagen-adhered platelets, i.e. those showing profound changes in shape and shedding microparticles (resembling apoptotic cells), appear to contribute to the procoagulant activity of platelets. The main signaling receptor for collagen, glycoprotein VI (GPVI), plays a key role in the platelet procoagulant response during thrombus formation; this is a reason why new anti-GPVI antibodies are promising antithrombotic tools.

2.1 Platelet activation and coagulation

The intricate connection between platelets and coagulation was first recognized at the end of the 19th century, when it was observed that fibrin strands co-localize to sites of platelet adhesion. The concept re-emerged about 25 years ago, when it was proved that collagen, in combination with thrombin, is the most potent physiological platelet agonist in potentiation of the coagulation process by causing scrambling of phospholipids in the plasma membrane (1). Scrambling results in the surface expression of two aminophospholipids, phosphatidylserine (PS) and phosphatidyl-ethanolamine (PE), both of which are active in coagulation.

Since agents that strongly elevate cytosolic Ca^{2+} in platelets, such as Ca^{2+} ionophores (ionomycin) and Ca^{2+} -ATPase inhibitors (thapsigargin), are even more potent than collagen in inducing phospholipid scrambling, it was envisaged that elevation in cytosolic Ca^{2+} must play a key role in this process (2). High cytosolic Ca^{2+} is considered to influence two enzymatic processes that control the scrambling process, i.e. inactivation of an aminophospholipid translocase and activation of a phospholipid scramblase (3). After a long search, candidate proteins for these activities have been proposed, but the identities of the translocase and scramblase are still unknown (4). This PS expression greatly enhances the assembly and activity of two major coagulation factor complexes, the tenase and prothrombinase complexes, which produce key regulators of the coagulation process, factor Xa and thrombin, respectively. Thus, PS-expressing platelets have a potent enhancing effect on thrombin generation in plasma, where the extrinsic coagulation pathway is triggered with tissue factor. The importance of PS expression is demonstrated by the hemorrhagic episodes in patients with the Scott syndrome (5) and in dogs with similar symptoms (6), whose platelets are deficient in PS expression and microparticle formation (see below). In platelets from Scott patients, the diminished collagen-induced PS expression is not accompanied by reduced Ca^{2+} responses, indicating that the defect lies downstream of Ca^{2+} signaling (7).

As reviewed elsewhere, phospholipid scrambling is required but perhaps not exclusively responsible for the coagulation-supporting property of platelets (4,8). For instance, platelet glycoproteins serve as binding sites for a number of coagulation factors such as

prothrombin (9), factor XI and factor V, of which the last is released from activated platelets themselves (10). A still pertinent question is how these protein receptors function in the regulation of platelet procoagulant activity. The debate is reopened with the finding that the elusive factor Xa receptor EPR-1 is not transcribed in cells, but that the 'EPR-1' gene encodes for a protein regulating apoptosis (11). A new, interesting possibility is that extracellular glycoprotein modification, e.g. by transglutaminases such as factor XIII, generates receptor sites on platelets for coagulation factors (see below at COAT platelets).

Since PS-expressing platelets enhance the coagulation and, in turn, the coagulation product thrombin stimulates platelets, both processes are in a strong positive feedback loop which can have a control function in hemostasis and thrombosis (12). Thrombin generation measurements with platelets in plasma indeed demonstrate synergistic activity of (PS-expressing) platelets and the coagulant system (12,13).

2.2 Platelet adhesion to collagen stimulates procoagulant activity

Platelet adhesion to collagen, especially to type I collagen fibers, causes PS expression and stimulates prothrombinase activity and thrombin formation after a delay of several minutes (14,15). The fibrillar structure of collagen appears to be important for the procoagulant response, as platelet interaction with protease-treated soluble collagen (with an intact triple helical structure) does not lead to significant activity (16). Other platelet agonists such as ADP, acting via the P2Y₁₂ receptor, and thrombin, acting particularly via the protease-activated receptor-1 (PAR1), can enhance the procoagulant activity of collagen-activated platelets (e.g. see (17)). Despite being strong platelet-aggregating agonists, ADP and thrombin are only weak generators of procoagulant activity by themselves, pointing to a divergence between the aggregation and procoagulant responses of platelets.

The need of platelets to adhere to collagen may explain why in suspension the combination of collagen and thrombin is a stronger procoagulant stimulus than collagen alone (3). Thrombin, for instance, enhances platelet interaction with collagen by increasing the affinity of its adhesive receptor, integrin $\alpha 2\beta 1$ (18). On the other hand, it is proposed that the combination of collagen and thrombin induces a specific platelet reaction resulting in the formation of so-called COAT (collagen- and thrombin-activated) platelets (19). Such platelets are characterized by transglutaminase-mediated cross-linking of serotonin to platelet-derived procoagulant proteins (e.g., factor V), which bridge to fibrinogen and thrombospondin, forming a two-dimensional lattice. The precise contribution of COAT platelets to platelet-dependent coagulation is a current subject of research.

Figure. 2.1. Morphology of procoagulant platelets adhering to collagen under static conditions. Microscopic images of human platelets adhered to fibrinogen, collagen, collagen-related peptide (CRP) or convulxin. **A.** Phase-contrast bright-field images; note heterogeneity of spread and blebbing morphology. **B.** Scanning electron microscopic images; note sponge-like structures. Bars represent 2 μm . Modified after Siljander et al. (14).

2.3 Collagen induces dramatic morphological changes in procoagulant platelets

Platelet interaction with collagen fibers appears to lead not to a predetermined sequence of responses, but to several types of changes. In static adhesion, most collagen-adhering platelets first develop pseudopods, which extend to broader lamellipods. These platelets can attract further platelets to initiate aggregate formation. However, some of the platelets (depending on the experimental conditions) suddenly transform into balloon-shaped cells with membrane blebs on their surface (Fig. 2.1). The blebbing platelets as well as the microparticles they shed express a procoagulant, PS-containing surface (14). Intravital fluorescence-microscopic observations of platelets adhering to fibrillar collagen or collagen-related peptide (CRP) have indicated that the PS expression (detected with fluorescently labeled annexin A5) is preceded by a potent and prolonged rise in cytosolic Ca^{2+} (14,20). This Ca^{2+} signal, induced by GPVI signaling, is also a prerequisite for the dramatic morphological

Figure 2.2. Properties of procoagulant platelets adhering to collagen under static conditions. Two-photon fluorescence images of collagen-adherent platelets labeled with OG488 annexin A5 (staining surface exposed PS) or Fluo-3 (upper panel right, indicating elevated cytosolic Ca^{2+}). Bars represent

change (Fig. 2.2). Others have similarly observed that collagen-bound platelets with high cytosolic Ca^{2+} respond by membrane contraction, cell fragmentation and microvesiculation (21). Such platelets were therefore designated as having SCIP (for **sustained calcium-induced platelet**) morphology. In many cell types, expression of PS, formation of membrane blebs and shedding of vesicles are signs of the apoptotic process. However, normal apoptosis (like in other cells of the cardiovascular system) is unlikely to occur in bleb-forming/vesiculating platelets since, for instance, the protease calpain rather than caspases controls the changes in platelet morphology (22).

During bleb formation, actin fibers become confined to the plasma membrane skeleton, implicating that degradation of cytosolic actin fibers contributes to the changed morphology of the cells (23). Protein tyrosine dephosphorylation also plays a role in bleb formation (24), as does the p38 mitogen-activated protein kinase pathway (SAPK2/3), known to be involved in actin rearrangement (14). Little more is known of the signaling prerequisites for this dramatic loss of normal platelet shape. So far, it seems that above a certain threshold of cytosolic Ca^{2+} concentration calpain and perhaps other proteases begin to degrade actin cytoskeletal proteins, resulting in the loss of main structural and membrane-anchoring cytoskeletal components. In scanning electron micrographs (Fig. 2.1B), the bleb-forming platelets on collagen or CRP appear as sponge-like structures (16), most likely because of the loss of lipid material by chemical fixation (25). The increase in size of blebbing platelets (balloons of about 5 μm in diameter *versus* 2-3 μm disks of resting platelets) is best explained by disappearance of the

surface-connected canalicular system upon cytoskeleton degradation. This fits with the notion that this change in morphology is rather due to a loss of structure than to an active gain in 'procoagulant' shape.

Earlier, Zwaal and colleagues already concluded that microparticle (bleb) formation is related but not the cause of phospholipid scrambling and PS expression (26). It is clear that PS expression and bleb formation are separate processes, e.g. calpain is not involved in PS expression (2), but under most experimental conditions bleb-forming platelets on collagen also bind annexin A5, indicating an overlap in the regulation of these processes (4).

2.4 Collagen induces formation of procoagulant microparticles

Interestingly, platelets that bind to a collagen fiber, CRP or convulxin (with little surface contact) tend to develop into round, blebbing cells, whereas platelets that are spread and then stimulated via GPVI eventually fully fragment into smaller particles (Fig. 2.1) (14). Time-lapse measurements indicate that the vesiculation of retracting lamellipods of 'procoagulant' platelets can occur rapidly, i.e. in a time scale of 1-3 min (21). High-resolution microscopy images further show that blebbing platelets have a translucent appearance.

Microparticles derived from collagen-adherent platelets are capable of associating with fibrin (27). As these microparticles, like the blebbing cells from which they are shed, express PS on their surface, their shedding provides a mechanism for dissemination of procoagulant activity in the circulation. This can be physiologically relevant, as (collagen/thrombin-induced) microparticle formation is perturbed in patients with moderate bleeding disorders such as the Scott syndrome (5). Although more research is needed, there is growing evidence that the presence of platelet-derived microparticles in the circulation correlates with pathological conditions (28). Of interest in this context is that most drugs that block the $\alpha\text{IIb}\beta 3$ integrin inhibit microvesiculation, but differently affect (collagen-induced) PS expression.

2.5 Glycoprotein VI as a key player in the regulation of platelet procoagulant activity during thrombus formation

Experiments with human and mouse blood that is flown over collagen fibers have pointed out that some collagen-adherent platelets tend to develop into blebbing platelets within a developing thrombus. The experiments using blocking antibodies, inhibitors and knock-out mice, have also established that GPVI, a member of the immunoglobulin receptor family, plays a key role in the regulation of procoagulant activity during the thrombus-forming process.

Under conditions of high, arterial shear rates, platelets initially adhere to von Willebrand factor (vWF), which avidly binds to fibrillar collagen via the glycoprotein Ib-V-IX complex and subsequently via integrin $\alpha\text{IIb}\beta 3$ (29,30). Binding to vWF can predispose platelets

Figure 2.3. Thrombus formation and procoagulant activity under flow. Microscopic images of platelet thrombi formed during high-shear flow of human blood over collagen: **A.** Differential interference contrast image of aggregated platelets (4 min). **B.** Two-photon fluorescence image after labeling with calcein (4 min). **C.** Two-photon fluorescence image after labeling with OG488 annexin A5 (8 min). Bars = 10 μ m

to collagen-induced activation and facilitate the procoagulant response, particularly under conditions of reduced GPVI activity (31). The subsequent order of events appears to differ from platelet to platelet both for human and mouse (32). Some platelets on collagen become rapidly activated by the main activating collagen receptor, GPVI. Subsequent signaling via GPVI involves its interaction with the Fc receptor γ -chain, activation of Src kinases, and a series of tyrosine phosphorylation events leading to activation of phospholipase C- γ 2 and Ca^{2+} mobilization (33). In addition, the integrins α 2 β 1 (collagen receptor) and α IIb β 3 (fibrinogen and vWF receptor) assume an active conformation, which stabilizes the adhesion (29,34). Autocoids like ADP and thromboxane are secreted, which activate passing platelets (35-37). However, some of the platelets, perhaps interacting with different motifs in collagen, follow a different order of events by first stably adhering to collagen via integrin α 2 β 1 before showing activation responses. This heterogeneity in platelet responses may explain the apparent differences between current adhesion-activation models for human and murine platelets (32).

A further difference in responses of platelets from either species is displayed at a later stage. At first, collagen-adherent platelets trap other flowing platelets to form a growing aggregate, but after several minutes some platelets on collagen start to form blebs and expose PS (Fig. 2.3). GPVI plays a key role in both types of responses - aggregation and PS expression - as mouse platelets lacking GPVI or antibodies inhibiting mouse or human GPVI fail to form aggregates on collagen and no longer form blebs or express PS (Fig. 2.4) (35,37). Absence of GPVI activity blocks Ca^{2+} signaling, autocrine secretion, and partially inhibits integrin activation. As expected from these findings, GPVI blockade also suppresses collagen-induced prothrombinase activity (38). Nevertheless, but in agreement with the dual adhesion-activation model described above, adhesion under flow is affected variably by GPVI

Figure 2.4. Function of collagen receptors in thrombus formation and procoagulant activity under flow. Microscopic images of human (A) and mouse (B) thrombi formed during high-shear flow of blood over collagen. Blood was pretreated with anti-human or anti-mouse GPVI or $\alpha 2\beta 1$ antibodies, as indicated. Representative images of phase-contrast ($120 \times 120 \mu\text{m}$) and fluorescent-labeled annexin A5 ($150 \times 150 \mu\text{m}$). Modified after (35,37).

antagonism, probably depending on the experimental conditions (36,37,39). A different situation is achieved by absence or blocking of integrin $\alpha 2\beta 1$, which only decreases but does not abolish aggregate formation and PS expression, reducing the stability of thrombi (Fig. 2.4) (35,37,40). These results agree with the proposal that GPVI and $\alpha 2\beta 1$ determine collagen-induced platelet activation in concert, such that integrin adhesion mostly serves to amplify GPVI signaling. These results point to an interplay of GPVI and $\alpha 2\beta 1$ in aggregation and procoagulant activity during thrombus formation, but they do not explain the observation that only some platelets develop to a 'procoagulant' shape. It is likely that differences in Ca^{2+} signaling explain the different fate of these platelets (4,21).

Taken together, these results indicate that collagen provides a substrate on which the processes of platelet aggregation and coagulation are integrated but also separated. Especially, GPVI controls the multi-receptor interplay implicated in procoagulant activity upon thrombus formation (Fig. 2.5).

Figure 2.5. Diagram of function of collagen receptors in platelet procoagulant activity. Platelets adhere to a vWF-collagen surface via various receptors. GPVI has a central role by activating integrins, which enhance the adhesion and activation of platelets. Depending on the activation state, platelets transform into round structures with membrane blebs and shedding microparticles (increased cytosolic Ca^{2+} , calpain and SAPK2/3 activation). These platelets (increased Ca^{2+} , PS exposed) are also active in thrombin formation and coagulation.

2.6 Collagen-induced coagulation as new target of antithrombotic therapy

The presence of procoagulant platelets is not sufficient to induce plasma coagulation. Although in literature collagen is considered to be a trigger of the contact activation system of (intrinsic) coagulation, the current view is that for rapid onset of coagulation also tissue factor is needed. The precise localization of tissue factor in blood is still discussed. There is evidence that activated platelets themselves contain tissue factor (41), but this finding needs confirmation. On the other hand, it is clear that tissue factor is present close to all sites where (collagen-induced) procoagulant platelets are expected. For instance, tissue factor is found in the damaged wall of veins and arteries, and also within thrombi with fibrin-rich domains close to the vessel wall (42). Along with collagen, it is also found within human plaque tissue (43). Recent evidence indicates that thrombus formation in the macro-vasculature is primarily driven by tissue factor from the blood vessel wall (44), as opposed to (blood-borne) tissue factor from leukocytes, e.g. in contact with platelets. Together, this suggests that tissue factor and procoagulant platelets often co-localize at thrombotic sites and thus contribute synergistically to the thrombotic process.

Recent *in vivo* studies highlight the importance of platelet-collagen interaction and, thus, procoagulant activity in arterial thrombosis. In a variety of murine models, abolition of GPVI function suppresses thrombosis in arteries, but does not result in major bleeding (36,45). Similarly, patients lacking GPVI do not suffer from a high bleeding tendency (18), but the

number of patients is too small to estimate their thrombotic protection. Another promising finding for the usefulness of GPVI receptor blockade comes from perfusion studies, indicating that thrombus formation and PS expression on collagen-containing human atherosclerotic plaques is significantly suppressed by anti-GPVI antibodies (43,46). Meanwhile, a number of novel anti-human GPVI antibodies, generated by various approaches, are available for studying the function of GPVI in thrombosis (38,47). In vitro, all these antibodies avidly suppress PS expression and thrombus formation (34,37) along with thrombin generation (P. Siljander and C. Lecut, unpublished data), which are promising features for in vivo studies.

Inherently, the collagen-induced procoagulant and aggregating responses via GPVI are linked to platelet adhesion to collagen that is exposed in the damaged or (ruptured) atherosclerotic vessel wall. Anti-GPVI antibodies will thus interfere at thrombus-forming and coagulant processes at sites close to the vessel wall. From a mechanistic point of view, it can be anticipated that anti-GPVI treatment can suppress not only the acute assembly of thrombi on exposed collagen, but in addition the ongoing thrombus growth due to the locally generated thrombin on PS-expressing platelets. Accordingly, these novel tools may provide a locally effective antithrombotic therapy targeting adhesion-dependent platelet activation and coagulation.

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Chapter 3

Complementary roles of glycoprotein VI and $\alpha 2\beta 1$ integrin in collagen-induced thrombus formation in flowing whole blood ex vivo

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Abstract

Platelets vigorously interact with subendothelial collagens exposed upon injury or pathological damage of a vessel wall. The collagen-bound platelets trap other platelets to form aggregates and expose phosphatidylserine (PS) required for coagulation. Both processes are implicated in the formation of vaso-occlusive thrombi. We previously demonstrated that the immunoglobulin receptor, glycoprotein VI (GPVI), but not $\alpha 2\beta 1$ integrin is essential in priming platelet-collagen interaction and subsequent aggregation. Here, we report that these receptors have a complementary function in *ex vivo* thrombus formation during perfusion of whole-blood over collagen. With mice deficient in GPVI or blocking antibodies, we found that GPVI was indispensable for collagen-dependent Ca^{2+} mobilization, exposure of PS and aggregation of platelets. Deficiency of integrin $\beta 1$ reduced the GPVI-evoked responses, but still allowed the formation of loose platelet aggregates. By using mice deficient in $\text{G}\alpha_q$ or specific thromboxane A_2 and ADP antagonists, we show that these autocrine agents mediated aggregation, but not collagen-induced Ca^{2+} mobilization or PS exposure. Collectively, these data indicate that $\alpha 2\beta 1$ facilitates the central function of GPVI in the platelet-activation processes that lead to thrombus formation, whereas autocrine thromboxane A_2 and ADP mainly serve to trigger aggregate formation.

Introduction

Vessel wall injury triggers sudden platelet activation and formation of a platelet plug. Subsequent coagulant activity leads to the formation of fibrin-containing thrombi, which occlude the injured vessel. Hemostasis thus starts with platelets adhering to activating components of the subendothelial matrix such as collagen. Similarly, thrombus formation, resulting from the rupture or erosion of an atherosclerotic plaque, is triggered by platelet interaction with extracellular matrix components.

Fibrous collagens are among the strongest reactive matrix components involved in platelet adhesion and activation (1). Such collagens avidly bind other proteins such as von Willebrand factor (vWF) that are implicated in stable platelet immobilization (2, 3). It is recognized that platelet-collagen interaction initiates a number of distinct processes that are implicated in thrombus formation. First, collagen-adherent platelets change in shape, produce pseudopods and express activated integrin $\alpha \text{IIb}\beta 3$, which acts as a high-affinity ligand for fibrinogen. Platelet-bound fibrinogen then serves as a binding site for flowing platelets that are trapped to form a platelet aggregate. Second, collagen increases the "activation" state of the platelets, as apparent from a rise in their cytosolic $[\text{Ca}^{2+}]_i$. The Ca^{2+} response mediates release of thromboxane A_2 (TxA_2) and ADP, both of which act as potent autocrine agonists activating nearby platelets (4). Furthermore, the collagen-adherent platelets activate blood coagulation by

exposing phosphatidylserine (PS) at their surface; the procoagulant PS accomplishes the assembly of tenase and prothrombinase complexes and, thereby, stimulates the generation of thrombin by several orders of magnitude (5).

In recent years, several platelet collagen receptors have been identified. Among these, especially glycoprotein VI (GPVI) and glycoprotein Ia-IIa (integrin $\alpha 2\beta 1$) are considered to be clinically important (6). GPVI is the main signaling collagen receptor on platelets (7). It belongs to the immunoglobulin superfamily (8, 9), and is non-covalently associated with the Fc receptor (FcR) γ -chain (10, 11). Patients lacking platelet GPVI suffer from a mild bleeding diathesis and their platelets respond poorly to collagen or collagen-related peptide (12, 13). The importance of the FcR γ -chain is exemplified by the fact that mice lacking this protein fail to express GPVI on their platelets and do not respond to collagen (14, 15). Signaling through the FcR γ -chain activates phospholipase $C\gamma 2$ via tyrosine kinases, causing a prolonged increase in cytosolic $[Ca^{2+}]_i$ (7, 16). This Ca^{2+} response mediates not only exposure of procoagulant PS but also converts the platelets into blebbing cells, from which procoagulant microvesicles are shed (16, 17).

Unlike the role of GPVI, the role of the integrin $\alpha 2\beta 1$ collagen receptor in platelet adhesion and activation has been controversial. Few patients lacking $\alpha 2\beta 1$ and showing prolonged bleeding have been reported (18). Some authors found that collagen-induced adhesion and aggregation responses was greatly diminished in the absence of functional $\alpha 2\beta 1$ (19, 20). Others however, found only minor effects of $\alpha 2\beta 1$ inhibition on these responses both under stasis and under flow conditions (21-24). By using $\beta 1$ -null mice with a conditional inactivation of the integrin $\beta 1$ gene in megakaryocytes and thus a complete lack of integrin $\alpha 2\beta 1$ on platelets, we recently demonstrated that integrin $\alpha 2\beta 1$ is dispensable for both platelet adhesion and activation on fibrillar collagen (25).

Ex vivo flow studies provide an elegant tool to evaluate platelet adhesion to collagen fibrils and all subsequent processes involved in thrombus formation under physiologically relevant conditions. A current model proposes that at high wall shear rates (as in arteries) platelets initially interact via glycoprotein Ib-V-IX (GPIb-V-IX) with vWF that is absorbed to the collagen, after which the collagen receptors accomplish irreversible adhesion (2, 3, 26). Subsequent aggregation in such a system is caused by the soluble agonists TxA_2 and ADP. Because mice deficient in the GTP-binding protein α -subunit, $G\alpha_q$ display defective platelet activation in response to TxA_2 and ADP (27), these animals are an interesting model to study aggregate and thrombus formation under flow conditions. We have previously shown that these mice have normal GPVI function (28).

In the present study, we set out to resolve the current discrepancy with respect to the function of integrin $\alpha 2\beta 1$ in collagen-dependent platelet activation by using mice with platelets deficient in either GPVI or $\alpha 2\beta 1$. We studied the different hemostatic processes contributing to collagen-induced thrombus formation with an ex vivo whole blood flow system and real-time

video imaging techniques. These methods allowed us to monitor shear-dependent adhesion and aggregate formation, as well as platelet activation and platelet procoagulant activity (PS exposure). We show that the integrin has a secondary yet complementary role to GPVI in all these responses. Furthermore, we demonstrate that aggregate formation, but not procoagulant activity, depends on $G\alpha_q$ stimulation by the autocrine mediators released from GPVI-stimulated platelets. These results provide novel insights into the differential roles of these platelet receptors in the hemostatic cascade. In addition, given the general importance of immunoglobulin receptors (such as GPVI) and integrins in cell biology, we infer that similar receptor interplay may also exist in other cellular systems expressing members of these two receptor families.

Materials and methods

Animals

Control NMRI mice were obtained from Charles River (Sulzfeld, Germany), while control C57BL/6 and C57BL/6 x 129/Sv mice were obtained from Iffa Credo (Somerens, the Netherlands). C57BL/6 x 129/Sv mice carrying a floxed $\beta 1$ integrin gene, $\beta 1(f/f)$ (29) were cross-bred with mice carrying the Mx-cre transgene (Mx-cre⁺). Deletion of the $\beta 1$ gene was induced in 4-5 week old $\beta 1(f/f)/Mx-cre^+$ mice by three intra-peritoneal injections with polyinosinic-polycytidylic acid at 2-day intervals (25). The animals with a C57BL/6 x 129/Sv background, were used at least two weeks after the last injection. C57BL/6 mice deficient in FcR γ -chain (30) were obtained from Taconics (Germantown, USA). To produce virtually complete GPVI-depleted animals, control mice were injected with a single dose of 100 μ g anti-GPVI antibody JAQ1 (31). Mice deficient in $G\alpha_q$ (mixed C57BL/6 x 129/Sv background) were generated as described (27). All mice had similar platelet counts, as determined with a thrombocounter (Coulter Electronics, Luton, UK). Expression levels of integrin $\alpha 2\beta 1$, integrin $\alpha IIb\beta 3$, GPIb-V-IX and GPVI were checked with antibodies directed against mouse glycoproteins using flow cytometry on a FACScalibur (Becton Dickinson, Heidelberg, Germany) (15). Mice were specific pathogen-free. Experimental protocols were approved by the Institutional Animal Care and Use Committees.

Materials

Fibrillar Horm-type collagen from equine tendon (Horm type-I) was purchased from Nycomed (Munich, Germany). Native type-I collagen fibers were extracted from bovine tendon as described (32). Non-fibrillar 'soluble' type-I collagen from rat tail (Sigma type VII) came from Sigma (St. Louis, MO), as well as apyrase, high molecular weight heparin and MRS2179.

Annexin A5 (Apoptest) labeled with Oregon green 488 (OG488) was from Nexins Research (Hoeven, the Netherlands); fluo-3 acetoxymethyl ester was from Molecular Probes (Leiden, the Netherlands); H-Phe-Pro-Arg chloromethyl ketone (PPACK) was from Calbiochem (La Jolla, CA). AR-C69931MX, an antagonist of the P2Y₁₂ purinergic receptor, was purchased from AstraZeneca R&D (Charnwood, UK). Sources of other chemicals are mentioned elsewhere (32).

Fab fragments of rat anti-mouse GPIIb/IIIa antibody p0p/B and of rat anti-mouse GPVI antibody JAQ1 were generated, produced and modified as described before (15).

Platelet preparation and labeling

Mice were bled retro-orbitally, and blood (1 vol) was collected in 0.5 vol of saline containing 5 U/ml heparin and 40 μ M PPACK. Anticoagulated blood (1 vol) was diluted in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (hepes) buffer pH 7.45 (0.5 vol) containing 137 mM NaCl, 5.6 mM glucose, 5 mM hepes, 2.7 mM KCl, 2 mM MgCl₂, 0.42 mM NaH₂PO₄, 0.1% (w/v) bovine serum albumin (BSA), which was supplemented with 1 U/ml heparin and 2 mM CaCl₂. The blood was used within 2 h. Washed mouse platelets in hepes buffer, pH 7.45 were prepared from platelet-rich plasma, as described for rat blood (33). For Ca²⁺ measurements, the washed platelets were incubated with 5 μ M fluo-3 acetoxymethyl ester in the presence of pluronic F-127 (0.2 mg/ml) and apyrase (0.1 U ADPase/ml) at room temperature for 45 min.

Flow experiments and vital imaging recording

Adhesion experiments under flow conditions were performed with PPACK and heparin anticoagulated mouse blood, basically as described previously (25). Rectangular coverslips (24 x 60 mm) were coated with Horm-type collagen fibers (12.5 μ g/cm²), rinsed with saline, and blocked with hepes buffer containing 1% (w/v) BSA. Where indicated, coating was with fibrillar or soluble type-I collagens from other sources (12.5 μ g/cm²). A coverslip was placed in a parallel-plate transparent flow chamber (slit depth of 50 μ m), which was provided with polyethylene tubing (diameter of 1 mm). To prevent coagulation, chamber and tubing were pre-washed with hepes buffer supplemented with 1 U/ml heparin. The blood was perfused through the flow chamber using a 1-ml syringe and a pulse-free pump (34), at high or low wall-shear rate (1000 or 150 s⁻¹, respectively) for 4 min. During the perfusion, high-resolution microscopic transmission and/or fluorescent fluo-3 images were recorded in real-time with a Visitech imaging system (Sunderland, UK). Digital images were captured with two parallel-placed intensified, charge-coupled device cameras, recording phase-contrast light and epifluorescence (16). After 4 min, the flow chamber was perfused with hepes buffer supplemented with 1 U/ml heparin at the same shear rate for 10 min. Exposure of PS was detected with OG488-labeled annexin A5 (1 μ g/ml). Phase-contrast and fluorescent images were obtained from at least nine different collagen-containing microscopic fields, which were randomly chosen.

Where indicated, blood was incubated for 10 min before perfusion with pOp/B or JAQ1 Fab fragments (40 $\mu\text{g/ml}$), aspirin (100 μM), MRS2179 (20 μM) and/or AR-C69931MX (50 μM). The same antibodies or antagonists were added to the rinse buffer. To measure changes in cytosolic $[\text{Ca}^{2+}]_i$, fluo-3-labeled platelets (see above) from mice of the same genotype were added to the anticoagulated blood, so that about 5% of total platelets were labeled. Labeling of only a small fraction of the platelets in blood has as advantage that individual platelets can be distinguished from each other in fluorescence images, even when they are trapped in multiplatelet aggregates.

Surface area coverage from phase-contrast images was analyzed off-line using ImagePro software (Media Cybernetics, Silver Spring, MD). Data from at least 9 different microscopic images were averaged per experiment. Platelet coverage of the coverslip increased linearly around the standard perfusion time of 4 min at 1000 s^{-1} . Area coverage by platelets staining with OG488-annexin A5 (9 images/experiment) was determined with Quanticell software (Visitech).

Real-time changes in fluo-3 fluorescence from single platelets, recorded during the flow experiment (5 images/second), were analyzed with Quanticell software and compared with translocation of the platelets, as deduced from the recorded images. Changes in fluo-3 fluorescence of individual platelets were converted into levels of $[\text{Ca}^{2+}]_i$ using a common pseudo-ratio calibration procedure (35). Normalized maximal and minimal fluorescence values per cell (F_{max} and F_{0} , respectively) were obtained by activating fluo-3-loaded mouse platelets with ionomycin and thapsigargin, using the same optical and camera settings. Traces representing averaged Ca^{2+} responses were constructed from time-adjusted responses of 10-15 different platelets, observed during a period of at least 60 s (2 or 3 mice per experimental condition). Note that the fluo-3 images were captured during flow, and individual labeled platelets adhered and responded to the collagen surface at different time points and different sites in the microscopic field.

Statistical analysis

Data were tested on significance using the statistical package for social sciences (SPSS 8.0, Chicago, IL). Significant differences compared to control condition were evaluated with a Mann-Whitney U test.

Results

Integrin $\alpha_2\beta_1$ supports GPVI-dependent formation of stable aggregates under flow and potentiates platelet procoagulant activity

The role of collagen receptors GPVI and integrin $\alpha_2\beta_1$ in the process of collagen-dependent platelet aggregation and procoagulant activity was examined in mice lacking either receptor on their platelets. Two different models for GPVI-deficiency were used. First, FcR γ chain-deficient mice which also lack expression of GPVI on platelets (15), and second, wildtype mice injected with the anti-GPVI antibody JAQ1. The latter treatment induces virtually complete internalization and proteolytic degradation of GPVI on circulating platelets, which results in a GPVI knockout-like phenotype for at least two weeks (31). As shown in Table 3.1, GPVI was undetectable on the platelets of either group of mice. Mice lacking integrin $\alpha_2\beta_1$ on their platelets were generated by crossing mice carrying a floxed β_1 gene with mice expressing cre recombinase under control of an Mx promoter (29). Two weeks after injection of polyinosinic-polycytidylic acid, Mx-cre-mediated deletion was almost complete in bone marrow and platelets. Integrin $\alpha_2\beta_1$ was undetectable on the platelets from these mice, but their GPVI level was normal (Table 3.1). Mice lacking G α_q , but expressing normal levels of platelet collagen receptors (Table 3.1) were used to determine the involvement of G α_q /phospholipase C β -mediated signaling implicated in TxA $_2$ - and ADP-dependent events in aggregation.

Table 3.1 Surface expression of different glycoproteins on platelets from control and modified mice^a.

Glycoprotein	Mean log fluorescence (AU) $\pm$ SD				
	Control	GPVI-def	FcR γ -/-	β_1 -null	G α_q -/-
GP1a (integrin β_1)	123.1 $\pm$ 5.6	122.0 $\pm$ 7.8	129.0 $\pm$ 5.7	6.6 $\pm$ 1.1*	124.5 $\pm$ 8.6
GP1a (integrin α_2)	63.3 $\pm$ 6.3	67.0 $\pm$ 8.2	64.3 $\pm$ 8.0	6.2 $\pm$ 0.8*	69.2 $\pm$ 7.2
GP1c' (integrin α_6)	72.8 $\pm$ 7.9	64.4 $\pm$ 6.5	68.6 $\pm$ 8.3	6.0 $\pm$ 0.8*	67.4 $\pm$ 8.6
GPVI	47.3 $\pm$ 4.0	5.8 $\pm$ 0.7*	5.1 $\pm$ 0.5*	50.5 $\pm$ 5.0	48.3 $\pm$ 4.8
GP1Ib/IIIa (integrin α IIb β_3)	359.0 $\pm$ 15.5	368.0 $\pm$ 14.7	379.9 $\pm$ 8.8	366.0 $\pm$ 12.0	369.9 $\pm$ 19.4
GP1b-V-IX	289.5 $\pm$ 10.8	283.9 $\pm$ 12.4	285.0 $\pm$ 15.3	290.2 $\pm$ 11.2	278.2 $\pm$ 15.4

^aExpression levels were determined by flow cytometry using fluorophore-labeled monoclonal antibodies, as described previously (15). Platelets were gated by their forward scatter/side scatter characteristics. Control group refers to platelets from NMRI mice, but similar results were obtained with control C57BL/6 and C57BL/6 $\times$ 129/Sv mice. GPVI-def indicates GPVI-deficient mice. Results are expressed as mean log fluorescence (AU) $\pm$ SD for 6 mice per group. * p <0.01 compared to control.

Figure 3.1. Platelets lacking GPVI, integrin $\alpha 2\beta 1$ or $G\alpha_q$ differed in collagen-mediated aggregate formation and procoagulant activity under flow conditions. (A-D) Phase-contrast microscope images after 4 min perfusion of whole mouse blood at a wall shear rate of 1000 s^{-1} over a collagen-coated surface (Horm type I) in a parallel plate flow chamber. Image areas are $120 \times 120\text{ }\mu\text{m}$. (E-H) Fluorescence images after staining with OG488-labeled annexin A5, for detecting PS-exposing platelets. Image areas are $150 \times 150\text{ }\mu\text{m}$. (A, E) NMRI control mice; (B, F) $FcR\gamma^{-/-}$ mice; (C, G) β_1 null mice; (D, H) $G\alpha_q^{-/-}$ mice. Inserts are 300% magnifications of platelets at a collagen fiber; arrows indicate one blebbing platelet (A) and three unstimulated platelets (B). Data are representative for 4-9 independent experiments.

