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REVIEW

Targeting platelet receptor function in thrombus formation: The risk of bleeding

Frauke Swieringa, Marijke J.E. Kuijpers, Johan W.M. Heemskerk *, Paola E.J. van der Meijden

Department of Biochemistry, CARIM, Maastricht University, Maastricht, The Netherlands

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ABSTRACT

In this review, we presume that the process of thrombus formation, as assessed in whole blood flow studies and in experimental (murine) thrombosis studies, reflects the platelet responses in human haemostasis and thrombosis. Following this concept, we give an up-to-date overview of the main platelet receptors and signalling pathways that contribute to thrombus formation and are used as targets in (pre)clinical intervention studies to prevent cardiovascular disease. Discussed are receptors for thrombin, thromboxane, ADP, ATP, prostaglandins, von Willebrand factor, collagen, CLEC-2 ligand, fibrinogen and laminin. Sketched are the consequences of receptor deficiency or blockage for haemostasis and thrombosis in mouse and man. Recording of bleeding due to (congenital) platelet dysfunction or (acquired) antiplatelet treatment occurs according to different protocols, while common laboratory methods are used to determine platelet function.

1. Introduction

Platelets are essential for normal haemostasis by forming a primary plug or thrombus after vascular injury, thus preventing further blood loss. Quantitative or qualitative platelet defects explain a considerable part of spontaneous or induced abnormal bleeding events in the population. Prospective studies suggest that the prevalence of bleeding due to platelet defects is high and comparable to that of von Willebrand disease [1]. Although the most severe platelet disorders are identified at childhood, the majority of patients with milder platelet disorders remain undiagnosed until excessive bleeding occurs after specific challenges, as in surgery or trauma. On the other hand, undesired platelet activation contributes to arterial thrombotic diseases, and antiplatelet medication is the common therapy for secondary prevention, as in cardiovascular disease and stroke. Risk of bleeding is a well-known side effect of this suppression of platelet activation.

The premise of this paper is that the process of thrombus formation, such as assessed in whole blood flow studies and in experimental (murine) thrombosis studies, mirrors the platelet responses that determine haemostasis and thrombosis. In this scenery, we aim to give an up-to-date overview of the main platelet receptors and signalling pathways that contribute to thrombus formation and are used as targets in (pre) clinical intervention studies to attack cardiovascular disease. Since platelet dysfunction and treatment with antiplatelet therapy may both lead to a higher bleeding risk, we also sketch the current views of assessment of normal haemostasis by bleeding scores and discuss current methods to measure platelet function impairment.

2. Platelet receptors, antagonists and thrombus formation

2.1. Thrombin receptors, PAR1, 3 and 4

Thrombin, a short-living proteolytic enzyme generated from prothrombin by coagulation factor Xa, is not only a strong platelet agonist, but also a main effector of the coagulation cascade, inducing fibrin clot formation [2]. Thrombin is generated at phosphatidylserine-exposing membranes from the damaged vessel wall and highly activated platelets [3]. Its formation and inactivation can precisely be measured in platelet-rich plasma or blood by thrombin generation assays [4]. Antithrombin in plasma binds and inactivates thrombin, a process that is enhanced by heparins.

In human platelets, thrombin cleaves and activates the protease-activated receptors (PAR)1 and PAR4. In comparison to PAR4, the former displays a higher affinity to thrombin, transmitting signals at sub-nanomolar thrombin concentrations. Accordingly, PAR1 functions as the key thrombin receptor of human platelets, while PAR4 rather sustains the action of PAR1 [5]. Platelets do not express the factor Xa receptor, PAR2. Both expressed receptors, PAR1 and 4, signal *via* the G-proteins G12/13α and Gqα, which evoke the majority of functional platelet responses (Fig. 1). Current view is that both thrombin receptors only indirectly signal *via* Giα, *i.e.* through ADP secretion and autocrine effects [6]. The PAR-induced activation of G12/13α results in platelet shape change by activation of Rho-associated protein kinase (ROCK) followed by actin cytoskeletal changes, whereas the activation of Gqα

* Corresponding author at: Department of Biochemistry, CARIM, P.O. Box 616, 6200 MD, Maastricht, The Netherlands. Tel.: +31 43 3881671; fax: +31 43 3884159.
E-mail address: jwm.heemskerk@maastrichtuniversity.nl (J.W.M. Heemskerk).

