Platelet Activation by Extracellular Matrix Proteins in Haemostasis and Thrombosis

Steve P. Watson*

Centre for Cardiovascular Sciences, Institute of Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, Birmingham, B15 2TT, UK

Abstract: The prevention of excessive blood loss to avoid fatal haemorrhage is a pivotal process for all organisms possessing a circulatory system. Increased circulating blood volume and pressure, as required in larger animals, make this process all the more important and challenging. It is essential to have a powerful and rapid system to detect damage and generate an effective seal, and which is also exquisitely regulated to prevent unwanted, excessive or systemic activation so as to avoid blockage of vessels. Thus, a highly specialised and efficient haemostatic system has evolved that consists of cellular (platelets) and protein (coagulation factors) components. Importantly, this is able to support haemostasis in both the low shear environment of the venous system and the high shear environment of the arterial system. Endothelial cells, lining the entire circulation system, play a crucial role in the delicate balance between activation and inhibition of the haemostatic system. An intact and healthy endothelium supports blood flow by preventing attachment of cells and proteins which is required for initiation of coagulation and platelet activation. Endothelial cells produce and release the two powerful soluble inhibitors of platelet activation, nitric oxide and prostacyclin, and express high levels of CD39 which rapidly metabolises the major platelet feedback agonist, ADP. This antithrombotic environment however can rapidly change following activation or removal of endothelial cells through injury or rupture of atherosclerotic plaques. Loss of endothelial cells exposes the subendothelial extracellular matrix which creates strong signals for activation of the haemostatic system including powerful platelet adhesion and activation. Quantitative and qualitative changes in the composition of the subendothelial extracellular matrix influence these prothrombotic characteristics with life threatening thrombotic and bleeding complications, as illustrated by formation of atherosclerotic plaques or the disorder Ehler-Danlos syndrome, which is caused by a defect in collagen synthesis and is associated with fragile blood vessels. This review will focus on the role of the subendothelial matrix in haemostasis and thrombosis, highlighting its potential as a target for novel antithrombotics.

THE COMPOSITION OF THE EXTRACELLULAR MATRIX

The intima extracellular space is localized between the endothelial basement membrane and the internal elastic lamina representing the border to tunica media. The vascular endothelial basement membrane (50-100 nm) provides the primary physical support for endothelial cells and is the first matrix constituent to be exposed to the flowing blood following denudation of the endothelium. The basement membrane is primarily composed of laminin (typically α4 laminin subtypes), which is the most abundant protein, fibronectin, enactin, several proteoglycans (including heparin sulphate proteoglycan) and collagen type IV [1-3]. Of these, laminin and fibronectin have been shown to support adhesion and activation of platelets (see below). In contrast, the role played by collagen type IV in regulating platelet activation is unclear (see below).

The extracellular matrix that lies between the basement membrane and the internal elastic lamina is primarily layed down by smooth muscle cells and has been termed the vascular extracellular matrix. The major components of the vascular matrix are type I and III fibrillar collagens, as exemplified in the human aorta where they make up 50% of its dry weight [4,5]. These two fibrillar collagens capture circulating VWF and mediate powerful adhesion and activation of platelets through GPVI and integrin $\alpha 2\beta 1$ as discussed below [6,7]. Other important components of the vascular extracellular matrix include proteoglycans, hyaluronate, elastin and vitronectin. Elastins are highly abundant in the vascular extracellular matrix, often making up to as much as 50% of its dry weight, and are essential to preserve elasticity [8]. In addition the proteoglycan chondroitin sulphate contributes to the tensile strength of the vessel wall, particularly in the aorta.

The major components within the vascular extracellular matrix that interact directly with platelets are laminin, fibronectin, vitronectin and collagen. Thus, the molecular basis of the interaction of these proteins with platelets is discussed in more detail below. Several other structural proteins also have significant roles in thrombus formation and are recruited to the extracellular matrix upon damage to the endothelium, including VWF [9], fibrinogen, thrombospondin and proteins involved in the coagulation response, and these are discussed as well. For a representative structure of an arterial wall showing the location of extracellular matrix proteins, see Fig. (**1**) in the Chapter by Eble and Niland (this volume).

*Address correspondene to this author at the Centre for Cardiovascular Sciences, Institute for Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, Birmingham, B15 2TT, UK; Tel: 00 44 121 4146514; E-mail: s.p.watson@bham.ac.uk

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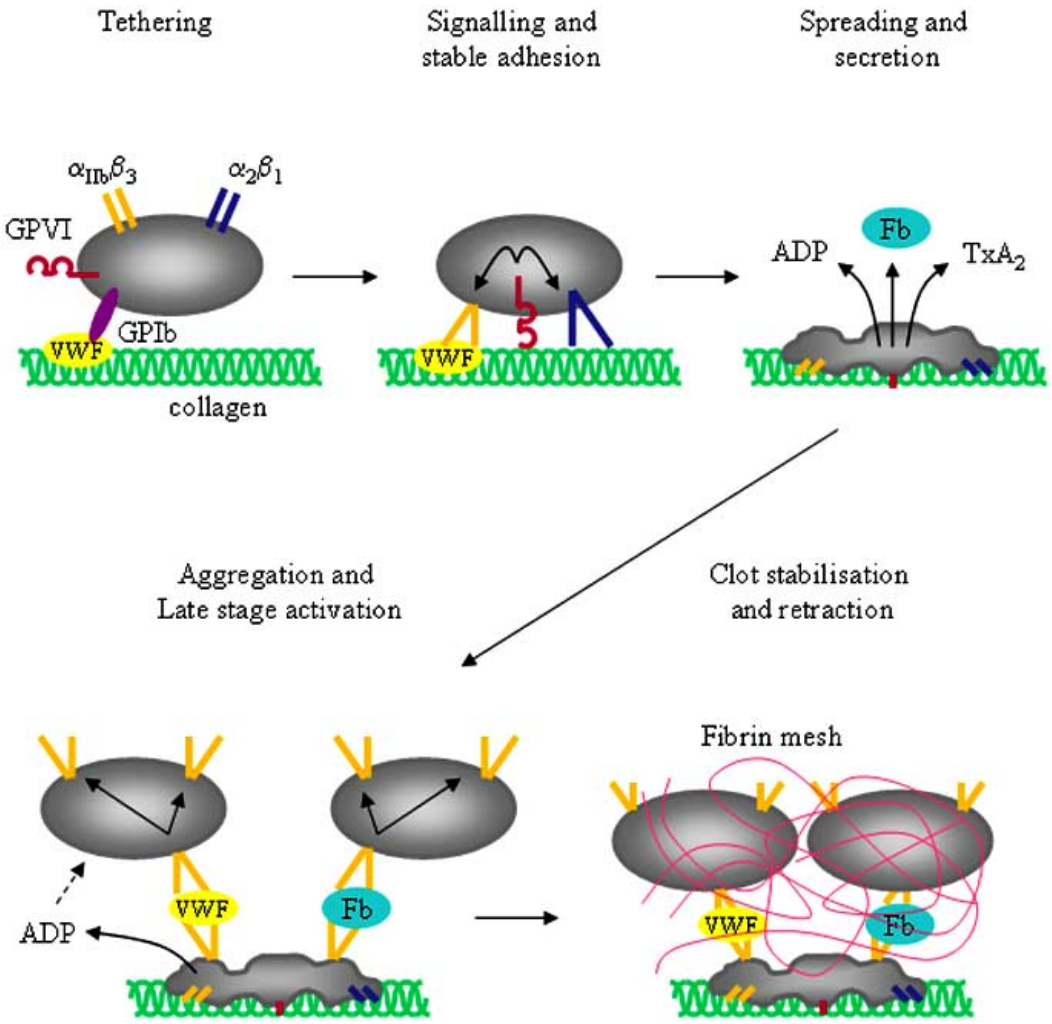

Fig. (1). Steps of platelet adhesion, activation and thrombus formation.