Whole blood from control mice was perfused over fibrillar (either Horm type-I or native type-I from bovine tendon) or non-fibrillar type-I collagen at a defined wall shear rate. During and after the flow video (fluorescence) microscopic images were continuously taken at the optical plane of the collagen. Blood was anticoagulated with PPACK and heparin to maintain physiological, millimolar Ca^{2+} and Mg^{2+} concentrations. At a high shear rate of 1000 s^{-1} , platelets from control mice adhered to Horm collagen fibers and formed pseudopods within 30 s. The adhered platelets served as starting sites for large platelet aggregates that assembled within 2 min. After about 4 min, some collagen-attached platelets rounded off and developed into balloon-shaped, blebbing cells (Fig. 3.1A, inset). Platelet deposition and aggregate formation on collagen was independent of the genetic background of the control mice; i.e. blood from wildtype NMRI, C57BL/6 or C57BL/6 x 129/Sv mice, all with similar platelet counts, was equally reactive (see below). At a low shear rate of 150 s^{-1} , the rate of platelet adhesion to the collagen surface and subsequent assembly of aggregates was lower than at 1000 s^{-1} , as described before (25).

Figure 3.2. Both GPVI and integrin $\alpha 2\beta 1$ contributed to collagen-mediated platelet procoagulant activity. Blood from different types of mice was perfused over collagen as described for Fig. 3.1. Control 1, NMRI mice; control 2, C57BL/6 $\times$ 129/Sv. Data represent percentages of area coverage of all platelets (white bars) or PS-exposing platelets (gray bars) after 4 min of perfusion. Means $\pm$ SD ($n=3-7$; ¶ $n=1$). * $p<0.05$ compared to NMRI control mice; # $p<0.05$ compared to C57BL/6 $\times$ 129/Sv control mice.

Perfusion of blood from FcR γ -chain $-/-$ mice at high shear stress resulted in only few platelets adhering to the Horm collagen; these did not show morphological signs of activation (Fig. 3.1B). Blood from mice depleted in GPVI by JAQ1 injection gave similar results. However, when using $\beta 1$ -null blood, many platelets adhered and formed aggregates (Fig. 3.1C), confirming that integrin $\alpha 2\beta 1$ is not essential for collagen-induced aggregate formation (25). Yet, the $\beta 1$ -null aggregates consistently had a looser structure and they disintegrated more frequently later in the experiment as compared to wildtype. Also at low shear rate (150 s^{-1}) and/or when native type-I collagen was used, GPVI depletion resulted in nearly complete suppression of platelet adhesion, whereas $\alpha 2\beta 1$ absence prevented neither adhesion nor aggregation. However, in the latter case, again aggregates were consistently looser packed in comparison to those of control platelets.

High shear flow experiments with blood from G αq $-/-$ mice resulted in normal platelet-collagen interaction and normal pseudopod and bleb formation, but aggregates remained small and were no more than two cell layers thick (Fig. 3.1D). Because of the greatly reduced aggregate size in the absence of G αq , total platelet deposition was reduced by 70% as compared to the wildtype control (Fig. 3.2).

The appearance of balloon-shaped, blebbing platelets on collagen fibers was indicative of surface exposure of procoagulant PS, a process that usually occurs simultaneously with the blebbing response (17). Indeed, after the perfusion a significant number of the control platelets was in procoagulant state, as demonstrated by their staining with OG488-labeled annexin A5

(Fig. 3.1E, and quantified in Fig. 3.2). Only platelets in the focal plane of the collagen were positive for annexin A5, which demonstrates that direct contact of the cells with collagen was essential for PS exposure. This finding was confirmed by the virtually complete absence of annexin-V positive cells after perfusion of blood from FcR γ -chain $-/-$ or GPVI-depleted mice. Even the few adherent platelets of these mice did not bind annexin A5, in agreement with their apparently unstimulated morphology. Strikingly, $\beta 1$ -null blood, despite forming platelet aggregates, showed markedly reduced annexin A5 positivity of platelets after the perfusion, as compared to the controls (Fig. 3.2). Reduced PS exposure was observed independent of the shear rate (1000 or 150 s^{-1}) or type of collagen fibers. This result is in contrast to the situation with blood from $G\alpha_q$ $-/-$ mice, where the number of PS-exposing platelets was unchanged in spite of fairly reduced aggregate formation (1000 s^{-1}). Together, these results confirm the essential role of GPVI in platelet-collagen interaction (25), but they also demonstrate that $\alpha_2\beta_1$ integrin, but not $G\alpha_q$, is required to potentiate GPVI-dependent PS exposure, i.e. procoagulant activity.

Integrin $\alpha_2\beta_1$ but not $G\alpha_q$ contributes to sustained, GPVI-induced Ca^{2+} responses under flow conditions

To determine the activation state of platelets interacting with collagen under flow conditions, whole blood was spiked with fluo-3-loaded platelets derived from the same mouse type, to reach a concentration of 5% labeled platelets. This partial labeling allowed visualization of the Ca^{2+} responses in individual cells even within large aggregates. Platelet labeling with fluo-3 had no effect on in vitro aggregate formation (data not shown). Microscopic fluorescence images were captured at 5 Hz to monitor the changes in cytosolic $[\text{Ca}^{2+}]_i$ of individual adherent platelets during the flow at 1000 s^{-1} (35). The fluo-3-labeled platelets in blood from control mice stably attached to collagen fibrils and exhibited a potent increase in $[\text{Ca}^{2+}]_i$, as shown in the averaged trace of Fig. 3.3A. Traces from most single platelets indicated one or more transient spikes in $[\text{Ca}^{2+}]_i$ that was followed by a persistent, elevated plateau level (41/42 cells); see the single-cell trace of Fig. 3.3A. In contrast, in flow experiments with FcR γ $-/-$ blood, the few labeled platelets that stably adhered remained low in $[\text{Ca}^{2+}]_i$ (Fig. 3.3B), indicating that the Ca^{2+} signal generation was effectively abolished.

With blood from $\beta 1$ -null mice, a different situation was obtained in that many platelets adhered transiently and then translocated downstream. About 90% of the labeled platelets performed such translocation (26 of 29 cells). The averaged Ca^{2+} response of $\beta 1$ -null platelets fluctuated and was somewhat lower than the control response (Fig. 3.3C). Traces from single cells showed highly spiking rises in $[\text{Ca}^{2+}]_i$ that occurred upon contacts with collagen for more than several seconds (Fig. 3.3C). A minority of the labeled platelets displayed prolonged binding to collagen, in which case a sustained $[\text{Ca}^{2+}]_i$ rise was observed (3 of 29 cells). In contrast,

Figure 3.3. Prolonged Ca^{2+} responses of platelets interacting with collagen under flow required both GPVI and integrin $\alpha 2\beta 1$. Whole blood was spiked with fluo-3-loaded platelets from the same mouse type, and perfused over a collagen surface (Horm type I) at $1000\ s^{-1}$. Upper traces show averaged changes in $[Ca^{2+}]_i$ of 15–20 platelets that could be observed during at least 60 s (2–3 mice per condition). Lower traces show Ca^{2+} responses of representative, single platelets (black lines) as well as their downstream translocation over the collagen surface (gray lines). Types of mice: (A) NMRI control; (B) $FcR\gamma$ -chain $-/-$; (C) $\beta 1$ null; (D) $G\alpha q^{-/-}$.

absence of $G\alpha q$ influenced neither stable adhesion to collagen nor the potent Ca^{2+} signal. The averaged Ca^{2+} response from $G\alpha q^{-/-}$ platelets resembled that of control platelets (Fig. 3.3D); individual knock-out platelets showed a normal initial $[Ca^{2+}]_i$ spike followed by a persistent increase (18 of 22 cells). These results demonstrate that GPVI is required for the generation of the Ca^{2+} signal once the platelets are deposited from the flowing blood, while integrin $\alpha 2\beta 1$ serves to facilitate and prolong GPVI-induced Ca^{2+} signaling. These data further show that $G\alpha q$ -dependent stimulation of phospholipase C β is not involved in the generation of this Ca^{2+} signal.

Secretion of TxA_2 and ADP mediates formation of large platelet aggregates but not the primary GPVI-dependent Ca^{2+} responses and procoagulant activity under flow conditions

With blood from wildtype mice, we used blocking antibodies against the adhesive receptors and agents inhibiting TxA_2 - and ADP-mediated effects to further delineate the process of shear-dependent thrombus-formation. Initial platelet adhesion and subsequent aggregate formation depended on GPIb-vWF interaction, as a Fab fragment of p0p/B blocking antibody (40 $\mu g/ml$), directed against mouse GPIb α , almost completely inhibited these events (Fig. 3.4). The few adhering platelets, however, were activated, because they spread, formed blebs and bound annexin A5. These data are in line with the hypothesis that GPIb mediates platelet tethering under these shear-rate conditions by interacting with vWF deposited on the collagen

Figure 3.4. Different effects of anti-glycoprotein antibodies and inhibitors of TxA_2 and ADP action on platelet-collagen interaction under flow conditions. (A-D) Phase-contrast microscopic images after 4 min perfusion of whole blood at 1000 s^{-1} over a collagen-coated surface (Horm type I). Image areas are $120 \times 120 \text{ }\mu\text{m}$. (E-H) Fluorescence microscopic images after staining with OG488-labeled annexin A5, for detecting PS-exposing platelets. Image areas are $150 \times 150 \text{ }\mu\text{m}$. Blood from C57BL/6 control mice was untreated (A, E) or was incubated with the following substances for 10 min prior to perfusion: (B, F) Fab fragments of p0p/B antibody against GPIIb/IIIa ($40 \text{ }\mu\text{g/ml}$); (C, G) JAQ1 Fab fragment against GPVI ($40 \text{ }\mu\text{g/ml}$); (D, H) aspirin ($100 \text{ }\mu\text{M}$) together with MRS2179 ($20 \text{ }\mu\text{M}$) and AR-C69931MX ($50 \text{ }\mu\text{M}$). Insets are 300% magnifications of platelets at collagen; arrows point to one spreading platelet (B) and two unstimulated platelets (C). Representative images from four to six experiments are shown.

fibers (3, 23), but they also point to unaltered GPVI- and integrin-mediated activation of those platelets that immobilized independently of GPIIb/IIIa.

Preincubation of wildtype blood with Fab fragments of the anti-GPVI antibody JAQ1 ($40 \text{ }\mu\text{g/ml}$) almost completely blocked adhesion and aggregate formation on either Horm, native or non-fibrillar type-I collagens, as expected. The few adherent platelets, presumably binding through GPIIb/IIIa, showed neither morphological signs of activation nor staining with annexin A5 (Fig. 3.4). These results again confirm the pivotal role of GPVI in all collagen-induced responses. Different results were obtained with a new monoclonal rat anti-mouse antibody against integrin $\alpha 2\beta 1$, LEN1 (rat IgG1), which specifically blocks $\alpha 2\beta 1$ -mediated platelet adhesion to non-fibrillar collagen under steady-state conditions (V. Schulte & B. Nieswandt, unpublished results). In high-shear experiments over Horm type-I collagen, LEN1 ($20 \text{ }\mu\text{g/ml}$) failed to abolish platelet adhesion and aggregate formation, but lowered the number of annexin A5-positive platelets to $21 \pm 20\%$ (mean $\pm$ SD, $n=4$) compared to the control condition. Together these results are in line with those obtained with $\beta 1$ -null platelets.

Figure 3.5. Contribution of GPVI-stimulated TxA_2 and ADP secretion to collagen-mediated aggregate formation and procoagulant activity under flow conditions. Flow experiments over collagen surface were performed exactly as indicated for Fig. 3.4. Data represent percentages of area coverage of all platelets (A) or PS-exposing platelets (B) after 4 min of perfusion. Mean percentages of control condition $\pm$ SD ($n=4-11$; ¶ $n=1$). * $p<0.05$ compared to control.

In the absence of thrombin, i.e. coagulation, it is known that $\text{G}\alpha_q$ mainly mediates Ca^{2+} and other platelet responses that are evoked by TxA_2 and ADP through the $\text{TP}\alpha$ and P2Y_1 receptors, respectively (27, 36). Both TxA_2 and ADP are released from collagen-stimulated platelets and contribute to platelet aggregation *in vitro* (37). To mimic the effects of $\text{G}\alpha_q$ abolishment, we treated blood from wildtype mice with aspirin (blocking TxA_2 formation) and added MRS2179 (a specific P2Y_1 antagonist). After 4 min of perfusion over Horm collagen, the treated platelets formed no more than small aggregates (Fig. 3.4); area coverage of platelets on the coverslip was half of the control condition (Fig. 3.5). Many of the collagen-adherent platelets

Figure 3.6. Different effects of GPVI antibody and inhibitors of TxA_2 and ADP action on Ca^{2+} responses induced by collagen interaction. Whole blood from C57BL/6 control mice was spiked with fluo-3-loaded platelets from the same mouse type, and perfused over a collagen surface at 1000 s^{-1} . Upper panels show averaged changes in $[\text{Ca}^{2+}]_i$ of 12–20 platelets, which could be observed during at least 60 s, from at least two different mice. Lower panels show representative platelets (black lines) as well as their downstream translocation over the collagen surface (gray lines). Blood was either untreated (A) or incubated for 10 min prior to perfusion with the following substances: (B) JAQ1 Fab fragment against GPVI (40 $\mu\text{g}/\text{ml}$); (C) aspirin (100 μM) plus MRS2179 (20 μM) and AR-C69931MX (50 μM).

were still blebbing, and the area coverage with annexin A5-positive platelets was only slightly reduced. Treatment of the blood with aspirin alone had no influence on platelet adhesion, aggregate formation, or PS exposure (data not shown). Interestingly, concurrent inhibition of the second platelet ADP receptor, P2Y_{12} (coupled to G_i), by the potent antagonist AR-C69931MX in combination with aspirin/MRS2179 still resulted in small aggregates (Fig. 3.4), but reduced the amount of annexin A5-binding platelets with about 50% (Fig. 3.5). The decreased PS exposure by additional P2Y_{12} blockage may point to an anticoagulant effect, which agrees with earlier demonstrated inhibitory effects of AR-C69931MX on platelet function (38).

The contribution of TxA_2 and ADP to aggregate formation under flow conditions may depend on the type of collagen or the shear rate. To investigate this, we performed experiments with native fibrillar and non-fibrillar type I collagen as adhesive surfaces. Area coverage of platelets with these collagens was $22.5 \pm 3.7\%$ and $14 \pm 8.5\%$, respectively (mean $\pm$ SD, $n=3$), which is in the same range as the coverage observed with Horm type-I collagen (Fig. 3.2). Preincubation with aspirin, MRS2179 and AR-C69931MX resulted in deposition of single platelets and small aggregates of no more than two cell layers thick; the coverage was lowered to $37 \pm 11\%$ and $28 \pm 11\%$, respectively, compared to untreated platelets (100%). This preincubation reduced the area coverage with annexin A5-positive platelets to 60% of control.

Similar effects of these antagonists were obtained in low-shear experiments (150 s^{-1}) with Horm type-I collagen as adherent surface. Thus, the contribution of TxA_2 and ADP appears to be independent of the type of collagen surface and the shear rate.

Together, these results suggest that TxA_2 and ADP, both secreted from collagen-activated platelets during flow conditions, are essential for the formation of large platelet aggregates, but of moderate importance for platelet-collagen interaction and subsequent activation. This hypothesis was confirmed when we examined the contribution of TxA_2/ADP to the Ca^{2+} signal of platelets flowing over Horm collagen. As shown in Fig. 3.6 for averaged and representative single-cell Ca^{2+} responses, treatment of wildtype blood with aspirin, MRS2179 and AR-C69931MX did not alter the abrupt translocation of platelets or their sustained increase in $[\text{Ca}^{2+}]_i$. This result was in sharp contrast to the situation where JAQ1 Fab fragments were added. In the latter case, the very few adherent platelets showed only minimal increases in $[\text{Ca}^{2+}]_i$ upon adhesion (Fig. 3.6). This confirms the idea that GPVI, and not the released autocrine substances, mediates activation of the collagen-bound platelets.

Discussion

GPVI is the key receptor in platelet-collagen interaction under flow conditions but needs integrin $\alpha 2\beta 1$ to stabilize adhesion and potentiate activation

Our data provide novel, detailed insight into the different roles of two important collagen receptors on platelets, which are both implicated in normal hemostasis and (arterial) thrombosis. We used a combination of various genetic mouse models as well as specific antibodies and drugs directed to the platelet receptors to investigate the complementary roles of GPVI and integrin $\alpha 2\beta 1$ in a physiologically relevant test system of whole blood perfusion over collagen fibers at high shear rate. The data thereby provide a first example of interplay of immunoglobulin receptors (GPVI) and integrins ($\alpha 2\beta 1$) in cellular activity. Given the importance of either receptor type in cell biology, similar cross-talk may also be operative in other cellular types.

It is generally considered that, at high wall-shear rates ($>500 \text{ s}^{-1}$), initial platelet tethering to plasma-exposed collagen critically involves the interaction between GPIb and vWF deposited from plasma on collagen (1,39). Recently, we provided evidence that stable platelet-collagen adhesion under these conditions is strictly dependent on active GPVI receptors (25). The GPVI-dependent adhesion, for instance, shifts the integrins $\alpha 2\beta 1$ and $\alpha \text{IIb}\beta 3$ to a higher affinity state. The present paper confirms and extends the key position of GPVI as central collagen receptor on platelets. Adhesion to collagen of platelets lacking GPVI (from $\text{FcR}\gamma^{-/-}$ mice) or platelets in which GPVI activation is blocked with JAQ1 antibody is considerably

inhibited under conditions of high shear stress. The few GPVI-deficient or -inactivated platelets that adhere remain low in $[Ca^{2+}]_i$, and cannot spread, collect other platelets (thus forming aggregates), or form membrane blebs and expose procoagulant PS. All these responses are essential for hemostatic plug and thrombus formation.

Using mice with $\beta 1$ -deficient platelets, we previously demonstrated that $\alpha 2\beta 1$ -collagen interaction is dispensable for adhesion to collagen and for collagen-dependent aggregate formation (25). This result was confirmed in the present study. However, close examination of the aggregates formed under flow conditions revealed that, in $\beta 1$ -null platelets, these were less firmly bound to the collagen and tended to disaggregate more frequently than aggregates of wildtype platelets. Similar results were obtained with the blocking anti- $\alpha 2\beta 1$ antibody LEN1. That integrin $\alpha 2\beta 1$ plays a critical role in the stabilization of platelet-collagen interactions became also evident, when $\beta 1$ -null platelets were loaded with fluo-3 and their Ca^{2+} responses were measured under flow conditions. Most labeled $\beta 1$ -null platelets exhibited transient periods of adhesion which were typically accompanied by short periods of spiking increases in $[Ca^{2+}]_i$. This phenomenon coincided with a marked reduction in PS exposure of the $\beta 1$ -null platelets. The latter effect is most likely related to the reduced Ca^{2+} responses of many of the platelets, because prolonged elevation in $[Ca^{2+}]_i$ is required for PS exposure and bleb formation (17,40). Others have reported that GPVI agonists activate $\alpha 2\beta 1$ integrin (24). This is probably also true for the present flow experiments, because adhesion to collagen of GPVI-deficient platelets, expressing normal levels of $\alpha 2\beta 1$, was severely impaired.

The exact role of integrin $\alpha 2\beta 1$ in platelet-collagen interactions has been debated, which is in part related to different experimental conditions. This is exemplified by the apparent discrepancy between our study with whole mouse blood and the study by Chen and co-workers with isolated platelets from $\alpha 2$ -deficient mice (41). These authors reported that $\alpha 2$ -deficiency abolishes adhesion of washed platelets to type I collagen under either static or flow conditions. Although this approach allows precise examination of direct platelet-collagen interactions, it has clearly limitations. First, in flow experiments, red cells are needed to bring flowing platelets in close contact with the collagen surface, and vWF – which binds to collagen – is required to enhance platelet-collagen contact via GPIb and integrin $\alpha IIb\beta 3$ even under stasis (3). Second, Chen et al. performed their experiments in the presence of millimolar Mg^{2+} but no added Ca^{2+} , which greatly promotes the contribution of integrin $\alpha 2\beta 1$ -dependent adhesion (42). This situation contrasts with the physiological cationic condition with millimolar concentrations of free Mg^{2+} and Ca^{2+} as used in the present paper.

Collectively, these data suggest that, after activation by GPVI and, possibly, released ADP (see below), the integrin serves to facilitate GPVI-dependent signaling events by stabilizing the adhesion to collagen, with as a consequence prolonged and magnified activation responses. Accordingly, both receptors have different, yet complementary and cooperative roles

in collagen-induced thrombus formation and procoagulant activity: GPVI as an initiating and signaling receptor, and activated $\alpha_2\beta_1$ integrin as an inducible, high-affinity adhesive receptor allowing GPVI to signal.

GPVI-mediated release of TxA_2 and ADP enables aggregate formation on collagen-adherent platelets

The $\text{G}\alpha_q$ heterotrimer is a major signaling molecule in platelet responses evoked by TxA_2 and ADP through the $\text{TP}\alpha$ and P2Y_1 receptors, respectively (36,37). This is best highlighted by the finding that $\text{G}\alpha_q$ -deficient platelets display defective aggregation in response to these agonists and collagen (27). However, $\text{G}\alpha_q$ -deficient platelets are normally activated by agonists specific for GPVI (28), suggesting that receptors other than GPVI are involved in collagen-induced platelet aggregation at least under conditions of standard aggregometry. In the current study, we found that $\text{G}\alpha_q$ -deficient platelets normally adhered to collagen under high shear conditions, and elicited normal rises in $[\text{Ca}^{2+}]_i$, followed by PS exposure. We thus demonstrate for the first time that $\text{G}\alpha_q$ is not involved in collagen-dependent adhesion or Ca^{2+} and procoagulant responses of platelets. However, $\text{G}\alpha_q$ -deficient platelets failed to form large aggregates under flow conditions; only two-layered platelet deposits could be formed on collagen. This result strongly suggests that, under normal (wildtype) conditions, collagen-adherent platelets activated via GPVI produce TxA_2 and/or ADP, which causes rapid trapping and aggregation of other flowing platelets, most likely through the fibrinogen receptor, integrin $\alpha\text{IIb}\beta_3$.

Indeed, adhesion and aggregation similar to those of $\text{G}\alpha_q$ -deficient platelets was obtained with wildtype platelets when TxA_2 formation was inhibited with aspirin and the ADP receptor P2Y_1 was blocked using MRS2179. The combination of aspirin, MRS2179 and AR-C69931MX had a partial but significant suppressive effect on the activation state of adherent platelets, as PS exposure was reduced in comparison to the control condition. This result suggests that the P2Y_{12} -evoked G_i signaling pathway, which is blocked by AR-C69931MX but not in $\text{G}\alpha_q$ -deficient platelets, has a potentiating effect on GPVI signaling and consequent autocrine $\alpha_2\beta_1$ activation, i.e. stabilization of GPVI-collagen interactions.

Taken together, our data provide novel, detailed insight into the different roles of two important receptors that are implicated in normal hemostasis as well as arterial thrombosis. They can resolve the apparent discrepancy in the literature whether integrin $\alpha_2\beta_1$ is or is not primarily involved in platelet-collagen interaction. Platelet binding and activation via GPVI primes for integrin-mediated adhesion. Conversely, the activated integrin $\alpha_2\beta_1$ (also perhaps by other agonists) stabilizes the platelets on collagen, so that GPVI can complete its signaling task. Although GPVI depletion has been shown to lead to antithrombotic protection in mice (31), these findings yet suggest that agents inhibiting $\alpha_2\beta_1$, perhaps in combination with anti-GPVI

effects, can be useful for platelet-directed anti-thrombotic therapies. The possible relevance of such treatment may appear from the evidence that subject-dependent variation in integrin $\alpha 2\beta 1$ density on platelets influences shear-dependent platelet adhesion to collagen (43), and correlates with the GPVI content on platelets (44).

Acknowledgments

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Chapter 4

Role of integrin $\alpha 2 \beta 1$ in thrombus stabilization and embolization

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Abstract

Upon vessel wall damage, platelets interact with exposed collagen via two receptors, glycoprotein (GP)VI and $\alpha 2\beta 1$ integrin. In contrast to the established platelet-activating role of GPVI, the function of $\alpha 2\beta 1$ in thrombus formation has remained unclear. We report that using a murine thrombosis model, induced by extracellular matrix exposure, in arteries from $\alpha 2$ -null mice smaller thrombi were formed with more embolization compared to the vessels from wildtype mice. In vitro, perfusion of blood from $\alpha 2$ -null mice over collagen also resulted in formation of thrombi that were smaller and looser in appearance than those obtained with wildtype blood, regardless of the shear rate (low/high) and the presence of coagulation. Two-photon laser scanning microscopy showed that under flow the absence of $\alpha 2\beta 1$ reduced fibrinogen binding to platelets and lowered exposure of procoagulant phosphatidylserine. In platelet-rich plasma from $\alpha 2$ -null mice, thrombin generation in response to tissue factor and collagen was delayed and reduced. Because also thromboxane A_2 release from platelets was reduced in the absence of $\alpha 2\beta 1$, blood from $\alpha 2$ -null mice was co-perfused with thromboxane A_2 analogue U46619 over collagen. This led to normalization of the thrombus size. In vivo, application of the thromboxane analogue abrogated the frequent embolization of $\alpha 2$ -null thrombi. Together, these novel data indicate that $\alpha 2\beta 1$ has a moderate but qualitatively important effect to stabilize thrombi by enhancement of the following GPVI-dependent mechanisms: (i) release of thromboxane A_2 , (ii) activation of $\alpha IIb\beta 3$ leading to irreversible aggregation, and (iii) phosphatidylserine exposure leading to generation of thrombin and fibrin clots. The increased embolization in the absence of $\alpha 2\beta 1$ argues against use of $\alpha 2\beta 1$ integrin inhibitors for antithrombotic therapy.

Introduction

Vascular damage triggers platelet adhesion and aggregation at the exposed extracellular matrix, leading to thrombus formation and cessation of bleeding. In vivo studies with a variety of murine thrombosis models have shown that the low-affinity platelet collagen receptor, glycoprotein VI (GPVI) is required for thrombus formation on injured arteries (1,2). In addition, integrins on the platelet surface, i.e. the fibrinogen receptor $\alpha IIb\beta 3$ and the collagen receptor $\alpha 2\beta 1$, are involved in stable platelet adhesion to the damaged vessel wall. Both integrins can be activated via inside-out signaling by a conformational switch mechanism, which shifts the extracellular domain to a higher affinity state with increased ligand interaction (3-5).

Whereas the role of activated $\alpha IIb\beta 3$ in platelet aggregation is well-established, the precise function of $\alpha 2\beta 1$ in platelet-collagen interaction is still debated. About twenty years ago, it was reported that two patients with deficiency in $\alpha 2\beta 1$ experienced greatly prolonged bleeding times (6-8). However, later it was questioned whether these clinical symptoms were related to

the proposed platelet defect (9). Further interest in the $\alpha 2\beta 1$ integrin came from the observation that its level on platelets can vary up to 10-fold in association with the C807T genetic polymorphism of $\alpha 2$ (10). Since 1986, many investigations have been carried out to determine whether absence or blocking of $\alpha 2\beta 1$ may protect from thrombosis. Therefore, mice have been generated with conditional $\beta 1$ -integrin deficiency (1) or lacking the $\alpha 2$ -integrin (11). These mice had normal tail bleeding times and unaltered thrombosis tendency (12). However, in a different strain of $\alpha 2$ -deficient mice (13), arterial thrombosis tendency was reported to be diminished (14).

In vitro whole blood perfusion studies at high shear have indicated that platelets which are adhering to collagen and von Willebrand factor (vWF) are activated by interplay of the low-affinity collagen receptor GPVI with integrin $\alpha 2\beta 1$ (15,16). Again the precise role of $\alpha 2\beta 1$ is debated, as some in vitro studies show that platelets from $\alpha 2$ -null mice are reduced in adhesion on collagen (13), while other studies with $\alpha 2$ - and $\beta 1$ -null mice point to normal adhesion and aggregate formation under flow (17). Recent insight, however, indicates that both collagen receptors have additive effects on platelet adhesion (18,19), likely by two mechanisms of stable platelet adhesion, one via $\alpha 2\beta 1$ and one via GPVI, both of which are conserved in mouse and man. Earlier, we have proposed that the facilitating and consolidating role of $\alpha 2\beta 1$ explains why aggregates are less compact in the absence of $\alpha 2\beta 1$ (17).

In the present study, we hypothesized that $\alpha 2\beta 1$ has a role in thrombus stabilization on collagen rather than in mediating thrombus formation per se. We used intravital and two-photon fluorescence microscopy to determine thrombus growth and stability both in vivo, at sites of vascular injury, and in vitro, on collagen/vWF surfaces under conditions of flow and coagulation. The data reveal that $\alpha 2$ -null mice have a subtle but clinically relevant phenotype, in which unstable thrombi tend to shed from the adhesive surface as emboli, due to three mechanisms: reduced thromboxane formation, reduced platelet aggregation, and diminished coagulant activity.

4.3 Materials and Methods

Animals

Mice deficient in the $\alpha 2$ -integrin subunit were produced and checked for absence of the integrin on platelets, as described (11). Both mutant and wildtype control animals were on a 129/Sv x C57BL/6 genetic background. Animals were used at the age of 4-5 weeks for intravital experiments, and at 12-16 weeks for perfusion and thrombin generation experiments. The local Animal Care and Use Committees approved the studies.

Materials

Fibrillar type-I collagen (Horm) was purchased from Nycomed (Munich, Germany). Annexin A5 labeled with fluorescein isothiocyanate (FITC) 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). 5-carboxyfluorescein diacetate (DCF) succinimidyl ester was from Invitrogen (Karlsruhe, Germany). Monoclonal antibody against the $\alpha 2$ -integrin subunit, SAM.G4, was from Emfret Analytics (Würzburg, Germany). 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) and FeCl_3 from Merck (Darmstadt, Germany). Ketamine and xylazine were from Eurovet (Bladel, the Netherlands). Other materials were obtained from Sigma (St. Louis, MO).

Preparation of platelets for intravital microscopy

Mouse blood (1 vol) was drawn from the retro-orbital plexus and collected into 0.5 vol 5 mM Hepes buffer pH 7.14 containing 20 U/ml heparin. Blood was centrifuged at 250 g for 5 minutes, and platelet-rich plasma (PRP) was gently transferred to a fresh tube. After labeling with DCF (5 $\mu\text{g}/\text{ml}$ for 2 minutes), platelets were centrifuged at 2000 g for 10 minutes. The pellet was resuspended in phosphate-buffered saline (PBS) and platelet concentration was adjusted to a final concentration of 200×10^6 platelets/250 μl (12).

Animal preparation, intravital microscopy and thrombus formation in vivo

Four- to 5-week old mice were anesthetized by intraperitoneal injection of 2,2,2-tribromoethanol and 2-methyl-2-butanol (0.15 ml/10g of body weight from 2.5% solution). A mouse was positioned on a plastic petridish, and a segment of the ileum was exteriorized through a right side abdominal incision. The mesentery was spread out on the petridish and kept wet with a buffered Tyrode's 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) supplemented with 1 $\mu\text{g}/\text{ml}$ isoprenaline. The exteriorized ileum was kept moist with overlying wet gauze. Fluorescent platelets ($200 \times 10^6/250 \mu\text{l}$) were infused through the retro-orbital plexus.

Vessel wall damage was induced as described (20). Briefly, FeCl_3 (30 μl , 500 mM) was topically applied on selected mesenteric vessels. Wildtype arterioles (diameter $66 \pm 7 \mu\text{m}$, $n=15$) and $\alpha 2$ -null arterioles (diameter $93 \pm 5 \mu\text{m}$, $n=20$, mean $\pm$ SEM) each constricted by 15-20% upon application of FeCl_3 , while venular diameters (wildtype $155 \pm 14 \mu\text{m}$, $n=15$; $\alpha 2$ -null $182 \pm 10 \mu\text{m}$, $n=19$) did not change. Prior to and following vascular injury, fluorescent platelets in mesenteric vessels were visualized in vivo by video microscopy using a Zeiss Axiovert 200

inverted microscope, equipped with 10 $\times$ (0.25 Numerical Aperture) and 20 $\times$ (0.40 NA) objective lenses (Zeiss, Göttingen, Germany) and a 100-W HBO mercury lamp for epi-illumination. Platelet adhesion and thrombus formation were observed for at least 10 minutes and recorded on videotape (Panasonic AG-7355, Matsushita Electric, Osaka, Japan). Recorded images corresponded to 410 $\times$ 308 μm . Per vessel, the following parameters were determined off-line: (i) time to first platelet-vessel wall interaction (tethering), (ii) time to first stable platelet adhesion (i.e. fixed adhesion for at least 30 seconds), (iii) number of thrombi in the recorded vessel segment, (iv) height of thrombi ($\geq 10 \mu\text{m}$ in the same segment) at 2, 5 and 10 minutes after FeCl_3 application, measured perpendicular to the vessel wall and expressed as percentage of vessel diameter, and (v) number of emboli during 10 minutes. Thrombus height was measured relative to the local vessel diameter, as described (21). If separate thrombi were detected, the cumulative thrombus height was calculated as summation of the short thrombus axes, perpendicular to the vessel wall. Heights of individual thrombi were categorized as $<25\%$, between 25-50%, or $\geq 50\%$ of vessel diameter.

Thrombus formation on collagen under flow

Thrombus formation was measured in flow chamber experiments in which mouse blood was perfused over a collagen surface as described (17). Briefly, an area of 5 $\times$ 5 mm in the centre of a glass coverslip was coated with fibrillar Horm type-I collagen (50 $\mu\text{g}/\text{ml}$ for 20 minutes at 37°C), blocked with Hepes buffer pH 7.45 containing 137 mM NaCl, 5.6 mM glucose, 5 mM Hepes, 2.7 mM KCl, 2 mM MgCl_2 , 0.42 mM NaH_2PO_4 and 1% (w/v) bovine serum albumin (BSA), and then placed in a parallel plate flow chamber (slit depth 50 μm). Blood was incubated for 10 minutes with various inhibitors and/or fluorescent probes, placed in a syringe and perfused over the coverslip at a shear rate of 150-1000 s^{-1} for 4 minutes. Thereafter, the coverslip was perfused with Hepes buffer pH 7.45 containing 137 mM NaCl, 5.6 mM glucose, 5 mM Hepes, 2.7 mM KCl, 2 mM MgCl_2 , 0.42 mM NaH_2PO_4 , 0.1% (w/v) BSA with 2 mM CaCl_2 and 1 U/ml heparin plus fluorescently labeled annexin A5 (0.5 $\mu\text{g}/\text{ml}$) for 4 minutes. Phase contrast images and non-confocal fluorescence images of adherent platelets were recorded using a two camera system (15).