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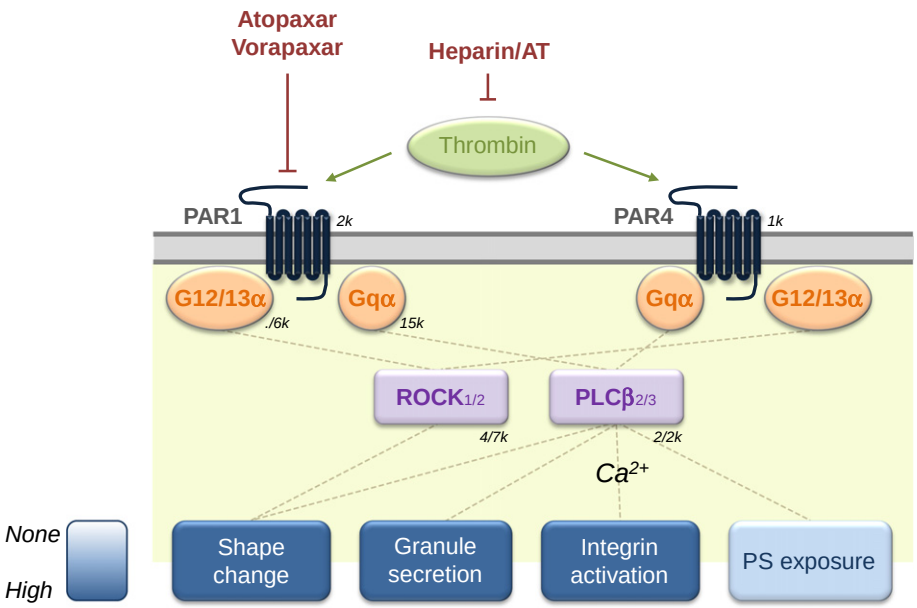

Fig. 1. Signalling and intervention *via* the protease-activated receptors (PARs) for thrombin. Shown are established agonists and receptors on human platelets that are involved in normal haemostasis and are (potential) targets of antithrombotic treatment. Only key signalling proteins are indicated, as far as they associate with the receptors and act as essential molecular switches or second messenger-generating proteins (isoforms in smaller font). Asterisks point to the known presence of dysfunctional mutations in man. Numbers in italic refer to copy numbers (×1000) per platelet, as described in handbooks and proteomic analyses [162,163]. For instance, for G12/13α the indication *./6k* indicates expression levels of 'unknown' (G12α) and ~6000 (G13α) copy numbers per platelet. Dashed lines show networks of signal transmitting proteins linking to the indicated platelet responses (lower boxes). Activation strength of specific responses is represented by a heat map with colour codes from white to blue. Pharmacological inhibitors used in clinic or laboratory are indicated in red, physiological agonists in green. *Abbreviations*: AT, antithrombin; Gq, GTP-binding protein Gq; PLC, phospholipase C; PS, phosphatidylserine; ROCK, Rho-associated protein kinase.

results in Ca^{2+}-dependent integrin $\alpha_{IIb}\beta_3$ activation and secretion *via* the effector enzyme, phospholipase Cβ (PLCβ). Thrombin is a strong platelet agonist, which *via* PAR1/4 evokes maximal shape change, secretion and integrin activation, but thrombin by itself has little effect on platelet procoagulant activity (Fig. 1) [3]. *In vitro*, the various PAR receptors can be activated by specific thrombin receptor-activating peptides (TRAP). Patients with mutations in the genes encoding for PAR receptors have not yet been described, but a patient is reported with platelet Gqα deficiency suffering from mucocutaneous bleeding [7].