At sites of vessel wall damage where ECM is exposed, initial tethering of platelets occurs *via* GPIb-VWF interaction. VWF is bound to collagen *via* its A3 domain and interacts with GPIb on the surface of platelets *via* its A1 domain. Rolling platelets undergo stable adhesion through α2β1-collagen and αIIbβ3-VWF interaction. At this stage both integrins become activated mainly through inside-out signaling triggered by GPVI engagement to collagen. Platelet activation and integrin engagement cause platelet spreading as well as secretion of prothrombotic agents (ADP, TxA2, Fibrinogen). This process recruits additional platelets and stabilizes the growing thrombus. Highly activated platelets within this thrombus provide a 'procoagulant' surface leading to massive thrombin generation, which further reinforces platelet activation but also generates a rigid fibrin mesh and leads to clot retraction, which is based on αIIbβ3-fibrin interaction.

THE FUNCTION OF THE SUBENDOTHELIAL MATRIX IN HAEMOSTASIS AND THROMBOSIS

The subendothelial extracellular matrix provides structural support to endothelial cells and is therefore important in the organization and maintenance of the shape of the vessel. The cylindrical shape of the vessel is required for unobstructed laminar blood flow. The structural function of the extracellular matrix is illustrated by the massive remodeling that occurs in processes such as restenosis or atherosclerotic plaque development which cause a narrowing of the vessel lumen [10,11]. This may cause a disturbance to laminar flow and a significant increase in shear force, and eventually lead to obstruction of blood flow giving rise to life-threatening thrombotic complications. Indeed, it is noteworthy that atheroma are frequently found at sites of vessel bifurcation, where flow is disturbed.

The subendothelial matrix is also involved in the regulation of endothelial cells [12] through integrin-mediated 'outside-in' signalling in response to components of the vessel wall such as collagen, laminin, fibronectin, vitronectin and fibrinogen. Thus, changes in the composition of the subendothelial matrix have the potential to modulate composition and the response of endothelial cells to other stimuli, including shear [13,14]. Activation of endothelial cells induces the expression of the pro-thrombotic proteins, VWF, P-selectin and tissue factor, and adhesion receptors, such as E-selectin, ICAM-1 and VCAM-1. Expression of VWF and P-selectin on the endothelial surface contribute to the development of atherosclerotic plaques through recruitment of platelets and leukocytes [15]. Furthermore, once exposed to the flowing blood, the subendothelial matrix plays a critical role in platelet adhesion and activation, and thrombus formation, as discussed in further detail below.

THE REGULATION OF THROMBUS FORMATION BY THE SUBENDOTHELIAL EXTRACELLULAR MATRIX

The events underlying thrombus formation at the high shear rates of the arterial system that occur following vascular damage or rupture of atherosclerotic lesions have been subject to intensive research over a number of years, and have led to the development of a multistep model. The trigger is the denudation of endothelial cells causing exposure of the subendothelial extracellular matrix to the flowing blood. This is followed by capture (or tethering) of platelets, which is a specialized process that serves to counter the very high shear rates of the arterial system, including those found at sites of stenosis. Tethering of platelets is accomplished by binding of VWF to subendothelial collagen which leads to a conformational change that enables it to bind to the GPIb-IX-V complex on the platelet surface (for review [16]). The interaction between VWF and GPIb-IX-V has a very fast on-rate of association that leads to the formation of an effective bond at high shear and therefore platelet capture [17]. Strikingly, the effectiveness of the VWF conformational change and subsequent binding to GPIb binding increases with increasing shear. The interaction between VWF and GPIb-IX-V has a very fast off-rate, however, thereby leading only to the transient capture of platelets [17]. This is illustrated by the rolling of platelets in the direction of flow on a monolayer of VWF, with stable aggregates only being formed after several minutes through the weak activation of integrins by the GPIb-IX-V complex [18].

The tethering of platelets to VWF enables other matrix proteins with a slower on-rate of association to their receptors to interact with platelet surface receptors. Crucially, these interactions would not occur in the absence of VWF [19]. The most important interaction is that of the fibrillar collagens, type I and III with the low affinity immunoglobulin surface receptor, GPVI. Clustering of GPVI by collagen or by laminin, which has an approximate tenfold lower affinity for the glycoprotein receptor compared with collagen [20], generates powerful signals that lead to activation of platelets, including inside-out activation of platelet integrins, cytoskeletal remodeling (leading to platelet spreading), granule secretion and thromboxane A_2 (TxA_2) formation [6]. Activation of integrins converts the tethering of platelets to stable adhesion. In contrast, the affinity of the interaction between collagen or laminin with GPVI is not sufficient to mediate stable adhesion. The platelet integrins that interact with the extracellular matrix are $\alpha IIb\beta 3$, $\alpha 2\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$ and $\alpha v\beta 3$. Of these, the most important two are considered to be $\alpha IIb\beta 3$, which is expressed at 80,000 - 120,000 copies per platelet, and $\alpha 2\beta 1$, which has 1500 - 3000 copies. Integrin $\alpha IIb\beta 3$ mediates stable adhesion through binding to VWF and fibronectin, and also mediates platelets aggregation through interaction with above ligands and also fibrinogen [21]. Integrin $\alpha 2\beta 1$ mediates stable adhesion through binding to collagen, although this role can be masked by the interaction of VWF with integrin $\alpha IIb\beta 3$. Thus, the importance for $\alpha 2\beta 1$ in supporting adhesion to collagen is increased under conditions of reduced VWF plasma concentration [22]. Integrins $\alpha 5\beta 1$ and $\alpha 6\beta 1$ have also been shown to support stable adhesion of platelets under high shear through binding to fibronectin and laminin, respectively, although this role is redundant with that of integrin $\alpha IIb\beta 3$ [23-25].