For flow experiments without coagulation, blood collected on PPACK/heparin was used. For experiments in the presence of coagulation, citrated blood was mixed during perfusion with 10% volume of 20 pM tissue factor in 200 mM CaCl_2 upon entry in the flow chamber. This flow protocol allows thrombin generation at millimolar free Mg^{2+} and Ca^{2+} concentrations (22). Platelet surface coverage of images was analyzed using ImagePro software (Media Cybernetics, Silver Spring, MD) for phase contrast images and Qanticell 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

For two-photon laser scanning microscopy (TPLSM), coverslips with thrombi were observed with a Bio-Rad 2100 multiphoton system (23). 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 using appropriate wavelength filters. Thrombi in flow chambers, double labeled with OG488-fibrinogen and AF647-annexin A5, were scanned at the end of perfusions. Optical sections were recorded in Kalman filtering mode; no further image processing was performed.

Thrombin generation measurements

Mouse PRP and platelet-poor plasma (PPP) collected on citrate were used to measure thrombin generation with the thrombogram method (24), which was adapted for murine plasma. Briefly, normalized PRP (1.5×10^8 platelets/ml) was preincubated with inhibitors for 10 minutes, followed by 10 minutes stimulation with collagen (5 μ g/ml). Subsequently, 40 μ l of PRP was added to a 96-well plate, containing per well 10 μ l tissue factor (6 pM) in buffer A (20 mM Hepes, 140 mM NaCl and 5 mg/ml BSA, pH 7.35). Final tissue factor concentration was 1 pM, at which thrombin formation depends on the presence of platelet-expressed phosphatidylserine (PS). Thrombin formation was started by adding 10 μ l of CaCl_2 (16.6 mM) and Z-GGR-AMC (2.5 mM) in buffer A. Changes in fluorescence were continuously measured at 37°C with a Fluoroscan Ascent well-plate reader (Thermolab Systems, Helsinki). First-derivative curves of accumulation of fluorescence were generated as described (25). Curves were not processed for α_2 -macroglobulin correction, as this parameter in murine blood is unknown; calibrations were with human thrombin. Assays were run in duplicates or triplicates.

Thromboxane A₂ measurements

Citrated PRP was prepared as described for thrombin generation measurements and activated with collagen in the presence and absence of SAM.G4. Then, PRP was centrifuged at 22,000 g and plasma was stored at -80°C. Thromboxane B₂, the stable breakdown product of thromboxane A₂, was measured with a competitive enzyme immunoassay (Cayman, Ann Arbor, MI). Samples were diluted to the optimal detection range, and were analyzed in duplicate according to the manufacturer's instructions. Thromboxane B₂ concentrations were calculated from calibration curves.

Statistical analysis

Data are given as means $\pm$ SEM. Differences between experimental groups were tested with the non-parametric Mann-Whitney U test, using the statistical package for social sciences (SPSS 11.0). Level of significance was set at 5%.

Results

Mice deficient in $\alpha 2$ -integrin show subtle changes in arterial thrombus formation in vivo

Injury of the mesenteric arterioles and venules of anesthetized mice was induced by topical application of FeCl_3 . This free radical-forming compound causes disruption of endothelial cells (26), resulting in collagen exposure in the sub-endothelial matrix (27). Before vascular injury, DCF-labeled platelets were injected into the mice. During and after FeCl_3 application, dynamic platelet accumulation in arterioles and venules was measured in vivo by fluorescence microscopy. In damaged arterioles, platelets adhered stable after 53 ± 21 seconds in wildtype and after 99 ± 36 seconds in $\alpha 2$ -null mice (mean $\pm$ SEM, $n = 11-13$, $p = 0.18$). In most arterioles of either animal type, thrombi were formed which gradually increased in size (Fig. 4.1). After 10 minutes, thrombi were present in 73% of wildtype arterioles and in 60% of $\alpha 2$ -null arterioles (Table 4.1), indicating that the thrombotic process persisted in the absence of the integrin. In most wildtype and knockout venules, platelets adhered almost instantly after FeCl_3 application, and assembled together to form often occlusive thrombi within 10 minutes (Fig. 4.1).

Continuous recording of the thrombus-forming process indicated that a significant proportion of the fluorescent thrombi in $\alpha 2$ -null arterioles was unstable, meaning that the aggregates moved to downstream sites after seconds or minutes (Fig. 4.2). This instability was rare in vessels from wildtype animals. Quantitation showed that significantly more thrombi were shed as emboli in arterioles from $\alpha 2$ -null mice than in vessels from wildtype mice (Fig. 4.2B).

Table 4.1: Percentage of vessels with thrombi and number of thrombi per arteriole and venule from wildtype and $\alpha 2$ -null mice at 2, 5 and 10 minutes after FeCl_3 application. Data are means ($n=15-20$).

	Wildtype			$\alpha 2$ -null		
	2 min	5 min	10 min	2 min	5 min	10 min
Arterioles with thrombi (%)	47	67	73	50	50	60
Venules with thrombi (%)	87	93	100	74	95	95
Mean number of thrombi per arteriole	4.6	4.7	4.9	5.0	8.1	6.0
Mean number of thrombi per venule	10.2	13.5	14.3	7.3	12.1	12.3

Figure 4.1. Thrombus formation in arterioles and venules from $\alpha 2$ -null mice. Damage of adjacent mesenteric arterioles and venules was induced by topical application of FeCl_3 at $t = 0$ seconds (baseline). Fluorescence images of aggregating DCF-labeled platelets in representative arterioles (upper panels) and venules (lower panels) at 2, 5 and 10 minutes after FeCl_3 application. Bar represents 50 μm .

The cumulative height of thrombi (measured as % of vessel diameter) increased similarly in time for arterioles from wildtype and $\alpha 2$ -null mice (Fig. 4.3A). Typically, when the height of individual thrombi was compared, arterioles from knockout animals had a much larger fraction of small (<25%) thrombi (Fig. 4.3B). While in vessels from wildtype mice the average number of thrombi did not change during the measurement time of 10 minutes, in $\alpha 2$ -null mice it peaked at 5 minutes and then decreased due to embolization (Table 4.1). Thus, in this *vivo* thrombosis model, $\alpha 2\beta 1$ appears to have a subtle but consistent role in the dynamics of thrombus formation, by stabilizing platelet adhesion and consolidating the formed thrombi.

Application of U46619 stabilizes thrombi in arterioles from $\alpha 2$ -null mice

We hypothesized that the absence of $\alpha 2\beta 1$ may cause platelets to release less secondary mediators such as thromboxane A_2 , which may affect both thrombus size and

Figure 4.2. Decreased thrombus stability in arterioles and venules from $\alpha 2$ -null mice. Damage of adjacent mesenteric arterioles and venules was induced by topical application of FeCl_3 at $t = 0$ seconds (baseline). (A) Fluorescence images of appearance and disappearance (embolization) of thrombi formed in an arteriole of an $\alpha 2$ -null mouse. Arrows indicate direction of flow; bar represents $50 \mu\text{m}$. For dynamic images, see supplemented videos. (B) Number of emboli produced in 10 minutes per vessel segment (means $\pm$ SEM, $n=4-9$). * $p = 0.08$.

stability. Indeed, we found that inhibition of $\alpha 2 \beta 1$ resulted in a reduced thromboxane B_2 production with $31 \pm 6 \%$ in collagen-stimulated PRP (mean $\pm$ SEM, $n=6$, $p=0.002$). To investigate whether reduced thromboxane formation could restrict the size and stability of thrombi in vivo, a solution of U46619 ($30 \mu\text{l}$ of $100 \mu\text{M}$) was topically applied on the mesentery of wildtype and $\alpha 2$ -null mice at 6 minutes after FeCl_3 application, a time point when thrombus formation was fully active. Both in wildtype and $\alpha 2$ -null arterioles - the latter with much stronger embolization tendency - this intervention stopped embolization, while it continued in control arterioles (Fig. 4.4A). Quantitative analysis of all data indicated that embolus formation was greatly reduced after U46619 application in $\alpha 2$ -null arterioles (Fig. 4.4B).

Figure 4.3. Smaller thrombi in arterioles from $\alpha 2$ -null mice. Damage of adjacent mesenteric arterioles and venules was induced by topical application of FeCl_3 at $t = 0$ seconds (baseline). (A) Cumulative thrombus height at 2, 5 and 10 minutes after FeCl_3 application (expressed as % of vessel diameter). Data are mean $\pm$ SEM ($n=11-13$). (B) Number of thrombi per vessel measured at 2, 5 and 10 minutes after FeCl_3 application. Thrombi were categorized as <25%, between 25 and 50%, and >50% of vessel diameter.

Reduced thrombus formation and platelet activity on collagen in flowing blood from $\alpha 2$ -null mice

Knowing that the process of thrombus formation in the damaged arterioles is driven by collagen exposure, thrombin generation and coagulation (27,28), we investigated how integrin $\alpha 2\beta 1$ contributes to collagen-dependent thrombus formation under conditions of shear and coagulation. First, PPACK/heparin-anticoagulated blood from wildtype and $\alpha 2$ -null mice was perfused over collagen at a shear rate of 1000 s^{-1} , i.e. representative of that found in arterioles (17). As observed before, platelets from $\alpha 2$ -null mice formed smaller sized, round-shaped aggregates (Fig. 4.5A). When compared to perfusion with wildtype blood, adhered $\alpha 2$ -null platelets showed 49% reduced staining with FITC-annexin A5, indicative for diminished PS exposure (Fig. 4.5A, B). Interestingly, during flow 6.8 ± 3.9 emboli were shed from $\alpha 2$ -null

Figure 4.4. Effect of U46619 application on thrombus formation in vivo. Damage of adjacent mesenteric arterioles and venules was induced by FeCl_3 at $t = 0$ seconds (baseline). U46619 or vehicle was topically applied ($30 \mu\text{l}$ of $100 \mu\text{M}$) at about 6 minutes after FeCl_3 application. (A) *Left panels:* cumulative number of emboli in a wildtype and $\alpha 2$ -null arteriole with many emboli, *right panels:* cessation of embolization after U46619 application (arrow) at embolizing wildtype and $\alpha 2$ -null arterioles. (B) Quantification of embolus production before (white bars) and after (grey bars) U46619 application; data are means $\pm$ SEM.

aggregates compared to 0.8 ± 0.8 emboli in wildtype blood (mean $\pm$ SEM, $n = 4-6$, $p = 0.046$), indicating that thrombus stability was also compromised in vitro. When $\alpha 2$ -null blood was perfused at a low shear rate of 150 s^{-1} , representative of that found in venules, this resulted in reduced aggregate formation and in less PS exposure of platelets (Fig. 4.5C).

To determine the contribution of coagulation, citrated blood was co-perfused with tissue factor/ CaCl_2 during flow over collagen (1000 s^{-1}). With wildtype blood, this resulted in formation of platelet-fibrin clots. Using blood from $\alpha 2$ -null mice, aggregate-containing clots were still formed but were again smaller in size, while PS exposure was decreased compared to wildtype (Fig. 4.5A, D). Similar results were obtained when wildtype blood was preincubated with the anti- $\alpha 2$ antibody, SAM.G4 (data not shown).

Absence of integrin $\alpha 2 \beta 1$ may lead to diminished activation of integrin $\alpha \text{IIb} \beta 3$, restricting the size of thrombi in $\alpha 2$ -null blood. To investigate this, fluorescently labeled fibrinogen in combination with annexin A5 was added to the blood prior to perfusion. The thrombotic process was visualized using the high resolution power of TPLSM. In thrombi from $\alpha 2$ -null blood less fibrinogen staining was present, pointing to reduced activation of $\alpha \text{IIb} \beta 3$ (Fig. 4.6). Interestingly, the annexin A5 labeling did not co-localize with fibrinogen labeling, but restricted to single, collagen-bound platelets around the aggregates. This heterogeneity within thrombi was not influenced by the presence or absence of $\alpha 2 \beta 1$, indicating it is not controlled by this integrin.

Figure 4.5. Reduced thrombus formation in flowing blood from $\alpha 2$ -null mice in the absence or presence of coagulation. (A) PPACK/heparin-anticoagulated blood was perfused over a collagen surface or citrated blood was perfused over collagen together with tissue factor (2 pM, f.c.) and CaCl_2 (20 mM) to allow coagulation. Perfusion time was 4 minutes at a shear rate of 1000 s^{-1} , followed by 4 minutes wash with buffer in the presence of fluorescently labeled annexin A5 (0.5 $\mu\text{g}/\text{ml}$). *First column:* representative phase-contrast images after buffer perfusion without coagulation; *second column:* images of FITC-annexin A5 fluorescence without coagulation; *third column:* representative phase contrast images after buffer perfusion with coagulation; *fourth column:* images of FITC-annexin A5 fluorescence with coagulation. Phase-contrast ($120 \times 120 \mu\text{m}$) and fluorescence ($150 \times 150 \mu\text{m}$) images are representative of 5-12 experiments. (B-D) Percentages of area coverage of all platelets (phase contrast, white bars) or PS-exposing platelets (annexin A5-FITC, grey bars) after 4 minutes of perfusion in the absence of coagulation at shear rates of 1000 s^{-1} (B) or 150 s^{-1} (C) or in the presence of coagulation at 1000 s^{-1} (D). Data are means $\pm$ SEM, * $p \leq 0.05$, + $p = 0.06$.

In these flow studies, the reduced thrombus formation and $\alpha \text{IIb}\beta 3$ activation may be also mediated by impaired autocrine release of thromboxane A_2 . To investigate this, PPACK/heparin-anticoagulated blood was co-perfused with a solution of U46619. With both wildtype and $\alpha 2$ -null blood, this resulted in an about 2-fold increased platelet deposition, whereas typically PS exposure did not increase (Fig. 4.7). Similar results were obtained with U46619 co-perfusion using wildtype blood that was preincubated with anti- $\alpha 2$ antibody (data not

Figure 4.6. Reduced fibrinogen binding in flowing blood from $\alpha 2$ -null mice. PPACK/heparin-anticoagulated blood was perfused over a collagen surface as described for Fig. 4.5. Prior to perfusion OG488-fibrinogen (0.25 mg/ml) and AF647-annexin A5 (0.5 μ g/ml) were added to the blood. TPLSM images were taken of OG488-fibrinogen and AF647-annexin A5 (indicated by Δ) fluorescence (77 $\times$ 77 μ m).

Figure 4.7. Effect of U46619 co-infusion on thrombus formation in flowing whole blood from wildtype and $\alpha 2$ -null mice. PPACK/heparin-anticoagulated wildtype blood was perfused over collagen for 4 minutes at a shear rate of 1000 s^{-1} , as indicated for Fig. 4.5. Thromboxane A_2 analogue U46619 (1 μ M, f.c.) was co-infused where indicated. (A) Representative phase-contrast (panels 1 and 3, 120 $\times$ 120 μ m) and FITC-annexin A5 (panels 2 and 4, 150 $\times$ 150 μ m) images after buffer perfusion. Images are representative of 6-10 experiments. (B) Percentages of area coverage of all platelets (phase contrast, white bars) or PS-exposing platelets (FITC-annexin A5, grey bars) perfused for 4 minutes at 1000 s^{-1} with or without U46619 co-infusion. Data are means $\pm$ SEM ($n=3-10$). * $p \leq 0.05$, + $p < 0.06$.

shown). Thus, insufficient thromboxane A_2 production can explain reduced thrombus formation in the absence of $\alpha 2 \beta 1$.

Figure 4.8. Contribution of $\alpha 2\beta 1$ to collagen-mediated thrombin generation in PRP. Mouse PRP was preincubated with vehicle (control) or 5 $\mu\text{g/ml}$ collagen for 10 minutes, after which coagulation was initiated with 1 pM tissue factor (f.c.) and 16.6 mM CaCl_2 . (A) Representative thrombin generation curves of wildtype and $\alpha 2$ -null mice in the presence or absence of collagen. (B) Effect of collagen on thrombin peak height, (means $\pm$ SEM, $n=3-4$). (C) Effect of collagen on time to peak. Collagen compared to control: * $p \leq 0.05$, + $p < 0.08$; $\alpha 2$ -null compared to wildtype: # $p \leq 0.05$

Diminished collagen-enhanced thrombin generation in PRP in the absence of $\alpha 2\beta 1$

The results of reduced PS exposure point to a role of $\alpha 2\beta 1$ in collagen-induced platelet procoagulant activity. This was directly measured in thrombin generation experiments with PRP. Upon triggering with tissue factor/ CaCl_2 , collagen enhanced the thrombin-forming process in PRP from wildtype and knockout mice (Fig. 4.8A). However, in $\alpha 2$ -null PRP this collagen effect was smaller (Fig. 4.8B), and the time to maximal thrombin generation was delayed (Fig. 4.8C). Preincubation of wildtype PRP with $\alpha 2$ antibody SAM.G4 resulted in similar changes (data not shown). These results indicate that $\alpha 2\beta 1$ supports and enhances the platelet function in thrombin generation and, thus, promotes the coagulation process.

Discussion

In this report, we investigated the function of $\alpha 2\beta 1$ in collagen-induced thrombus stability and growth. In an *in vivo* model where thrombosis was induced by exposure of the collagen-containing extracellular matrix, thrombi in $\alpha 2$ -null arterioles were smaller in size than in wildtype arterioles, and had a significantly increased tendency to detach from the vessel and form emboli. In agreement with this, the *in vitro* flow experiments indicate that $\alpha 2\beta 1$ has a role in the consolidation of thrombi, although the integrin is dispensable for thrombus formation as such. The integrin $\alpha 2\beta 1$ appears to be involved in release of autocrine mediators, integrin activation and procoagulant activity. These are all platelet reactions known to be triggered primarily by GPVI (15,17,29), and thus point to support of GPVI activity via $\alpha 2\beta 1$. Importantly, we now describe that $\alpha 2\beta 1$ retains this effect in the presence of flow and (tissue factor-induced) thrombin generation and coagulation. This knowledge is particularly relevant, since thrombin and fibrin play a significant role in the current (M. Kuijpers, submitted, (26)) and other thrombosis models (30). Accordingly, in the *in vivo* situation, $\alpha 2\beta 1$ can regulate thrombus size and stability via a number of pathways: enhanced release of autocrine agents, increased $\alpha IIb\beta 3$ activation, and enhanced procoagulant activity of collagen-bound platelets, resulting in fibrin deposition.

The absence of the integrin $\alpha 2$ -subunit from the currently used $\alpha 2$ -null mice was confirmed by flow cytometry and the levels of $\beta 1$ were reduced by 30% (see also (11)). The expression levels of other glycoproteins were not altered in mutant platelets. In mice with a conditional $\beta 1$ -deficiency, dependent on Cre-mediated excision (1), we observed the appearance of loose platelet aggregates on the collagen surface (17). In the present study, we see that $\alpha 2\beta 1$ -deficient aggregates are intrinsically less stable structures, with increased tendency to embolize. Such instability might also explain the partially reduced aggregate formation – especially at high shear – seen with blood from another strain of $\alpha 2$ -deficient mice (19).

The moderate effect of $\alpha 2$ deficiency sharply contrasted to the strongly delayed and reduced thrombus formation of GPVI-deficient platelets in the same thrombosis model (27). In various collagen-induced thrombosis models $\alpha 2$ -deficiency did not result in impaired thrombus formation (12,14,31). However, in some of these models, a moderate delay in the thrombotic process has been observed (14), which may be compatible with the presently detected thrombus instability. The present continuous recordings of the thrombotic process in response to vascular damage point to a subtle but important function of $\alpha 2\beta 1$: the integrin appears to determine the growth rate and stabilization of the thrombi, rather than to trigger the thrombotic process as such. Keeping in mind that platelets may react differently when encountered with a different stimulus, differences between the two $\alpha 2$ -deficient mouse strains may be only minimal.

The $\alpha 2\beta 1$ integrin has a moderate but qualitatively important effect to stabilize thrombi and reduce embolization, most likely by enhancement of GPVI-dependent mechanisms.

The reduced thromboxane A_2 formation provides a mechanistic explanation for earlier studies with $\alpha 2$ -null mice, which showed a delay in collagen-induced aggregation in the absence of the integrin (11). We found that the thromboxane A_2 analogue U46619 could normalize the thrombus-forming process in $\alpha 2$ -deficient mice in vivo and under flow in vitro. Application of this compound increased platelet aggregation in vitro and immediately stopped the embolizing process in arterioles in vivo. Noteworthy, the platelet TP receptors for thromboxane A_2 couple to G_q and $G_{12/13}$, both of which signaling proteins have been implicated in the formation of stable murine thrombi (32-34). Since U46619 can induce activation of $\alpha 2\beta 1$ (5) and $\alpha IIb\beta 3$ (35), our results provide additional support to the conclusion that $\alpha 2\beta 1$ plays a role in shear-resistant platelet adhesion particularly under conditions of weak integrin activation (31). Furthermore, thromboxane release mediates ADP secretion (36), implicating that absence of $\alpha 2\beta 1$ will indirectly suppress ADP-evoked $\alpha IIb\beta 3$ activation and platelet aggregation. Together with reduced physical contact with collagen in the absence of $\alpha 2\beta 1$, this can enforce the detachment of formed thrombi.

An important question is why such a thrombus-destabilizing effect is not observed with aspirin, which completely suppresses thromboxane formation. One explanation is that aspirin will affect the activation of all platelets and not only those that adhere to collagen, thus leading to an overall reduced capability of the platelets to aggregate. Further, it should be considered that $\alpha 2\beta 1$ is not the only platelet protein regulating thrombus stability. Increased platelet-platelet interaction is achieved, e.g., via ADP receptors (21,37), CD40L (38), ephrins (39), SLAM (40), and also fibronectin (41).

In summary, the present study reveals that (platelets from) $\alpha 2$ -null mice have a subtle but clinically relevant phenotype upon vascular damage, in which unstable thrombi have an increased tendency to embolization, likely due to three mechanisms: reduced release of autocrine mediators, reduced platelet activation and lower coagulation activity. This thrombus-stabilizing role of $\alpha 2\beta 1$ is likely to be relevant for the clinic, as antithrombotic therapy directed against $\alpha 2\beta 1$ might be beneficial in reducing the size of thrombi, but also lead to an undesired effect of increased embolization.

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Chapter 5

Facilitating roles of murine platelet glycoprotein Ib and $\alpha\text{IIb}\beta 3$ in phosphatidylserine exposure during vWF-collagen-induced thrombus formation

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Abstract

Vessel wall damage exposes collagen fibres, to which platelets adhere directly via the collagen receptors glycoprotein (GP)VI and integrin $\alpha 2\beta 1$, and indirectly by collagen-bound von Willebrand factor (vWF) via GPIb-V-IX and integrin $\alpha IIb\beta 3$ receptor complexes. Platelet-collagen interaction under shear stimulates thrombus formation in two ways, by integrin-dependent formation of platelet aggregates and by surface exposure of procoagulant phosphatidylserine (PS). GPVI is involved in both processes complemented by $\alpha 2\beta 1$. In mouse blood flowing over collagen, we investigated the additional role of platelet-vWF binding via GPIb and $\alpha IIb\beta 3$. Inhibition of GPIb as well as blocking of vWF binding to collagen reduced stable platelet adhesion at high shear rate. This was accompanied by delayed platelet Ca^{2+} responses and reduced PS exposure, while micro-aggregates were still formed. Inhibition of integrin $\alpha IIb\beta 3$ with JON/A antibody, which blocks $\alpha IIb\beta 3$ binding to both vWF and fibrinogen, reduced PS exposure and aggregate formation. The JON/A effects were not enhanced by combined blocking of GPIb-vWF binding, suggesting a function for $\alpha IIb\beta 3$ downstream of GPIb. Typically, with blood from FcR γ -chain +/- mice, expressing 50% of normal platelet GPVI levels, GPIb blockage almost completely abolished platelet adhesion and PS exposure. Together, these data indicate that, under physiological conditions of flow, both adhesive receptors, GPIb and $\alpha IIb\beta 3$, facilitate GPVI-mediated PS exposure by stabilising platelet binding to collagen. Hence, these glycoproteins have an assistant procoagulant role in collagen-dependent thrombus formation, which is most prominent at reduced GPVI activity and is independent of thrombin.

Introduction

Collagen fibres, exposed upon vessel wall damage, are strongly platelet-adhesive. Binding of platelets to collagen triggers a chain of activating events and leads to the assembly of platelet aggregates and the formation of fibrin-containing thrombi. The thrombus-forming reaction is essential in haemostasis, but detrimental in the progression of atherothrombosis and plaque rupture. This process of thrombus formation has widely been studied *in vitro*, using flow chambers where whole blood is perfused over a collagen-containing surface at (patho)physiological shear conditions, even in the absence of coagulation. From this work, it has appeared that multiple receptors are involved in the interaction of platelets with collagen.

Collagen rapidly adsorbs von Willebrand factor (vWF), which is present in plasma as a multimeric protein (1). vWF acts as a bridging molecule in platelet-collagen interactions, since it can bind to both collagen and the receptor complexes, glycoprotein (GP)Ib-V-IX and integrin $\alpha IIb\beta 3$, through its A3, A1 and C1 domains, respectively. High shear stress induces conformational changes of vWF, which result in a reversible interaction with GPIb (2). This reduces the velocity of platelets flowing over collagen-bound vWF and results in transient

attachment to the collagen surface (3). Subsequent, irreversible binding to vWF is mediated by the α IIb β 3 integrin. This integrin also needs conformational changes for ligand interaction (inside-out signalling), which can be achieved e.g. by vWF-GPIb binding (4,5) or following stimulation of the ADP, thromboxane A_2 or collagen receptors (6,7). Both GPIb and α IIb β 3 also mediate vWF/fibrinogen dependent platelet aggregate formation under shear (7,8).

Direct platelet-collagen contact is established by the collagen receptors, GPVI and integrin α 2 β 1 (8-10). GPVI acts as a major signalling receptor, while α 2 β 1 is required for stable adhesion to collagen. Ligand-induced clustering of GPVI results in its non-covalent association with the Fc receptor (FcR) γ -chain, which leads to signalling via tyrosine-phosphorylation (11,12). As a result, phospholipase $C\gamma$ 2 becomes phosphorylated and activated, which causes a prolonged increase in cytosolic $[Ca^{2+}]_i$ (13). This Ca^{2+} response contributes to the release of feedback agonists such as ADP and thromboxane A_2 , which sustain platelet aggregate formation.

Previous in vivo and in vitro flow studies with mice have indicated that the α 2 β 1 integrin is dispensable for platelet-collagen adhesion and subsequent thrombus formation (14,15). Using mice deficient in α 2 β 1 or GPVI, it was furthermore shown that under high, arterial shear conditions this integrin enhances GPVI signalling and, thereby, stabilises the platelet aggregates on collagen (16). This has led to a model of interplay between the collagen receptors, in which the α 2 β 1 integrin supported by release products functions to enhance GPVI-induced platelet activation. Such a model is now proposed by several groups (10,17-19), although it is still unclear to what extent the synergistic effect of α 2 β 1 on GPVI is due to intracellular signaling by the integrin itself (9,20) or to stabilization of collagen-GPVI contact by an activated integrin form.

Apart from aggregate formation, platelet-collagen interaction stimulates the coagulation process. Collagen or collagen-related peptide provoke, in a Ca^{2+} dependent way, exposure of phosphatidylserine (PS) at the platelet outer membrane surface (21,22). The availability of PS greatly potentiates the conversion of prothrombin into coagulant thrombin and, thus, enhances thrombin generation (23-25). PS exposure is one of the early platelet responses in shear-dependent thrombus formation upon perfusion of human or murine blood over vWF/collagen (16,19). In both species, it is a consequence of GPVI activity, while α 2 β 1 mainly potentiates the GPVI effect. There is evidence, mostly from experiments with coagulating plasma, that GPIb, α IIb β 3 and also vWF have discrete functions in stimulating platelet-dependent thrombin generation (26,27). However, whether these factors also contribute to the PS-exposing, procoagulant response of platelets interacting with vWF/collagen is still unresolved.

Since platelet-collagen interaction under high shear is dependent on vWF, GPIb and α IIb β 3, we focused here on the contribution of these proteins in the generation of the platelet procoagulant response under flow conditions. Using real-time video imaging techniques, we

show that at high shear rates GPIb and vWF – and downstream of it $\alpha\text{IIb}\beta 3$ – enhance GPVI-mediated PS exposure, especially under conditions of reduced GPVI expression and activity. This novel procoagulant effect of GPIb is independent of thrombin action and is additional to its function in platelet aggregation. It is likely to be of physiological relevance, given the large variation in GPVI expression between humans. As $\alpha 2\beta 1$ also stimulates GPVI activity, our results reveal receptor interplay in the regulation of platelet procoagulant activity that is not limited to collagen receptors, but extends to the GPIb-V-IX complex.

Methods

Animals

Pathogen-free C57BL/6 mice were obtained from Charles River (Maastricht, The Netherlands); mice deficient in FcR γ -chain (28) were obtained from Taconics (Germantown, NY, USA). Heterozygous FcR γ -chain deficient mice were generated by crossing wildtype mice with homozygous deficient mice of C57BL/6 background (14). Reduced expression of both GPVI and FcR γ -chain was checked at the protein level by Western blots, and by flow cytometric measurement of surface expression of GPVI, as described (29). Experimental protocols were ethically approved by the animal care and use committee of the University of Maastricht.

Materials

Fab fragments of rat anti-mouse antibodies were produced and modified in our laboratories (30). This concerned p0p/B antibody, which binds to the vWF binding site on GPIb α ; and JON/A antibody, which selectively binds to activated integrin $\alpha\text{IIb}\beta 3$ and inhibits integrin interaction with both vWF and fibrinogen. Polyclonal rabbit anti-human vWF antibody, cross-reacting with mouse vWF, was obtained from Dako (Glostrup, Denmark), and FITC-labeled goat anti-rabbit IgG (GAR-FITC) was from Southern Biotech (Birmingham, AL, USA).

Fibrillar Horm collagen from equine tendon (Horm type-I) was purchased from Nycomed (Munich, Germany). Apyrase, high molecular weight heparin and MRS2179, an antagonist of the P2Y₁ purinergic receptor, came from Sigma (St. Louis, MO, USA). Annexin V (annexin A5) labeled with fluorescein isothiocyanate (FITC) was from Nexins Research (Hoeven, The Netherlands); calcein and Fluo-3 acetoxymethyl esters, Pluronic F-127, and phalloidin labeled with Texas red X were from Molecular Probes (Leiden, the Netherlands); H-Phe-Pro-Arg chloromethyl ketone (PPACK) was from Calbiochem (La Jolla, CA, USA). AR-C69931MX, an antagonist of the P2Y₁₂ purinergic receptor, was kindly provided by AstraZeneca

R&D (Charnwood, UK). Recombinant saratin was produced in the yeast *Hansenula polymorpha*, as described (31). Sources of other chemicals are mentioned elsewhere (32).

Platelet preparation and labeling

Under full anesthesia, mice were bled retro-orbitally and sacrificed by cervical dislocation. The blood (1 volume) was collected in 0.5 volumes of saline containing 5 units ml^{-1} heparin and 40 μM PPACK. Anticoagulated blood (1 volume) was diluted in Hepes buffer pH 7.45 (0.5 volumes) containing 137 mM NaCl, 5.6 mM glucose, 5 mM Hepes, 2.7 mM KCl, 2 mM MgCl_2 , 2 mM CaCl_2 , 0.42 mM NaH_2PO_4 , 0.1% (w/v) bovine serum albumin (BSA), which was supplemented with 1 unit/ml heparin. The blood was used within 2 h. Where indicated, blood was incubated with 2.5 μM calcein acetoxymethyl ester for 30 min, as described (14).

Washed murine platelets in Hepes buffer pH 7.45 were prepared from platelet-rich plasma, as described for rat blood (33). For Ca^{2+} measurements, washed platelets were incubated with Fluo-3 acetoxymethyl ester (5 μM) in the presence of Pluronic F-127 (0.2 mg/ml) and apyrase (0.1 units ADPase/ml) at room temperature for 45 min.

Real-time video imaging

Adhesion experiments under flow conditions were performed with mouse blood anticoagulated with PPACK and heparin, basically as described earlier (14). Briefly, coverslips (24 × 60 mm) were partly coated with Horm collagen type I fibers (1.25 mg/50 mm^2), rinsed with saline, and blocked with Hepes buffer containing 1% (w/v) BSA. Coverslips were placed in a parallel-plate transparent flow chamber (slit depth of 50 μm), which was connected by polyethylene tubing (diameter 1 mm), and mounted on an inverted Nikon (Tokyo, Japan) microscope. To prevent coagulation, chamber and tubing were pre-washed with Hepes buffer containing 1 unit/ml heparin. Blood was perfused through the flow chamber using a 1 ml syringe and a pulse-free pump, at the desired shear rate (1000 or 1500 s^{-1}) for 4 min. During perfusion, high-resolution microscopic images of transmission or fluorescence (Fluo-3 or calcein) were recorded in real-time with a Visitech imaging system (Sunderland, UK). Digital images were captured with two parallel-placed intensified, CCD cameras, recording infrared (0.3 Hz) and epifluorescence (5 Hz) light at desired wavelengths (21). After perfusion, the flow chamber was rinsed with Hepes buffer supplemented with 1 unit/ml heparin at the desired shear rate. Exposure of PS was detected with FITC-labeled annexin A5 (0.5 $\mu\text{g/ml}$) added to the rinse buffer.

Where indicated, blood was incubated for 10 min prior to perfusion with a saturating concentration of p0p/B or JON/A Fab fragment (40 $\mu\text{g/ml}$), saratin (10 $\mu\text{g/ml}$) or MRS2179 (20 μM) plus AR-C69931MX (50 μM). These antagonists were also added to the rinse buffer. To measure changes in cytosolic $[\text{Ca}^{2+}]$, Fluo-3-labelled platelets from mice of the same genotype

were added to the anticoagulated blood, so that 5-10% of total platelets were labeled. Labeling a small fraction of the platelets in blood had the advantage that individual, collagen-bound platelets could be distinguished from each other, even when trapped in multi-platelet aggregates.