Mouse platelets are devoid of PAR1 but express the isoform PAR3, which serves as thrombin-binding co-factor for PAR4 promoting the activity of this receptor [8]. Studies with *Par3*$^{-/-}$ and *Par4*$^{-/-}$ mice revealed marked protection in experimental arterial thrombosis, which was associated with prolonged bleeding times upon challenge [9]. In *Par4*$^{-/-}$ mice, thrombus formation *in vivo* was reduced compared to wildtypes, but knockout platelets showed normal adhesion and normal support of fibrin deposition [10]. These *in vivo* observations suggest a beneficial effect of blocking thrombin receptors in platelets.

In accordance with a key role of PAR1 in human platelet activation, clinical trials have been performed with PAR1 antagonists like Vorapaxar and Atopaxar. Vorapaxar has been evaluated in two phase III clinical trials. The TRACER study did not reveal superiority of Vorapaxar over standard therapy in the primary endpoint, which was a composite of death from cardiovascular causes, myocardial infarction, stroke, recurrent ischemia with rehospitalization, or urgent coronary revascularization [11]. In the TRA-2P TIMI-50 study, where patients with prior stroke were excluded, Vorapaxar was superior to placebo on top of standard care [12,13]. This benefit was at the expense of an increased risk of intracranial bleeding, which was observed in both studies. The other PAR1 antagonist, Atopaxar, has been tested in several phase II trials, showing similar outcomes as Vorapaxar in terms of safety and efficacy [14].

One consideration for the clinical practice, when prescribing PAR1 antagonists in combination with other antiplatelet agents is that, although an extra bleeding risk would be acceptable in comparison to gained antithrombotic protection, the patients need to take even more medication at extra costs, with lower compliance as a side effect [15].

2.2. Thromboxane-prostanoid receptor, TP

The TP receptor (one gene product, previously split into α and β forms) is activated by the fatty acid derivative, thromboxane A_2 [16]. This prostanoid is released from activated platelets as a very unstable metabolite, hence providing a rapid shut-off action mechanism upon stimulation of the TP receptors. Thromboxane A_2 formation requires the release of arachidonic acid from membrane phospholipids, a process catalysed by the Ca^{2+}-dependent cytosolic phospholipase A_2. Arachidonate acts as a substrate for cyclooxygenase 1 (COX1) to produce prostaglandin H_2, which is converted by thromboxane synthase into thromboxane A_2. Signalling *via* the TP receptor takes place *via* G12/13α and Gqα, similarly as for thrombin, but at a lower extent (Fig. 2) [17]. Activation of G12/13α again triggers platelet shape change *via* ROCK activation, while the low activation of Gqα/PLCβ is still sufficient for integrin activation and secretion.

In the laboratory, the stable thromboxane analogue U46619 is used to specifically trigger TP receptors. By itself, U46619 is a weak agonist evoking limited functional responses, but it enhances the effects of other platelet agonists. In agreement with this, collagen-induced platelet activation relies for a considerable extent on the release of thromboxane A_2 and ADP, and ensuing TP and $P2Y_{12}$ receptor activation, respectively [18]. The few patients described with mutations in the thromboxane receptor experience mild bleeding [7]. This agrees with the finding that also in *Tp*$^{-/-}$ mice bleeding times are prolonged [19].

The COX1 complex is irreversibly inhibited by Aspirin and other non-steroid anti-inflammatory drugs (NSAIDs), such as Indomethacin, Diclofenac, Ibuprofen and Naproxen (Fig. 2). The classical test to check for inhibited COX1 activity is measurement of arachidonic acid-induced (*i.e.*, thromboxane-dependent) platelet aggregation. Benefit of Aspirin in the treatment and secondary prevention of cardiovascular disease has clearly been shown in early clinical trials [20,21]. However, Aspirin has side effects experienced by some patients, particularly renal insufficiently, gastrointestinal symptoms and haemorrhagic complications [22]. In accordance with this, also patients with an inherited