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Review Article

The role of VWF-collagen interaction in acute platelet thrombus formation

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CONTENTS

Abstract

The first step in the formation of an arterial platelet thrombus consists of the interaction between collagen-bound VWF and the platelet glycoprotein Ib/IX/V complex. This event results in a further interaction of the collagen receptors with the damaged vessel wall, leading to platelet activation and platelet aggregation, a process mediated by the platelet GPIIb/IIIa receptor. Current antiplatelet agents interfering with platelet activation steps (*e.g.*, acetylsalicylic acid, clopidogrel) or blocking the GPIIb/IIIa receptor (*e.g.*, abciximab, eptifibatide, lamifiban, tirofiban) have proven their clinical usefulness. However, their efficacy is not optimal and therefore the search for new antiplatelet drugs continues. Here we review data on a new approach for preventing arterial thrombosis, *i.e.*, by blocking the initial platelet adhesion step.The *in vitro* and *in vivo* antithrombotic effects of inhibiting collagen-VWF binding are summarized and the anticipated benefits of specifically interfering with this interaction are highlighted.

Introduction

Platelets in hemostasis and thrombosis

When a normal vessel wall is damaged, platelets interact with the exposed subendothelium to preserve hemostasis. Formation of the initial platelet layer involves the interaction of platelet receptors with components of the damaged vessel wall, *e.g.,* collagens. In the initial steps of platelet adhesion, von Willebrand factor (VWF) forms a bridge between the damaged vessel wall and the platelet receptor complex glycoprotein (GP)Ib/IX/V at sites of rapid blood flow (1). The interaction between VWF and GPIb is characterized by a high association/dissociation rate resulting in platelet rolling over the damaged blood vessel. Stabilization of platelet adhesion proceeds via collagen receptors, such as GPIa/IIa and GPVI, and results in platelet activation. Activated platelets secrete amongst others thromboxane A_2 and ADP that reinforce the activation process after release into the extracellular space. Other resting platelets in the circulation are activated and bind to the adhered platelets resulting in platelet aggregation, a process mediated mainly by GPIIb/IIIa. This platelet aggregation finally results in a firm hemostatic plug, which seals the endothelial lesion from the blood flow (2).

When platelets interact with a diseased vessel, this same process may result in pathologic vascular occlusion which is responsible for cardiovascular diseases, the major cause of death in the world. The pathophysiological basis for acute coronary syndromes like unstable angina, non-Q-wave infarct and Q-wave infarct is an acute plaque rupture with subsequent platelet adhesion, aggregation and formation of a thrombus (3).

Antiplatelet agents

The introduction of thrombolysis and mechanical recanalization procedures were the first techniques used to treat coronary occlusion. However, one problem with these techniques is the risk for recurrent thrombosis mainly due to platelet aggregate formation. Therefore, in addition to anticoagulants, antiplatelet agents are being administered as adjunctive therapy. Of these, most recently platelet GPIIb/IIIa antagonists, interfering with the final step of platelet aggregation, were introduced (4). Clinically useful antagonists of the platelet GPIIb/IIIa receptor were derived from disintegrins found in venomous secretion of snakes and from inhibiting murine monoclonal antibodies (MAbs). To date 4 GPIIb/IIIa

antagonists have been tested in large clinical trials: the humanized chimeric MAb abciximab (ReoPro®), the cyclic RGD containing peptide eptifibatide (Integrilin®) and the nonpeptide antagonists lamifiban and tirofiban hydrochloride (Aggrastat®) (4, 5). They are well tolerated and different clinical trials have demonstrated that the use of fibrinogen receptor antagonists for acute coronary syndromes can significantly improve the standard drug therapies. However, these advantages become especially important when PTCA is performed (5-9). The main side effect of GPIIb/IIIa antagonists, especially in combination with other antithrombotic agents, is the occurrence of unexpected bleeding.

Other antiplatelet substances interfere with different earlier steps in platelet activation. Acetylsalicylic acid (ASA) irreversibly inhibits cyclooxygenase and thus thromboxane synthesis. ASA is used as an effective antithrombotic agent in cardiovascular diseases, such as angina pectoris, myocardial infarction and ischemic stroke (10). Side effects of ASA are hemorrhagic and gastrointestinal complications. The thienopyridines ticlopidine and clopidogrel are prodrugs that are not active *in vitro* but, after oral administration, are transformed into active metabolites that block the ADP receptor P2Y1 and by this interfere with ADP-induced platelet activation (11). Both drugs are more effective then ASA in the treatment of arterial thrombosis. The use of clopidogrel in combination with ASA resulted in a highly significant benefit in treatment of acute coronary syndromes (12). A severe side effect of ticlopidine is the occasional occurrence of neutropenia and thrombocytopenia, effects hardly seen with clopidogrel (13). Bleeding problems are as frequent with ASA as with thienopyridines.