Real-time changes in Fluo-3 fluorescence from single platelets, recorded during the flow experiment, were analyzed with Quanticell software. Changes in Fluo-3 fluorescence of individual platelets were converted into levels of $[Ca^{2+}]_i$ using a pseudo-ratio calibration procedure, exactly as described (34). Traces representing averaged Ca^{2+} responses were constructed from time-adjusted responses of at least 13 single platelets that could be observed for at least 60 s (2-4 mice per condition).

Confocal fluorescence microscopy

Immediately after perfusion, collagen-coated coverslips with aggregates were removed from the flow chamber, fixed with 2% formaldehyde during 15 min, and rinsed with tap water. Further procedures were performed in a humid chamber at room temperature. The samples were first blocked with 15% (w/v) BSA in phosphate-buffered saline (PBS) for 30 min. Samples were then incubated with rabbit anti-human vWF antibody (10 μ g/ml) for one h, washed in PBS 3 times for 5 min, and stained with GAR-FITC (10 μ g/ml) for one h. Samples were counterstained for actin to stain all platelets. After a triple wash in PBS and permeabilisation with 0.005% SDS in PBS for 10 min, they were blocked with 1% BSA in PBS. The samples were then incubated with Texas red-labeled phalloidin (3 units/ml) in blocking buffer for 1 h and, after another wash, they were mounted in 9 volumes of glycerol plus 1 volume of 0.2 M Tris-HCl, 0.02% NaN_3 with 2% 1,4-diazabicyclo(2,2,2)-octane (Dabco), pH 8.0. Double stained aggregates were observed with a BioRad MRC-600 confocal scanning laser microscope (BioRad, Richmond, CA, USA) equipped with a krypton/argon mixed gas laser and a red diode laser (Ion Laser Technology, Salt Lake City, UT, USA) with two separate wavelengths for excitation of fluorescein (488 nm) and Texas red X (568 nm).

Image analysis

Data were compared off-line from at least nine different randomly chosen, microscopic images, taken at the collagen surface. Platelet deposition was determined from phase-contrast images, as described before (16). Area coverage by platelets staining with calcein or FITC-annexin A5 fluorescence was determined with Quanticell software (Visitech). Procoagulant area was corrected for fluorescent glare in the optics by comparison of camera images in fluorescence and transmission modes.

Statistical analysis

Data were tested on significance using the statistical package for social sciences (SPSS 11.0, Chicago, IL, USA). Significant differences compared to control condition were evaluated with a Mann-Whitney U test.

Results

Roles of GPIb and vWF in PS exposure of platelets adhering to collagen under shear

Whole blood from wildtype mice, anticoagulated with PPACK/heparin, was used to investigate the contribution of GPIb-vWF interaction to platelet deposition on collagen and subsequent PS exposure. The blood was perfused over immobilized type-I collagen at an intermediate, arterial wall shear rate (1000 s^{-1}) to achieve GPIb dependency (16). Video microscopy showed that most wildtype platelets firmly adhered to the collagen fibers. Part of these platelets assembled to platelet aggregates, while others remained single and transformed

Figure 5.1. Effect of blocking GPIb on platelet deposition and PS exposure upon flow over vWF/collagen. Whole blood from C57BL/6 wildtype mice was perfused over type I collagen at a wall shear rate of 1000 s^{-1} . A, C, E, phase-contrast microscopic images ($120 \times 120\text{ }\mu\text{m}$) after 4 min of perfusion. B, D, F, fluorescence images ($150 \times 150\text{ }\mu\text{m}$) after staining with FITC-labeled annexin A5. A and B, untreated control blood. C and D, blood pre-treated with $40\text{ }\mu\text{g/ml}$ anti-GPIb p0p/B Fab fragment (10 min). E and F, blood pre-treated with $10\text{ }\mu\text{g/ml}$ saratin (10 min).

Figure 5.2. Roles of GPIb and shear stress in platelet deposition and PS exposure. Whole blood from wildtype mice was perfused over collagen for 4 min at a shear rate of 1000 or 1500 s⁻¹. Effect of pre-treatment with p0p/B Fab (40 µg/ml) or saratin (10 µg/ml) on area coverage of all platelets (white bars) and PS-exposing platelets (gray bars). Data are percentages of control condition at indicated shear rate (mean ± S.E.M., n = 6-20). *p < 0.05 compared to control.

into round, blebbing structures. The latter morphological change is an indicator for PS exposure and procoagulant activity (16,22). The deposition of platelets, measured as surface area coverage, increased about linearly for at least 6 min. After 4 min perfusion, $25 \pm 2\%$ (mean ± S.E.M., n = 32) of the surface was covered with platelets (Fig. 5.1A). A similar, linear increase in platelet deposition was observed, when blood containing calcein-labeled platelets was perfused over the collagen surface (data not shown, but see (14)). Staining with FITC-labeled annexin A5 showed that, at the 4-min end point, a considerable proportion of single, collagen-binding platelets had exposed PS, with an area coverage with fluorescence of $9 \pm 1\%$ (n = 20) (Fig. 5.1B). Experiments where FITC-annexin A5 was added to the blood indicated that PS exposure of deposited platelets started after a lag time of 2 min and then increased continuously up to 10 min.

To block GPIb-vWF interaction, wildtype blood was pre-incubated with a saturating concentration of anti-GPIbα p0p/B Fab fragment (40 µg/ml), which specifically abolishes the GPIb binding to mouse vWF (30). This treatment led to increased translocation velocity of platelets over the collagen surface and to diminished stable adhesion, while micro-aggregates were still formed (Fig. 5.1C). Platelet deposition was reduced by p0p/B to $66 \pm 13\%$ (n = 5) of the control condition. The number of single, PS-exposing platelets (Fig. 5.1D) was more strongly reduced to about 20% (p = 0.02) of control (Fig. 5.2A). As an alternative way to eliminate the contribution of vWF, blood was treated with 10 µg/ml saratin, a leech protein which blocks vWF binding to collagen (31). Again, platelets were moving over the collagen surface and made fewer stable contacts (Fig. 5.1E). With some of the platelets still forming aggregates, the total area coverage was $34 \pm 6\%$ (n = 11) of control. The number of PS-exposing platelets (Fig. 5.1F) was similarly reduced to $30 \pm 13\%$ of control (Fig. 5.2A).

Figure 5.3. Effect of saratin on vWF binding to collagen and platelets. Blood from wildtype mice was perfused over collagen in the absence (A) or presence (B) of saratin (10 $\mu\text{g/ml}$) at 1000 s^{-1} for 4 min. Representative confocal fluorescence images are given after fixation with 2% formaldehyde and staining for vWF and actin. Micrographs show contours of platelets (actin) and deposited vWF as brighter punctuated spots; arrow indicates vWF covered collagen fiber. Image sizes are 20 $\times$ 25 μm .

To achieve higher GPIb dependency, additional experiments were performed at a higher shear rate of 1500 s^{-1} . In this case, blocking GPIb with p0p/B further reduced area coverage of platelets to $15 \pm 6\%$ and of annexin A5-binding platelets to $26 \pm 7\%$ of the control (Fig. 5.2B). Similarly, the presence of saratin decreased the coverage by platelets to $21 \pm 5\%$ and by annexin A5 fluorescence to $36 \pm 17\%$ of control. Thus, inhibition of the GPIb-vWF interaction by GPIb blocking (p0p/B) or absence of vWF (saratin) caused a substantial but incomplete reduction in platelet deposition and PS exposure at shear rates up to 1500 s^{-1} .

The similarities in effects of p0p/B and saratin treatment suggested that GPIb interaction with collagen-bound vWF could control PS exposure by allowing stable platelet adhesion. To confirm this, platelets on collagen-coated coverslips were stained post-perfusion for vWF and then counterstained with phalloidin to visualize actin filaments. In the control situation, confocal microscopy showed bright vWF staining on the collagen fibers as well as on platelet aggregates, where it appeared as clustered spots (Fig. 5.3A). With saratin present, the collagen fibers no longer stained for vWF, whereas the platelets in aggregates had unaltered vWF staining (Fig. 5.3B).

Role of GPIb in Ca^{2+} responses of platelets adhering to vWF-collagen

The activation state of platelets interacting with vWF/collagen was investigated by adding 10% Fluo-3 labeled murine platelets to the blood. This labeling of platelets did not influence collagen-induced aggregate formation *in vitro*, as measured by aggregometry (data not shown). Under control conditions of flow (1000 s^{-1}), the large majority of Fluo-3 labeled platelets stably attached to collagen fibrils. The platelets showed a potent and prolonged Ca^{2+} response (Fig. 5.4A), as is required for PS exposure (21). Traces from single platelets indicated a rapid, prolonged rise in $[\text{Ca}^{2+}]_i$ that was occasionally preceded by one high spike in $[\text{Ca}^{2+}]_i$ (Fig. 5.4B). As we have shown before, this Ca^{2+} signal under flow conditions is almost completely dependent on GPVI signaling, with minute, spiking $[\text{Ca}^{2+}]_i$ increases remaining in the absence of GPVI (16).

Figure 5.4. Potentiating role of GPIIb in Ca^{2+} response of collagen-adhering platelets under shear. Whole blood was supplemented with 10% Fluo-3-loaded platelets, and perfused over collagen as indicated for Fig. 5.1. Upper panels show averaged changes in $[\text{Ca}^{2+}]_i$ of 13-32 platelets, observed during at least 60 s, from at least two different mice. Lower panels show changes in $[\text{Ca}^{2+}]_i$ of representative single platelets. Blood was either untreated (A, B) or incubated for 10 min prior to perfusion with 40 $\mu\text{g}/\text{ml}$ p0p/B Fab (C, D) or 10 $\mu\text{g}/\text{ml}$ saratin (E, F).

When the blood was treated with anti-GPIIb p0p/B, many of the Fluo-3 labeled platelets moved over the surface before stopping. Once adhered, the Ca^{2+} signals of many of the p0p/B-treated platelets were reduced in comparison to the control (Fig. 5.4C, D). Individual cells often showed a series of Ca^{2+} spikes before reaching a maximal level, which was on average reduced by 35% ($n = 13-26$ platelets, $p = 0.02$ unpaired, measured after 60 s). Treatment of the blood with saratin caused similar but not identical effects. Again, many of the Fluo-3 labeled platelets moved over the surface before adhering stably. The Ca^{2+} responses of these platelets were typically delayed (Fig. 5.4E, F). The median lag time (time measured from first collagen contact until $[\text{Ca}^{2+}]_i$ rise) was notably increased from 9 to 13 s compared to control platelets. Maximal $[\text{Ca}^{2+}]_i$ rises after the delay were unchanged with saratin. These data indicated that the blocking of GPIIb and absence of vWF delayed and destabilized platelet-collagen contact and subsequent signaling. This points to a stimulating role of GPIIb and vWF in GPVI-induced Ca^{2+} signaling. Since PS exposure relies on a persistent $[\text{Ca}^{2+}]_i$ rise, the reduced or delayed Ca^{2+} signal with p0p/B or saratin can explain the lower PS exposure under these flow conditions (see Fig. 5.2A).

Figure 5.5. Role of integrin α IIb β 3 in platelet deposition and PS exposure. Whole blood from wildtype mice was perfused over type I collagen as indicated for Fig. 5.1. Upper panels: representative phase contrast images ($120 \times 120 \mu\text{m}$) after 4 min of perfusion. Lower panels: fluorescence images ($150 \times 150 \mu\text{m}$) after staining with FITC-labeled annexin A5. Blood was untreated (A, B), or treated with 40 $\mu\text{g/ml}$ anti- α IIb β 3 JON/A Fab fragment alone (C, D), with JON/A plus 40 $\mu\text{g/ml}$ p0p/B Fab fragment (E, F), or with 10 $\mu\text{g/ml}$ saratin (G, H) 10 min prior to perfusion. I, Surface area coverage of all platelets (white bars) and PS-exposing platelets (gray bars) of conditions described in panels A-H. Data are given as percentages of control condition (mean values $\pm$ S.E.M., $n = 3-11$). * $p < 0.05$ compared to control.

Role of integrin α IIb β 3 and GPIb in PS exposure of platelets adhering to vWF-collagen

Stable adhesion to vWF requires platelet interaction with both GPIb-V-IX and activated integrin α IIb β 3 (5,35-37). To interfere with the contribution of α IIb β 3, the murine blood was incubated with Fab fragments (40 $\mu\text{g/ml}$) of JON/A, which is a unique, recently characterized antibody that blocks α IIb β 3 binding sites for vWF and fibrinogen (38,39). This is in contrast to anti-aggregatory peptide inhibitors of α IIb β 3, which only react with the fibrinogen binding site. Upon perfusion of blood in the presence of JON/A, platelets adhered to vWF/collagen as single cells or two-layered micro-aggregates (Fig. 5.5A-D). Surface coverage with platelets was

reduced to $44 \pm 15\%$ ($n = 7$) of control (Fig. 5.5I). PS exposure of platelets on collagen was greatly lowered to $7 \pm 3\%$ of control. In comparison, when effects of autocrine ADP were blocked with the P2Y₁ and P2Y₁₂ receptor antagonists, MRS2179 (20 μ M) and AR-C69931MX (50 μ M), respectively, this gave a similar decrease in platelet deposition (area coverage with platelets to $48 \pm 9\%$ of control; not shown), but less strong decrease in PS exposure ($38 \pm 17\%$ of control).

To test whether the inhibitory effects of JON/A were dependent on GPIb and vWF, combined treatments were carried out with either p0p/B or saratin (Fig. 5.5E-H). The addition of p0p/B did not further reduce platelet deposition nor PS exposure in comparison to JON/A alone (Fig. 5.5I). Perfusion with saratin plus JON/A resulted in a somewhat lower platelet deposition, and an unchanged reduction in PS exposing platelets. These data suggest that GPIb and integrin α IIb β 3 influenced platelet activation at least in part via a common route involving vWF. Increased role of GPIb in PS exposure at reduced expression of GPVI

Since GPVI activity is a limiting factor in collagen-dependent platelet activation and aggregation (18), the effect of GPIb inhibition was also examined under conditions of reduced GPVI expression. Therefore, blood was used from mice which were partially or completely deficient in FcR γ -chain. As expected, homozygous deficiency resulted in undetectable levels of both FcR γ -chain and GPVI on platelets, while heterozygous deficiency gave 50% reduction of both proteins (Table 5.1). Expression levels of GPIb-V-IX and α IIb β 3 were unchanged in these animals. When blood from heterozygous mice was perfused over vWF/collagen (1000 s⁻¹), platelet deposition was about linear with time. In a typical experiment, the increase in surface area coverage was 2.3%/min ($R^2=0.44$) in comparison to 5.5%/min ($R^2=0.93$) for wildtype blood. At the 4-min endpoint, this resulted in an about 42% lower platelet adherence for the FcR γ -chain +/- mice (Fig. 5.6A-D). Exposure of PS was also about 40% reduced in comparison to

Table 5.1. Surface expression of different glycoproteins on platelets from wildtype and FcR γ -chain deficient mice

Glycoprotein(s)	Wildtype	FcR γ +/-	FcR γ -/-
GPVI	48.3 $\pm$ 8.7	27.2 $\pm$ 9.3*	6.4 $\pm$ 1.9*
GPIIa (β 1)	135.6 $\pm$ 11.2	131.7 $\pm$ 13.1	133.3 $\pm$ 12.8
GPIa (α 2)	47.2 $\pm$ 8.5	45.5 $\pm$ 7.3	44.8 $\pm$ 7.9
GPIIb/IIIa (α IIb β 3)	395.8 $\pm$ 30.4	416.2 $\pm$ 32.5	399.4 $\pm$ 33.7
GPV	187.2 $\pm$ 19.3	179.4 $\pm$ 16.7	185.1 $\pm$ 20.5
CD9	532.3 $\pm$ 38.2	535.7 $\pm$ 41.4	528.7 $\pm$ 35.6

Expression levels were determined by flow cytometry using fluorophore-labeled monoclonal antibodies, as described (54). Platelets were gated by their forward scatter/side scatter characteristics. Results are expressed as mean log fluorescence (arbitrary units) $\pm$ S.D. for 6 mice per group. * $P < 0.01$ compared to wildtype.

Figure 5.6. Increased role of GPIb in platelet deposition and PS exposure upon reduced expression of GPVI. Blood from wildtype, FcR γ -chain +/- and -/- mice was perfused over collagen (see Fig. 5.1). Wildtype and FcR γ -chain +/- blood were pre-incubated with 40 μ g/ml p0p/B Fab as indicated. Upper panels: representative phase-contrast images ($48 \times 48 \mu$ m) after 4 min perfusion. Inserts are 200% magnifications showing platelet morphology. Lower panels: fluorescence images ($150 \times 150 \mu$ m) after staining with FITC-labeled annexin A5. (I) Surface area coverage of all platelets (white bars) and PS-exposing platelets (gray bars). Data are mean values $\pm$ S.E.M. (n = 3-5).

wildtype (Fig. 5.6I). Strikingly, blockage of GPIb with p0p/B fragments in the heterozygous blood nearly completely abolished stable platelet adhesion, while PS-exposing platelets were hardly detectable (Fig. 5.6E-F). In agreement with earlier results (14,16), perfusion of blood from homozygous FcR γ -chain -/- mice under these conditions resulted in diminished platelet adhesion to the collagen. During flow, most FcR γ -chain -/- platelets adhered only transiently to the surface (putatively via GPIb) and then moved on. After 4 min, a limited number of stably adherent platelets was detected (Fig. 5.6G-H), interestingly especially at sites of higher density of collagen fibers. These platelets neither showed morphological signs of activation nor exposed PS, confirming the essential role of GPVI in these platelet responses.

Discussion

In this study with mouse blood, we have evaluated the contribution of GPIb and collagen-bound vWF to shear-dependent platelet adhesion and procoagulant activity during thrombus formation. The results extend earlier work, demonstrating that GPVI has a conditional role in platelet-collagen interaction and subsequent activation both *ex vivo* (14,16) and in denuded artery *in vivo* (40). In the present study we find that, at high shear rates up to 1500 s^{-1} (mimicking those at the arterial wall), GPIb interaction with vWF deposited on collagen enhances irreversible platelet binding and GPVI-induced PS exposure, along with stimulation of the rise in $[\text{Ca}^{2+}]_i$, which is a prerequisite for exposure of procoagulant PS. This response must be regulated separately from platelet aggregate formation, as the PS exposing cells are usually not found in aggregates. Importantly, at reduced levels of GPVI we find that the procoagulant response is highly dependent on GPIb. Inhibitor studies indicate that $\alpha\text{IIb}\beta 3$, which also binds to vWF, has an overlapping role with GPIb, underlining the important adhesive function of this integrin, which is mostly considered subordinate to its function in platelet aggregation. As exposed PS potentially enhances thrombin generation, in platelet-collagen interaction either of the two adhesive glycoproteins appears to potentiate the two haemostatic processes contributing to thrombus formation: aggregate formation and coagulant activity.

Human and murine vWF and GPIb-V-IX play an initial role in tethering and adhesion to collagen under high shear flow conditions, and thereby mediate platelet aggregate formation on collagen (8,36,41). In addition, both vWF and the GPIb-V-IX complex are involved in thrombin generation and coagulation, perhaps because of the binding to multiple coagulation proteins, including factor VIII, factor XI, thrombin and fibrin (26,27,42,43). Here, we report on a procoagulant effect of vWF and GPIb, i.e. PS exposure, linked to collagen receptor stimulation, which is independent of the formation of thrombin. Whether vWF and GPIb may also contribute to platelet PS exposure in coagulating plasma is still unknown. However, recent data indicate that GPIb can stimulate thrombin formation (in the absence of collagen) in a shear-dependent way (27). It is thus conceivable that the function of GPIb in thrombin formation also involves a shear-dependent stimulation of PS exposure, similarly as we report here for platelet-collagen interaction.

The data with anti-GPIb p0p/B Fab fragment or saratin (which prevents vWF binding to collagen) show that platelets can still bind to collagen in the absence of vWF-GPIb interaction up to a shear rate of 1500 s^{-1} . Others have described that GPIb, in the absence of vWF, is still free to bind thrombospondin, which also mediates platelet adhesion under flow (44). Thus, GPIb can still have a role at intermediate shear rates independently of collagen-bound vWF. However, we find that residual, vWF-independent adhesion is mostly due to GPVI or integrin $\alpha 2\beta 1$, as it is abolished in the absence of functional collagen receptors (M. Kuijpers, unpublished results, 2003). On the other hand, p0p/B or saratin treatment did lead to a

decreased deposition of platelets (Fig. 1), and in a reduced or delayed Ca^{2+} response and PS exposure (Fig. 5.4). This could indicate that the GPIb-dependent spiking elevations in $[\text{Ca}^{2+}]_i$ reported in platelets binding to immobilized vWF (5,45,46) also contribute to the Ca^{2+} signal in the presence of collagen. We have earlier shown that collagen-induced Ca^{2+} signaling in murine platelets is almost completely dependent on the presence of the GPVI/FcR γ -chain complex, with minute spiking $[\text{Ca}^{2+}]_i$ increases remaining in FcR γ $-/-$ platelets (16), which might be the result of vWF-GPIb interaction. Accordingly, GPVI must be responsible for the majority of the Ca^{2+} signal, while GPIb has only a small contribution. Taken together, this points to an enhancement of GPVI signaling by GPIb/vWF, by direct signaling and/or allowing full engagement of the GPVI receptors.

The Fab fragment JON/A blocks binding of the α IIb β 3 to fibrinogen and vWF (39), and thus provides a unique tool to study the role of this integrin in adhesion and aggregation. Incubation with JON/A resulted in stronger inhibition of platelet deposition and PS exposure than inhibition of ADP function by receptor antagonists. This confirms the concept that α IIb β 3 is involved in stable adhesion to this surface (35). Furthermore, combined blockage of GPIb and integrin α IIb β 3 did not further reduce platelet activation in comparison to α IIb β 3 inhibition alone. This places the function of integrin α IIb β 3 downstream of GPIb, which is in good agreement with the current model of vWF-platelet interaction. This model presumes initial shear-dependent tethering via GPIb-V-IX, subsequent stable adhesion and enhanced signaling via activated integrin α IIb β 3 (5,35). This is also in line with *in vivo* findings with mice that platelet adhesion to the vessel wall involves multiple integrin-ligand interactions, none of which are essential by themselves (39), and that β 3-deficiency results in defective stable platelet adhesion to the vessel wall (47).

For mice with reduced levels of GPVI/FcR γ -chain it is demonstrated that, under static conditions, neither platelet adhesion to immobilized collagen nor protein tyrosine phosphorylation induced by collagen is affected compared to wildtype (29). Here we show, with mice expressing half of the normal GPVI/FcR γ -chain levels, that under flow conditions both platelet adhesion on collagen and platelet activation – as apparent from PS exposure – are reduced by about 50%. This indicates that the level of GPVI expression determines (and is a limiting factor for) signaling under shear. This conclusion is in agreement with the findings that collagen-induced Ca^{2+} responses of murine platelets are dependent on the GPVI expression level (48). Similarly, in human, partial blockage of GPVI has been shown to suppress aggregate formation and PS exposure under flow (19). Thus, the regulatory function of this collagen receptor in platelet activation and procoagulant activity appears to be conserved between species.

Interestingly, in FcR γ -chain heterozygous mice, expressing normal GPIb and α IIb β 3 levels on platelets, the blockage of GPIb caused almost complete abolition of stable platelet

adhesion and PS exposure under shear (Fig. 6). This indicates that GPIb plays a more prominent role in platelet-collagen interaction at reduced levels of GPVI/FcR γ -chain. Apparently, there is some redundancy in GPIb and GPVI receptor function. It is likely to be of physiological relevance, given the large variation in GPVI expression between humans (48-50). As also $\alpha 2\beta 1$ stimulates GPVI activity, the present results point to receptor interplay in the regulation of platelet procoagulant activity that is not restricted to collagen receptors, but extends to the GPIb-V-IX complex.

Earlier it has been proposed that the FcR γ -chain mediates GPIb-induced platelet activation (51,52). However, our data do not support this concept, as we find that partial knock-out of the γ -chain enhances the GPIb functioning. On the other hand, our findings are fully in line with those of Mangin *et al.* (2003), who propose that in mice GPIb signaling to Ca^{2+} flux and filopod extension relies on Src kinases via a pathway distinct from FcR γ -chain derived signaling.

Perfusion of blood from homozygous FcR γ -chain deficient mice resulted in only few adhering platelets, especially at sites where the collagen density was relatively high (Fig. 6). This is in apparent contrast to recent findings with GPVI knockout mice, where full adhesion to collagen was detected under flow conditions (53). The difference with the present data is likely explained by differences in methodology (collagen surface, anticoagulant, mepacrine labeling, etc.). Preliminary data in our laboratory indicate that the few GPVI/FcR γ $-/-$ platelets adhere in a GPIb/ $\alpha 2\beta 1$ -dependent way (unpublished results). This is similar to the situation with human platelets, where it also has been shown that $\alpha 2\beta 1$ is adhesive in the absence of GPVI signaling (19).

In summary, the current data, together with the earlier established role of integrin $\alpha 2\beta 1$ (10,14,16), confirm that murine GPVI is the primary trigger of activation in platelet-collagen interaction under shear. In addition it is shown that GPVI is facilitated by GPIb-V-IX in addition to integrins $\alpha 2\beta 1$ and $\alpha \text{IIb}\beta 3$. Platelet adhesion and subsequent activation evolves as a series of multi-receptor events with redundancy, facilitation and mutual interactions, rather than as a linear process. Specifically, concerning the procoagulant platelet reaction, both GPIb and $\alpha \text{IIb}\beta 3$ appear to control PS exposure under these shear conditions, likely by enhancing GPVI-dependent activation. The finding that these adhesive receptors in concert with GPVI have a procoagulant role in collagen-dependent thrombus formation indicates that interplay does not only occur between the collagen receptors, but also extends to the GPIb-V-IX complex.

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Chapter 6

Regulation of tissue factor-induced coagulation and platelet aggregation in flowing whole blood

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Abstract

Photochemically induced thrombosis (a thrombin-dependent process) was measured in rats treated with moderate doses of anticoagulants, but appeared to be unchanged. We considered the possibility that platelet-inhibiting agents which also indirectly inhibit coagulation, would act as more potent antithrombotic agents. Inhibitors used as such were prostaglandin E_1 (PGE_1), which elevates cyclic AMP levels, and the $P2Y_{12}$ ADP-receptor antagonist, AR-C69931MX. Effects of these agents were investigated in an *ex vivo* model system, in which whole blood under coagulant conditions was perfused over fibrinogen at defined wall shear rate. Perfusion of blood (rat or human) in the presence of tissue factor resulted in deposition of activated platelets and subsequent aggregate formation, along with exposure of procoagulant phosphatidylserine (PS) on the platelet surface and formation of fibrin fibers. In the presence of PGE_1 aggregation was completely inhibited, but platelet adhesion and PS exposure were only partly reduced, while fibrin formation was hardly affected. Treatment with AR-C69931MX caused similar, but less complete effects. These results indicate that in tissue factor-triggered blood under conditions of flow: (i) the platelet procoagulant response is independent of aggregate formation; (ii) the platelet-inhibiting effect of PGE_1 and AR-C69931MX is sufficient to suppress aggregation, but not platelet adhesion and coagulation. These platelet inhibitors thus maintain their aggregation-inhibiting effect at sites of thrombin formation.

Introduction

Hemostasis involves formation of a vaso-occlusive platelet plug and coagulation of blood plasma. Although usually studied separately, it is known that platelet activation and the formation of thrombin in the coagulation cascade are mutually dependent processes. The extrinsic coagulation pathway is initiated by tissue factor (TF), either blood-borne or exposed at the surface of a damaged vessel wall (1). Thrombin, once formed from prothrombin, has multiple amplifying effects. It facilitates its own generation by activating the coagulation factors V, VIII, and XI. Thrombin converts fibrinogen into fibrin and, furthermore, stimulates platelets to change shape and assemble into aggregates and fibrin-containing thrombi.

Platelets, in turn, support and maintain the coagulation process in different ways. They bind coagulation factors like prothrombin and factor XI (2,3), and they secrete coagulation factors when stimulated by agonists that induce rises in cytosolic $[Ca^{2+}]_i$. Activated platelets also expose procoagulant phosphatidylserine (PS) at their outer membrane surface (4,5), which serves as a site for the assembly and activation of tenase and prothrombinase complexes, forming factor Xa and thrombin, respectively (6-8). Exposed PS thereby is the main binding site on platelets for the γ -carboxyglutamate-containing enzymes, factor IXa and factor Xa (8,9). The significance of PS exposure becomes apparent in patients suffering from the Scott syndrome, a

rare bleeding disorder in which platelets are partly deficient in PS exposure upon activation (10,11).

Platelet-rich plasma (PRP) triggered with TF enters a potent reinforcement loop of thrombin generation, fibrin formation and platelet activation (12,13). In such a plasma system, platelet-dependent thrombin generation can be suppressed by antibodies against the platelet-adhesive receptors, integrin $\alpha\text{IIb}\beta 3$ (fibrinogen receptor) and glycoprotein Ib/IX/V (von Willebrand factor receptor) (14,15). Similarly, thrombin generation is reduced in the PRP from patients with Glanzmann's thrombasthenia (deficient in $\alpha\text{IIb}\beta 3$) or von Willebrand disease (deficient in von Willebrand factor) (12,15). Together, this suggests that the mutual interaction of platelets and coagulation is of physiological importance in hemostasis, and that these glycoproteins herein play an important role.

A number of platelet inhibitors can also suppress thrombin generation and coagulation in platelet-containing plasma. Particularly active in this respect are cyclic AMP elevating agonists, such as prostaglandin E_1 (PGE_1), and P2Y_{12} receptors antagonists like AR-C69931MX (12,16,17). The latter receptor comprises the pharmacological target of clopidogrel and ticlopidine, and contributes to ADP-mediated platelet aggregation (18-22). As these compounds also downregulate integrin $\alpha\text{IIb}\beta 3$ activation and platelet aggregation, it is possible that their effects on thrombin generation are via regulation of integrin activation.

The rat is a widely used laboratory animal to study thrombosis *in vivo*. Photochemical injury of the rat femoral artery is an established model for thrombosis with limitedly invasive effect, which relies on endothelial damage and subsequent initiation of coagulation and platelet activation (23,24). In earlier *in vitro* studies with platelet-containing plasma, we have established that, also in rat, thrombin generation triggered by the TF pathway relies on the activation of platelets and the coagulation system (25,26). If this interaction of platelets and coagulation is of (patho)physiological relevance, it is expected that thrombin inhibitors would be effective in such a thrombosis model.

In the present paper we studied the effect of thrombin inhibition on photochemically induced thrombosis tendency in this animal model. To this end, rats were subjected to moderate treatment with heparin or warfarin. The inability of these anticoagulants to influence the thrombotic process prompted us to investigate the effects of the anti-platelet agents, PGE_1 and AR-C69931MX, which indirectly affect coagulation via their platelet-inhibiting activity. To mimic physiological conditions, we studied the effects of these agents in whole blood triggered with tissue factor on the formation of platelet-fibrin thrombi under conditions of flow and coagulation.

Methods

Materials

H-Phe-Pro-Arg chloromethyl ketone (PPACK) was from Calbiochem (La Jolla, CA, USA). Bovine fibrinogen, bovine serum albumin (BSA), MRS 2179, PGE₁, rat thrombin, rose bengal and unfractionated heparin and were from Sigma (St. Louis, MO, USA). Recombinant human TF was from Dade (Düdingen, Switzerland); Oregon green 488 (OG488)-labeled and unlabeled annexin A5 (annexin V) from Nexins Research (Hoeven, The Netherlands); benzyloxycarbonyl Gly-Gly-Arg 7-amido-4-methyl-coumarin (Z-GGR-AMC) and Gly-Pro-Arg-Pro (GPRP) from Bachem (Bubendorf, Switzerland); Fluo-3 acetoxymethyl ester and human OG488-fibrinogen from Molecular Probes (Leiden, The Netherlands). Collagen ('Horm' type I) was from Nycomed (Munich, Germany). Warfarin (coumarin) was bought from Dupont Merck and heparin (enoxaparin) from Rhone-Poulenc Rorer. FITC-labeled monoclonal antibody PAC1, against the activated human α IIb β 3 was obtained from Becton and Dickinson. AR-C69931MX was kindly provided by AstraZeneca R&D (Charnwood, UK). Other materials were from sources described before (25).

Animals

Forty eight male Wistar rats (Charles River, Sulzfeld, Germany) were used for the intervention study. Of these, 24 rats were subjected to photochemically induced thrombosis; 24 other rats were used for ex vivo measurements. In each cohort, 3 groups of 8 rats (selected at random) were formed. The animals were subjected to one of the following interventions: none (control group), heparin (2 mg/kg enoxaparin, subcutaneous injection at 49, 25 and 1 h before experiment), or warfarin (0.3 mg/kg, intravenous injection at 24 h before experiment). Subsequent experiments were carried out with blood from adult Wistar rats. The animals were anesthetized by intra-peritoneal injection with Nembutal (50 mg/kg body weight) and subjected to abdominal aortic puncture. Animals were sacrificed after blood collection (10-12 ml). The studies were approved by the institutional animal care committees (Unilever Research Vlaardingen and University of Maastricht).

In vivo thrombus formation

Rats were subjected to a standard protocol for measurement of photochemically induced thrombosis (27). Briefly, the rats were anaesthetized with ketamine and domitor (intramuscular, 40 and 500 mg/kg body weight, respectively), and fixed on a heating pad at 36°C. The femoral artery and vein were separated from surrounding tissue, and a catheter was inserted through the jugular vein. An ultrasound flow probe was placed against the femoral

artery to measure blood flow. Green light (540 nm) from a xenon lamp was led through an optical fiber positioned at 5 mm from a part of the femoral artery proximal to the flow probe. Light was switched on after stable flow for 3 min; 1 min later, rose bengal (10 mg/kg) was infused over a period of 1.5 min; at 4 min after rose bengal infusion the light was switched off. Blood flow was recorded during 30 min. In this model, endothelial injury and thrombosis are caused by free radical formation via a photochemical reaction between rose bengal and green light (23). Thrombus formation was evaluated by measuring the time to vascular occlusion and the area under the curve of blood flow, calculated over 30 min. The latter gives an indication of the overall blood flow response during the whole measurement period (27).

Collection and preparation of rat and human blood

For most experiments, rat blood was collected into 0.1 vol of 129 mM trisodium citrate. Blood was used as such, or centrifuged to obtain platelet-rich plasma (PRP) and platelet-free plasma (PFP) (26). To prepare Fluo-3-labeled platelets, blood was collected into 0.2 vol of 183 mM glucose, 80 mM trisodium acetate and 52 mM citric acid. Washed platelets (5×10^8 /ml) were loaded with 5 μ M Fluo-3 acetoxymethyl ester in the presence of 0.5 g/l Pluronic F-127 for 45 min (room temperature). Platelets were suspended at 1×10^9 /ml in Hepes buffer pH 7.45 (in mM: NaCl 136, glucose 5.6, Hepes 5, KCl 2.7, $MgCl_2$ 2, NaH_2PO_4 0.42, and 0.1% BSA), and re-added to citrate-anticoagulated blood from the same animal to give a fraction of 2% labeled platelets.