Stable adhesion of matrix proteins to their respective integrins serves to increase the net affinity of collagen for GPVI leading to enhancement of signals through the immunoglobulin receptor. In addition, clustering of the integrins themselves generates outside-in signals that serve to reinforce platelet activation, albeit these are much weaker than those of GPVI [21]. Thus, platelet glycoprotein receptors generate a repertoire of signals that mediate platelet adhesion and spreading at sites of vessel injury, with the latter serving to strengthen platelet adhesion.

The secretion of platelet dense granules and activation of phospholipase A_2 leads to the release of ADP and formation of TxA_2, respectively, the two major feedback mediators of platelets. These two agonists synergise with each other and with signals from platelet glycoprotein receptors to mediate further platelet activation. Crucially, the two feedback messengers, along with VWF and fibrinogen, mediate formation of a platelet aggregate through a continual cycle of platelet tethering (*via* VWF), platelet activation (primarily *via* ADP and TxA_2) and platelet crosslinking (*via* fibrinogen or VWF binding to integrin $\alpha IIb\beta 3$). Moreover, this scheme of events is further enhanced by generation of thrombin through the coagulation cascade which activates platelets through binding to the protease activated receptors, PAR1 and PAR4, as well as converting fibrinogen to fibrin, which serves to further strengthen the growing thrombus. The growing thrombus is also consolidated by low affinity interactions between several platelets surface proteins, including binding of CD40 ligand (CD40L) to integrin $\alpha IIb\beta 3$, and binding of ephrins to Eph kinases. Furthermore, thrombus formation is completed by an integrin-driven clot retraction, a process through which contraction of the actin-myosin cytoskeleton is channelled *via* integrins to fibrin [26].

Alongside the above events, platelets provide a 'procoagulant' surface through exposure of phosphatidylserine leading to a burst of thrombin generation, which further reinforces platelet activation and generates fibrin, leading to clot retraction, as described above. A simultaneously activated fibrinolytic response prevents excessive thrombus formation (for review [27]).

The above model emphasizes that platelet adhesion to the subendothelium represents the first step in thrombus formation following damage to the arterial system. The early events in this cascade have attracted much interest for pharmacological intervention in anticipation that this may overcome limitations of current ant-platelet regimes. In this review, we shall discuss what is known about the structural features of the major matrix proteins that mediate platelet binding and platelet activation, as well as the possibility of pharmacological intervention and therapeutic potential.

COMPONENTS OF THE VASCULAR EXTRACELLULAR MATRIX AND PLATELET ACTIVATION/ADHESION

A). Collagen

There are at least 43 distinct collagen α chains in the human genome, assembling into 28 collagen types [28].

Their common feature is the Col-domain, a triple-helical core. Three polyproline helices form a superhelical structure, characterized by the sequence glycine-X-Y. This sequence may be repeated more than 300 times, so that a triple-helical domain is often more than 1,000 amino acids in length. The α chains form a right-handed triple helix and may be identical or different, depending on the type of collagen. The X-position is often taken by proline (Pro), while the Y position is frequently occupied by 4-hydroxyproline (Hyp) [29]. This regular repetition and high glycine content is found in very few other fibrous proteins. Glycine, being the smallest amino acid, is required at every 3rd position where it occupies the interior of the helix, since there is no space for side chains. The rings of Pro and Hyp point outward, thermally stabilizing the triple helix. More than 90% of the collagen in the body is type I, II, III and IV.

Collagen Types in the ECM

While collagen types I, III, IV, V, VI, VIII, XV, XVIII and XIX have been found in the vessel wall, types I, III and V are the most prominent [30]. Collagen I and III form fibres, confer tensile strength as well as flexibility. Collagen V is found as co-polymer in association with other collagens and may also form filaments by itself. Collagens IV and VIII, forming 2D or 3D networks, are believed to lie closer to the actual vessel lumen than other collagens [4,31]. Collagen type IV is found directly beneath the endothelial cells in the vascular endothelial basement membrane, often in association with laminin around smooth muscle cells. Collagen type IV is a non-fibrillar collagen and is considerably less potent than the fibrillar collagens in supporting activation of the major signalling receptor for collagen on platelets, glycoprotein VI (GPVI), or supporting adhesion through recruitment of von Willebrand factor (VWF) [32]. On the other hand, there are reports that collagen type IV is able to support GPVI-mediated platelet aggregation in a low shear environment, or platelet adhesion and thrombus formation at arteriolar rates of flow [33,34]. The explanation for these contradictory observations may be related to the method of collagen preparation. Thus, the importance of basement membrane collagen type IV in supporting platelet activation and thrombus formation warrants further investigation. Further into the ECM, collagen VI is found, forming beaded filaments. Following superficial vessel wall damage, these sub-endothelial collagens are the first ones to contact patrolling platelets. On the other hand, increased damage reaches the internal elastic lamina and deeper layers of the media, exposing collagens type I and III and also tissue factor which activates the coagulation cascade.

Collagen I is present in abundance in the cap of plaques [35]. Remodelling of the vessel wall, including collagen turnover, induced by proteolytic activity of macrophages, is important for the ability of smooth muscle cells to migrate and proliferate within the intima. This contributes to the development of plaques.

Direct and Indirect Binding of Platelets to Collagen

The main collagen receptors on platelets are considered to be the integrin α2β1 (VLA-2, GPIa/IIa) and GPVI [36,37]. Over the last years the binding regions of collagen receptors on different types of collagen have been analyzed by an approach based on the use of toolkits of triple-helical peptides (summarized in [38]). From these very elegant studies, it has become clear that although α2β1 binds with highest affinity to GFOGER (single amino code), it binds with varying affinities to more general sequences of GXXGER, thus allowing for a controlled adhesion and cell migration over a collagen surface. For GPVI it has become clear that the spacing of the GPO-triplets plays a crucial role in GPVI-collagen interaction [39]. The VWF binding sequence on collagen has been identified as GPRGQOGVMGFO [40]. The intact sequence is found in collagen types II and III, but not in intact collagen type I. However, VWF binding to type I collagen has been shown. It has been suggested that a binding region for VWF on collagen type I could be formed by contributions of both (α1 and α2) chains [38].

The first step in platelet adhesion and activation following damage to the vessel wall is the interaction of plasma VWF with subendothelial collagen under shear, as discussed above [41]. Platelets are tethered by the interaction of the GPIb-IX-V complex with the A1 domain of VWF, which is exposed by shear [42,43]. VWF is immobilized on collagen types I and III in the subendothelial matrix *via* its A3 domain [44,45]. In addition, the A1 domain has also been reported to mediate binding to collagen type VI in the subendothelial matrix [46,47]. Platelets roll over the collagen-VWF surface, forming new GPIb-VWF bonds at the front while releasing bonds at the tail. During rolling under high shear conditions, platelets also shed parts of their membranes, which are in firm contact with VWF leaving trails of procoagulant microparticles [48]. Firm attachment of platelets to collagen is achieved *via* the α2β1-collagen interaction or indirectly *via* binding of VWF to integrin αIIbβ3. Both integrins have to be in an active conformation to mediate binding. Activation is mediated by binding of talin and kindlin-3 to their cytosolic tails (inside-out signalling) following agonist-induced platelet activation [49,50]. Collagen is the most powerful agonist in the subendothelial matrix mediating activation, although weak signals from GPIb-IX-V also contribute to activation.