Although a number of antiplatelet drugs are available for the treatment of cardiovascular diseases, their effectiveness is not optimal and controlling the occasional side effects, especially the bleeding problems, remains a great challenge. Therefore, the search for new antiplatelet drugs continues. One new approach that we and others are pursuing is to determine the effect of inhibiting the first step in arterial thrombosis, *i.e.*, platelet adhesion instead of platelet activation or aggregation. The anticipated benefits of blocking platelet adhesion are 3-fold: (i) as platelet adhesion is the obligatory first step in arterial thrombus formation (1), a marked effect is expected, perhaps comparable with blocking GPIIb/IIIa; (ii) the interaction between the damaged vessel wall, VWF and GPIb is especially important under high shear conditions, hence inhibition at this level would be targeting the effect to the arterial bed, with little influence on the venous side and thus less bleeding problems might be expected; and (iii) blocking platelet adhesion would in addition result in less platelet activation with a decrease in the release of growth factors from platelets, less smooth muscle cell activation, and thus an effect on (re)stenosis could be expected.

Several products that interfere with the interaction between VWF and GPIb have been studied in animal models. Substances interacting with VWF, such as the MAb AjvW2 (14, 15), the organic compound aurin tricarboxylic acid (ATA) (16, 17) or substances inhibiting GPIb such as MAb 6B4 (18, 19) or VCL (20, 21), a recombinant fragment of VWF, all were found to be potent inhibitors of arterial thrombosis *in vivo*. Moreover, administration of all these compounds revealed no or only minor prolongations of bleeding time, especially when compared to the GPIIbIIIa antagonists. For AjvW-2 (22), VCL (23) and ATA (24, 25) beneficial effects on stenosis formation were also demonstrated.

Another way of interfering with platelet adhesion is to block the interaction of VWF with the damaged vessel wall. Four compounds have been tested *in vivo*: calin (26), saratin (27), rLAPP (28) and MAb 82D6A3 (29). The goal of this review is to summarize the available evidence on the role of VWF-collagen interaction in thrombus formation both *in vitro* and *in vivo* and to demonstrate the potential usefulness of blocking this interaction in coronary artery disease.

VWF-collagen interaction in thrombus formation

The vessel wall

Different kinds of collagens are found in the different layers of the vessel wall. Collagen type IV, present in the basement membrane of the endothelial cells, is composed of 2 monomers that overlap in an antiparallel fashion to form dimers which then aggregate to tetramers stabilized by disulfide bridges to produce a spider web-like structure (30, 31). Collagen type VI, a glycoprotein with a short triple helical-core and large globular domains, is found in the superficial portion of the subendothelium and was shown to be associated with VWF mainly (32). Collagens type I and III are present in the media and adventitia, and form fibrils due to lateral interaction between homologous regions within the triple helical domains. These interactions result in the alignment of staggered collagen molecules and are responsible for the visualization of striated fibrils in electron microscopy (33).

von Willebrand factor

von Willebrand factor (VWF) is a large multimeric glycoprotein synthesized in endothelial cells and megakaryocytes. Endothelial VWF is either constitutively secreted to the subendothelium and into the plasma or stored in Weibel Palade bodies for regulated secretion (34). Platelet VWF is stored in platelet alpha-granules and is secreted when platelets are activated. The VWF gene is located on chromosome 12 and its mRNA encodes a preproprotein consisting of a 22 amino acid signal peptide, a 741 amino acid propeptide and a mature subunit of 2050 amino acids. During synthesis, the VWF subunit assembles into multimers of up to 20 million Da (34, 35). The preproVWF is composed almost entirely of 4 types of domains (A to D), present in 2 to 5 copies each. VWF binds through its A1-domain with GPIb (35, 36) and

through its A3-domain with fibrillar collagens type I and III (37). The A1-domain also contains a binding site for heparin and collagen type VI (38). Within the A2 domain a proteolytic cleavage site between Tyr 842 and Met 843 for the VWF-cleaving protease ADAMTS13 (39) is present, and finally also an RGD sequence is present at position 1744-1746 within the C1-domain, essential for binding to GPIIbIIIa (40).