Human blood was collected from healthy volunteers into 0.1 vol of 129 mM trisodium citrate. For some experiments PRP and (defibrinated) PFP were prepared, as described elsewhere (15).

Coagulation factors and platelet aggregation

In rat PFP, prothrombin time, and activity of fibrinogen, prothrombin, factors VII and antithrombin were measured as previously described, and the anticoagulant effect of heparin in rat plasma was quantified from the anti-thrombin activity (26). Platelet aggregation in citrated whole blood was measured as change in impedance, while stirring at 37°C in a Chronolog whole blood aggregometer (Chronolog Corporation, Havertown, PA, USA) (28). Platelet aggregation in recalcified PRP under coagulant conditions was measured by turbidometry with/without 2 mM GPRP in siliconized cuvettes, while stirring at 37°C.

Thrombin generation

Thrombin generation was measured in freshly obtained rat or human PRP using low-affinity fluorescent substrate, Z-GGR-AMC (0.42 μM), following the procedure described for human plasma (29). PRP (1.0×10^8 platelets/ml, f.c.) was triggered with 0.5–2 pM TF and 16.7 mM CaCl_2 in 96-well microtitre plates (37°C). Fluorescence from cleaved amidomethyl coumarin was followed in time, and first-derivative curves were constructed. The curves were converted to nanomolar thrombin by using internal calibrator (26).

Perfusion of whole blood under coagulant conditions

Rat or human whole blood was perfused over a glass surface covered with fibrinogen, which is an adhesive protein abundantly present in plasma and involved in platelet-platelet contact. Immobilized fibrinogen, as an excellent starting point for fibrin fiber formation, was used for the detection of initial stages of coagulation. Fibrinogen-coated coverslips were blocked with Hepes buffer pH 7.45 containing 2% (w/v) BSA (5). The coverslips were mounted in a transparent, parallel-plate flow chamber (9). The chamber (flow-cell volume of 60 μl) was connected, via siliconized plastic tubes and a three-way adapter, to plastic syringes placed in two pulse-free perfusion pumps (Harvard Apparatus, South Natick, MA, USA). Pump 1 contained a syringe filled with blood (normal perfusion rate of 27 ml/h). Pump 2 contained a syringe with 200 mM CaCl_2 , 153 mM NaCl and 20 pM TF (perfusion rate 3 ml/h⁻¹). Together, these pumps produced a flow rate of 30 ml/h, resulting in a wall shear rate at the flow chamber surface of 250 s⁻¹. Other experiments were carried out at 1500 s⁻¹ by increasing the rate of pumping. Experiments were stopped before occlusion of the flow chamber by clotting. Rinsing was at the same flow rate using heparinized Hepes buffer pH 7.45 containing, as indicated, 2 mM CaCl_2 and 0.5 $\mu\text{g/l}$ OG488-annexin A5.

Phase-contrast and fluorescence images were taken from the perfusion chamber, placed on the stage of an inverted microscope (Nikon Diaphot 200, Tokyo, Japan). Intensified, charge-coupled device cameras were connected to the microscope via a beam splitter (5). Digital images were obtained with a recording system controlled by Qanticell software (Visitech, Sunderland, UK). Excitation and emission wavelengths were 485 ± 11 and 530 ± 15 nm, respectively.

Changes in Fluo-3 fluorescence from single platelets were converted into pseudo-ratio values and converted into nanomolar concentration of cytosolic $[\text{Ca}^{2+}]_i$ as described (30). Fluorescence and phase-contrast transmission images (10 microscopic fields per experiment) were analyzed on coverage by platelets or label using Qanticell and ImagePro software, respectively (9).

Statistics

Differences between intervention groups and the control group were analyzed with the Dunnett's test (mean square error) taking into account all values obtained. Effects of antagonists were compared to control condition with a Mann-Whitney U-test (SPSS, Chicago, IL, USA). Data are mean values $\pm$ SEM.

Results

Effect of moderate anticoagulation treatment on in vivo thrombosis tendency in rats

Thrombus formation involves platelet activation and coagulation, which are mutually stimulatory processes, both in human and rat (13,26). To study the importance of this interaction, we used a limitedly invasive thrombosis model in rats, which relies on photochemically induced exposure of tissue factor (TF) and subsequent activation of coagulation and platelets (23,31). In a standardized way, thrombus formation was induced in the femoral artery by infusion of rose bengal and controlled illumination with green light (27). To achieve moderate anticoagulation, the rats were injected at 1-2 days before the experiment with intermediate doses of heparin (enoxaparin, a low molecular-weight heparin increasing the activity of antithrombin) or warfarin (inhibiting the synthesis of vitamin K-dependent prothrombin, factors VII, IX and X and proteins C and S). As shown in Table 6.1, intervention with heparin or warfarin led to a slight, insignificantly prolonged occlusion time of the artery

The efficacy of the treatments was verified using blood samples from rats that were similarly treated, but not used for thrombosis induction. Thrombin generation was measured in citrated PRP, triggered with TF/CaCl₂, using the thrombogram method. The endogenous thrombin potential (ETP, area-under-the-curve) was substantially decreased by heparin and warfarin treatment with 87% and 52.5%, respectively (Table 6.1). Platelet aggregation, measured in anticoagulated whole blood was not significantly changed. After heparin treatment, levels of coagulation factors in plasma were unaltered, whereas increased antithrombin activity was detected. After warfarin treatment, levels of the vitamin K-dependent coagulation factors prothrombin and factor VII were reduced by 40-60%. This was accompanied by a prolonged prothrombin time, indicative of delayed coagulation. Taken together, these moderate anticoagulant treatments led to a marked suppression in thrombin generation which, apparently, was not sufficient to significantly reduce the in vivo thrombosis tendency.

Earlier studies with human PRP showed that the platelet-inhibiting agents, PGE₁ (which elevates cyclic AMP) and AR-C69931MX (which specifically blocks P2Y₁₂ receptors for ADP), caused a substantial decrease in thrombin generation (12,16), which was similar in size as detected in the rats subjected to the anticoagulant interventions. This raised the question of

Table 6.1 Effect of heparin or warfarin intervention on thrombosis tendency and hemostatic parameters in rat.

	Control	Heparin	Warfarin
Thrombosis tendency			
- time to occlusion (min)	17.4 ± 3.6	19.7 ± 3.5	21.5 ± 3.1
- area under flow curve (AU*)	1160 ± 241	1293 ± 128	1361 ± 213
Thrombin generation in PRP			
- time to peak (min)	8.63 ± 0.25	22.4 ± 0.78**	10.2 ± 0.65
- peak height (AU)	36.6 ± 1.3	3.62 ± 1.4**	18.3 ± 4.2**
- ETP (AU × min)	186 ± 8.3	23.6 ± 8.9**	88.4 ± 23**
Whole blood aggregation (% aggregation/min)	39.7 ± 4.2	31.1 ± 4.8	31.1 ± 7.5
Prothrombin time (% of pool)	99.1 ± 1.7	98.6 ± 2.1	75.4 ± 8.6*
Fibrinogen (g/l)	2.19 ± 0.04	2.27 ± 0.06	2.27 ± 0.07
Prothrombin (% of pool)	96.9 ± 2.1	91.4 ± 2.0	54.5 ± 7.6**
Factor VII (% of pool)	95.6 ± 5.5	85.5 ± 3.2	36.6 ± 9.6**
Antithrombin (% of pool)	107 ± 2.5	106 ± 2.8	105 ± 3.1
Thrombin inhibiting activity (/mol/s)	n.d.	53009 ± 3000	n.d.

Animals were untreated (control) or subjected to intervention with heparin (2 mg/kg enoxaparin, injection at 49, 25 and 1 h before measurement) or warfarin (0.3 mg/kg, injection at 24 h before measurement). Thrombosis tendency was measured in the femoral artery following rose bengal injection and photochemical induction. Other rats were subjected to the same interventions, and blood was taken on citrate to determine: thrombin generation in PRP (triggered with 16.7 mM CaCl_2 and 1 pM TF), giving time to thrombin peak, thrombin peak height and endogenous thrombin potential (ETP); whole blood aggregation (1 $\mu\text{g/ml}$ collagen); prothrombin time; individual coagulation factors (data as percentages of rat pool plasma). Mean $\pm$ SEM (n=8); * p < 0.01 and ** p < 0.001 compared to control. *AU, arbitrary units; n.d., not determined.

possible advantages of such agents, which combine antiplatelet with indirect anticoagulant activity, in comparison to moderate anticoagulant treatment. We investigated this under a variety of physiologically relevant conditions.

In vitro effects of platelet inhibitors on thrombin generation and aggregation in rat plasma

First we determined the effects of PGE_1 and AR-C69931MX in coagulating rat PRP in the absence of flow. In recalcified PRP, addition of TF (2 pM) greatly accelerated the generation of thrombin (Fig. 6.1A). We verified that this thrombin generation was almost completely abolished by preincubation with a saturating dose of heparin (reducing the ETP by >95%). Furthermore, thrombin generation fully relied on activated platelets, because it was abolished

Figure 6.1. Effect of PGE₁ and AR-C69931MX on thrombin generation and aggregation in PRP triggered with tissue factor. (A) Thrombin generation was measured in citrated rat PRP triggered with 0.2 pM TF and 16.7 mM CaCl₂. Heparin (0.05 U/ml) was present, where indicated. (B) Effect of PGE₁ (10 μ M), AR-C69931MX (10 μ M) or annexin A5 (30 μ g mL⁻¹) on thrombin generation with TF (2 pM) and CaCl₂. (C) Effect of PGE₁ or AR-C69931MX (each 10 μ M) on turbidity change in PRP triggered with TF (2 pM) and CaCl₂ under stirring conditions. Experiments were performed in the presence or absence of GPRP (2 mM); cl = formation of clot. Traces are representative for 5-7 experiments.

when PFP instead of PRP was used (data not shown), and it was fully blocked in the presence of 30 μ g/ml PS-scavenging annexin A5 (peak height $1.6 \pm 0.5\%$ of control, Fig. 6.1B).

Preincubation of rat PRP with 10 μ M PGE₁ resulted in greatly reduced but not abrogated thrombin formation with a thrombin peak height of $28.0 \pm 8.6\%$ ($n=5$, $p<0.05$) compared to control (Fig. 6.1B). Typically, this peak was reached at an earlier time point, i.e. 2.1 ± 0.5 min earlier than in the control condition. Preincubation of rat PRP with 10 μ M AR-C69931MX gave a smaller reduction in peak height to $53.9 \pm 3.1\%$ ($n=5$, $p<0.05$); the peak was now delayed with 3.1 ± 1.0 min compared to control. The effects of PGE₁ and AR-C69931MX did not add up: combined preincubation gave a peak height of $22.0 \pm 4.3\%$ of control. Furthermore, the AR-C69931MX effect was not influenced by addition of 20 μ M MRS 2179 (an antagonist of the second platelet ADP receptor, P2Y₁), giving a peak height of $61.7 \pm 3.5\%$ of control. This indicates that, similarly as reported for human platelets (17), the procoagulant effect of ADP is mediated by P2Y₁₂ receptors.

Figure 6.2. Effect of annexin A5 on thrombus and fibrin formation in flowing blood triggered with tissue factor. Citrated rat blood containing 0.2 mg/ml OG488-fibrinogen was perfused during 5 min over a fibrinogen surface at a shear rate of 250 s^{-1} . During perfusion coagulation was triggered with 2 pM TF and 16.7 mM CaCl_2 . Blood was pretreated with a high dose of 30 $\mu\text{g/ml}$ unlabeled annexin A5, where indicated. Shown are representative phase-contrast (A, B) and fluorescent images (C, D), captured after 8 min ($n=3$). Bar=10 μm .

The efficacy of AR-C69931MX and PGE_1 in inhibiting platelets under conditions of thrombin generation was evaluated by turbidometry. Triggering of citrated PRP with TF/ CaCl_2 in presence of GPRP, the shape change in response to TF/ CaCl_2 was followed by rapid aggregation of platelets. Preincubation with AR-C69931MX delayed both shape change and aggregation, as was observed in the presence of GPRP (Fig. 6.1C). Preincubation with PGE_1 further delayed shape change and completely inhibited aggregation. Thus, in TF-triggered rat PRP, both compounds had an aggregation-delaying effect in addition to their inhibiting effect on thrombin generation.

Effects of platelet inhibitors on thrombus formation and coagulation in rat blood under flow

To study the effects of platelet inhibitors under conditions of flow and coagulation, whole rat blood was triggered with TF/ CaCl_2 and perfused over a fibrinogen surface at a moderately low wall-shear rate of 250 s^{-1} . Perfusion of blood with CaCl_2 plus 2 pM TF resulted in early platelet deposition and formation of fibrin fibers, which started after about 2 min. Platelets later assembled into aggregates, leading to $29.7 \pm 2.6\%$ ($n=12$) of the fibrinogen surface area covered with multi-layered thrombi after 5 min of flow (Fig. 6.2A). At a more advanced stage (7–8 min), the chamber became occluded by large fibrin-platelet thrombi. Addition to the blood of 30 $\mu\text{g/ml}$ annexin A5 (chelating exposed PS), completely abolished aggregate formation, while

Figure 6.3. Contribution of thrombin to thrombus formation and PS exposure in tissue factor-triggered, flowing blood. Citrated rat blood was perfused during 5 min over a fibrinogen surface at a shear rate of 250 s^{-1} . During perfusion, coagulation was triggered with 2 pM TF and 16.7 mM CaCl_2 (A, D); with 16.7 mM CaCl_2 alone (B, E); or with TF/ CaCl_2 and 0.05 U/ml heparin (C, F). Representative images are presented, which were taken after 5 min ($n=4-10$ experiments). (A-C) Phase-contrast images; (D-F) fluorescence images after staining post-perfusion with 0.5 $\mu\text{g}/\text{ml}$ OG488-annexin A5. (G) Accumulation of fluorescence at fibrinogen surface during perfusion of blood, labeled with 0.5 $\mu\text{g}/\text{ml}$ OG488-annexin A5, triggered with TF/ CaCl_2 . Heparin (0.05 U/ml) was present as indicated. Bar=10 μm ; inserts show $2\times$ magnifications.

only few platelets adhered (Fig. 6.2B). That annexin A5 acted as an anticoagulant was confirmed by experiments where OG488-fibrinogen was added. In the control condition, networks of OG488-labeled fibrin and platelets were observed after perfusion (Fig. 6.2C). With annexin A5 present, fluorescent fibers remained absent and only few single platelets displayed fibrinogen labeling (Fig. 6.2D). This indicated that the formation of aggregates and fibrin during flow relied on PS exposure and subsequent thrombin formation, putatively on activated platelets.

Further flow experiments confirmed that platelet activation and aggregate formation were enhanced by TF. Perfusion of blood without TF resulted in a delayed adhesion of mostly

Figure 6.4. Platelet Ca^{2+} responses in flowing blood triggered with tissue factor. Citrated rat blood containing 2% Fluo-3 loaded platelets from the same animal was triggered with TF/ CaCl_2 during perfusion (see Fig. 6.3). Rises in $[\text{Ca}^{2+}]_i$ in single adherent platelets were determined after about 3 min of flow. Coagulation was triggered during perfusion with 2 μM TF and 16.7 mM CaCl_2 . (A) Control condition, (B) triggering without TF. Further, pretreatment of the blood with 0.05 U/ml heparin (C), 30 $\mu\text{g}/\text{ml}$ annexin A5 (D), 10 μM PGE_1 (E), or 10 μM AR-C69931MX (F). Single-platelet Ca^{2+} responses are representative for those of 16-45 cells.

individual platelets. First traces of fibrin fibers did not appear before 5 min of flow (Fig. 6.3B). To show that the TF-induced process relied on thrombin formation, a saturating dose of heparin was added, which indeed completely abolished platelet deposition and fibrin formation during the experimental time (Fig. 6.3C). The activation state of adhered platelets was verified by post-staining with OG488-annexin A5 to detect PS-exposing platelets (Fig 6.3D-F). The few adherent platelets hardly exposed PS in the presence of heparin. When OG488-annexin A5 label was added to the flowing blood (0.5 $\mu\text{g}/\text{ml}$, a low dose not influencing coagulation), it could be verified that heparin prevented the appearance of PS-exposing platelets during the whole flow experiment (Fig. 6.3G).

To monitor the changes in activation state of platelets during adhesion, blood was supplemented with 2% Fluo-3 loaded platelets from the same animal. Upon perfusion with TF/ CaCl_2 , adherent fluorescent platelets displayed a prolonged increase in $[\text{Ca}^{2+}]_i$, starting at

Figure 6.5. Effects of PGE₁ and AR-C69931MX on thrombus formation and platelet activation in flowing rat blood triggered with tissue factor. Flow experiments were carried out in the presence of TF/CaCl₂, as described for Fig. 6.3. Rat blood was pretreated with PGE₁ or AR-C69931MX, each 10 μM as indicated. (A) Representative phase-contrast images taken after 5 min of perfusion. *Upper panels*: images taken slightly above the coverslip surface; *lower panels*: images of fibrin fibers at the focal plane of the surface (bar=10 μm). (B) Surface area coverage with platelets or OG488-annexin A5 fluorescence after 5 min of perfusion. Mean±SEM (n=4-6 experiments).

Figure 6.6. Effects of PGE₁ and AR-C69931MX on thrombus formation and platelet activation in flowing human blood triggered with tissue factor. Flow experiments were carried out in the presence of TF/CaCl₂, as indicated for Fig. 6.3. Human blood was pretreated with 10 μM PGE₁ or 10 μM AR-C69931MX, as indicated. (A) Representative phase-contrast images and (B) surface area coverage after 6 min of perfusion at a shear rate of 250 s⁻¹ (N.D., not determined). *Upper panels*: images taken slightly above the coverslip surface; *lower panels*: images of fibrin fibers at the focal plane of the surface (bar=10 μm). Surface area coverage is given with platelets or OG488-annexin A5 fluorescence (stained post-perfusion). (C) surface area coverage after 4 min of perfusion at 1500 s⁻¹. Mean±SEM (n=2-3 experiments).

the moment of adhesion or after a short lag-time (Fig. 6.4A). This pointed to a high activation state of the cells. In the absence of TF, [Ca²⁺]_i in adherent platelets initially remained low. However, after 5 min of flow, most cells responded by series of spiking rises in [Ca²⁺]_i (Fig. 6.4B). Further control experiments indicated that with heparin or annexin A5 present (at concentrations completely blocking thrombin generation), platelets remained low in [Ca²⁺]_i except for incidental spikes (Fig. 6.4C,D). The majority of the Ca²⁺ responses thus were

secondary to generation of thrombin. Together, these data indicate that under flow the TF/CaCl₂-triggered formation of aggregates and fibrin is driven by the procoagulant action of activated, PS-exposing platelets and subsequent thrombin generation. Indeed, without coagulation (heparin) or procoagulant platelets (annexin A5), thrombus formation was absent.

Effects of the platelet inhibitors PGE₁ and AR-C69931MX were studied in this flow model in the presence of TF-triggered coagulation. Pretreatment of blood with PGE₁ (10 μ M) resulted in deposition of mostly single platelets (Fig. 6.5A). Fibrin fibers were still formed. In comparison to the control condition, area coverage by platelets was not reduced after 5 min of flow (Fig. 6.5B). PGE₁ influenced the activation state of adhered platelets: the Ca²⁺ responses showed a consistent lag-time (Fig. 6.4E) and the surface coverage with PS-exposing platelets was about halved (Fig. 6.5B). Nevertheless, platelets gradually spread over the surface and some formed lebs, which are morphologic indications for (delayed) activation. When blood was pretreated with AR-C69931MX (10 μ M), similar effects were seen as with PGE₁: platelets mostly adhered as single cells, but sometimes assembled into micro-aggregates and fibrin was still formed (Fig. 6.5A). Furthermore, the AR-C69931MX treatment reduced PS exposure (Fig. 6.5B) and gave transient Ca²⁺ responses (Fig. 6.4F). This indicated that both agents effectively suppressed platelet aggregate formation under flow, but only partially inhibited the activation state of the deposited platelets in terms of [Ca²⁺]_i elevation and PS exposure.

Effects of platelet inhibitors on thrombus formation and coagulation in human blood under flow

To investigate the relevance of these findings for the human system, we performed a similar set of flow experiments with human blood under conditions of coagulation at a shear rate of 250 s⁻¹. The onset of stable platelet adhesion and the formation of platelet aggregates and fibrin fibers was relatively slow when TF/CaCl₂-triggered human blood was perfused over fibrinogen in comparison to rat blood. After several min of perfusion, the human platelets adhered and became activated so that, after 6 min, aggregated platelets had covered 21 $\pm$ 2% (n=3) of the fibrinogen surface (Fig. 6.6A). After staining for PS exposure with labeled annexin A5, 2.6 $\pm$ 0.7% of the surface was covered with fluorescent platelets, which was lower than seen with rat blood. Control experiments showed that heparin addition (at a concentration abrogating thrombin generation), as with rat blood, completely inhibited platelet aggregation and PS exposure as well as fibrin formation; only few single platelets adhered. In the presence of PGE₁ (10 μ M), aggregate formation was completely prevented. However, with human blood, many single platelets adhered, which had an activated shape and spread over the surface; fibrin fibers were still formed. Surface coverage of OG488-annexin A5 fluorescence was only partly reduced by 30% compared to the control (Fig. 6.6B), similar as seen with rat platelets.

When the flow experiments were performed at a higher (arterial) shear rate of 1500 s⁻¹, essentially the same effects of PGE₁ treatment were obtained (Fig. 6.6C). Inhibition of the ADP

receptor P2Y₁₂ by AR-C69931MX (10 μ M) resulted in similar, but less potent effects: reduced aggregate formation and still fibrin formation and PS exposure. Under static conditions, using TF-triggered, defibrinated human PRP, we found that PGE₁ treatment (10 μ M) suppressed integrin α IIb β 3 activation with 83% (platelet binding of FITC-labeled PAC1, an antibody against activated α IIb β 3, as measured by flow cytometry), while thrombin was still formed (peaking at 40 nM). Assuming that PGE₁ has the same effect under flow, we can conclude that a substantial but incomplete suppression of integrin activation can abolish platelet aggregation, but still allow platelet adhesion to fibrinogen. Taken together, these data show that in flowing, coagulating blood from rat and human, either inhibitor suppressed platelet aggregation, but no more than delayed formation of thrombin and fibrin, or PS exposure on activated platelets.

Discussion

Photochemically induced thrombus formation is considered to be a sensitive method to detect hypocoagulant conditions in laboratory animals, e.g. as induced by dietary fish oil (27). Surprisingly, using this thrombosis model in rats, we did not find effects of moderate anticoagulant treatment, with either heparin (inactivating factor Xa and thrombin) or warfarin (reducing levels of vitamin K-dependent coagulation factors). Nevertheless, this treatment caused major reduction in thrombin generation, when measured *ex vivo* in tissue factor-triggered PRP. Because these results suggested that the mere down-regulation of coagulant activity is insufficient to reduce thrombosis tendency, we further explored the effects of two antiplatelet agents which, in addition, are known to reduce thrombin generation.

First, PGE₁ was used, which causes EP/IP receptor-mediated, G_s-dependent elevation in cyclic AMP in platelets, resulting in down-regulation of Ca²⁺ responses, inactivation of α IIb β 3 integrin, and inhibition of platelet aggregation (32-34). Second, AR-C69931MX was used to block the P2Y₁₂ receptor (21,22). This ADP receptor, coupled to G_i, contributes to ADP-mediated platelet aggregation as well as thrombin generation (17,18,20). In human PRP, the reducing effects of these agents on thrombin generation are due to inhibition of appearance of procoagulant PS at the platelet surface (16). We thus assessed the effects of PGE₁ and AR-C69931MX on procoagulant activity of rat platelets by performing static thrombin generation experiments with PRP and, more physiologically, *ex vivo* flow experiments with coagulating whole blood. In both sets of experiments, the extrinsic coagulation pathway was triggered with TF. Most flow studies were performed at relatively low shear rate (250 s⁻¹), as thrombin is considered to play a prominent role under this condition.

Perfusion of whole rat blood over a fibrinogen surface upon simultaneous triggering with TF/CaCl₂ allowed detection of initial and late coagulant effects: platelet adhesion and activation, PS exposure, fibrin formation and platelet aggregation. Adhered platelets showed high, prolonged Ca²⁺ responses that were accompanied by bleb formation and surface exposure

of procoagulant PS. These platelet responses were thrombin-mediated, as they were absent in control experiments with heparin. Labeling experiments with OG488-annexin A5 showed a good correlation between the appearance of PS-exposing (blebbing) platelets and fibrin. Blocking experiments with high annexin A5 demonstrated that the exposed PS is critical for the formation of thrombin and fibrin, proving that procoagulant platelets play a key role in this process. The above experiments thereby illustrate that the positive feedback loop, in which initial thrombin formation stimulates platelet aggregate formation and exposure of coagulation-stimulating PS, is also operative under conditions of flow.

We note that in the initial *in vivo* experiments heparin and warfarin were used at moderate concentrations to partially suppress thrombin generation. On the other hand, in the flow experiments with whole blood, heparin was applied *in vitro* at maximally effective dose, sufficient to completely block thrombin/fibrin formation, to verify that the observed platelet responses were thrombin-dependent. However, as *in vivo* also the vessel wall plays a regulatory role in thrombus formation, the apparent inability of anticoagulants to influence *in vivo* thrombosis can also be due to the presence of activating substances at the (damaged) vessel wall.

The flow results obtained with platelet inhibitors under conditions of TF-triggered coagulation are unexpected. In both rat and human blood, PGE₁ prevents the formation of platelet aggregates in flowing blood (Figs. 6.5-6.6), even until occlusion of the flow chamber by massive fibrin deposition. AR-C69931MX has a similar, though more moderate effect. In agreement with the notion that PGE₁ and AR-C69931MX considerably but incompletely inhibit PS-dependent thrombin generation under stasis in PRP from rat (Fig. 6.1B) and human (16), these agents also only partially reduce PS exposure and fibrin formation in flowing blood (Figs. 6.5-6.6). Consistent with these results, other authors have described that PGE₁ delays fibrin clot formation in TF-triggered whole blood under static conditions (35). Overall, we can conclude that PGE₁ and AR-C69931MX inhibit the various aspects of coagulation-induced platelet activation in a different way. They extensively reduce integrin activation, but only delay Ca²⁺ signaling and PS exposure in response of the thrombin that is formed upon coagulation. That platelets deposited from PGE₁- and AR-C69931MX-treated blood are in an activated state is also apparent from their changes in morphology, i.e. spreading over the surface and forming blebs. The final result is abolished (with PGE₁) or suppressed (with AR-C69931MX) platelet aggregation, yet leaving sufficient PS exposure on activated platelets to maintain platelet-dependent coagulation (thrombin and clot formation). Both rat and human platelets behave similarly in the presence of PGE₁ or AR-C69931MX under coagulant conditions. Yet, subtle differences are observed between the rat and human systems, as in human blood deposition of platelets is reduced in the presence of PGE₁ or AR-C69931MX, while in rat blood this remains

unchanged. This can be explained, for instance, by the higher platelet count of rat blood and different levels of adhesive receptors on the platelets from this species.

The present results thus indicate that platelet aggregation is more sensitive to platelet inhibitors such as PGE₁ than PS exposure and subsequent formation of thrombin and fibrin. In agreement with this, others have observed that prostaglandin-induced down-regulation of integrin α IIb β 3 activation is of longer duration than the platelet Ca²⁺ signal (34). Our findings, using whole blood in which coagulation is triggered with TF/CaCl₂ under flow conditions, are a clear indication that this differential regulation of platelet responses is of physiological relevance.

Interestingly, for the human system, we find that treatment of TF-triggered blood with PGE₁ gives reduced platelet aggregation with only partial reduction of PS exposure upon perfusions at low and high shear rates of 250 and 1500 s⁻¹, respectively. This finding is of potential clinical interest. For many years, patients with peripheral arterial obstructive disease are being treated by intravenous administration of PGE₁ (alprostadil). The mechanism for the therapeutic efficacy is not fully clear yet; moderate increases in blood flow have been measured as well as reduced expression of endothelial activation markers and increased production of pro-angiogenic factors (36-38). The present results suggest that part of the beneficial effect of alprostadil can lay in a reduction of aggregate and thrombus formation under conditions where coagulation is still sufficiently active. If this proves to be true, it argues for the use of inhibitors which suppress both platelets and thrombin generation as antithrombotic agents. These positive effects of substances with anti-aggregatory as well as anti-coagulatory properties thus need to be taken further in future human studies.

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Chapter 7

Platelet-dependent coagulation regulates arterial and venous thrombus formation in vivo

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Abstract

The interactions between platelet activation and coagulation have extensively been studied *in vitro*, but not *in vivo*. The present study investigated the contribution of procoagulant platelets to thrombin generation and thrombus formation in a mouse model of arterial and venous thrombosis, where vascular damage was induced by FeCl_3 . In either vessel type, thrombus formation appeared to be triggered by tissue factor-factor VII(a) interaction and collagen exposure. Moderate thrombin inhibition or platelet inhibition suppressed arterial thrombus formation, while high thrombin inhibition or combination with platelet inhibition was necessary to suppress thrombosis in veins. Thrombus formation in either vessel type was characterized by the presence of PS (PS)-exposing platelets, detected with fluorescently labeled annexin A5. Shielding of exposed PS by injected annexin A5 completely abolished the formation of arterial and venous thrombi, while mutant M1234-annexin A5 was ineffective. Thus, in this thrombosis model, initiation occurs by tissue factor and collagen exposure, followed by propagation of thrombin generation by PS-exposing platelets. The latter appears to be a key regulatory process in veins and arteries. Platelet-dependent thrombin generation, which is most active in venous thrombus formation, may constitute a clinically relevant target to reduce thrombosis.

Introduction

Undesired thrombus formation both in veins and arteries can be life-threatening. The current understanding is that activated platelets on one side and the coagulation system on the other side contribute in different ways to pathological thrombosis in veins and arteries. 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 that thrombin is one of the most potent platelet agonists (1,2), which implicates that platelets must become activated at sites of thrombin generation in veins. On the other hand, arterial thrombosis is associated with undesired platelet activation; thus, 'white' thrombi formed in atherothrombotic arteries are rich in aggregated platelets, although fibrin is detectable here as well. 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. In recent years, however, there is increasing interest in combination therapy of anticoagulant and antiplatelet drugs (3). This urges for better knowledge of the interaction mechanisms of platelet activation and coagulation *in vivo*.

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 $\alpha\text{IIb}\beta_3$ antagonists, have an anticoagulant effect by slowing down the formation of thrombin and fibrin

(4,5). Although there is strong evidence that (activated) platelets stimulate the coagulation process (6,7), the mechanism behind this effect is still disputed partly because in vivo evidence is lacking. Studies in vitro have pointed to specific binding of coagulation factors (prothrombin and factors V, X and XI) to platelet integrins and other receptor proteins (8,9). There is also evidence that tissue factor (TF) binding to platelet aggregates and thrombi can stimulate the coagulation process (10,11). Another proposed mechanism concerns the exposure of procoagulant phosphatidylserine (PS) at the outer surface of activated platelets. In vitro, PS serves as a site for the assembly of coagulation factor complexes, which greatly enhance thrombin generation and, hence, coagulation (12,13). Platelet interaction with immobilized collagen is a strong trigger of this procoagulant response (14,15), while platelet stimulation via the thrombin receptors is less effective (16). How important these processes, such as TF accumulation and PS exposure, are in pathological thrombus formation in vivo is completely unclear.

The present work was directed to resolve this question. We studied the procoagulant function and mechanism of platelets in an established murine thrombosis model of FeCl_3 -induced vascular damage, known to be sensitive to platelet inhibition as well as anticoagulant activity (2,17-19). The data show that PS exposure plays a prominent role in the thrombotic process in both veins and arteries. In either vessel type, procoagulant platelets, in part activated by collagen, together with triggering of the TF pathway appear to mediate local thrombin generation and fibrin formation. These findings suggest that the 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 Fc γ -chain were from Taconics (Germantown, NY, USA) (20). Experiments were approved by the local animal care and use committee.

Materials

Fibrillar Horm collagen from equine tendon (Horm type-I) was purchased from Nycomed Pharma GmbH (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 VIIa (active site-inhibited seven, ASIS) (21) were kindly provided by Dr. P. Tijburg (Novo Nordisk, Bagsværd, Denmark).

Perfusion of whole blood under coagulant conditions

Mice were bled retro-orbitally under full anaesthesia by subcutaneous injection of 0.1 mg/g body weight (bw) ketamine and 0.02 mg/g bw xylazine. Blood (9 volumes) was collected in 1 volume of citrate (0.129 M). Anticoagulated blood (1 volume) was diluted with Hepes buffer pH 7.45 (0.5 volume) containing 137 mM NaCl, 5.6 mM glucose, 5 mM Hepes, 2.7 mM KCl, 2 mM MgCl_2 , 2 mM CaCl_2 , 0.42 mM NaH_2PO_4 , 0.1% (w/v) bovine serum albumin (BSA). Adhesion experiments under coagulant flow conditions were performed, as described earlier (22). Briefly, coverslips (24×60 mm) were coated with Horm collagen type I fibers (50 $\mu\text{g}/50\text{ mm}^2$), rinsed with saline, and blocked with Hepes buffer pH 7.45 containing 1% (w/v) BSA. Coverslips were placed in a parallel-plate transparent flow chamber (slit depth 50 μm) and mounted on an inverted Nikon microscope. The chamber was connected via a three-way siliconized polyethylene plastic tube (diameter 1 mm) to plastic 1 ml syringes placed in two pulse-free perfusion pumps (Harvard Apparatus). Pump 1 contained a syringe filled with blood (normal perfusion rate of 4.5 ml/h); pump 2 contained a syringe with 200 mM CaCl_2 and 20 pM TF in Hepes buffer pH 7.45 (perfusion rate 0.45 ml/h). Together, these pumps produced a wall shear rate at the flow chamber surface of 1000 s^{-1} . Where indicated, blood was labeled with 0.5 $\mu\text{g}/\text{ml}$ FITC-annexin A5 before perfusion. During perfusion, high-resolution microscopic images of transmission or fluorescence were recorded in real-time with a Visitech imaging system (Sunderland, UK). Per experiment, at least 10 representative images were captured with two parallel-placed intensified, CCD cameras, recording infrared (0.3 Hz) and epifluorescence (0.5 Hz) light (14). Excitation and emission wavelengths were 485 ± 11 and 530 ± 15 nm, respectively. 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.