The collagen-binding region of α2β1 is the I-domain, also called A-domain due to its analogy to the vWF A1-domain [51]. Crystallography, using mutant α2 I-domains, has enabled identification of the critical residues that support binding of a synthetic collagen triple helix peptide containing GFOGER [52]. The glutamate coordinates the divalent cation (under physiological conditions Mg^{2+}) in the integrin metal ion dependent adhesion site (MIDAS). The MIDAS is not exposed in the inactive conformation and only becomes accessible to collagen l upon integrin activation [53]. Helix 7 of the integrin undergoes a dramatic movement during activation suggesting that this is a mechanism for affinity regulation of the integrin leading to agonist binding and possibly also outside-in signalling, which involves clustering of the integrin [54,55]. Further regulation of integrin α2β1 is achieved *via* rearrangement of disulphide bonds, catalyzed by protein disulphide isomerases on the platelet surface [56], although this remains controversial. Studies with recombinant collagen peptides have shown that only GFOGER (found in collagen type I, II and IV) is capable of binding to the inactive conformation of integrin α2β1 [57]. The binding to the other GXXGER sequences depends upon inside-out

signalling. The main role of integrin $\alpha 2\beta 1$ in platelet collagen interaction is to support firm adhesion, with GPVI playing a crucial role in platelet activation.

Studies using mice deficient in the GPVI signal transduction subunit, the Fc receptor γ chain, or treatment of mice with a GPVI-depleting monoclonal antibody, JAQ1, suggest that under certain experimental conditions, GPVI is the only collagen receptor of importance in thrombosis [58]. Indeed, treatment with JAQ1 leads to a complete loss of the thromboembolism induced by collagen and epinephrine. On the other hand platelets from $\beta 1$-deficient mice develop less stable aggregates under flow, pointing to an important role of $\alpha 2\beta 1$ in thrombosis [59]. Experimental and species (e.g. mouse platelets are much smaller than human) differences might be one reason for the sometimes conflicting results. However, the focus on certain aspects of artificial thrombus formation might lead to overestimation of the role of the two receptors, as exemplified in the study of Auger *et al.* This study suggested two distinct mechanisms underlying stable adhesion to collagen, namely *via* VWF bridging or through direct binding, possibly *via* integrin $\alpha 2\beta 1$ to the GFOGER motif, as this does not require inside-out activation of the integrin [60].

B). Laminin

Laminins are heterotrimeric glycoproteins consisting of three polypeptide chains (α, β, γ) [61,62]. 5 genes encode for the α-chains and 3 each for the β- and γ-chain [62]. The chains can assemble in various combinations and 15 different laminin subtypes have been described [2]. The expression of the different laminin subtypes is tissue specific. Basement membranes of all blood vessels are characterized by the presence of $\alpha 4$ laminin subtypes [61].

The central part of the laminin molecule is characterized by a triple α-helical assembly of the α, β, and γ chain [63]. While the C-terminal parts of the β– and γ-chain are within the triple-helical assembly, the α-chain extends further to form five globular motifs, called g-domains. This domain is an important mediator of laminin - cell interaction because of its involvement in binding to various integrins [62,64]. The N-terminus of the molecule is not triple helical and consists of short arms of the 3 chains, forming distinctive domains. These domains are critical for the self-assembly of the laminin into the polymer that is found in endothelial basement membranes [65-67].

Laminin Receptors

The integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$, $\alpha 7\beta 1$, $\alpha 9\beta 1$ and $\alpha v\beta 3$ have been shown to interact with laminins, with different preferences for the various laminin subtypes. However, the only reported laminin-binding integrin on the platelet surface, $\alpha 6\beta 1$, is known to bind all laminins subtypes [68,69]. On the other hand, $\alpha 2\beta 1$ does not appear to support binding of platelets to laminin [70,71].

Several additional cell surface molecules on platelets and other cell types have also been described to interact with laminin. Among them is the Lutheran glycoprotein, a blood group glycoprotein expressed on erythrocytes [72]. The Lutheran glycoprotein mediates adhesion of red cells to laminin, specifically to α5-chain laminins [73,74]. Based on an increased expression of the Lutheran glycoprotein, sickle cells have been shown to have increased affinity for binding to laminin relative to normal red cells, possibly contributing to vasoocclusive episodes in these patients [75,76]. Other laminin receptors include dystroglycan, a heterodimeric polypeptide particularly important for the function and structure of muscle fibres, the 'collagen' receptor GPVI and an uncharacterised platelet protein of 67 kDa receptor [20,77, 78].

Laminin - Platelet Interactions

Adhesion of platelets to laminin was first described in 1983 and was subsequently shown to be due to binding to integrin $\alpha 6\beta 1$ (also known as VLA-6) [79-81]. The involvement of GPVI in laminin- induced platelet activation was only recently reported [20]. The 67 kDa laminin receptor, that was first identified on human breast carcinoma and muscle cells [82,83] is also expressed on platelets, although almost nothing is known about its function [81,84,85]. Thus, it seems that the principle laminin receptor on platelet is the integrin $\alpha 6\beta 1$, while GPVI functions as a downstream receptor, contributing to laminin induced platelet activation. In the absence of GPVI platelets are still able to adhere normally to laminin but spreading and lamellipodia formation are strongly reduced [20]. Biacore studies have revealed that the interaction between laminin and GPVI has a ten-fold lower affinity compared to binding to collagen. This lower affinity may explain why $\alpha 6\beta 1$ binding is a prerequisite for GPVI binding and why laminin, in contrast to collagen, is unable to induce significant tyrosine phosphorylation or platelet aggregation in suspension [20]. The concept of $\alpha 6\beta 1$ and GPVI as the platelet laminin receptors is very appealing since it mirrors the cooperation of $\alpha 2\beta 1$ and GPVI in the interaction of collagen [6].

Again in analogy to platelet-collagen interaction, VWF is required for platelet adhesion to laminin under flow conditions [24]. Anti-VWF antibody treatment blocks platelet adhesion to laminin completely under high (1500 s^{-1}) but not under low shear (300 s^{-1}) conditions. Similar results were obtained with VWF deficient blood (type III VWF disease) or blood from Bernard-Soulier syndrome patients (GPIb deficiency) [24]. Therefore, in whole blood under flow conditions, the interaction of VWF with laminin seems to be the initial step. Recruitment of platelets by VWF may enable integrin $\alpha 6\beta 1$ and GPVI to interact with laminin and thereby support integrin activation giving rise to stable adhesion and formation of small aggregates. The interaction of VWF with components of the extracellular matrix other than collagen has been suggested for several years [86,87], but laminin is the first non-collagen to be able to mediate direct binding of VWF, a result that has been confirmed by Biacore [24].