Under normal physiological conditions, VWF and platelet GPIb do not interact. Only when VWF is bound to the subendothelium and under conditions of high shear stress, an interaction between bound VWF and GPIb is observed. It is believed that VWF undergoes a conformational change when bound. However, this is still a matter of debate as 2 studies using atomic force microscopy obtained apparently conflicting results: Siedlecki *et al.* (41) did demonstrate a major conformational change of VWF when coated an a glass surface and subjected to shear stress, whereas Novak *et al.* (42) could not demonstrate an overall conformational change in collagen bound VWF under high shear stress.

In vitro *role of the VWF-collagen interaction in thrombus formation*

Initially, the role of VWF in platelet adhesion was demonstrated in perfusion studies using an annular perfusion chamber where abnormal adhesion of platelets to subendothelium was found with VWF deficient blood (43-46) and on to collagen fibrils with blood from patients with von Willebrand's disease (VWD) (47). Later, also on cross sections of arteries platelet adhesion was demonstrated with a major platelet deposition on the luminal site of the artery and in the adventitia (48). These data corresponded with the detection of VWF binding sites in the adventitia but not in the media (49). Studies on cross sections of atherosclerotic plaques demonstrated increased platelet deposition in areas where collagen type I and III were enriched although no platelet adhesion was seen at other sites rich in these collagens (48).

In addition, MAbs were developed that inhibit platelet adhesion by interacting with VWF. Although most of these MAbs interfered with the VWF-GPIb axis (50, 51), CLB-Rag-201 inhibited the binding of VWF to purified collagen type I and III but did not inhibit the binding of VWF to collagen type VI containing subendothelium (51). One MAb, CLB-RAg 38 completely inhibited the binding of VWF and platelet adhesion to the subendothelium from human umbilical arteries or to the extracellular matrix of endothelial cells, but not to purified collagens (52). Another MAb, RU5, was shown to inhibit VWF binding to human fibrillar collagens type I and III (53) and was used to determine the collagen binding epitope in the VWF A3-domain (54). Our own group developed MAb 82D6A3 which is directed against the VWF A3-domain and inhibits the binding of VWF to human fibrillar collagens type I and III, but not to human collagen type VI (38). Moreover, it is able to displace VWF already bound to collagen (29). Of all these antibodies only MAb 826A3 was tested *in vivo*.

Studies on the interaction sites in VWF for collagen revealed the presence of different binding sites for collagen. Studies using a VWF mutant with the A3-domain deleted (ΔA3-VWF) proved that the A3-domain is indeed the major binding site for fibrillar collagen type I and III (37). This was confirmed with inhibiting anti-A3-domain VWF MAbs (29, 53), moreover, recently a patient was identified with a S1731T mutation in the A3 domain resulting in a decreased binding to fibrillar collagens type I and III (55). The binding site within the A3-domain was further refined by cocrystalizing the A3-domain with MAb RU5. Based on the position of the epitope of RU5, Ala mutants were constructed and revealed that His1023, which is located close to the bottom of the A3-domain, is essential in collagen binding. The position of this binding site for collagen is different from the one in the I-domain of integrins, where it is situated at the top face of the domain, containing the MIDAS motif (54). However, the A3-domain is not involved in VWF-binding to human collagen type VI under static conditions. Inhibition experiments using the anti-A1 VWF MAb AJvW2 or ATA, demonstrated that VWF binds to collagen type VI and to collagen type VI containing extracellular matrix of endothelial cells through its A1-domain (38).

Other compounds interfering with the VWF-collagen axis by binding to collagen have been identified in the saliva of leeches. Calin, a product isolated from the saliva of the leech *Hirudo medicinalis* binds to collagen and inhibits VWF binding to collagen under both static, low (300 s^{-1}) and high (1300 s^{-1}) shear conditions (56). Calin had an effect on platelet adhesion under shear conditions to equine tendon (Horm) collagen, human collagen type I and III and to extracellular matrices. It also inhibited collagen-induced platelet aggregation and static platelet adhesion to collagen, which are collagen receptor-dependent (26).