Animal preparation and intravital microscopy

Four to 5-week old mice were anesthetized by subcutaneous injection of 0.1 mg/g body weight (bw) ketamine and 0.02 mg/g bw 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. Dehydration during the experiment was prevented by a subcutaneous bolus injection of saline (1.5 ml). Body temperature was kept at 37°C using an infrared heating lamp, controlled by a thermo-analyzer 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 intravital microscope, and continuously superfused with a buffered Tyrode's solution (130 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 0.56 mM MgCl₂, 11.1 mM glucose, 13.1 mM sucrose, 25 mM NaHCO₃ and 1.2 mM NaH₂PO₄; pH 7.35 at 37°C) supplemented with 1 µg/ml isoprenaline and saturated with 95% N₂ and 5% CO₂. The exteriorized ileum was kept moist with overlying wet gauze. Mesenteric tissue was visualized by intravital microscopy, using an LL25 objective lens (numerical aperture 0.35) and a CCD camera (Hamamatsu) as described previously (23).

Where indicated, (fluorescently labeled) compounds were injected (bolus, 100 µl) via the tail vein catheter, either immediately before or after application of FeCl₃. Control mice received the same volume of saline (vehicle). High-resolution fluorescence images were observed by epi-illumination with a Xenon lamp, using an SW50 objective (numerical aperture 1.0), a sensitive, high-resolution fluorescence imaging system and an intensified CCD camera (Photonic Sciences), as described (23).

Thrombus formation in vivo

After selection of a fat-free mesenteric arteriole with a proximate venule, superfusion with Tyrode's buffer was stopped, and the remaining fluid was carefully removed from the mesentery. Vessel wall damage was induced by topical application of FeCl₃ (30 µl, 500 mM), which never caused bleeding. Arterioles constricted by 19% upon application of FeCl₃, while venular diameters did not change (Table 7.1). After FeCl₃ application, vessels were observed for 600 s, either under trans-illumination by bright-field microscopy and/or under epi-illumination by fluorescence microscopy. 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 local vessel diameter) at 2, 5 and 10 min after FeCl₃ application, and time to occlusion. Where indicated, acridine red (30 µl of 5 mg/ml) was injected intravenously for higher resolution fluorescence imaging of labeled thrombi. After

intravital microscopy, blood was drawn from the mice by retro-orbital puncture and collected into citrate (0.129 M). Isolated plasmas were frozen at -80°C .

To determine wall shear rates (WSR) in representative mesenteric arterioles and venules, two mice were injected with rhodamin 6G (30 μl of 1 mg/ml solution, intravenous) for labeling of circulating leukocytes. Fluorescence microscopy was applied to quantify centerline blood flow velocities in 8 arterioles and 8 venules, using the fastest passing leukocyte as a marker (24). WSR were calculated according to the Newtonian definition: $\text{WSR} = 8 \times (\text{mean velocity} / \text{vessel diameter})$, where mean velocity represents the centerline velocity / 1.6 (25). The resulting WSR represent least estimates of the actual WSR (26).

Thrombin Generation Measurements

Murine platelet poor plasma (PPP) or platelet rich plasma (PRP) was prepared from blood from 12 week old mice as described (27). Thrombin generation was measured using the thrombogram method (28), adapted for murine plasma. Briefly, normalized PRP (1.5×10^8 platelets/ml) or PPP (supplemented with 4.0 μM vesicles of phosphatidylserine : phosphatidylcholine : phosphatidylethanolamine = 1 : 3 : 1) was preincubated with inhibitors for 10 min at room temperature, as indicated. Subsequently, 40 μl of PRP or PPP was added to a 96-well plate, containing per well 10 μl TF (6 pM) in Hepes buffer (20 mM Hepes, 140 mM NaCl, 5 mg/ml bovine serum albumin, pH 7.35). TF was diluted to a concentration (1 pM) at which thrombin formation depended on the presence of platelet expressed PS. Thrombin formation was started by adding 10 μl 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 (29), but were not processed for α_2 -macroglobulin correction, as the activity of α_2 -macroglobulin-bound thrombin in murine blood is unknown. Assays were run in duplicates or triplicates; calibrations were performed with human thrombin.

Immunohistochemical staining

After intravital experimentation, intestine with attached mesentery was collected from mice for histology. Subsequently, the removed tissues were fixed in freshly dissolved paraformaldehyde (1% in phosphate buffered saline) at 4°C for 24 h, routinely processed and embedded in paraffin. Sections of 4 μm were cut and stained with hematoxylin and eosin.

Statistical analysis

Data from in vivo experiments displayed nonsymmetrical distribution and are presented as median values with interquartile ranges. Data from in vitro flow chamber and thrombin

generation experiments are presented as mean $\pm$ SEM. Differences between experimental groups were tested with the non-parametric Mann-Whitney U test, using the statistical package for social sciences (SPSS 11.0). Level of significance was set at 5%.

Results

Tissue factor-induced thrombin formation and collagen contribute to platelet phosphatidylserine exposure in flowing blood

To evaluate the importance of the interaction between platelet activation and coagulation in thrombus formation, perfusion studies were performed with mouse blood, which was recalcified and activated with a low dose of TF (2 pM), directly before entering a collagen-containing flow chamber (shear rate 1000 s^{-1}). This resulted in early platelet deposition, subsequent aggregate formation and appearance of fibrin fibers after a lagtime of 2-3 minutes. At a more advanced stage (7-8 minutes), the chamber became occluded by large fibrin-platelet thrombi with trapped red cells (Fig. 7.1A). The activation state of adhered platelets was monitored by labeling blood with FITC-annexin A5 (0.5 $\mu\text{g}/\text{ml}$, a low dose not influencing

Figure 7.1. Contribution of coagulation to thrombus formation and platelet phosphatidylserine exposure in flowing blood triggered by tissue factor and collagen. Citrated mouse blood was perfused for 4 minutes over a collagen surface at a shear rate of 1000 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 of 5 $\mu\text{g}/\text{ml}$ melagatran (C, D). Representative phase contrast images (A, C) and fluorescence images (B, D; labeled phosphatidylserine-exposing platelets) are given, taken after 4 minutes of perfusion of blood and 4 minutes wash with 0.5 $\mu\text{g}/\text{ml}$ FITC-annexin A5 ($n=3$). (E) Accumulation of fluorescence at the collagen surface during perfusion of blood prelabeled with 0.5 $\mu\text{g}/\text{ml}$ FITC-annexin A5 and triggered with tissue factor/ CaCl_2 . Control condition (●), 5 $\mu\text{g}/\text{ml}$ melagatran (○).

Figure 7.2. FeCl_3 application results in endothelial denudation of mesenteric arterioles and venules. (A) Control and (B) FeCl_3 -treated paraffin sections of mesenteric arterioles (a) and venules (v) were stained with hematoxylin and eosin. Nuclei of endothelial cells stained strongly in untreated vessels (A) indicated by the arrows, while almost no endothelial cells were seen after FeCl_3 treatment (B). Note that vessels and thrombi were not fixed prior to removal from the mouse.

coagulation) to detect PS-exposing platelets. After 8 minutes, many collagen-adherent and aggregated platelets exposed PS, covering about 20% of the surface area (Fig. 7.1B). Perfusion was also performed in the presence of melagatran (5 $\mu\text{g}/\text{ml}$), a high affinity, active-site thrombin inhibitor (30). In this case, aggregate formation still occurred, but fibrin fibers were absent, as were occlusive thrombi containing red cells (Fig. 7.1C). In this case, only the collagen-bound platelets exposed PS, covering 5% of the surface area (Fig. 7.1D). Quantitative analysis indicated that melagatran significantly but incompletely reduced the appearance of PS-exposing platelets during the whole flow experiment (Fig. 7.1E). In the absence of collagen, aggregates or PS-exposing platelets were not formed in this time interval (not shown). These results with melagatran resemble earlier flow data obtained with wildtype murine blood anticoagulated with PPACK/heparin (27). Together, these data indicate that, under these *in vitro* flow conditions, platelet-collagen interaction and activation of the TF pathway synergize in generating procoagulant platelets and stable platelet-fibrin thrombi.

Tissue factor and collagen trigger thrombus formation in arterioles and venules

Using an established mouse model of arterial and venous thrombus formation, intravascular thrombi were generated by topical application of FeCl_3 on adjacent arterioles and venules in a mesenteric intestinal loop of anesthetized mice. This model of vascular damage is known to rely on local free radical formation (31). FeCl_3 application resulted in almost complete disruption and denudation of the endothelial cells and full exposure of the sub-endothelial matrix, as demonstrated by hematoxylin/eosin stained paraffin sections of the mesenteric tissue (Fig. 7.2).

Intravital microscopic trans-illumination images indicated that, in venules, FeCl_3 induced local accumulation of platelets into large thrombi within a few seconds, while in arterioles this process started only after a lagtime of several minutes (Fig. 7.3A). However, thrombus build-up was more rapid in arterioles than in venules; at 10 min after FeCl_3 application the incidence of total occlusion was comparable for arterioles (33%) and venules (42%) (Table 7.1). Median wall shear rates were 1336 s^{-1} in arterioles and 285 s^{-1} in venules. Some mice were injected intravenously with acridine red, which labels platelets and leukocytes *in vivo*, to confirm the more rapid onset of thrombus formation in venules compared to arterioles, using a sensitive fluorescence video imaging approach (23). Again, application of FeCl_3 caused rapid

A **B**

Figure 7.3. Progression of thrombus formation in arterioles and venules. Damage of adjacent mesenteric arterioles and venules was induced by topical application of FeCl_3 at $t = 0$ sec (baseline). (A) Transillumination images of thrombus formation in venule (V; upper panels) and arteriole (A; lower panels). Dotted lines indicate contours of a thrombus (T). (B) Fluorescence images of thrombus formation with platelets labeled with acridine red in venule (upper panels) and arteriole (lower panels); dotted lines indicate vessel wall. Arrows indicate direction of flow; bars represent $50 \mu\text{m}$.

accumulation of fluorescently labeled platelets in venules, and in arterioles platelet deposition was delayed (Fig. 7.3B). Topical application of the hydrophilic molecule acridine red, which has a similar molecular weight as FeCl_3 (about 300 Da), on the mesenteric vessels resulted in almost simultaneous appearance of the fluorescent dye in arterioles and venules (data not

Table 7.1. Control parameters of arterioles and venules from wildtype mice before and after FeCl₃ application

	Arterioles	Venules
Number of vessels (mice)	30 (23)	20 (16)
Vessel diameter before FeCl ₃ (μm)	70 (60-80)	110 (80-130) *
Wall shear rate (s ⁻¹)	1336 (906-1400)	285 (219-434) *
After application of FeCl ₃ :		
constriction (% of diameter)	19 (8-30)	0 (0-10) *
occlusion during 10 min (% of vessels)	33	42
occlusion time of occluded vessels (s)	473 (441-546)	361 (113-392) *

Data are median values (and interquartile ranges). Wall shear rate was determined in 8 vessels. * $p \leq 0.05$; + $p = 0.08$ as compared to arterioles.

Figure 7.4. Effects of tissue factor and factor VIIa inhibition and GPVI deficiency on thrombus formation in arterioles and venules in vivo. Wildtype mice were treated systemically with vehicle (control), 3 μg/g bw anti-mouse tissue factor antibody (anti-mTF) or 10 μg/g bw factor VIIa (FVIIa-i). Mesenteric vessels of wildtype and FcRγ-chain ^{-/-} mice were damaged by topical application of FeCl₃. (A) Lagtime to thrombus formation after FeCl₃ application. (B) Thrombus height measured at 2, 5 and 10 min after FeCl₃ application (% of vessel diameter). Data are median values with interquartile ranges (n = 6-12). * $p \leq 0.05$ as compared to control; + $p \leq 0.1$ compared to control.

Figure 7.5. Effects of melagatran and heparin on tissue factor-triggered thrombin generation in vitro. (A) Citrated PPP, supplemented with 4 μ M phospholipids, or (B) PRP containing 1.5×10^8 platelets/ml was incubated with 0.1–10 μ M melagatran or 0.05–5 U/ml heparin. Thrombin generation was determined after activation with 1 pM (f.c.) tissue factor and 16.6 mM CaCl_2 by measuring fluorescence accumulation from cleaved Z-GGR-AMC. Upper panels show representative thrombograms for 3 independent experiments; lower panels show effects on thrombin peak height relative to control condition without thrombin inhibitor (mean $\pm$ SEM, $n = 3$ –5). * $p \leq 0.05$ as compared to control.

shown). This suggests that the wall of either vessel type is similarly permeable for such small molecules.

To determine the involvement of TF in this thrombosis model, we inhibited TF activity or, alternatively, its ligand factor VII(a) in wildtype mice. Intravenous administration of 3 μ g/g bw anti-murine TF antibody significantly delayed and reduced the thrombotic process in both arterioles and venules (Figure 7.4). Injection of 10 μ g/g bw mouse active site-inhibited factor VII (factor VIIai) had a similar inhibitory effect. In vitro experiments showed that thrombin generation in mouse plasma was completely blocked with anti-TF antibody or factor VIIai (each 20 μ g/ml, not shown). Similarly, in plasma samples taken from mice injected with factor VIIai, thrombin generation was delayed and reduced to $6.8 \pm 2.4\%$ of control level. Thus, TF, probably present in the extracellular matrix, had an initiating role in the thrombus-forming process in arterioles and venules by interaction with factor VII(a). Other experiments showed that platelet interaction with exposed collagen also contributed in this mouse model. Mice deficient in the FcR γ -chain, which do not express the activating collagen receptor GPVI on platelets (32), showed a delayed and significantly reduced thrombus build-up in arterioles and venules (Figure 7.4). Injection of

Figure 7.6. Effects of melagatran and heparin on thrombus formation in arterioles and venules in vivo. Mice were treated systemically with vehicle (control), 0.175–0.500 $\mu\text{g/g}$ bw melagatran or 0.05–0.5 U/g bw heparin. Mesenteric vessels were damaged by topical application of FeCl_3 . (A) Lagtime to thrombus formation after FeCl_3 application. (B) Thrombus height measured at 2, 5 and 10 min after FeCl_3 application (% of vessel diameter). Data are median values with interquartile ranges ($n = 6$ –9). * $p \leq 0.05$ as compared to control. (C) Effect of in vivo infusion of melagatran or heparin on thrombin generation in PPP, supplemented with 4 μM phospholipids. PPP was prepared from blood collected at the end of in vivo measurements. Left panel shows representative thrombograms; Right panel shows bar graph of measured thrombin peak height (mean $\pm$ SEM; $n = 1$ –9).

mice with the inhibitory anti-GPVI antibody JAQ1 gave similar results (33). Thus, in this model, both activation of coagulation by exposed TF/factor VII(a) and platelet activation by collagen appear to initiate the thrombotic process.

Thrombin inhibition differently affects thrombus formation in damaged arterioles and venules

Before intravenous administration into mice, the efficacy of two thrombin inhibitors in murine plasma was tested in thrombin generation measurements using the thrombogram method. The high-affinity, active-site thrombin inhibitor melagatran was used (30), as well as unfractionated heparin, which acts indirectly by enhancing the antithrombin-mediated inactivation of thrombin and factor Xa (34). Each compound substantially reduced thrombin generation in murine plasma activated with TF both in the presence of procoagulant phospholipids (Fig. 7.5A) and in the presence of platelets (Fig. 7.5B). Effects on peak height of thrombin generation curves (maximal levels) were measured, which parameter is proportional to the maximal rate of thrombin generation (35). Dose-response experiments indicated a 50% reduction in maximal thrombin levels in the presence of phospholipids at about 0.5 μ M melagatran and < 0.05 U/ml heparin. In the presence of platelets, the reducing effect of melagatran on thrombin peak height was similar. However, a six-fold higher dose of heparin (0.2 U/ml) was needed for 50% inhibition; this difference is probably due to an activating effect of heparin on platelets (36). Typically, considerable (nanomolar) concentrations of thrombin were still generated, with both phospholipids and platelets, at high heparin (5 U/ml) but not high melagatran (10 μ M) doses (Fig. 7.5, lower panels).

Mice were injected with a moderate dose of melagatran or heparin to give about three-fold prolongation in clotting time (as determined in vitro), and used to study thrombus formation in vivo; higher doses were used as well. In comparison to vehicle controls, injection of 0.175 μ g/g bw melagatran did not influence the lagtime to thrombus formation in arterioles (Fig. 7.6A), but thrombus height was significantly reduced (Fig. 7.6B). After intravenous administration of a three-fold higher melagatran dose (0.5 μ g/g bw), the arteriolar lagtime to thrombus formation was prolonged (>600 s, $p = 0.05$, Fig. 7.6A), while thrombus build-up was again greatly reduced (Fig. 7.6B). Venular thrombus formation was inhibited only by the higher dose of melagatran (Fig. 7.6A, B). None of the arterioles and venules occluded within 10 min with the moderate and high dose of melagatran. At the end of the experiments, plasma samples were taken and used for thrombin generation measurements. As indicated in Fig. 7.6C, thrombin peak height was diminished to 18% and 4% of control, respectively, after administration of these melagatran doses.

Figure 7.7. Effects of melagatran, PGE₁ and GPRP on tissue factor-triggered thrombin generation in vitro. Thrombin generation was measured in mouse PRP with the thrombogram method, as described for Fig. 7.5. PRP was pretreated for 10 min with prostaglandin E₁ (PGE₁, 10 μ M), PGE₁ plus melagatran (1 μ M), GPRP (1 mg/ml), or PGE₁ plus GPRP, as indicated. Presented is the effect on thrombin peak height as % of control condition (mean $\pm$ SEM). * $p \leq 0.05$ as compared to control.

In mice injected with 0.05 U/g bw heparin, thrombus formation was only influenced in arterioles (Fig. 7.6A, B). Injection of the higher heparin dose (0.5 U/g bw) similarly reduced this process in arterioles, but - intriguingly - led to increased thrombus formation in venules (Fig. 7.6B). Thrombin generation, measured in plasma samples taken from mice treated with moderate or high heparin, showed an incomplete reduction in peak height to 25% and 9% of control, respectively (Fig. 7.6C). 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 venular compared to arteriolar thrombus formation.

Additive effect of platelet and thrombin inhibitors on venous thrombus formation

To inhibit platelet activation, the cAMP-elevating agent prostaglandin E₁ (PGE₁) was used. In isolated mouse PRP, 10 μ M PGE₁ substantially reduced platelet aggregation (not shown) and platelet-dependent thrombin generation (Fig. 7.7, peak height reduced to 73 $\pm$ 5%, $P=0.003$). This corresponds to results obtained with human PRP (4,29). In vivo treatment of mice with 0.4 mg/g bw PGE₁ (giving a plasma concentration of about 10 μ M) significantly delayed and reduced thrombus formation in arterioles, but not in venules (Fig. 7.8).

Administration of PGE₁ (0.4 mg/g bw) together with the lower dose of melagatran (0.175 μ g/g bw) resulted in significant retardation and reduction of the thrombotic process, not only in arterioles, but also in venules (Fig. 7.8). Thus, in venules, this combined administration was more effective than that of PGE₁ or moderate melagatran alone. Platelet inhibition by PGE₁ or moderate thrombin inhibition by melagatran was sufficient to inhibit arterial thrombus formation, but the combined action was needed for reducing venous thrombosis.

Figure 7.8. Effects of melagatran, PGE₁ and GPRP on thrombus formation in arterioles and venules in vivo. Mice were treated intravenously with vehicle (control) or with: 0.4 mg/g bw prostaglandin E₁ (PGE₁), PGE₁ plus 0.175 µg/g bw melagatran, 135 µg/g bw GPRP or PGE₁ plus GPRP. Mesenteric arterioles and venules were damaged by topical application of FeCl₃. (A) Lagtime to thrombus formation after FeCl₃ application. (B) Thrombus height measured at 2, 5 and 10 min after FeCl₃ application (% of vessel diameter). Data are median values with interquartile ranges (n = 4-9). * p ≤ 0.05; * p ≤ 0.07 as compared to control.

Inhibition of fibrin formation in arterial and venous thrombus formation

Fibrin fibers can bind thrombin and protect it from inactivation (37). We considered the possibility that the capture of thrombin by abundant fibrin formation is responsible for the relative resistance of venular thrombi to thrombin antagonism. This was investigated with GPRP, a peptide which blocks the polymerization of fibrin monomers. In vitro experiments indicated that GPRP (1 mg/ml) abolished the formation of fibrin clots (not shown), but was of only little effect on platelet-dependent thrombin generation (Fig. 7.7). Mice were subsequently infused with 135 µg/g bw GPRP, resulting in a plasma concentration of about 1 mg/ml. Surprisingly, the infused GPRP greatly delayed thrombus formation in arterioles, but enhanced this process in venules (Fig. 7.8). With GPRP, only one of the arterioles, but 75% of the venules occluded within 10 minutes. No extra effect was reached in either vessel type by combined infusion of GPRP and PGE₁. This suggests that fibrin polymerization restricts rather than facilitates the action of thrombin in venules, possibly by limiting thrombin diffusion.

Figure 7.9. Detection of phosphatidylserine exposure in arterial and venous thrombi formed in vivo. Vessel wall damage was induced by topical FeCl_3 application, directly after which FITC-labeled annexin A5 ($0.16 \mu\text{g/g bw}$) was infused intravenously. Trans-illumination images were taken at baseline; fluorescence images were taken at 5 and 10 min after FeCl_3 application. Dotted lines indicate vessel wall, arrows indicate direction of flow; bar represents $50 \mu\text{m}$.

Thrombus formation in arterioles and venules relies on exposure of procoagulant phosphatidylserine by platelets

To investigate whether procoagulant platelets play a role in the thrombin-dependent thrombus-forming process, mice were injected with a tracer amount of FITC-labelled annexin A5, which detects cell surface-exposed PS, after the onset of FeCl_3 -induced thrombus formation. Thrombi in both arterioles and venules incorporated labeled annexin A5 (Fig. 7.9), indicating 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).

The importance of PS exposure for the thrombus-forming process was further elucidated by injecting mice with a saturating dose ($7.7 \mu\text{g/g bw}$) of unlabelled annexin A5. Quadruple-mutated annexin A5 (M1234-annexin A5) was used as a negative control. The M1234-annexin A5 has single amino acid mutations in each of the four Ca^{2+} binding sites, which greatly reduces its affinity for binding to PS (38). Whereas M1234-annexin A5 infusion had no significant effect on thrombus formation as compared to vehicle controls ($P \geq 0.18$), wildtype annexin A5 almost completely inhibited this process in both arterioles and venules (Fig. 7.10A, B). In fact, in none of the 7 damaged arterioles any platelet deposition was detected within the 10-min time frame, while in only 3 out of 6 venules limited adhesion of platelets assembling into very small thrombi was observed.

In thrombin generation assays in vitro, addition of $10 \mu\text{g/ml}$ annexin A5 to PRP was sufficient to reduce the thrombin peak to $11 \pm 1.6 \%$ of control, while M1234-annexin A5 was without effect (Fig. 7.10C). In plasma collected from mice injected with annexin A5 ($7.7 \mu\text{g/g}$

Figure 7.10. Effect of annexin A5 on thrombus formation in arterioles and venules in vivo. Mice were infused with 7.7 $\mu\text{g/g}$ bw annexin A5 or mutated M1234-annexin A5, resulting in an estimated plasma concentration of 70 $\mu\text{g/ml}$. Mesenteric vessels were damaged by topical application of FeCl_3 . (A) Lagtime to thrombus formation after FeCl_3 application. (B) Thrombus height measured at 2, 5 and 10 min after FeCl_3 application (% of vessel diameter). Data are median values with interquartile ranges ($n = 6-7$); * $p \leq 0.05$ as compared to control and M1234-annexin A5. (C) In vitro effect of annexin A5 on thrombin generation in PRP. Thrombin generation was measured in PRP, treated with a moderate dose (10 $\mu\text{g/ml}$) mutated M1234-annexin A5 or wildtype annexin A5. Given is thrombin peak height from thrombograms as % of control (mean $\pm$ SEM, $n = 3-4$). * $p \leq 0.05$ as compared to control and M1234-annexin A5.

bw), thrombin generation was reduced to $17 \pm 4\%$ ($n=4$) of control plasma. Together, these data show that in either vessel type, thrombus formation appears to rely on PS exposure and ensuing thrombin generation by (activated) platelets.

Discussion

In this paper, we investigated the contribution of platelets to the coagulant process under *in vivo* conditions, using a murine microvascular thrombosis model known to be sensitive to platelet and coagulation inhibitors. Both in arterioles and venules, we were able to visualize externalized PS on platelets trapped in thrombi, using fluorescently labeled annexin A5. This probe has previously been used for *in vivo* monitoring of apoptotic, PS-exposing cardiomyocytes in a mouse model of ischemia/reperfusion of the heart (39). The present data indicate that the PS-exposing platelets contributed to thrombin and fibrin generation and build-up of a stable thrombus. Intravenous administration of a high dose of annexin A5, sufficient to suppress PS-dependent thrombin generation in plasma, resulted in a profound delay in initiation and in almost complete suppression of thrombus formation in the observed vessels. The specificity of the intervention was demonstrated by administration of point-mutated M1234-annexin A5, with a low affinity for PS, which was without notable influence on the thrombotic process. In agreement with these results, abundant appearance of PS-exposing platelets under conditions of thrombin generation and formation of platelet-fibrin clots was observed in flow chamber experiments. Together, to our knowledge, this is first evidence for *in vivo* importance of PS exposure and platelet-dependent coagulation in arterial and venous thrombosis.

This work thereby significantly extends the knowledge from experiments in test tubes, that activated platelets enhance thrombin generation by exposure of PS (7,13). Indirect evidence for the clinical importance of platelet procoagulant activity is given by patients with a rare bleeding disorder, the Scott syndrome, who have a deficiency in PS exposure upon activation (40,41). Furthermore, patients with the Stormorken syndrome, a thrombophilic disorder, appear to have platelets with increased PS exposure (42).

The *in vivo* findings also provide novel insight into thrombus-forming mechanisms that can take place in different parts of the circulation. The interventions with anti-TF antibody and active site-inhibited factor VIIa, both of which inactivate TF exposed to blood, significantly reduced the thrombotic process in arterioles and venules. Moreover, the experiments with mice deficient in GPVI/ FcR γ -chain indicate that also platelet-collagen interaction via GPVI contributes to the thrombus forming process in both vessel types. This agrees well with the *in vitro* observation that platelet activation by GPVI is a key, initiating event in PS exposure and platelet aggregation in mice (27,43) and man (15). Thus, in the present model, in both arterioles and venules the thrombotic process appears to be initiated by TF and collagen.

We also note similarities in the propagation phase of arteriolar and venular thrombus formation. In either case, the thrombotic process was abrogated by infusion of melagatran at a concentration sufficiently high to completely inhibit thrombin generation; a lower melagatran dose had similar effects provided that it was given in combination with platelet-inhibiting PGE $_1$. Together with the inhibitory effect of annexin A5, this is strong evidence that the thrombotic

process is driven by sequential loops of thrombin generation, platelet activation, PS exposure and prothrombinase activity. This is in agreement with published evidence that FeCl_3 -induced thrombus formation in murine arteries is diminished in the absence of one of the two platelet thrombin receptors, PAR-3 or PAR-4 (2,44).

Despite these mechanistic similarities, also typical differences between arterioles and venules are noted. Suppression of the venous thrombotic process required a more profound thrombin inhibition compared to suppression of thrombus formation in adjacent arterioles. Moderate thrombin inhibition with melagatran or platelet inhibition with PGE_1 was sufficient to block arterial thrombus formation. However, the combination of both was needed to suppress venous thrombosis. Along the same line, blocking of fibrin polymerization (by GPRP) was antithrombotic in arterioles, but it did not affect thrombosis in venules. An attractive explanation is that thrombin accumulates at relatively high levels in (slowly flowing) venules, and that this process needs to be suppressed more strongly to come below the threshold level where thrombin enhances its own formation (via activated platelets).

In venules, administration of a high dose of unfractionated heparin increased rather than decreased the formation of thrombi. This contrasts with the inhibitory effect of melagatran infusion. Unfractionated heparin is an indirect thrombin inhibitor, which effectively enhances the inactivation of formed factor Xa and α -thrombin by antithrombin. This indirect way of action may explain why, even at a high heparin concentration, thrombin generation and coagulation are incompletely inhibited, in contrast to the direct antagonistic effect of melagatran. A recent report indicates that heparin promotes clot formation by interacting with platelet factor 4 (45), which may explain why heparin enhances FeCl_3 -induced in venules. Another effect of unfractionated heparin is enhancement of the platelet activation process, likely in a way involving autocrine ADP release (36). Thus, in addition to incomplete inhibition of thrombin generation, increased thrombotic activity in the presence of high heparin doses can also be due to a platelet-activating effect.

In vitro, platelets respond to minute (sub-nanomolar) concentrations of thrombin via their receptors (PARs), i.e. well before fibrin starts to be formed. The recognition that more complete suppression of thrombin generation is needed to inhibit venous than arterial thrombosis suggests that local thrombin generation in venules is too high to be antagonized by platelet inhibitors like PGE_1 and aspirin. Indeed, in flow experiments, we have recently observed that PGE_1 is unable to completely suppress PS exposure and ensuing coagulation at low shear rate (22).

Typically, we observed a significantly faster onset of thrombus formation in venules than in arterioles. Such a difference between vessels has also been found in other thrombosis models (reviewed in Ref. 46). There are a number of explanations for the rapid progression of the thrombotic process in venules. First, the thicker arterial wall may delay the penetration of

FeCl_3 . However, this is unlikely, as the walls of arterioles and venules are similarly permeable for the hydrophilic, fluorescent compound acridine red, which has a molecular weight similar to that of FeCl_3 . Also, no correlation was found between vessel diameter (which relates to wall thickness) and lagtime to thrombus formation in arterioles ($R^2 = 0.007$) or venules ($R^2 = 0.003$), suggesting that vessel diameter and, hence, wall thickness does not influence the start of thrombus formation. Second, different antithrombotic properties between endothelial cells in arterioles and venules (46) may lead to a different degree of antagonism. Arterial endothelium may have a higher antithrombotic activity, e.g. increased activity of platelet-inhibiting substances like prostacyclin and NO (47). However, this is not consistent with the fact that endothelium in both vessel types was almost completely removed by FeCl_3 . Third, damaged venules may express more TF, which is known to be distributed through the vascular system in a non-uniform way (48). Fourth, the higher flow rate in arterioles may accomplish faster dilution of locally active prothrombotic substances (e.g. factor VIIa, ADP and thrombin). In fact, our finding that higher doses of thrombin inhibitors are needed to antagonize venular than arteriolar thrombosis is compatible with either of the last two explanations.

In another mouse thrombosis model, applying laser-induced vascular injury, there is evidence that TF, platelets and fibrin gradually accumulate into developing thrombi (10). The current data cannot answer the question whether (49) or not (50) blood-borne TF is involved in the thrombotic process in arteries and veins. However, they support the proposal that inhibition of TF interaction with factor VII(a) is a promising target for new antithrombotic treatment of the thrombotic process (51,52). Furthermore, the recognition that PS exposure by platelets is a key propagating step in thrombus formation in veins and arteries indicates that this process is also therapeutically interesting. Indeed, a number of new platelet inhibitors, i.e. P2Y_{12} antagonists like clopidogrel (53) and $\alpha\text{IIb}\beta 3$ blockers like abciximab and eptifibatide (54), inhibit the platelet procoagulant response in addition to their anti-aggregatory action.

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.

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Chapter 8

General discussion

Atherothrombosis, thrombus formation at the surface of ruptured atherosclerotic plaques in arteries, is a major cause of cardiovascular disease. The thrombotic process starts with platelet interaction with the damaged atherosclerotic vessel wall. As an important tool in the fight against atherosclerosis, a better understanding is needed of the mechanisms involved in platelet adhesion to vessel wall components and subsequent events leading to thrombus formation. The main goal of the studies presented in this thesis was to investigate the contribution of key platelet receptors to collagen-induced platelet activation, in particular the platelet procoagulant response. Part of the experiments were performed using an *in vitro* model of thrombus formation under controlled conditions of blood flow and coagulation. Also, an *in vivo* mouse model of intravascular thrombus formation was used. The role of the established key receptors for collagen as well as the relationship between collagen-induced platelet aggregatory and procoagulant activity will be discussed below. Furthermore, the importance of the vessel wall in thrombus formation will be addressed. In Figure 8.1, current understanding of the interaction between platelet activation and coagulation is schematically depicted; this figure provides a framework for discussing the results from this thesis.

8.1 Roles of GPVI and integrin $\alpha 2\beta 1$ in platelet adhesion, activation and thrombus formation

Platelet interaction with subendothelial collagen is a key initial step in thrombus formation following vessel wall damage. At high shear rates, the von Willebrand factor (vWF) receptor glycoprotein (GP) Ib-V-IX initially tethers platelets to vWF deposited on collagen fibers. Subsequent stable adhesion involves two collagen receptors, the immuno-globulin receptor GPVI and the integrin $\alpha 2\beta 1$ (GPIIb/IIIa). Their relative contribution has been the subject of considerable debate in the past decades. A critical role for $\alpha 2\beta 1$ was identified in the mid 1980s, when two $\alpha 2$ -deficient patients were described, who presented with a bleeding phenotype and impaired platelet aggregation in response to collagen (1-3). However, later it was questioned whether the clinical symptoms were related to the proposed platelet defect (4). Further studies in the 1980s and 1990s established the critical role of $\alpha 2\beta 1$ in mediating stable adhesion on collagen (5). Nevertheless, a second, unidentified collagen receptor was proposed, that mainly regulated platelet activation. This sequence of events, i.e. adhesion followed by activation was termed the two-site, two-step model of platelet-collagen interaction (6,7). The model was challenged after the discovery of the GPVI-Fc-receptor (FcR) γ -chain complex as the principal receptor for platelet activation by collagen (4,8). Especially studies with GPVI- or $\alpha 2\beta 1$ -deficient mice were important since they demonstrated a critical role for GPVI in both adhesion and aggregation on collagen (9,10). In addition, these studies showed that $\alpha 2\beta 1$ was not required for these events; the integrin $\alpha IIb\beta 3$ mediated stable adhesion on collagen-bound vWF in the

Figure 8.1 Schematic overview of platelet-dependent coagulation in thrombus formation. In the initiation phase, tissue factor/factor VII(a) interaction leads to formation of traces of thrombin, while platelets interact with collagen/vWf via GPIb (arteries) and the collagen receptors GPVI and integrin $\alpha 2 \beta 1$. Platelet activation is characterized by integrin $\alpha II \beta 3$ activation and exposure of phosphatidylserine accompanied by blebbing morphology. This results in two platelet populations with aggregation on the one hand and platelet procoagulant activity on the other hand. In the propagation phase of thrombus formation, platelet aggregates as well as massive amounts of thrombin are formed. A fibrin network stabilizes the developing thrombus.

absence of $\alpha 2 \beta 1$. As a consequence, the role of $\alpha 2 \beta 1$ in platelet-collagen interaction remained unclear.