In conclusion, laminin seems to interact with platelets both indirectly *via* VWF and directly through integrin $\alpha 6\beta 1$ and GPVI. The role of the 67kDa protein in the interaction of laminin with platelets is not known. The sequence of events that results in adhesion of platelet to a laminin surface under flow conditions is similar to that seen on collagen. The overall significance of the interaction of platelets with laminin however, would appear to be minimal due to collagen exposure. On the other hand, it can be speculated for example that the interaction of platelets with laminin plays a key role fol-

lowing superficial injuries that lead only to disruption of the endothelial lining or during the final stages of angiogenesis as a mechanism for completing the endothelial monolayer through release of growth factors. In such cases the deeper, underlying collagen may not be exposed.

C). Fibronectin

Structure of Fibronectin

Fibronectin is a homodimeric glycoprotein consisting of 250 kDa subunits linked by two disulphide bonds in their COOH-termini [88]. In contrast to laminin and collagen, fibronectin is present in blood plasma at a concentration of 300-400μg/ml (~10% of the fibrinogen concentration) as well as in platelet α-granules [89-91]. Fibronectin RNA is alternatively spliced at three sites generating EIIIA(EDA), EIIIB(EDB) and V (CS-1) variants. EIIIA and EIIIB variants are mostly found in the extracellular matrix of blood vessels, but are also present in platelet granules [92,93]. In contrast plasma fibronectin mostly lacks these domains, although the level of EIIIA fibronectin in plasma seems to be increased in disease states associated with an increased thrombotic risk such as atherosclerosis, diabetes and stroke [94-96]. Plasma fibronectin is a soluble dimer, while extracellular matrix fibronectin is assembled into insoluble aperiodic matrix fibrils. The latter is a cell-mediated process, under involvement of integrins and cell surface LAMMs (molecules of large apparent molecular mass). Cells dedicated to fibronectin assembly are fibroblasts, vascular smooth muscle cells and endothelial cells but also platelets [97]. Furthermore, several binding sites for adhesive proteins such as fibrin, collagen, VWF and integrins have been identified in the fibronectin molecule, underlining its capability to crosslink molecules and cells [97].

Fibronectin Receptors

Cell surface receptors for fibronectin belong to the integrin family and several different integrins have been described to bind fibronectin (α3β1, α5β1, α4β1, α4β7, αIIbβ3 and αvβ3) [98-103]. While most of the above integrins interact *via* a characteristic RGD- containing sequence, α4β1 and α4β7 bind to a site characterized by a LDV motif [104]. Of the above, only α5β1, αIIbβ3 and αvβ3 are expressed on platelets.

The Interaction of Cell Matrix Fibronectin with Platelets

The interaction of fibronectin with platelets and a role for subendothelial fibronectin in the regulation of platelet responses has been suggested since the late seventies, although this still remains poorly understood [105,106]. This is in part due to the presence of plasma fibronectin and the embryonic lethality of the fibronectin-deficient mice [107], as these have hampered investigation of the role of extracellular matrix fibronectin in thrombosis and haemostasis.

The first evidence for the presence of fibronectin receptors on platelets came from the observation that platelets from a patient with Glanzmann's thrombasthenia showed a strongly reduced binding to fibronectin [100,108]. Subsequently, by using chemical cross-linking and monoclonal antibodies, αIIbβ3 was shown to bind fibronectin directly [109]. Binding of fibronectin to αIIbβ3 involves the RGD sequence [110], although additional αIIbβ3-binding sites in fibronectin have been identified. This multisite interaction of αIIbβ3 with fibronectin seems to be necessary for a tight interaction [111], with RGD-mediated integrin binding being essential for platelet adhesion under shear to fibronectin [112]. Platelets also express two additional fibronectin receptors α5β1 and αvβ3 [79,113]. α5β1 has been shown to have non-redundant role in platelet adhesion to fibronectin, since antibodies to α5 are able to inhibit platelet deposition under flow ($300s^{-1}$) by approximately 50%. In comparison RGD-containing peptides or antibodies to αIIbβ3 blocked this interaction by over 85% [25,112].

While αIIbβ3 supports platelet adhesion as well as activation in response to fibronectin, α5β1 and αvβ3 seem to mainly function as adhesion receptors. Platelets adhere and spread on fibronectin coated surfaces under static conditions [80,114,115]. Platelet adhesion to fibronectin induces formation of filopodia and lamellipodia through binding to integrin αIIbβ3, whereas binding to integrin α5β1and αvβ3 induces only filopodia formation [23,116]. Robust Ca^{2+} mobilization is dependent on the interaction of αIIbβ3 with fibronectin [23], causing outside-in signaling as observed for the binding of fibrinogen to integrin αIIbβ3 [117]. Interestingly, adhesion of platelets to fibronectin is not inhibited in the absence of functional αIIbβ3, supporting a role for α5β1 and αvβ3 in this process [23,113]. However, neither α5β1 nor αvβ3 are able to induce a similar level of spreading to fibronectin in comparison to αIIbβ3, which may be due to an almost two orders of magnitude higher expression level of the latter.

Although all these studies support a function of purified fibronectin in platelet adhesion and spreading, an important question is the relative role of fibronectin within the subendothelial extracellular matrix, particularly taking into account the presence of other platelet-regulatory proteins. This has been addressed using human umbilical artery subendothelium and antibodies against fibronectin [118]. Incubation of the subendothelium with an anti-fibronectin antibody resulted in a significant inhibition of platelet adhesion. Interestingly, this inhibition is dependent on the wall shear rates used, being most pronounced at shear rates above $800s^{-1}$ and absent at $400s^{-1}$ [118]. In contrast to the situation on collagen or laminin, VWF is not able to bind fibronectin and therefore does not mediate platelet adhesion to fibronectin under flow.

Fibronectin regulates platelet activation and thrombus formation at several checkpoints. Soluble, plasma fibronectin significantly contributes to thrombus stability and thrombus growth (see below). Fibronectin within the endothelial extracellular matrix also supports platelet adhesion and spreading [25]. Interestingly, this interaction seems to be more important under high shear conditions [118], typically reflecting a pathological situation of a stenosed artery.

D). Vitronectin

Structure of Vitronectin

Human vitronectin is a 75kDa protein which has a similar distribution to fibronectin [119]. Thus, it can be found in plasma (200-500μg/ml), in the extracellular matrix and in platelet α-granules where it is taken up from the plasma [120-122].

Vitronectin Receptors

Vitronectin binds many of the proteins involved in thrombosis and haemostasis, including plasminogen activator inhibitor-1 (PAI-1), plasminogen and heparin. Vitronectin posseses a RGD site that enables it to bind to αIIbβ3 and the minor platelet integrin, αvβ3 [123]. However, the role of integrin αvβ3 in supporting platelet activation is unclear. Moreover, the ability of vitronectin to bind to integrin αIIbβ3 is masked by the presence of other ligands, including fibrinogen, fibronectin and VWF.