Another collagen-binding protein isolated from the saliva of *H. medicinalis*, is saratin (molecular weight of 12000) which almost exclusively interfered with the VWF-collagen interaction. Its recombinant form dose-dependently inhibited the binding of purified human VWF to human type I and III collagens and to calf skin collagen under static conditions with an IC_{50} of 0.23, 0.81 and 0.44 μg/ml respectively. Under conditions of elevated shear rates (2700 s^{-1}), inhibition of platelet adhesion to human collagen type III was observed with an IC_{50} of 0.96 μg/ml. High and low affinity-binding sites for saratin on human collagen type III were demonstrated (27).

A collagen-binding protein with a molecular weight of 16000 was isolated from the salivary glands of *Haementeria officinalis* which inhibits the collagen-induced platelet aggregation (57). The protein was cloned, named rLAPP (leech antiplatelet protein) and further demonstrated to inhibit collagen receptor-mediated platelet adhesion under static conditions with an IC_{50} of 80 nM (58). rLAPP completely inhibits platelet adhesion on collagen type I, III and IV but only partially on collagen

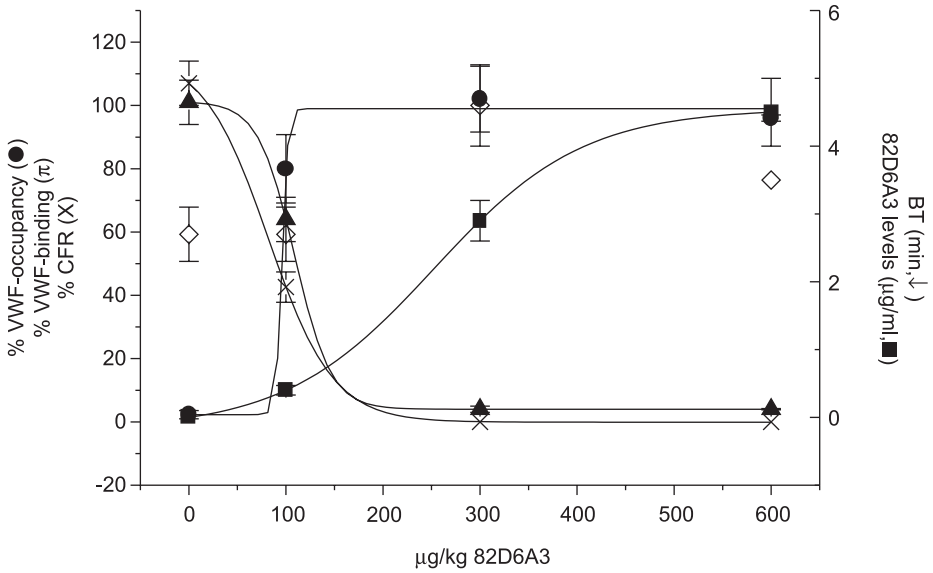

Fig. 1. Study of the antithrombotic effect of 82D6A3 in an arterial thrombosis model in baboons. The % CFR (X) (see text) demonstrates the *in vivo* antithrombotic effect of 82D6A3, data that corresponds well with the *ex vivo* VWF-binding to collagen (π) and VWF occupancy (●). Also bleeding times (↓) and plasma levels (■) are demonstrated (data points obtained 30 min after injection of 82D6A3) (29).

type VI under flow conditions (1600 s^{-1}) (59). In addition, the shear rate-dependent inhibition of platelet adhesion by rLAPP occurred through interference with VWF-binding to collagen (59). The observation that rLAPP blocks both the interaction of the collagen receptor GPIa/IIa and of VWF with collagen was later hypothesized to be due to the blocking of rLAPP of an overlapping binding site for VWF and GPIa/IIa on collagen (60). rLAPP was not only able to prevent VWF-binding to collagen but was also able to displace collagen bound VWF. Finally, rLAPP inhibits platelet deposition to cross-sections of human atherosclerotic coronary arteries, but not to matrices of human umbilical vein endothelial cells (48).