In chapter 3, experiments are described with mice deficient in FcR γ -chain (lacking GPVI), $\beta 1$ -integrin (lacking $\alpha 2 \beta 1$) or G α_q (defective responses to thromboxane A₂ (TxA₂) and ADP). The results show that GPVI and $\alpha 2 \beta 1$ have complementary functions in collagen-induced thrombus formation during perfusion of whole blood over collagen. Integrin $\alpha 2 \beta 1$ mainly facilitates the central role of GPVI in various platelet activation processes leading to thrombus formation, i.e. Ca²⁺ mobilization, exposure of phosphatidylserine (PS) and aggregation. The autocrine agents TxA₂ and ADP mainly serve to trigger aggregate formation. Several other mouse and human studies have confirmed these findings, thus re-establishing the role of $\alpha 2 \beta 1$ in platelet adhesion to collagen (11-14).

Regarding platelet adhesion, the critical role of GPVI has recently been questioned by observations that platelets from GPVI-deficient mice, as well human platelets inhibited with anti-GPVI antibodies, stably adhere to collagen (14-17). This issue is addressed by Auger et al. (18), who directly compared adhesion, activation and aggregation of human and mouse platelets. In this paper, identical or equivalent experimental tools were used with either species under the same experimental conditions. Two mechanisms of stable adhesion and activation on collagen

were shown to co-exist. GPVI-mediated platelet activation was demonstrated to either precede or follow adhesion mediated by both $\alpha 2\beta 1$ and GPVI. The two fundamental processes of adhesion and subsequent aggregate formation are preserved in mouse and human platelets (18). This view is supported by another study with $\alpha 2\beta 1$ -deficient mice, also indicating that GPVI and $\alpha 2\beta 1$ play an equal role in platelet adhesion to collagen (19). Because natural collagens contain different binding sites (high and low affinity) for $\alpha 2\beta 1$ and GPVI (20,21), it is likely that individual platelets detect different micro-environments when in contact with a collagen fiber, and then respond accordingly.

A surprising observation in chapter 3 was the formation of loose platelet aggregates upon perfusion of blood from $\beta 1$ -null mice over collagen. This finding raises the question whether $\alpha 2\beta 1$ plays a role in thrombus stability and embolization. This hypothesis was tested in chapter 4 by experiments with $\alpha 2$ -null mice. In an *in vivo* thrombosis model of FeCl_3 injured mesenteric arterioles, overall (cumulative) thrombus formation was similar between $\alpha 2$ -null and wildtype mice. However, individual thrombi were smaller in vessels from $\alpha 2$ -null mice compared to vessels from wildtype mice. Furthermore, the thrombi from $\alpha 2$ -null mice frequently embolized or even completely detached from the vessel wall. Also, perfusion of blood from $\alpha 2$ -null mice over collagen resulted in formation of smaller and looser thrombi, regardless of shear rate (low/high) or presence of coagulation. In addition, absence of $\alpha 2\beta 1$ reduced fibrinogen binding to platelets and lowered PS exposure. This was accompanied by delayed and reduced thrombin generation in response to tissue factor and collagen. In addition, TxA_2 release from platelets was reduced in the absence of $\alpha 2\beta 1$, which could explain the formation of smaller and unstable thrombi. These results provide a mechanistic explanation for earlier studies performed with the same $\alpha 2$ -null mice, which showed that aggregation of $\alpha 2$ -null platelets in response to fibrillar collagen was delayed (10). In these $\alpha 2$ -null mice *in vivo* thrombus formation was also similar to wildtype mice in ligation-induced collagen exposure in vessel wall of the carotid artery injury by ligation (22). In another strain of $\alpha 2$ -null mice, thrombus formation in the carotid artery was found to be moderately delayed, but the authors did not observe differences in the formation of pulmonary emboli following intravascular injection of collagen (23). Keeping in mind that platelets may react differently when encountered with a different stimulus, the apparent differences between the two $\alpha 2$ -deficient mouse strains may be only minimal. We conclude that $\alpha 2\beta 1$ has a moderate but qualitatively important effect to stabilize thrombi and reduce embolization, by enhancement of GPVI-dependent platelet activation.

The studies presented in chapter 3 and 4 supplement each other with regard to the establishment of GPVI as vital collagen receptor and $\alpha 2\beta 1$ as complementary partner mediating platelet adhesion and reinforcing GPVI-dependent activation and thrombus stabilization. This nicely fits with other studies with human platelets (12-14). Interestingly, some authors propose that $\alpha 2\beta 1$ also contributes to the signaling process by itself (24), but the present experiments

indicate that under shear this contribution is only minimal compared to that of GPVI. The moderate effect of $\alpha 2\beta 1$ deficiency sharply contrasted to the strongly delayed and reduced thrombus formation of murine GPVI-deficient platelets in the same flow and in vivo experiments (chapter 3, 7 and (25)).

In conclusion, in the initiation phase of platelet activation by collagen (Fig. 8.1), GPVI appears to be the essential receptor mediating all activation processes, but it needs to be complemented by integrin $\alpha 2\beta 1$ activity for stable adhesion to collagen, full platelet activation and subsequent stable thrombus formation.

8.2 Platelets and coagulation

Platelets that come into contact with collagen, the major platelet-activating constituent of the extracellular matrix, are activated via GPVI. This GPVI-collagen interaction induces a potent and prolonged rise in cytosolic Ca^{2+} , which is a prerequisite for PS exposure (26,27). The exposure of PS in the outer membrane is known to be necessary for accelerated thrombin formation (28,29). Chapter 2 describes that procoagulant platelets show typical morphological changes that accompany PS exposure; they can suddenly transform into balloon-shaped cells with membrane blebs. These so-called "blebbing" platelets shed microparticles, which are also known to express PS on their surface (27). That GPVI is the key receptor for this response is further demonstrated in chapter 3, in which it is shown that GPVI-deficient mouse platelets are deprived of collagen-induced calcium responses and PS exposure. Others have made complementary observations, i.e. that collagen-bound human platelets with high cytosolic Ca^{2+} respond by PS exposure (14), and by membrane contraction, fragmentation and microvesiculation (30). These platelets were termed to have SCIP morphology, being sustained calcium-induced platelets. In addition, the investigators demonstrated that SCIP morphology was accompanied by down-regulation of integrin $\alpha \text{IIb}\beta 3$ adhesive function, involving factor XIII and calpain (30). As PS-exposing platelets are known to be high in cytosolic Ca^{2+} , the SCIP morphology is likely to be related to platelet procoagulant activity. This points to the existence of two populations of activated platelets, one expressing procoagulant activity involved in coagulation, and another with activated integrin $\alpha \text{IIb}\beta 3$ involved in aggregation (see paragraph 8.3).

Although collagen-induced PS exposure appears to be mainly induced by GPVI complemented by $\alpha 2\beta 1$, which both bind collagen directly, this thesis describes that other receptors contribute to this platelet response in an indirect manner. The experiments in chapter 5 demonstrate that, under shear conditions, inhibition of GPIb, as well as blocking of vWF binding to collagen, reduced stable platelet adhesion, delayed Ca^{2+} responses and reduced PS exposure. Importantly, in mice expressing only 50% of normal GPVI levels, blockage of GPIb almost completely abolished platelet adhesion and PS exposure. Hence, GPVI-dependent

platelet activation is clearly facilitated by GPIb. In support of this, others have recently observed that GPIb and GPVI are both located in rafts, which are cholesterol-rich domains in the membrane. These rafts may physically concentrate receptors and downstream proteins involved in signaling pathways (reviewed in (31)). On the other hand, GPIb itself has also been reported to cause spiking Ca^{2+} -elevations in platelets binding to vWF (32-34). It is possible that these events contribute to the Ca^{2+} signal in the presence of collagen. The same may hold for integrin $\alpha\text{IIb}\beta_3$, as its binding to vWF under high shear can also cause spiking Ca^{2+} responses (33), which may be caused indirectly by ADP secretion and subsequent stimulation of ADP receptors. Thus, both GPIb and $\alpha\text{IIb}\beta_3$ promote collagen-induced PS exposure under shear conditions, either directly by causing Ca^{2+} signal generation or indirectly by enhancing GPVI-collagen contact. Together, this clearly indicates that initiation of platelet adhesion to collagen and subsequent activation evolves as a series of multireceptor events with redundancy, facilitation and mutual interactions, rather than as a linear process (Fig. 8.1).

GPVI-mediated platelet adhesion to collagen and subsequent activation is not the only direct cause of PS exposure. We find that in the propagation phase of thrombus formation, platelets can also be induced to expose PS in the absence of collagen. In chapter 6, perfusion studies were carried out with tissue factor-triggered rat and human blood over a fibrinogen surface. The results indicate that particularly under such coagulation conditions the ADP receptor P2Y_{12} enhances platelet procoagulant activity. Furthermore, in the presence of collagen, PS exposure can be strengthened via secondary mediators. Various platelet agonists have been shown to amplify PS exposure and subsequent thrombin generation by release of ADP and P2Y_{12} -receptor stimulation (35,36). In the presence of collagen, autocrine ATP can play a similar indirect role, as the ATP-triggered Ca^{2+} channel P2X_1 mediates enhancement of collagen-induced PS exposure (37). Thrombin, generated in the onset of coagulation, may play a similar stimulating role via PAR-1 activation (38). From these findings it can be concluded that the signaling events involved in platelet procoagulant activity (i.e. PS exposure) are not limited to GPVI, but extend to receptors that mediate responses to ADP, ATP and thrombin, in the propagation phase of thrombus formation (Fig. 8.1).

That thrombin generation and platelet activation are mutually stimulatory processes has been demonstrated by many in vitro studies with isolated platelets and plasma (39,40). However, in vivo evidence for interplay between thrombin generation and platelet activation, and also for a role of PS herein, was missing. This subject has been investigated in chapter 7, where thrombus formation in mouse mesenteric vessels was triggered by FeCl_3 -induced exposure of vascular collagen and tissue factor. Inhibition of platelets and thrombin activity revealed synergistic effects between platelet activation and coagulation during the thrombus-forming process in both arterioles and venules. Furthermore, coating of exposed PS by injection of annexin A5 almost completely abolished thrombus formation in the damaged arterioles and

venules. Staining with fluorescently labeled annexin A5 revealed the presence of exposed PS only at sites where thrombi were present. These studies may provide first in vivo support for the importance of PS exposure in thrombosis. They point to an important role of platelet-dependent coagulation in thrombus formation in vivo. However, a contribution of exposed PS in the injured vessel wall cannot be excluded. The clinical importance of the procoagulant platelet response is apparent in patients with a rare bleeding disorder, the Scott syndrome. These patients have a deficiency in PS exposure upon platelet activation (41,42).

Surprisingly, when thrombosis was induced by FeCl_3 in vivo, a high dose of thrombin inhibitor or a moderate dose in combination with a platelet inhibitor was needed to suppress thrombus formation in venules, whereas in arterioles a moderate level of thrombin inhibition was already sufficient. One explanation for this finding is that thrombin generation is locally higher in venules than in arterioles. Therefore, one may speculate that the synergy between platelets and coagulation may be more powerful under venous conditions. This could be a consequence of differences in subendothelial structures (as discussed in paragraph 8.4), resulting in different mechanisms of platelet adhesion in arteries and veins. Also, the lower blood flow in veins may allow platelets to interact with higher local agonist concentrations (thrombin) during longer time periods. In addition, thrombi were larger in size in venules compared to arterioles (at least in the initial phase of thrombus formation), and hence, more activated platelets with more available PS may cause the massive generation of thrombin in veins.

Availability of procoagulant phospholipids is essential for strong prothrombin and factor X activation (39). It is believed that these phospholipids act by generating binding sites for the vitamin K-dependent coagulation factors, II (prothrombin), VII, IX and X interacting via their Gla-domains to PS (43). While these classical interactions are essential in thrombin generation, it cannot be excluded that also PS-independent mechanisms exist (44,45). Indeed, evidence has emerged that platelet glycoproteins may serve as binding sites for certain coagulation factors, e.g. prothrombin binds to $\alpha\text{IIb}\beta_3$ (46) and factors IX and XI to GPIb (47,48). Also, transglutaminase-mediated cross-linking of serotonin is considered to generate receptor sites on platelets for coagulation factors (49,50). Furthermore, the vitamin K-independent factors V and VIII bind to activated platelets independently of PS, but how this occurs is presently unknown. Finally, the surface of procoagulant platelets may also activate factor XII, which may be caused by the negatively charged surface or by other structures exposed or released from platelets (51). These interesting novel mechanisms may indeed play a role in the alliance between platelets and coagulation, and might have been present during the experiments presented in this thesis. However, in contrast to PS exposure, their physiological role still needs to be established. Therefore, figure 8.1 does not refer to these mechanisms.

In short, platelet activation and PS exposure in response to collagen involves a series of multireceptor events with redundancy, facilitation and mutual interactions, in which GPVI is

essential. Moreover, PS exposure is not only mediated by adhesive receptors, but extends to receptors that mediate secondary responses to ADP, ATP and thrombin. This partnership between platelets and coagulation also plays an important role in thrombus formation *in vivo*.

8.3 Platelet heterogeneity in adhesion and thrombus formation

A striking observation throughout the studies in this thesis is the existence of different platelet populations after perfusion of blood over a thrombogenic surface. This is particularly visible in Figure 4.6, where fluorescently labeled fibrinogen (green), and annexin A5 (red), the latter binding to exposed PS, were added to mouse blood prior to perfusion over collagen. In wildtype blood, bright fibrinogen staining was observed in aggregates, indicating activation of integrin $\alpha\text{IIb}\beta 3$. Typically, annexin A5 labeling did not co-localize with fibrinogen labeling, but only single, collagen-bound platelets around the aggregates were annexin A5-positive. In all experiments in which mouse, rat or human blood was flown over collagen followed by post-perfusion staining with annexin A5, typically only a fraction of (single) platelets was positive for PS (chapters 2-6), similarly as reported earlier (52). In all conditions, these PS exposing platelets had a balloon-shaped, blebbing morphology. Thus, in this flow assay, clearly the platelets that participate in aggregation are not identical to the ones that contribute to coagulation.

This heterogeneity is partly explained by the different ways of platelet attachment to collagen, which is influenced by the local conditions, including the heterogeneity in collagen binding sites for platelets. Stable platelet adhesion under flow is mediated either by GPVI or $\alpha 2\beta 1$, and facilitated by $\alpha\text{IIb}\beta 3$ interaction with vWF, giving rise to complex receptor interplay which easily facilitates heterogeneity (18). Subsequent platelet activation by collagen involves different types of calcium responses, either spiking or prolonged elevations, depending on the particular receptor involved (chapters 3 and 5). In this activation process autocrine ADP may also play a role (chapter 6). The adherent platelets subsequently recruit platelets from the flowing blood, leading to the formation of aggregates. In this phase, heterogeneity is introduced when the initial adhesion of "vanguard" platelets to collagen is followed by attachment of "follower" platelets on the spread-out vanguard platelets; this latter process was shown to be $\alpha\text{IIb}\beta 3$ dependent (53). Furthermore, it was demonstrated that different agonists achieve different contributions of tyrosine and serine/threonine kinases in $\alpha\text{IIb}\beta 3$ activation and aggregation (54). It is likely that the relative contribution of each pathway towards platelets activation depends on microenvironmental conditions and results in platelet heterogeneity.

The concept of platelet heterogeneity is also implicitly assumed in a study directed to reveal the intercellular communication of platelets in a thrombus. It has been proposed that stationary (vanguard) platelets communicate their "calcium-activation-status" to subsequent tethering (follower) platelets, recruiting these platelets into developing aggregates (55). The

intercellular calcium communication was shown to be mediated via $\alpha\text{IIb}\beta_3$ and P2Y_{12} (55). Our findings from perfusion experiments however indicate that even in the presence of aggregates, platelets may still bind to collagen instead of adhering to other platelets, and then 'decide' to remain single.

The observation that some collagen-adherent platelets remain single is still puzzling. The formation of blebs occurs relatively late, i.e. after several minutes, suggesting that complex or slow signaling cascades precede this dramatic loss of normal platelet structure. It has been shown that degradation of cytosolic actin fibres contributes to the changed morphology of the procoagulant cells (56,57). Furthermore, protein tyrosine dephosphorylation (58) as well as the p38 mitogen-activated protein kinase (SAPK2/3) pathway are involved in actin rearrangement during the development of procoagulant activity and bleb formation (27). At present, little more is known about the signaling prerequisites for the blebbing morphology associated with PS exposure. It seems that above a certain threshold of cytosolic Ca^{2+} concentration, calpain and other proteases degrade actin, resulting in the destruction of platelet shape, which may even alter the aggregatory function of integrin $\alpha\text{IIb}\beta_3$. As a consequence, these platelets are unable to interact with other platelets, leaving them as single platelets. The present findings strongly suggest that this PS-exposing platelet population plays an important physiological role in thrombin generation and, hence, is quite distinct from the platelets recruited into aggregates. The *in vitro* observations that these platelets remain single, and thus are excluded from aggregates, stress their unique importance in the stimulation of coagulation.

Taking this reasoning further, it is also likely that within developing aggregates different microenvironments exist, due to locally different concentrations of released or formed ADP, TxA_2 and thrombin. Recruited platelets will react to these agonists accordingly, resulting in heterogeneity in platelet activation within a thrombus. That such heterogeneity in thrombi naturally occurs *in vivo* has been observed by van Gestel et al. (59) who showed that most platelets that adhered to a stationary thrombus, exhibited a prolonged rise in intracellular Ca^{2+} , accompanied by shape change and degranulation. In contrast, in platelets that adhered to a growing embolus the rise in intracellular Ca^{2+} was transient, while shape change and degranulation were absent (59). Besides the micro-environmental differences, platelet heterogeneity may also be the result of different receptor expression on platelets even within individuals. In addition, the platelet's age may also play a role. For instance, old platelets may cluster GPIb on their surface, similar to chilled blood platelets (60). This GPIb clustering may make older platelets more susceptible for tethering on (collagen-bound) vWF, or for aggregation mediated via GPIb-vWF interaction. Probably all of the explanations above are involved in the formation of heterogeneous platelet populations, and other unidentified mechanisms may contribute as well.

8.4 Flow chamber versus in vivo mouse models: importance of the vessel wall

Platelet adhesion and thrombus formation have been studied using both in vitro and in vivo models. In the present thesis a parallel-plate flow chamber was used to address the mechanisms of platelet activation and aggregation, using mostly collagen as platelet-activating surface. The major advantage of this perfusion approach is the ability to collect data under well-controlled conditions of exposure time, thrombogenic surface and reproducible shear stress. This setup allowed real-time monitoring of shear-dependent platelet adhesion and aggregate formation on collagen, as well as platelet activation and generation of platelet procoagulant activity using suitable fluorescent probes. Another advantage is its direct applicability to man, which can lead to a significant reduction in the need for in vivo animal models (61). Indeed, this perfusion assay allowed us to directly compare mouse, rat and human platelets under the same experimental conditions. For instance, experiments revealed that the fundamental processes of adhesion and aggregate formation on collagen are preserved in mouse and human platelets (18).

Although, the parallel-plate flow chamber is a valuable method to study (single) platelet activation and thrombus formation under controlled flow conditions, this model lacks the physiological environment of a blood vessel in the thrombotic process. Particularly the endothelial cells, lining the vessel wall, are eminently involved in the regulation of thromboembolism, and shear force is an important determinant of endothelial function (reviewed in (62)). The endothelium has many pro- and antithrombotic properties which significantly influence platelet behavior in vivo. Examples of prothrombotic substances produced and released by endothelial cells are ADP, ATP, vWF and fibrinogen. Antithrombotic substances are e.g. nitric oxide, prostacyclin and tissue factor pathway inhibitor. Furthermore, endothelial cells display different functions in different parts of the vascular bed (63), which may explain the differences we observed in thrombus formation between arterioles and venules (chapter 7). However, in the studies presented in this thesis the thrombotic process was induced by topical application of ferric chloride (FeCl_3), which causes endothelial denudation. Hence, the role of the endothelium in controlling thromboembolism was probably only limited in these studies. Heterogeneity in deeper vessel wall structures may also significantly influence the thrombotic process. For instance, tissue factor expression is not uniform throughout the vascular tree (64). Also collagen fibers vary greatly in type and thickness throughout the vessel wall, and variation in collagen content may coincide with diverse vWF adhesion sites. In our in vivo experiments, such differences may have contributed to the different thrombotic reactions in mesenteric arterioles and venules. In several in vivo mouse models, including our model (chapter 7), platelet collagen-interaction via GPVI has been shown to control thrombus formation in different

vessel types, pointing to a general importance of collagen exposure for platelet activation in vivo (65,66).

Nowadays, many animal models are being developed to study platelet-vessel wall interactions in vivo. Vascular damage can be inflicted in different vascular beds from different species using different methods (reviewed in (67)). In the studies presented in this thesis (chapters 4 and 7), the established FeCl_3 microvascular thrombosis model was used, which causes endothelial denudation. This model was chosen, because it is sensitive to both platelet inhibition and anticoagulation, with roles for fibrin(ogen), vWF and $\alpha\text{IIb}\beta_3$ (68-70). Platelet-dependent coagulation was anticipated to play a role in the thrombotic process. Both collagen and tissue factor were found to be important for the initiation of platelet activation and coagulation in this model.

In chapter 6, another chemical method was used to trigger thrombosis in vivo in the rat femoral artery. This method, the so-called light-dye technique (67), involves endothelial activation via release of reactive oxygen species. As a result, exposure of subendothelial collagen and tissue factor is probably limited, leading to low activation of coagulation and platelets (71). Typically, the endothelium remains present and thrombi consist primarily of platelets, while fibrin formation is minimal. Hence, thrombus formation in this model depends on platelet activation. This explains why in our experiments thrombin inhibition was found to be ineffective. In contrast, in the mouse FeCl_3 model used in this thesis, endothelial denudation results in the exposure of both collagen and tissue factor in the mesenteric arterioles and venules (chapter 7). In this model, inhibitor studies indicated that platelet activation by collagen synergized with thrombin generation initiated by tissue factor. Accordingly, upon comparing these two thrombosis models, it can be concluded that the relative contribution of platelets and coagulation depends on species, vessel type (large/small) and the type and severity of injury, as proposed by others (72).

More recently, various groups have used lasers to induce endothelial damage in the microcirculation (67,73). In the laser model, the accumulation of platelets in thrombi was found to coincide with the presence of fibrin and tissue factor (73). The mechanism responsible for the prothrombotic injury in this laser model remains to be elucidated (67). It will be interesting to find out whether platelet-dependent coagulation is also important in this model of laser-induced injury. Histological analysis of thrombogenic vascular tissue suggests that also in man exposed collagen and tissue factor play a role in the thrombosis process (74). However, it should be realized that in almost all murine injury models, thrombus formation is triggered in healthy vessels, whereas thrombosis in humans mostly occurs in diseased (atherosclerotic) areas of the vascular system. Therefore, in vivo research on platelet-vessel wall interactions should be extended to the atherosclerotic situation, e.g. in ApoE-deficient mice.

8.5 Clinical implications of the present research

An important question is to which extent the present data generated in mice can be extrapolated to the human situation, given the fact that clear differences exist in the thrombotic process of either species (see chapter 1). On the other hand, it must be noted that many platelet, coagulation and extracellular matrix proteins of the mouse show striking similarities in structure and function to their human counterparts. The introduction of targeted gene disruption in mice has provided unique opportunities to study platelet function, and of every known human platelet defect, a mouse deficient in the same receptor or ligand has been generated. Upon comparison of patients and transgenic mice, many similarities regarding the regulation of platelet activation and aggregation are observed (chapter 1). However, in most patients with a genetic platelet defect, procoagulant activity was not studied. Hence, the data regarding platelet procoagulant activity in mice may also be true for the human situation. Further, FeCl_3 -triggered thrombi in mouse blood vessels have been shown to have a similar morphology to thrombi found in human vessels (75). Hopefully, many of the observations in this animal model, including those presented in this thesis, will have direct relevance for human thrombosis and hemostasis.

Inhibition of platelet function has become an effective strategy for the control of cardiovascular disease. Lately, drugs have been developed that target the fibrinogen receptor $\alpha\text{IIb}\beta 3$ or the ADP receptor P2Y_{12} , acting in the propagation phase of thrombus formation (Fig. 8.1), and several of these compounds have been tested as promising in clinical trials. Unfortunately, many $\alpha\text{IIb}\beta 3$ -antagonists cause bleeding complications. The development of products that interfere with the initiation steps of hemostasis (Fig. 8.1), i.e. platelet adhesion and initiation of coagulation, has not been given much attention.

There seems to be a discrepancy between the observations that mice deficient in GPVI and/or $\text{Fc}\gamma\text{R}$ -chain are protected from arterial thrombosis (16,65,66) on the one hand, and the fact that GPVI-deficient mice (15,16) and humans (76,77) do not have a major bleeding defect on the other. Mice deficient in $\alpha 2\beta 1$ also do not have bleeding problems (10,11). The data from chapter 3 demonstrate that integrin $\alpha 2\beta 1$ has a complementary role to GPVI, which suggests that this receptor (together with aggregatory receptors) can substitute for GPVI during normal hemostasis. Since GPVI is a platelet-specific molecule and, hence, anti-GPVI agents are less likely to have side-effects, it may serve as an attractive antithrombotic target. Studies have indicated that GPVI-binding antibodies (such as JAQ1 used in chapter 3) can deplete GPVI from mouse platelets and protect the animals from thrombosis (78). Similar antibody-induced shedding of GPVI has recently also been demonstrated in humans (77). In addition, GPVI has also been implicated to be essential in plaque-induced thrombus formation (79,80). This seems to plea for GPVI-targeted antithrombotic treatment of atherothrombotic patients. In mice, however, side-effects have been seen of anti-GPVI treatment in combination with other anti-

platelet inhibitors, leading to markedly increased bleeding (19,81). The situation is quite different regarding targeting of $\alpha 2\beta 1$. Chapter 4 describes that absence of integrin $\alpha 2\beta 1$ resulted in frequent embolization of microvascular thrombi. This stabilizing role of $\alpha 2\beta 1$ argues against the use of $\alpha 2\beta 1$ -inhibiting agents, which may cause undesirable embolization also in humans.

Antibodies have also been developed against human GPIb. These antibodies were tested in a thrombosis model in a stenosed artery in baboons, where they showed potent antithrombotic activities, without a noteworthy prolongation of the bleeding time (82). This makes GPIb, similarly as GPVI, an interesting candidate for antithrombotic treatment. The studies in chapter 5 demonstrated that there is some redundancy in GPVI and GPIb receptor function. Therefore, simultaneous targeting of GPVI and GPIb may not be beneficial.

8.6 Conclusions

The main goal of the studies presented in this thesis was to investigate the contribution of key platelet receptors to collagen-induced platelet activation, in particular the platelet procoagulant response. The results indicate that in the initiation phase of platelet activation by collagen (Fig. 8.1), GPVI is the essential receptor mediating all activation processes, but it needs to be complemented by integrin $\alpha 2\beta 1$ (chapters 3 and 4), GPIb and integrin $\alpha IIb\beta 3$ (chapter 5) for stable adhesion to collagen, full platelet activation and procoagulant activity and subsequent stable thrombus formation. In the propagation phase of thrombus formation, platelets can also be induced to expose PS independently of collagen. At this level, the secondary mediators ADP (chapter 6), TxA_2 (chapters 3 and 4), ATP and thrombin play additive roles in PS exposure. A remarkable observation is that, during the process of thrombus formation, subpopulations of platelets display a different pattern of activation (either procoagulant or proaggregatory), which likely depends both on the microenvironment and on platelet characteristics, such as receptor density and age. Regarding the microenvironment, only few platelets make direct contact with collagen, while most platelets within a thrombus bind to each other. In vivo studies complement these findings by showing that synergism between platelet activation and coagulation is important in thrombus formation in both arterioles and venules in vivo (chapter 7).

Hopefully, many of the observations in the mouse studies presented in this thesis, will have direct relevance for human thrombosis and hemostasis. In the future, parallels between data obtained in experimental mouse models and data from clinical settings are very likely to become available. At present, both in vitro and in vivo mouse models to study platelet function have already achieved a key position in hemostasis and thrombosis research.

8.7 References

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Summary

Samenvatting

Summary

Platelet receptors and procoagulant activity in thrombus formation

In hemostasis and thrombosis, interactions of platelets with components from the vessel wall play a crucial role. After vascular damage, platelets adhere to the subendothelial extracellular matrix with collagen as important constituent, become activated and assemble into a platelet aggregate. Simultaneously, the coagulation process is activated, e.g. by exposed tissue factor in the injured vessel wall, which results in the generation thrombin and subsequently fibrin, consolidating the thrombus. The procoagulant response of platelets is considered to be responsible for the amplification of the coagulation process. This unique form of platelet activation involves exposure of the phospholipid phosphatidylserine (PS) on the outer platelet membrane. The exposure of PS strongly promotes the assembly of coagulation factors and the formation of thrombin. In turn, thrombin is also a potent platelet agonist. Hence, the processes of platelet activation and coagulation are closely entwined, and both thought to be essential for effective thrombus formation. The main goals of the present thesis was to investigate the involvement of key platelet receptors contributing to collagen-induced platelet activation, especially related to the platelet procoagulant response, and to demonstrate the importance of these processes *in vivo*.

Platelets can interact with collagen by several receptors. Current knowledge of platelet-collagen interaction is described in detail in **chapter 1**. First, von Willebrand factor (vWF), present in plasma and secreted by endothelial cells, binds to collagen. Under high shear conditions, platelets bind to vWF via glycoprotein Ib (GPIb). This is a weak interaction, which causes platelets to "roll" over the vWF/collagen surface. Following platelet rolling, direct interactions between collagen and platelets lead to strong adhesion. Platelets adhere to collagen via two receptors, GPVI and integrin $\alpha 2\beta 1$. This stable adhesion can be strengthened by the binding of GPIb and integrin $\alpha IIb\beta 3$ to vWF. The direct binding to collagen induces platelet activation, which is predominantly mediated by GPVI. Platelet activation involves a rise in the intracellular Ca^{2+} concentration. This causes platelets to change shape and to secrete activating substances, such as thromboxane A_2 (TxA_2) and adenosine diphosphate (ADP). These autocrine mediators recruit nearby platelets from flowing blood to form a platelet aggregate. Formation of a platelet aggregate is mediated by integrin $\alpha IIb\beta 3$, which binds to fibrinogen to form cross-links between platelets.

Chapter 2 addresses the present understanding of how platelet interaction with collagen supports the processes of thrombin generation and coagulation. Only part of the collagen-adhered platelets, namely those showing profound changes in shape and shedding microparticles, appear to accomplish procoagulant activity. The main signaling receptor for collagen, GPVI, plays a unique, key role in the platelet procoagulant response during collagen-

induced thrombus formation. This is one reason why new anti-GPVI antibodies are promising antithrombotic tools.

In most of the subsequent studies described in this thesis a perfusion model is used. This model consists of a parallel-plate perfusion chamber, which may be imagined as an artificial damaged blood vessel (chapter 1, figure 1.4). Coverslips coated with collagen or fibrinogen are mounted into the perfusion chamber. A pulse-free pump is used to perfuse isolated blood over the coverslip at a shear rate representative for arteries or veins. During and after perfusion the platelets attached to the collagen are observed with a microscope. This setup allows real-time monitoring of shear-dependent platelet adhesion and aggregate formation, as well as platelet activation and generation of platelet procoagulant activity using suitable fluorescent probes. However, the vessel wall, an important component in thrombus formation, is missing in this *in vitro* model. Therefore, platelet-vessel wall interactions and ensuing thrombus formation have also been studied *in vivo*, in an established murine microvascular thrombosis model. In this model, the thrombotic process is induced by topical application of ferric chloride (FeCl_3) on mouse mesenteric arterioles and venules, which results in endothelial denudation and the formation of intravascular thrombi. Thrombus formation has been studied in real-time using intravital microscopy.

In **chapter 3**, the roles of both collagen receptors GPVI and integrin $\alpha 2\beta 1$ in thrombus formation were directly compared during perfusion of whole blood over collagen. Using either mice deficient in GPVI or blocking antibodies against this receptor it was demonstrated that GPVI is indispensable for collagen-dependent Ca^{2+} mobilization, exposure of PS and aggregation. In contrast, deficiency in $\beta 1$ -integrin (i.e. mice lacking $\alpha 2\beta 1$) considerably reduced these GPVI-evoked responses, but still allowed the formation of loose platelet aggregates. Using mice deficient in G_{α_q} (an intracellular signaling G-protein coupled to thrombin, TxA_2 and ADP receptors) or specific TxA_2 and ADP antagonists it was shown that signaling via these autocrine agents is required for aggregation, but not for collagen-induced Ca^{2+} mobilization and PS exposure. Collectively, these findings indicate that $\alpha 2\beta 1$ facilitates the central function of GPVI in the platelet activation processes leading to thrombus formation, while TxA_2 and ADP mainly trigger aggregate formation.

In contrast to the established platelet-activating role of GPVI, the function of integrin $\alpha 2\beta 1$ in thrombus formation is still controversial in literature. Therefore, in **chapter 4**, the role of $\alpha 2\beta 1$ in thrombus growth and stability was investigated in $\alpha 2$ -deficient (-null) mice (lacking $\alpha 2\beta 1$) by *in vitro* and *in vivo* studies. Using the *in vivo* thrombosis model it was demonstrated that thrombi formed in arterioles from $\alpha 2$ -null mice were smaller in size and embolized more frequently compared to thrombi in vessels from wildtype mice. Second, perfusion of blood from $\alpha 2$ -null mice over collagen resulted in formation of thrombi that were smaller and looser in appearance than those obtained with wildtype blood. These data indicate that $\alpha 2\beta 1$ has a

moderate but qualitatively important effect to stabilize thrombi. Moreover, $\alpha 2\beta 1$ also assists GPVI in mediating fibrinogen binding to platelets, exposure of PS and TxA_2 formation. In addition, co-perfusion of blood and a stable TxA_2 analogue over collagen resulted in normalization of thrombus size in absence of $\alpha 2\beta 1$. In summary, these data indicate that $\alpha 2\beta 1$ enhances the following GPVI-dependent mechanisms: (i) release of TxA_2 , (ii) activation of $\alpha \text{IIb}\beta 3$ leading to irreversible aggregation, and (iii) PS exposure leading to generation of thrombin and fibrin clots. The frequent embolization in the absence of $\alpha 2\beta 1$ argues against the use of $\alpha 2\beta 1$ integrin inhibitors for antithrombotic therapy.