The Interaction of Cell Matrix Vitronectin with Platelets

The interaction of platelet αIIbβ3 and αvβ3 with cell matrix associated vitronectin may well have a supportive function in platelet adhesion to the subendothelium. However, experimental data to support this hypothesis are, to our knowledge, not available at the moment and studies investigating the role of vitronectin in thrombosis and haemostasis are mainly focused on the plasma fraction.

THE ROLE OF MATRIX PROTEINS PRESENT IN PLASMA AND PLATELET α-GRANULES

VWF and fibrinogen are the two major plasma proteins supporting platelet adhesion to the extracellular matrix and platelet thrombus formation through the formation of platelet - platelet contacts. However, unexpectedly mice deficient in both proteins are still able to form occlusive thrombi, although with strongly delayed kinetics [124]. This demonstrates that other plasma proteins or platelet granular components are able to substitute for VWF and fibrinogen and may therefore also play a role in thrombus formation.

Plasma Fibronectin

Early experiments investigating platelet thrombus formation under flow indicated a supportive role of plasma fibronectin in thrombus formation. Using collagen-coated surfaces, platelet adhesion as well as thrombus formation was reduced in fibronectin-depleted plasma. Interestingly, the diminished thrombus formation in fibronectin-depleted plasma was present at low and high shear rates [125-127]. *In vivo* investigations also support a role for fibronectin, as mice deficient in VWF and fibrinogen form occlusive thrombi. Fibronectin was proposed as the likely substituting candidate protein because of its structure and its ability to interact with αIIbβ3 [128,129]. Moreover, a role for fibronectin in thrombus formation *in vivo* was also shown in mice expressing less than < 2% of the plasma fibronectin and < 20% of platelet fibronectin. These animals showed delayed thrombus formation, increased embolization and an increased time to occlusion in the presence of normal amounts of fibrinogen and VWF [124]. The importance of fibronectin was further substantiated in heterozygous mice, having 50% of the normal fibronectin levels. This reduction was sufficient to have a profound inhibitory effect on thrombus formation *in vivo* following arterial injury [130]. In a very recent study Cauhan *et al.* showed, that the presence of a specific fibronectin splice variant (EIIIA) in plasma may have strong prothrombotic effects. Mice engineered to express EIIIA fibronectin only, have strongly accelerated thrombosis in two different *in vivo* thrombosis models, ferric-chloride injury and collagen/epinephrine induced thromboembolism, although the fibronectin plasma levels are only 20% of control mice. The enhancement of collagen/epinephrine induced thromboembolism strongly supports the role for plasma EIIIA fibronectin, since in this model no vessel wall injury is involved. Interestingly, venous thrombosis as well as coagulation parameters were unchanged in these mice, strongly suggesting that the prothrombotic effect is based on an altered EIIIA fibronectin - platelet interaction [131]. Although the molecular basis of this enhanced thrombosis is not clarified, enhanced EIIIA fibronectin plasma levels may represent a risk factor for thrombotic complications in certain pathologies [94-96].

Thus, it seems that fibronectin present in the plasma and/or platelet granules contributes to thrombus stabilization in the presence of VWF and fibrinogen. Possible mechanisms are the support of platelet - platelet contacts *via* integrins [132] as well as stabilizing the fibrin network and cross-linking of platelets to fibrin [133,134].

Another process that may support thrombus formation and stabilization is the assembly of plasma fibronectin into fibrillar extracellular matrix arrays, a process observed on several cell types including activated platelets [135-137]. Thus, activated, adherent platelets are able to assemble plasma fibronectin into an insoluble matrix on their surface which may support platelet cohesion and therefore thrombus strength. The importance of this process was shown using different proteins specifically interfering with this process. In flow chamber experiments, inhibition of fibronectin assembly resulted in a decrease of thrombus volume and the number of adherent platelets by 50-60% [134].

Plasma fibronectin is clearly an important player in thrombus formation, although its precise role remains undefined. Last, but not least, this is emphasised by the observation that increased fibronectin plasma levels are associated with an increased risk of coronary artery disease [138,139].

Vitronectin

Depending on the *in vivo* model and/or vascular bed, vitronectin-deficiency has a variable effect on thrombus formation. Thus, under certain conditions, there is increased embolization, reduced thrombus growth and stability and therefore increased time to vessel occlusion *in vitro*nectin-deficient mice [140-142]. Importantly, early platelet adhesion to the injured vessel wall was not altered in these mice [140], indicating that extracellular matrix vitronectin is not involved and that other components of the extracellular matrix are able to compensate. This suggests that the deficiency of plasma vitronectin is the cause for the phenotype.

The major role of plasma vitronectin in regard to haemostasis may be its ability to bind to plasminogen activator inhibitor (PAI-1). PAI-1 is the major physiological inhibitor of tissue-type plasminogen activator (tPA) and therefore an instrumental regulator of plasmin and fibrinolysis. Vitronectin binds PAI-1, stabilizes the active confirmation of the inhibitor and thereby increases the activity of PAI-1 [143-145]. This ultimately leads to a reduced fibrinolytic response and enhanced thrombus formation. Thus, vitronectin deficiency should cause reduced PAI-1 activity and

enhanced fibrinolysis, which nicely corresponds to the observed phenotype of the knockout mouse (increased embolization, reduced thrombus growth and stability). Interestingly, the thrombotic phenotype of vitronectin and PAI-1 double-deficient mice is indistinguishable from vitronectin or PAI-1 deficient mice [142]. This strongly suggest that both proteins serve the same mechanism, and that other vitronectin functions in thrombus formation are not significant, at least under the experimental conditions used. The connection between PAI-1 and vitronectin is further emphasised by their co-localisation in platelet granules [146,147]. Release of PAI-1 into the thrombus by activated platelets inhibits fibrinolysis, while simultaneous liberation of vitronectin stabilises this process based on the mechanism described above.

Thus, based on these data, plasma vitronectin is important for the regulation of thrombosis, most likely by regulation of PAI-1. The contribution of vitronectin associated with the extracellular matrix seems to be minor, although this has to be said with caution since few data are available.