In vivo *role of the VWF-collagen interaction in thrombus formation*

As the role of VWF in platelet adhesion at high shear rate was unequivocally demonstrated, some discussion on the role of the VWF A3 domain interaction with fibrillar collagens type I and III *in vivo* remained. Indeed, as described above, despite the abundance of collagen in the subendothelium and the affinity of VWF for fibrillar collagens it was thought that the major binding site for VWF in the basement membrane might not be collagen as some MAbs (CLB-RAg 38) did not inhibit the VWF-binding to collagen but did so to the extracellular matrix (52) and other MAbs had the opposite effect (50). However, we recently solved this issue by studying the inhibitory effect of the anti-A3 domain VWF MAb 82D6A3 in thrombus formation *in vivo* in a modified Folts model in baboons (29). In this arterial thrombosis model, the femoral artery is damaged, an external stenosis is applied and blood flow is measured. Due to platelet adhesion and aggregation, vessel obstruction occurs which is detected as a decline in blood flow. When the blood flow decreases by at least 50%, it is restored by mechanically dislodging the thrombus after which the process starts all over again. This repetitive pattern of decreasing blood flow after mechanically dislodging the thrombus is referred to as cyclic flow reductions (CFR). Administration of 100, 300 and 600 µg/kg MAb 82D6A3 to the baboons resulted in a clear *in vivo* inhibition of arterial thrombosis. During the 1 hour measurement period, reductions in CFRs of 58.3, 100 and 100% were observed with the respective doses which correlated well with the *ex vivo* inhibition of VWF-binding to collagen and the VWF-occupancy (Fig. 1). Moreover this *ex vivo* effect was observed for up to 5 h after the administration of 300 µg/kg 82D6A3 and for up to 24 h after administration of 600 µg/kg 82D6A3. At the latter time point, 88% of VWF was occupied with antibody, still resulting in an *ex vivo* 55% inhibition of VWF binding to collagen. Next to this potent *in vivo* antithrombotic effect of 82D6A3, no changes in VWF-antigen levels, platelet count or coagulation parameters were observed. Although some prolongation of bleeding time was measured, this never reached statistical significance (Fig. 1). This observation suggests a possible benefit for platelet adhesion inhibitors: a broader therapeutic window is observed which might result in a good antithrombotic effect with less bleeding problems. Indeed, when we used an anti-GPIIb/IIIa MAb in the same animal model, major bleeding time prolongations (> 15 min) were observed with antithrombotic doses (29). As mentioned above, similar minor effects on bleeding time have also been

demonstrated when using antithrombotic compounds that interfere with the VWF-GPIb axis.

The *in vivo* effect of collagen inhibition was tested in a venous thrombosis model in hamsters, where the femoral vein is damaged and thrombus formation is continuously monitored by transillumination. At 0.2 mg/kg, Calin inhibited thrombus formation by 99% with no prolongation of bleeding time. The effect of Calin on both platelet adhesion to collagen under static conditions and on VWF binding to collagen is possibly due to the presence of 2 active compounds in the calin preparation (60).

In vivo, recombinant saratin decreased platelet aggregation and intimal hyperplasia in a rat carotid endarterectomy model (61). Following arteriotomy including removal of the intima and media with microforceps, saratin was administered topically which resulted in a significant decrease in both platelet adhesion and in the development of intimal hyperplasia. No effect on platelet count or tail bleeding time was demonstrated.

rLAPP was tested in a thrombosis model in baboons (28) where a silastic arteriovenous shunt is mounted in the femoral artery containing a thrombogenic surface, here rat tail collagen type I, and the deposition of [^{111}In]-labeled platelets is measured. Unexpectedly, no *in vivo* antithrombotic effect was seen although administration of rLAPP did result in a profound inhibition of *ex vivo* collagen-induced platelet aggregation (28).

Conclusions

Collagens type I and III are exposed after either a type III vascular injury, which uncovers components of the medial layer, or plaque rupture. This kind of damage is induced by PTCA or stenting, but also in coronary artery diseases like unstable angina and myocardial infarction, where plaque rupture leads to the formation of a platelet-rich thrombus eventually resulting in coronary occlusion. The VWF-collagen interaction has been shown to play a dominant role *in vivo* in the formation of arterial platelet-rich thrombi. Hence, inhibiting the interaction of VWF with collagen and targeting to the high shear situation, is expected to be effective in these clinical settings.

As with the GPIIb/IIIa inhibitors, the first indications for VWF-collagen inhibitors would represent conditions in which the risk for acute thrombotic events is high, such as following PTCA or stenting or as adjunctive therapy for thrombolysis, where such inhibitors could both accelerate the restoration of vascular patency and help to preserve it. In addition, if the minor effects that are seen on bleeding times translate into less bleeding problems, additional therapeutic applications can be investigated such as stroke.

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