Besides direct adhesion to collagen via GPVI and integrin $\alpha 2\beta 1$, platelets also adhere to vWF bound to collagen fibers. In **chapter 5** the supportive role of platelet-vWF binding via GPIb and $\alpha \text{IIb}\beta 3$ in thrombus formation was investigated in mouse blood flowing over collagen at high shear rate. Inhibition of GPIb as well as blocking of vWF binding to collagen reduced stable platelet adhesion. This was accompanied by delayed platelet Ca^{2+} responses and reduced PS exposure, while micro-aggregates were still formed. Inhibition of integrin $\alpha \text{IIb}\beta 3$ with an antibody reduced PS exposure and aggregate formation. Interestingly, with blood from mice expressing 50% of normal platelet GPVI levels, GPIb blockade almost completely abolished platelet adhesion and PS exposure. Together, this work indicates that, under these conditions of flow, both adhesive receptors GPIb and $\alpha \text{IIb}\beta 3$, facilitate GPVI-mediated PS exposure by stabilizing platelet binding to collagen. Hence, these receptors have an assistant role in collagen-dependent thrombus formation, which becomes more prominent at reduced GPVI activity.

In chapter 6 thrombus formation was studied in rat and human blood. Rats were treated with a mild dose of classical coagulation inhibitor (heparin or warfarin), after which they were subjected to thrombosis in the femoral artery. The thrombosis tendency appeared not to be changed by these treatments. Therefore, it was considered that platelet-inhibiting agents that influence coagulation as well, may act as more potent anti-thrombotic agents compared to classical anticoagulants. This has been investigated in flow chamber studies, where whole blood was perfused over fibrinogen under coagulant conditions. Examined were the effects of the inhibitor prostaglandin E_1 (PGE_1), which elevates cyclic AMP levels and thereby inhibits platelet activity, and of the P2Y_{12} ADP-receptor antagonist, AR-C69931MX. Perfusion of rat or human blood in the presence of tissue factor resulted in deposition of activated platelets and subsequent aggregate formation, along with exposure of procoagulant PS on the platelet surface and formation of fibrin fibers. In the presence of PGE_1 aggregation was completely inhibited, but platelet adhesion and PS exposure were only partly reduced, while fibrin formation was hardly affected. Treatment with AR-C69931MX caused similar, but less complete effects. These results indicate that in tissue factor-triggered blood from various species under conditions of flow: (i) the platelet procoagulant response is independent of aggregate formation and (ii) the

platelet-inhibiting effect of PGE₁ and AR-C69931MX is sufficient to suppress aggregation, but not platelet adhesion and coagulation. These platelet inhibitors thus appear to maintain their aggregation-inhibiting effect at sites where platelets participate in thrombin formation.

The mutually stimulatory processes of platelet activation and thrombin generation in arterial and venous thrombus formation *in vivo* are investigated in **chapter 7**. In the murine FeCl₃ model, the thrombus-forming process appeared to be triggered by both collagen exposure and the complex of tissue factor and factor VII(a). Furthermore, arterial thrombus formation was suppressed by either partial thrombin inhibition or platelet inhibition. In contrast, suppression of venous thrombus formation required either the combination of partial thrombin inhibition plus platelet inhibition or full thrombin inhibition. Antagonism of platelet procoagulant activity, by intervention with PS-binding annexin A5, completely abolished the formation of thrombi in both vessel types, while a mutant M1234-annexin A5 (incapable of PS binding) appeared to be ineffective. The presence of procoagulant platelets in arterial and venous thrombi has been confirmed by *in vivo* staining with fluorescently labeled annexin A5. These findings demonstrate that the propagation of thrombin generation by PS-exposing platelets is a key regulatory process in both veins and arteries. In the venous circulation, very high thrombin levels may be reached locally, which explains why only platelet inhibition is ineffective.

The significance of the findings described in this thesis is discussed in **chapter 8**, particularly in the light of relevant new literature. In the initiation phase of platelet activation by collagen, GPVI is the essential receptor mediating all activation processes, but it needs to be complemented by integrin $\alpha 2\beta 1$, GPIb and integrin $\alpha IIb\beta 3$ for stable adhesion to collagen and subsequent responses: full platelet activation, procoagulant activity and stable thrombus formation. In the propagation phase of thrombus formation, platelets can also be induced to expose PS independently of collagen. At this level, "secondary mediators" like ADP and thrombin play additive roles in PS exposure. A remarkable observation from the perfusion chamber experiments is that, during thrombus formation, subpopulations of platelets display different patterns of activation (either procoagulant or proaggregatory). Finally, the *in vivo* studies show that the synergistic effects of platelets and coagulation are important in thrombus formation in both arterioles and venules.

Altogether, during the time in which the present thesis has been prepared, our knowledge on the roles of platelet (collagen) receptors in collagen-induced platelet activation, aggregation and PS exposure has greatly increased. Particularly, murine knock-out models have taught on the functions of these receptors. Although most of the studies of this thesis have been performed in mice, the observations in this thesis are likely to be of relevance for treating human thrombosis and maintaining normal hemostasis. It is anticipated that this knowledge will help to develop improved treatment of the numerous patients who still suffer from cardiovascular thrombotic disease.

Samenvatting

Bloedplaatjesreceptoren en procoagulante activiteit in trombusvorming

In een volwassen mens circuleert 5 à 6 liter bloed door de bloedvaten. Het bloed transporteert onder andere bloedcellen (rode en witte bloedcellen en bloedplaatjes), zuurstof, voedingsstoffen en hormonen. Het is dus van levensbelang dat de bloedcirculatie niet wordt verstoord. Het lichaam is in staat om enerzijds bloedverlies na beschadiging van de vaatwand tegen te gaan (hemostase), en anderzijds verstopping van intacte bloedvaten (trombose) te voorkomen. Bloedplaatjes, de kleinste cellen in het bloed, spelen een belangrijke rol in de hemostase, maar ook in de ongewenste trombose. Ze circuleren door de bloedvaten met zo'n 300 miljard per liter en bevinden zich relatief dicht bij de vaatwand. In een onbeschadigd bloedvat wordt hechting van bloedplaatjes aan de wand voorkomen door de anti-trombotische eigenschappen van endotheelcellen die de binnenbekleding van de vaatwand vormen. Wanneer het endotheel beschadigd raakt, gaan bloedplaatjes hechten (adhesie) aan de bestanddelen die zich onder de endotheellaag bevinden, zoals collageen. Collageen is een krachtige activator van bloedplaatjes. Geactiveerde bloedplaatjes gaan aan elkaar hechten (aggregatie), en er ontstaat op de plaats van beschadiging een bloedplaatjesprop of -plug die de beschadiging afdicht. Bij een vaatwandbeschadiging wordt tegelijkertijd de bloedstolling op gang gebracht. Dit proces wordt gestart door weefselfactor, een eiwit in de vaatwand, en verloopt via een aantal opeenvolgende reacties tussen eiwitten in het bloed (stollingsfactoren). De stollingsbevorderende (procoagulante) respons van bloedplaatjes is van groot belang voor de versnelling van de bloedstolling. Tijdens deze respons wordt het fosfolipide fosfatidylserine (PS) naar de buitenkant van het bloedplaatjesmembraan getransporteerd. Het verschijnen van PS katalyseert de vorming van trombine, dat zorgt voor de vorming van fibrine, het eindproduct van de bloedstolling. Fibrine vormt een netwerk van draden in de bloedplaatjesprop, waardoor deze een stevig geheel (trombus) wordt. Trombine kan echter ook bloedplaatjes activeren. Bloedplaatjesactivatie en bloedstolling zijn dus nauw met elkaar verweven, en beide processen zijn essentieel voor effectieve trombusvorming. Het doel van het onderzoek in dit proefschrift was om de activatie van bloedplaatjes door collageen onder stromingscondities te bestuderen, en de bijdrage van belangrijke bloedplaatjesreceptoren hierin te bepalen. Naast aggregatie en trombusvorming is met name de procoagulante respons van bloedplaatjes onderzocht.

Bloedplaatjes kunnen via een aantal receptoren interacties aangaan met collageen. Dit proces wordt in detail beschreven in **hoofdstuk 1**. Allereerst binden de bloedplaatjes op indirecte wijze aan collageen, via de von Willebrand factor (vWF). vWF is een eiwit in het plasma en de endotheelcellen, dat zelf hecht aan collageen. Bloedplaatjes kunnen binden aan vWF via glycoproteïne Ib (GPIb). Dit is echter een zwakke binding, waardoor de bloedplaatjes gaan 'rollen' over het collageen. Vervolgens kan stabiele adhesie van bloedplaatjes op collageen plaatsvinden via twee receptoren, namelijk GPIIb/IIIa en integrine $\alpha 2\beta 1$. De adhesie wordt

verder versterkt door de binding van GPIIb en integrine $\alpha IIb\beta 3$ aan vWF. Door de interactie met collageen worden bloedplaatjes geactiveerd, wat gepaard gaat met een stijging in de intracellulaire calcium-concentratie. Dit leidt vervolgens tot vormverandering van bloedplaatjes, waarbij ze ook allerlei activerende stoffen uitscheiden. Dit zijn onder andere tromboxaan A_2 (TxA_2) en adenosine difosfaat (ADP), die op hun beurt weer andere voorbij stromende bloedplaatjes kunnen activeren. Tenslotte aggregeren bloedplaatjes via interactie van integrine $\alpha IIb\beta 3$ met fibrinogeen.

Hoofdstuk 2 gaat in op de huidige kennis omtrent bloedplaatjes-interactie met collageen en de manier waarop die leidt tot trombinegeneratie en bloedstolling. Slechts een deel van de aan collageen gehechte bloedplaatjes slaagt erin om procoagulante activiteit te vertonen, namelijk dat deel dat zeer sterk van vorm verandert en microparticles afsnoert. GPVI, de belangrijkste signalerende receptor voor collageen, speelt een unieke rol in de procoagulante respons van bloedplaatjes tijdens collageen-geïnduceerde trombusvorming. Onder meer om deze reden vormen antilichamen tegen GPVI een veelbelovende vorm van antitrombotische medicatie.

In de meeste studies beschreven in de volgende hoofdstukken van dit proefschrift is gebruik gemaakt van een perfusiemodel. Dit model bestaat uit een perfusiekamer, die men zich zou kunnen voorstellen als een kunstmatig, beschadigd bloedvat (hoofdstuk 1, figuur 1.4). Hierin wordt een dekglasje aangebracht dat bedekt is met collageen of fibrinogeen. Met behulp van een pomp stroomt geïsoleerd bloed over het glasje met een stroomsnelheid representatief voor slagaders (arteriën) of aders (venen). Tijdens en na de perfusie worden de bloedplaatjes die gehecht zijn aan het collageen bestudeerd met een microscoop. Dit perfusiemodel is uitermate geschikt om bloedplaatjesadhesie, -activatie en trombusvorming onder gecontroleerde stromingscondities te bestuderen. Echter, in dit model ontbreekt een belangrijke component van de trombusvorming, namelijk de vaatwand. Daarom zijn ook experimenten gedaan in de levende muis, "in vivo", om bloedplaatjes-vaatwand-interacties te bestuderen in hun natuurlijke omgeving. In het gebruikte model is trombusvorming opgewekt in de bloedvaten van het darmvlies van de muis, door ijzertrichloride ($FeCl_3$) op het darmvlies te druppelen. Deze stof beschadigt de wand van de bloedvaten zodanig dat endotheelcellen verwijderd worden. De hieropvolgende trombusvorming kan vervolgens bestudeerd worden met een speciale lichtmicroscoop, een zogenaamde intravitaal microscoop.

In **hoofdstuk 3** wordt de bijdrage van de collageenreceptoren GPVI en integrine $\alpha 2\beta 1$ aan de trombusvorming vergeleken tijdens de perfusie van bloed over een collageen-oppervlak. Hierbij werd gebruik gemaakt van muizen die deficiënt zijn in GPVI, $\beta 1$ -integrine (waardoor $\alpha 2\beta 1$ ontbreekt), of $G\alpha_q$ (een intracellulair signaleringseiwit verbonden aan receptoren voor trombine, TxA_2 , en ADP), of van remmers of antilichamen die deze receptoren blokkeren. De resultaten toonden aan dat GPVI onmisbaar is voor de intracellulaire calciummobilisatie, de

expositie van PS en de aggregatie. In muizen deficient in het $\beta 1$ -integrine waren deze GPVI-afhankelijke processen aanzienlijk verminderd, hoewel de vorming van losse bloedplaatjes-aggregaten nog wel optrad. In muizen die $G\alpha_q$ missen, of in aanwezigheid van TxA_2 en ADP remmers, werd aangetoond dat signalering via deze secundaire mediators nodig is voor aggregatie, maar niet voor de collageen-geïnduceerde calciummobilisatie of PS-expositie. Uit deze resultaten kan geconcludeerd worden dat $\alpha 2\beta 1$ een faciliterende rol speelt bij de GPVI-afhankelijke activatie van bloedplaatjes en de daaropvolgende trombusvorming, terwijl TxA_2 en ADP hoofdzakelijk betrokken zijn bij de aggregatie.

Terwijl de rol van GPVI in de trombusvorming, namelijk bloedplaatjesactivatie, inmiddels algemeen geaccepteerd is, is de rol van het integrine $\alpha 2\beta 1$ controversieel in de literatuur. Daarom is in **hoofdstuk 4** de rol van $\alpha 2\beta 1$ in de trombusvorming en -stabiliteit opnieuw onderzocht, nu bij $\alpha 2$ -deficiente (-null) muizen (waardoor $\alpha 2\beta 1$ ontbreekt) middels in vitro en in vivo studies. Met het in vivo trombosemodel werd aangetoond, dat de trombi die werden gevormd in de arteriolen (kleine slagaders) van de $\alpha 2$ -null muizen kleiner waren vergeleken met de trombi in arteriolen van wildtype (normale) muizen. Bovendien waren de trombi in de $\alpha 2$ -null muizen instabiel: er werden meer aggregaten van de trombi afgebroken (emboli) dan in de wildtype muizen. Vervolgens bleek ook in het perfusiemodel dat trombi van $\alpha 2$ -null muizen kleiner en losser waren dan die van wildtype muizen. Deze resultaten geven aan dat $\alpha 2\beta 1$ een bescheiden, maar belangrijk effect heeft op de stabilisatie van trombi. Bovendien assisteert $\alpha 2\beta 1$ GPVI ook bij de binding van fibrinogeen aan bloedplaatjes, de expositie van PS en de vorming van TxA_2 . Perfusie van $\alpha 2$ -null bloed tezamen met een stabiele TxA_2 -analoog over collageen normaliseerde de grootte van de trombi. Samengevat laten deze resultaten zien dat $\alpha 2\beta 1$ de volgende GPVI-afhankelijke mechanismen versterkt: (i) vorming van TxA_2 , (ii) activatie van integrine $\alpha IIb\beta 3$, wat leidt tot irreversibele aggregatie, en (iii) PS-expositie, resulteert in de vorming van trombine en fibrine-stolsels. De frequente embolisatie die optreedt in afwezigheid van $\alpha 2\beta 1$ pleit tegen het gebruik van integrine $\alpha 2\beta 1$ -remmers als anti-trombotische medicijn.

Behalve via directe hechting aan collageen middels GPVI en $\alpha 2\beta 1$, hechten bloedplaatjes ook aan vWF, welke weer bindt aan collageen. In **hoofdstuk 5** werd de additionele rol van plaatjes-vWF-binding via GPIb en integrine $\alpha IIb\beta 3$ in de trombusvorming onderzocht in muizenbloed dat stroomde over collageen. Zowel remming van GPIb als het blokkeren van de binding van vWF aan collageen reduceerde de stabiele adhesie van bloedplaatjes bij hoge bloedstroomsnelheid (zogenoemde afschuifsneldheid). Dit ging gepaard met vertraagde calciumresponsen van bloedplaatjes en verlaagde PS-expositie, terwijl micro-aggregaatjes nog gevormd werden. Inhibitie van $\alpha IIb\beta 3$ met een antilichaam verlaagde de PS-expositie en aggregaatvorming. Vervolgens werd de trombusvorming onderzocht bij muizen met een 50% GPVI-expressieniveau. Boven verwachting resulteerde blokkade van GPIb in het

bloed van deze muizen in complete afwezigheid van bloedplaatjesadhesie en PS-expositie, terwijl in aanwezigheid van GPIb de trombusvorming onveranderd was. Deze experimenten duiden erop, dat onder fysiologische stromingscondities de twee adhesieve receptoren GPIb en $\alpha IIb\beta 3$ versterkend werken op de GPVI-gemedieerde PS-expositie door het stabiliseren van de plaatjesbinding aan collageen. Dus deze receptoren hebben een assisterende rol in de collageen-afhankelijke trombusvorming, die het meest prominent is bij verlaagde GPVI-activiteit.

In **hoofdstuk 6** werd de trombusvorming bestudeerd in humaan bloed en bloed van ratten. Ratten werden al dan niet behandeld met een milde dosis van klassieke anti-stollingsmiddelen (heparine of warfarine), waarna zij werden blootgesteld aan vaatwandschade in de slagader van de achterpoot. De daaropvolgende trombotische reactie bleek gelijk te zijn in behandelde en onbehandelde ratten. Dit leidde tot de vraag of de rol van geactiveerde bloedplaatjes, die eveneens de bloedstolling beïnvloeden, mogelijk erg belangrijk is tijdens deze reactie. Daarom werd het effect van bloedplaatjesremmende medicatie onderzocht en vergeleken met het effect van klassieke anti-stollingsmiddelen. Hiertoe zijn perfusie-experimenten uitgevoerd, waarbij bloed werd gestroomd over een fibrinogeen-oppervlak onder condities waarbij ook de bloedstolling op gang werd gebracht. De effecten van de remmer prostaglandine E_1 (PGE_1), die de intracellulaire cAMP-niveaus verhoogt en daardoor de bloedplaatjesactiviteit remt, en van AR-C69931 MX, een antagonist van de ADP-receptor $P2Y_{12}$, werden onderzocht. De perfusie van humaan of rattenbloed resulteerde in de adhesie van bloedplaatjes en daaropvolgende aggregaatvorming, tezamen met PS-expositie en de vorming van fibrinedraden. In aanwezigheid van PGE_1 werd de aggregatie volledig geremd, maar de adhesie en PS-expositie waren slechts deels gereduceerd. De vorming van fibrine was nauwelijks veranderd. Behandeling met AR-C69931 MX leidde tot vergelijkbare, maar minder complete effecten. Deze resultaten geven aan dat, in het bloed van mens en rat, de procoagulante respons van bloedplaatjes onafhankelijk is van de aggregaatvorming. Verder dat het plaatjesremmende effect van PGE_1 en AR-C69931 MX voldoende is om de aggregatie te onderdrukken, maar niet de plaatjesadhesie en bloedstolling. Deze plaatjesremmers lijken dus hun remmende effect op de aggregatie te behouden wanneer bloedplaatjes betrokken zijn bij de vorming van trombine.

De interactie tussen bloedplaatjesactivatie en trombinegeneratie in arteriële en veneuze trombusvorming in vivo is onderzocht in **hoofdstuk 7**. In het trombosemodel bleek na vaatwand-beschadiging het proces van trombusvorming op gang te worden gebracht door blootstelling van het bloed aan zowel collageen als weefselfactor. De trombusvorming in arteriolen kon worden geremd door ofwel een lage dosering van een trombine-remmer ofwel plaatjesremming. Echter, om de trombusvorming in de venulen te onderdrukken was een combinatie van beide nodig, ofwel een complete onderdrukking van trombinevorming. De procoagulante respons van bloedplaatjes kan worden onderdrukt door annexine A5, een eiwit

dat bindt aan PS. Toedienen van annexine A5 aan de muizen resulteerde in complete onderdrukking van de trombusvorming in beide typen bloedvaten. De aanwezigheid van procoagulante bloedplaatjes in arteriële en veneuze trombi werd bevestigd door deze in vivo aan te kleuren met annexine A5, waaraan een fluorescerend label gebonden was. Deze bevindingen demonstreren dat de versterking van trombine-generatie door PS-presenterende bloedplaatjes een belangrijk proces is in zowel venen als arteriën. In de veneuze circulatie lijkt het erop dat lokaal zeer hoge trombine-niveaus kunnen worden bereikt, waardoor bloedplaatjesremmers alleen niet effectief zijn in het onderdrukken van trombose.

In **hoofdstuk 8** wordt de significantie van de bevindingen beschreven in dit proefschrift bediscussieerd in het licht van recente literatuur. Tijdens de initiatiefase van bloedplaatjesactivatie door collageen is GPVI de essentiële receptor, die alle activatieprocessen medieert. Echter, GPVI wordt hierbij gecomplementeerd door integrine $\alpha 2\beta 1$, GPIb en integrine $\alpha 1\text{Ib}\beta 3$, zodat stabiele adhesie aan collageen optreedt met volledige plaatjesactivatie, procoagulante activiteit en uiteindelijk een stabiele trombus gevormd wordt. In de propagatiefase van trombusvorming kunnen bloedplaatjes ook onafhankelijk van collageen gestimuleerd worden om PS te exposeren. Hierbij spelen de "secundaire mediators" zoals ADP en trombine een aanvullende rol. Een opmerkelijke observatie tijdens de perfusiekamer-experimenten is dat tijdens de trombusvorming twee subpopulaties van bloedplaatjes ontstaan, die verschillen in hun activatiepatroon. De meeste bloedplaatjes gaan aggregeren en komen in trombi terecht, maar sommige bloedplaatjes worden procoagulant en blijven apart op het collageen liggen. Tenslotte laten de in vivo studies zien dat de synergistische effecten van bloedplaatjes en -stolling belangrijk zijn in de trombusvorming in zowel arteriolen als venulen.

Dit proefschrift is een toevoeging aan de kennis over de bijdragen van bloedplaatjes-(collageen)-receptoren in de collageen-geïnduceerde activatie van bloedplaatjes, aggregatie en PS-expositie. Hoewel het merendeel van de studies in dit proefschrift met muizen zijn uitgevoerd, zijn de uitkomsten naar verwachting ook relevant voor het handhaven van de normale hemostase en de behandeling van trombose bij de mens. Hopelijk zullen de resultaten bijdragen aan de ontwikkeling van nieuwe behandelingsmethoden van de vele patiënten die lijden aan cardiovasculaire aandoeningen.

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Op het lab heb ik de afgelopen jaren heel wat lief en leed gedeeld. In de eerste plaats had ik hier mijn kamergenoten voor. Marion, jou bijdrage aan onze groep is niet in woorden uit te drukken. Je staat altijd voor iedereen klaar en ik heb vooral je luisterend oor altijd erg gewaardeerd. Ik ben erg blij dat je mijn paranimf bent! En Jeffrey, met je nuchtere opmerkingen heb je ons regelmatig aan het lachen gemaakt. Ook met de andere AIO's uit de groep heb ik veel meegemaakt. Imke, jij kletst en lacht heel wat af! We hebben heel wat weken samen muizen geopereerd en experimenten gedaan. Dat was altijd erg gezellig. Maar ook naast het werk zijn we veel samen opgetrokken: aerobics, skaten, naar huis lopen, enz. Ik wil je bedanken voor je vriendschap en ik vind het leuk dat je mijn paranimf bent! Veel succes met het afronden van je eigen proefschrift. Sandra, Paola en Judith, stuk voor stuk dragen jullie bij aan de gezellige, motiverende sfeer op het lab. Ook jullie wens ik veel succes voor de rest van jullie promotietijd, en ook daarna nog natuurlijk! Kristof, ook al ben je al enige tijd vertrokken naar Humane Biologie, je kamer op de derde verdieping is makkelijk te vinden als we je hulp nodig hebben. Ook jou wens ik alle goeds in je verdere loopbaan.

Theo, jij bent altijd geïnteresseerd en betrokken bij het onderzoek van ons lab, dat we tot voor kort met jou en Simone deelden. Je stelde vaak moeilijke vragen, waardoor ik me vaak realiseerde dat het toch niet zo simpel was als ik dacht. Simone, al ben je klein van stuk (zeker naast mij), je staat je mannetje! Met praktische vragen kan ik altijd bij jou terecht, en tijdens de lunch weet je vaak leuke verhalen te vertellen. Mieke, met je (h)eerlijke uitspraken heb je ons vaak aan het lachen gemaakt! Hopelijk kunnen we nog vaak een mooie wandeling maken?

Ook mijn collega-AIO's van Biochemie: Heidi, Rory, Kristin, Jeroen, Kim, Sarah, Arne, Machiel, Hugo, Lisbeth, Kenneth, Svetlana, Erik en Rachelle wil ik veel succes wensen met hun promotie en/of verdere carrière. Berry, ik ben je ontzettend veel dank verschuldigd voor de ontelbare keren dat je me bij mijn computerproblemen hebt geholpen! Alle overige leden van de capgroep Biochemie wil ik bedanken voor de samenwerking en/of gezelligheid tijdens het werk en dagjes uit. Verder wil ik iedereen van Microcirculatie bedanken voor de plezierige samenwerking: Mirjam, Viviane, Dick, Marc, Wim, Miriam, Anita, Veerle, Jacqueline, Geertje, Alexandra, Remco en Sietze. Viviane, geduldig heb je mij geleerd om muizen onder anesthesie te brengen en vervolgens te opereren. Heel erg bedankt! Dick, bij koningin Beatrix is het vijf jaar geleden begonnen! Jij hebt me mede overgehaald om AIO te worden en nu ben je de voorzitter van mijn beoordelingscommissie! Dankjewel! Ook woorden van dank aan de medewerkers van de Centrale Proefdier Voorzieningen voor hun onmisbare verzorging van de muizen.

During my PhD period, PhD students from other labs came to work in our lab. Christelle from Paris, you stayed in Maastricht for one year. I really enjoyed the time we spent together, both in the lab and out! Jocelyn from Birmingham, you were with us for four months and I will always remember your enthusiasm! Amrei from Würzburg, you stayed in our lab for three months, and even took Dutch lessons! Ik vond het heel leuk dat je in Maastricht was! I wish all of you all the best for the future and hopefully we will meet again and maybe even work together some day!

I have visited the lab of Dr. Bernhard Nieswandt in Würzburg several times. Bernhard, thank you very much for all collaborations on several of my projects and also for the pleasant times I spent in your lab. Valerie, Sabine, Mirka, Rasti and Amrei, thank you all very much for your help with experiments and social events (I especially liked the Christmas market with Glühwein and Weinfest at the Residenz, even though it poured with rain!).

Marije, Yvonne en Else, sinds onze studietijd zijn we hele goede vriendinnen. Ook al wonen we nu in Montréal, Maastricht en Amsterdam, en zien we elkaar maar weinig, er wordt toch regelmatig "even" bijgekletst per telefoon of e-mail! Marije, heel veel succes bij het behalen van je PhD! Hopelijk kom je over niet al te lange tijd weer terug naar Europa. Yvonne, ik ben erg blij dat je na je tijd in Brussel nu je draai weer hebt gevonden in Maastricht! Veel succes bij je opleiding tot huisarts en veel plezier in je nieuwe huis! Ik vind onze "bijklets-zwem-avonden, eventueel met eten" altijd erg gezellig; die mogen van mij een wekelijkse traditie worden! Else,

onze stagetijd in Cambridge was echt geweldig en zal ik nooit vergeten! Jij ook heel veel succes voor de toekomst!

Papa en mama, ik zou nooit zover zijn gekomen als jullie mij niet altijd onvoorwaardelijk hadden gesteund. Jullie hebben mij de mogelijkheid gegeven om een goede opleiding te volgen, en hebben mij altijd vrij gelaten om mijn eigen keuzes te maken. Mijn dankbaarheid hiervoor is niet in woorden uit te drukken! Mijn zussen Ineke en Annelies: ontzettend bedankt voor jullie steun. Ik weet dat wij altijd op elkaar kunnen rekenen en ik ben ook geweldig trots op alles wat jullie hebben bereikt! Natuurlijk wil ik ook Jurgen en Arie bedanken, en ook mijn ooms en tantes: Jan en Anja, Els en Kees bedankt voor jullie steun!

Naast mijn eigen familie heb ik een leuke schoonfamilie. Han en Marianne, jullie hebben me meteen in jullie gezin opgenomen en dit betekent heel veel voor me! Vincent en Monique, jullie zijn voor mij bijna broer en zus, ook jullie bedankt voor alles! Heel veel plezier in jullie nieuwe huis! Naomi en Esmee, ook al zijn jullie nog klein, de wereld ligt voor jullie open en jullie kunnen altijd op mij rekenen! Ook wil ik oma, alle andere familieleden en mijn goede vrienden Tijs en Sietske, Joost en Regine, en Robbert bedanken voor alle steun "op afstand".

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Marijke Kuipers

februari 2006

Curriculum vitae

Marijke Kuijpers werd geboren op 20 juli 1977 te Weert. De eerste vier jaar van haar leven woonde zij in Maarheeze, in 1981 verhuisde zij naar Etten-Leur. Na het verlaten van de Gerardus Majella basisschool begon zij in 1989 aan het Voorbereidend Wetenschappelijk Onderwijs (VWO) op de Katholieke Scholengemeenschap te Etten-Leur. In 1995 behaalde zij het VWO-diploma, waarna ze startte met de opleiding Gezondheidswetenschappen aan de Universiteit Maastricht. Na ontvangst van het propedeuse in 1996 vervolgde zij deze studie in de richting Biologische Gezondheidskunde. Tijdens haar studie verwierf ze de status van artikel 9-deskundige volgens de Wet op de Dierproeven. Haar éénjarige afstudeerstage liep zij in 1999 bij de capgroep Fysiologie, laboratorium voor Microcirculatie onder begeleiding van Dr. M.G.A. oude Egbrink, Dr. A. Griffioen en Dr. J.W.M. Heemskerk. Voor een extra buitenlandstage vertrok ze begin 2000 voor zes maanden naar Cambridge, Engeland. Hier deed zij onderzoek bij the Department of Haematology, Division of Transfusion Medicine, University of Cambridge, onder begeleiding van Dr. N.A. Watkins en Dr. W.H. Ouwehand. Op 31 augustus 2000 mocht zij haar universitaire diploma in ontvangst nemen en in januari 2001 ontving zij de Studentenprijs 2000 voor Wetenschapsbeoefening van de Universiteit Maastricht, uitgereikt door h.k.h. Koningin Beatrix. Op 1 mei 2001 begon zij als Assistent in Opleiding (AIO) binnen het Cardiovascular Research Institute Maastricht (CARIM) bij de capgroep Biochemie van Prof. Dr. J. Rosing. Het onderzoek dat zij daar deed en wat beschreven staat in dit proefschrift vond plaats onder directe begeleiding van Dr. J.W.M. Heemskerk en Dr. M.G.A. oude Egbrink. Na het volgen van verscheidene cursussen werd haar in 2004 het CARIM PhD-Training Certificate uitgereikt. Tijdens haar AIO-periode bezocht zij congressen in onder andere Birmingham, Nottingham, Oxford, Eberbach, Erfurt en Sydney en won zij tweemaal een "young investigator award" voor haar abstracts of mondelinge presentatie. Zij bezocht verscheidene malen het laboratorium van Dr. B. Nieswandt in Würzburg, Duitsland, om daar proeven te doen. Tevens bezocht zij het laboratorium van Prof. Dr. S. Watson in Birmingham, Engeland. Op 21 oktober 2004 werd haar door de faculteit de Geneeskunde een "Kootstra Fellowship" toegekend, bedoeld voor talentvolle AIO's om de periode tussen het afronden van het proefschrift en de indienstreding als Post-doc, te overbruggen. Sinds 15 december 2005 is zij werkzaam als Post-doc bij de capgroep Biochemie van de Universiteit Maastricht.

Curriculum vitae

Marijke Kuijpers was born on July 20th 1977 in Weert, the Netherlands. The first 4 years of her life she lived in Maarheeze, and in 1981 she moved to Etten-Leur. After leaving the Gerardus Majella primary school in 1989, she started highschool (VWO) at the Catholic School in Etten-Leur. In 1995 she received her VWO-diploma, after which she started to study Biological Health Sciences at the University of Maastricht. During her study she obtained her status of article 9-expert according to the Law on Animal experiments. In 1999 she followed her graduation internship at the Department of Physiology, laboratory for Microcirculation under supervision of Dr. M.G.A. oude Egbrink, Dr. A. Griffioen and Dr. J.W.M. Heemskerk. In 2000 she spent six months in Cambridge, England, to perform research at the Department of Haematology, Division of Transfusion Medicine, University of Cambridge, under guidance of Dr. N.A. Watkins and Dr. W.H. Ouwehand. On August 31st 2000 she received her Master's Degree and in January 2001 she won the student award 2000 of the Faculty of Health Sciences on behalf of the Foundation for Practice of Science, University of Maastricht, procured by Her Majesty Queen Beatrix. On May 1st 2001 she started as PhD student at the Cardiovascular Research Institute Maastricht (CARIM) at the Department of Biochemistry of Prof. Dr. J. Rosing. The research that she performed there and which is described in this thesis, was supervised by Dr. J.W.M. Heemskerk and Dr. M.G.A. oude Egbrink. After taking several courses, she received the CARIM PhD-Training Certificate in 2004. During her PhD-period she visited congresses in among others Birmingham, Nottingham, Oxford, Eberbach, Erfurt and Sydney, and twice she won a "young investigator award" for her abstracts or oral presentation. She visited the laboratory of Dr. B. Nieswandt in Würzburg, Germany, several times to perform experiments. She also visited the laboratory of Prof. Dr. S. Watson in Birmingham, England. On October 21st 2004 she received a "Kootstra Fellowship talented candidate postdoc", from the Faculty of Medicine, intended for talented PhD-students to overcome the period between finishing the PhD thesis and starting as a Post-doc. Since December 15th 2005 she is working as a Post-doc at the Department of Biochemistry at the University of Maastricht.

List of publications

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Awards

- Student award 2000 of the Faculty of Health Sciences on behalf of the Foundation for Practice of Science, University of Maastricht, procured by Her Majesty Queen Beatrix (January 2001).
- Young investigator award of the International Society on Thrombosis and Haemostasis, XIXth Congress ISTH, Birmingham, UK (July 2003).
- Best contribution of young scientist, 10th Erfurt European Conference on Platelets, Erfurt, Germany (June 2004).

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- Kootstra Fellowship talented candidate postdoc, Faculty of Medicine, University of Maastricht, intended for the period between the accomplishment of the PhD degree and the appointment of a talented student as postdoc (May 2005).