PHARMACOLOGICAL INHIBITION OF PLATELET - EXTRACELLULAR MATRIX INTERACTION AS ANTI-THROMBOTIC MECHANISM

The critical role of platelets in arterial thrombosis has been well established. Furthermore, it becomes more and more evident that platelets also play a significant role in other pathological processes such as atherosclerosis [148]. Thus, anti-platelet regimes belong to the standard treatment in patients with atherothrombotic disease for acute and prophylactic prevention of thrombotic events. Landmark clinical studies for aspirin or clopidogrel have shown the enormous benefit of anti-platelet agents for the prevention of atherothrombotic events [149-151]. Moreover, dual anti-platelet therapy combining aspirin and the $P2Y_{12}$ antagonist clopidogrel has been shown to further reduce the risk of atherothrombotic complications, with a 20% relative risk reduction in patients with unstable angina or non - ST myocardial infarction as compared to aspirin mono-therapy [152]. Despite this significant achievement, a high number of patients experience thrombotic events while under anti-platelet treatment. Atherothrombosis is still a leading cause of death worldwide and, importantly, the absolute number of deaths from this disease continues to increase [153,154]. Thus, there is a large unmet clinical need and much effort is put into the improvement of existing drugs, e.g. the development of new $P2Y_{12}$ inhibitors, but also in the exploration of new platelet (thrombus) - inhibitory mechanisms. In this regard interference with primary platelet activation and adhesion to the extracellular matrix is considered to be a potentially attractive approach. This is based on the following assumptions and supporting experimental data.

i) Inhibition of the initial step of platelet activation prevents formation of activated platelets. This mechanism has the potential to be very effective in blocking thrombus formation and growth.

ii) The interaction of GPIb and VWF, and of GPVI and collagen, appear to have less redundancy with other pathways as compared to inhibitors of secondary platelet activation. Little redundancy (in the absence of tissue factor exposure) in the pathway suggest an effective and more uniform prevention of thrombus formation.

iii) Thirdly, several mechanisms seem to be more important under the high shear conditions that are typically found in stenosed arteries. Thus, selective interference of platelet activation in pathological high shear situations may lead to only minimal disturbance of normal haemostasis.

iv) Early platelet activation and adhesion is instrumental for the role of platelets in atherosclerosis development.

It is important to note, that most of the currently used platelet inhibitors (phosphodiesterase inhibitors, the cyclooxygenease inhibitor aspirin and $P2Y_{12}$ antagonists) do not interfere directly with these early events but are targeting late mechanisms or secondary platelet activation. Although, $\alpha IIb\beta 3$ antagonists can affect primary adhesion their main function being the inhibition of the final common pathway of platelet aggregation. Thus, several different approaches have been investigated to interfere with primary platelet activation and adhesion:

Collagen

The early steps of platelet-collagen interaction are seen as highly promising for anti-thrombotic intervention. None of the currently marketed anti-thrombotics target these events, last not least due to the fact that protein-protein interactions are not easy to inhibit, especially with small molecule compounds.

Biologics interacting with collagen that show some inhibitory effect on platelet binding in model settings include the recombinant leech proteins rLAPP (from Haementeria officinalis) and saratin (from Hirudo medicinalis), the mosquito salivary gland protein aegyptin (from Aedes aegypti) as well as CTRP-1, a C1qTNF-related protein from plasma [155-158]. All of them have been shown to act *in vivo* as inhibitors of platelet function. The exact collagen binding site of LAPP is not known. For saratin there is interference seen with both VWF and $\alpha 2$-I-domain binding to collagen, suggesting a certain overlap in binding epitopes on collagen [159]. Recently the 3D structure of saratin was solved. The data from docking experiments with collagen model peptides lead to the conclusion that despite a similar global fold of LAPP and saratin there is a significant difference in collagen binding [160]. CTRP-1 blocks the binding of vWF-A3 domain to collagen and thus inhibits collagen-induced platelet aggregation [157]. Aegyptin has been shown to block the interaction of the three glycoprotein receptors, GPIb, GPVI and $\alpha 2\beta 1$ with collagen, thereby showing that it is distinct from the other molecules described above [156]. Thus it might serve as a powerful tool to evaluate the relative importance of all three receptors in the very early steps of platelet adhesion and activation. Targeting collagen itself would limit the action of an inhibitor to sites where collagen is exposed but also limit the use of such inhibitors for systemic application.

An interesting mechanism is the use of the collagen binding domain of GPVI as competitor for the binding of platelet associated GPVI. Since only dimeric GPVI has a significant affinity for collagen the therapeutic protein is expressed as a human immunoglobulin Fc domain fusion protein [161].

Such GPVI-Fc fusion proteins have been shown to inhibit platelet adhesion to collagen and thrombus formation under flow. Moreover, they prevent platelet adhesion to the injured vessel and thrombus formation *in vivo* (endothelial denudation of mouse carotid artery by vigorous ligation) [162]. In contrast, Gruner *et al.* showed in the same *in vivo* thrombosis model an only partial inhibition at high concentration of the fusion protein, while antibodies against GPVI were highly inhibitory [163]. The reason for this discrepancy is unclear but may be based on slight but significant differences in the execution of the animal model. Support for the use of GPVI-Fc as therapeutic protein comes also from a study showing that local delivery of the protein to the site of an injured carotid artery (ballon injury in rabbits) is able to substantially attenuate thrombus formation [164,165]. This directed delivery is an interesting option since it ensures high local concentrations of the fusion protein plus it contains the antithrombotic effect to the targeted site, which should be favourable in terms of bleeding. The clinical development of GPVI-Fc (PR-15) is pursued by Trigen, Munich, Germany. A Phase I study has been successfully finalized [166].

GPVI

The crystal structure of the GPVI collagen binding domain has been recently described, which may open some avenues for the identification of small molecule inhibitors [167]. However, based on the large interaction surface between GPVI and collagen the use of biotherapeutic proteins (antibodies or fragments thereof) seems to be the most promising alternative to find potent GPVI inhibitors. Furthermore, anti GPVI antibodies are able to induce receptor down-regulation, which may reflect an additional anti-thrombotic mechanism. It has been shown *in vivo* that mice, treated with anti-GPVI antibodies are rendered GPVI-deficient and have significant antithrombotic characteristics in several thrombosis models [168]. This effect of anti-GPVI antibodies nicely explains much earlier observations in patients with autoantibodies against the collagen receptor. These patients also showed a defect in collagen-induced platelet activation and GPVI deficiency on their platelets [169]. However, the association of antibody-induced GPVI down-regulation with partial platelet activation and transient thrombocytopenia has been described, which makes this approach futile for clinical use [168]. Thus, more basic research in the principles of GPVI downregulation is warranted. Nevertheless, a recently published elucidation of the signalling pathways involved in GPVI downregulation and the description of an antibody which seems to cause a decrease in surface expression of the receptor without obvious signs of platelet activation or thrombocytopenia may open new avenues for the clinical use of this mechanism [170,171].

Another option is the classical competitive inhibition of the collagen binding site of GPVI. To avoid any platelet activation, monovalent proteins such as Fab fragments are the molecules of choice. A number of antibodies against human GPVI have been generated by several groups from academia and industry [171-174]. Characterization of these molecules *in vivo* has been often hampered by the very narrow cross-reactivity of anti-human GPVI antibodies generated in mice. To circumvent this, Matsumoto *et al.* used GPVI-knock out mice for the generation of anti GPVI antibodies, resulting in a series of molecules (OM) partly having crossreactivity to the rat protein [172]. Fab fragments of one of the generated antibodies, OM4, effectively inhibited thrombus formation in rats (modified Folts model of cyclic flow reduction in carotid artery). Importantly, the Fab fragment did not induce thrombocytopenia, GPVI depletion nor did treatment with OM4 prolong the bleeding [175]. This clearly supports the view, that anti-GPVI treatment in a competitive manner can be a promising antithrombotic mechanism, with an excellent benefit - safety window.

$\alpha 2\beta 1$

As the first receptor on platelets mediating firm adhesion to collagen, this integrin is also a potential target for pharmaceutical intervention. Derivatives of specific $\alpha 2\beta 1$ inhibiting snake venom components like rhodocetin might serve as starting points for such antagonists [176]. There are no reports of clinical trials with direct-acting $\alpha 2\beta 1$ antagonists. Eisai is investigating an $\alpha 2\beta 1$-expression inhibitor, E7820, for cancer indications in Phase II because of the role of the integrin in angiogenesis [177]. Small molecule antagonists of $\alpha 2\beta 1$ for cancer and thrombosis have been described [178] (Biotie website) but further development has not been reported.

VWF

As the interaction partner in the first step of platelet adhesion, VWF is a strong target for consideration of pharmacological intervention. Targeting VWF would also have the advantage of avoiding complications based on potential side-effects through targeting of GPIb-IX-V that might lead to thrombocytopenia given the critical role of GPIb-IX-V in platelet formation. As discussed above the binding region of VWF for the GPIb-receptor complex on platelets becomes only available under conditions of high shear.

AJW200 is a humanized anti-VWF-A1 antibody that has been reported to be well tolerated in a clinical Phase I study. This antibody had been shown to inhibit shear-induced platelet aggregation and to reduce thrombosis in coronary arteries in a canine coronary artery model [179,180]. Further, it showed a favorable effect - bleeding ratio versus the comparator, abciximab ($\alpha IIb\beta 3$ inhibitor), suggesting a safer pharmacological profile in dogs.

The two most advanced approaches in targeting VWF in clinical testing are an aptamer, a protein-binding oligonucleotide, as well as a nanobody, a “heavy-chain only” antibody derived from camelids (for review see [181,182]).

Archemix is investigating ARC1779, a pegylated aptamer binding to the VWF-A1 domain [183]. In a Phase I study in healthy volunteers it was well tolerated, with an apparent elimination half-life time of 2 hours and no bleeding episodes, while inhibiting platelet function dose-dependently. ARC1779 is currently in Phase II clinical development, assessing the safety, pharmacokinetics and pharmacodynamics in patients with VWF-mediated platelet function disorders, including TTP (Thrombotic thrombocytopenia purpura), as well as acute coronary syndrome patients undergoing PCI (Archemix website). Since TTP is associated with an increased plasma concentration of ultra-

large, highly prothrombotic multimers of VWF, a direct inhibition of VWF seems to be here particularly promising. However, clinical studies will show if these predictions hold true.

This is also the case for Ablynx' ALX-0081, an anti-VWF nanobody directed against VWF. The data from animal studies in a modified Folts-model in baboons showed a more pronounced effect on inhibition of thrombus formation than a combination of aspirin, heparin and clopidogrel, with less bleeding complications [184]. The half-life of ALX-0081 in baboons was similar to the half-life of its target, VWF-A1 (8 hours). A Phase I study in healthy volunteers has been successfully concluded and confirmed that ALX-0081 adopts at least the half-life of it's target. The nanobody showed no immungenicity, was safe and well tolerated at all doses tested, and no serious adverse events were reported. The anti-thrombotic effect was dose-dependent in a range from 2 - 12 mg. A Phase Ib study in patients with stable angina undergoing PCI is ongoing. Phase II is scheduled to start by the end of 2008 (Ablynx website).

GPIb

The receptor for VWF-A1 on platelets, the GPIb-V-IX complex, is another option for anti-thrombotic intervention. Besides VWF it also interacts with TSP, P-selectin, thrombin, Mac-1, high-molecular weight kininogen, FXI and FXII (reviewed in [185]). All these interactions may play a role under pathophysiological conditions, while their relative importance is still under discussion. The main concern in targeting GPIb is thrombocytopenia. Thus it will be crucial to monitor this potentially life-threatening condition closely while investigating GPIb inhibitory principles. For *in vivo* studies, Fab-fragments of anti-GPIb antibodies where successfully used, without causing thrombocytopenia. One example is the humanized anti GPIb antibody 6B4, which was applied in a model of arterial thrombosis (modified Folts model) in baboons [186]. While the bolus injection of 0.6 mg/kg or 2 mg/kg 6B4 Fab fragments led to a significant anti-thrombotic effect, platelet count and bleeding time were not significantly changed. When GPIb -/- mice were constructed they showed prolonged bleeding time, the formation of giant platelets and thrombocytopenia, providing a model of the Bernard-Soulier Syndrome [187]. Therefore, to investigate the effect of GPIb deficiency without these secondary effects, transgenic mice where most of the extracellular part of GPIb was replaced by IL-4Rα (IL-4Rα/GPIb-tg mice) were generated. These mice have platelets of normal size and only mild thrombocytopenia with an approx. 30% reduction in platelet count. In a $FeCl_3$-induced thrombosis model it was demonstrated that platelet adhesion to ECM in arterioles of these mice was completely inhibited [188].

Due to the diverse interactions of GPIb as mentioned above, the effect of GPIb blockade could be more marked than the effect of vWF inhibition. However it still has to be demonstrated in direct comparison in suitable animal models that this holds true and is not associated with thrombocytopenia. Several further approaches for targeting GPIb have been reported (for review [189,190], but none has yet reached phases of clinical testing.

CONCLUSIONS

The subendothelial extracellular matrix has been shown to be a powerful activator of platelets. Moreover, the interplay between components of the subendothelial matrix and specific platelet receptors causes a stable adhesion of activated platelets to the exposed subendothelium, which initiates the formation of a haemostatic plug but also localizes the haemostatic reaction to the site of injury. These properties underline a critical, life saving role of the subendothelial matrix in haemostasis. However, pathological changes within the vessel wall which include a remodelling of the subendothelial matrix and a dysfunctional endothelial layer give rise to atherosclerosis and life threatening thrombotic complications. Besides intensive medical treatment atherothrombosis is still a leading cause of death worldwide. Therefore, the development of new anti- thrombotic and anti-atherosclerotic mechanisms is needed. Because of the importance of the subendothelial matrix for these processes several components represent interesting drug targets. Platelets interact with vessel wall associated laminin, fibronectin, vitronectin and collagen. Of these, collagen and associated VWF seem, however, of particular importance for platelet activation and adhesion. Thus, blocking the interaction of platelets with collagen (*via* its receptors GPVI, α2β1 or collagen binding peptides) and VWF (GPIb inhibitors or molecules against VWF) seems to represent the most promising mechanism. Several approaches have been initiated and the first molecules have entered early clinical testing. The very promising investigational and (pre)clinical data raise hopes that these approaches may significantly improve the treatment of atherothrombotic diseases.

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