Proteinase-Activated Receptors (PARs), Platelets and Angiogenesis

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ABSTRACT

With respect to the role of proteinase-activated receptors (PARs), few cells have been as thoroughly studied as the platelet. PARs appear to act as the key receptors mediating the pro-aggregatory and pro-secretory effects of thrombin, but there is considerable variation from species to species in terms of which PARs are involved in these processes. In addition to contributing to hemostasis, platelets are increasingly being viewed as important contributors to healing and to tumor growth. This can be attributed to the many pro- and anti-angiogenic factors that are stored within platelets and are released as sites of injury and new vessel growth. There is emerging evidence for an important role for PARs in regulating the release of growth factors from platelets, raising the specter that PARs may be a rational target for new therapies that will modulate repair processes and tumour growth. Drug Dev. Res. 59:395–399, 2003. © 2003 Wiley-Liss, Inc.

Key words: protease-activated receptors; thrombin; wound healing; endostatin; vascular endothelial growth factor

INTRODUCTION

Platelets are lentil-shaped, anucleated fragments of megakaryocytes. Their organelles and subcellular compartments are inherited during the fragmentation process. There are three major types of granules in platelets, which are classified according to their ultrastructure, density, and content. Alpha-granules are the most abundant secretory granules, as well as the largest (200–500 nm) [Sixma et al., 1989; Harrison et al., 1990]. They contain cationic proteins, coagulation factors, adhesion molecules (P selectin, von Willebrand factor), growth factors, cytokines, and glycoproteins [Reed et al., 2000]. Alpha granules also contain molecules that are synthesized by other cells and are taken up into granules through endocytosis (e.g., fibrinogen) [Handagama et al., 1987, 1993; Harrison et al., 1989]. Dense granules contain high concentrations of small molecules such as adenosine diphosphate (ADP), adenosine triphosphate (ATP), calcium, magnesium, and serotonin that give these vesicles an electron-opaque ultrastructural appearance [King and Reed, 2002]. There are typically less than 10 dense granules per platelet in both humans and mice [White, 1969; Reddington et al., 1987]. The third type of granule, lysosomes, stores acid hydrolases.

Traffic of granule components appears to occur through membrane systems within the platelet. For example, the alpha granule membrane protein, P-selectin, can also be found on dense granule membranes [Israels et al., 1992; Youssefian et al., 1997] and the plasma membrane glycoproteins, Ib and IIb–IIIa, can be detected in both alpha granules and dense granules [Youssefian et al., 1997]. The main calcium reservoir is the dense tubular system. Despite the small

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size and limited capacity for synthesis, platelet function is controlled through complex mechanisms.

Resting platelets do not adhere to normal vascular endothelium. Areas of disrupted epithelium provide binding sites for adherence and activation. Once adherent to the endothelium, secretion of stored platelet constituents occurs and large platelet aggregates form a platelet plug. Additionally, platelet membrane sites become available for adsorption and concentration of coagulation factors, and plasma coagulation is then accelerated, forming a fibrin network that will reinforce the friable platelet plug.

**PROTEINASE-ACTIVATED RECEPTORS**

Platelet membranes express a number of receptors, the activation of which by various agonists regulates adhesion, aggregation, and secretion. Thrombin, the proteolytic enzyme that is a powerful agonist, acts at least in part through cleavage of proteinase-activated receptors (PARs). PARs represent a distinct subclass of G-protein-coupled receptors, the activation of which by a proteinase involves cleavage at a specific enzymatic site in the extracellular NH₂-terminus. This results in exposure of a new N-terminal domain that acts as a “tethered ligand,” binding and activating the receptor [Vergnolle et al., 2001]. Four PARs have been cloned to date. PAR₁, PAR₃, and PAR₄ can be activated by thrombin, while PAR₂ can be activated by trypsin or human mast cell tryptase. PARs can also be activated by small peptides that have structural homology with the tethered ligands that are revealed by proteolytic cleavage of the N-terminus. The “PAR-activating peptides” (PAR-AP) are very useful pharmacological tools for gaining an understanding of the physiological and pathophysiological roles of PARs (see Hollenberg, this Special Issue of Drug Development Research 59(4):336).

In terms of the regulation of platelet function, there is considerable interspecies variability with respect to the particular PARs that are involved. For example, human platelets express PAR₁ and PAR₄, and activation of either receptor by selective PAR-APs is sufficient to trigger aggregation and secretion [Vu et al., 1991; Kahn et al., 1999; Coughlin, 2000]. In contrast, mouse platelets do not express PAR₁, but their activation can be achieved through activation of PAR₄. Rat platelets express PAR₃ and PAR₄, while PAR₁ does not appear to be expressed [Hollenberg and Saiffedine, 2001]. PAR₁-APs can induce platelet aggregation and secretion in the rat, and thrombin-induced aggregation and secretion can be blocked by a selective PAR₄ antagonist [Hollenberg and Saiffedine, 2001; Ma et al., 2001]. Recent data suggest that an atypical PAR, possibly a PAR₁ subtype, is present on rat platelets and has the ability to inhibit aggregation when stimulated with a PAR₁ agonist [Ruef et al., 2000].

Is thrombin-induced aggregation of human platelets mediated via PARs? As mentioned above, activation of PAR₁ with PAR₁-APs can cause complete aggregation of human platelets. Like the aggregation induced by thrombin (Fig. 1), PAR₁-AP-induced aggregation is entirely dependent upon ADP release, as it is abolished by apyrase (an ADP scavenger) [Chung et al., 2002]. Subthreshold concentrations of PAR₁-AP can potentiate the aggregating effects of a PAR₄-AP [Chung et al., 2002], thus fitting with the model of thrombin activating platelets via a dual-PAR system [Coughlin, 2000]. Inhibition of PAR₁ activation, by an antagonist, immunoneutralization, or through receptor desensitization, markedly inhibits platelet aggregation at low, but not high concentrations of thrombin [Kahn et al., 1999]. Conversely, immunoneutralization of PAR₁ had no effect on platelet activation by thrombin, but when combined with PAR₁ blockade, even high doses of thrombin were unable to trigger aggregation. These findings suggest that PAR₁ mediates human platelet activation at low thrombin concentra-
tions, while high concentrations of thrombin are required to activate platelets via PAR4. However, PAR4 is not just a “back-up” system for PAR1. Cathepsin G, released by activated neutrophils, seems to play a role in platelet activation through PAR4 [Sambrano et al., 2000]. Furthermore, thrombin-induced calcium signaling elicited by PAR4 appears to have a different kinetic profile than that for PAR1. PAR4 activation is slowly activated and deactivated, while PAR1 activation is rapid and transient. This disparity is probably due to differences in receptor phosphorylation. Together, the kinetics of the responses to PAR1 and PAR4 agonists mimic the response observed when platelets are stimulated with thrombin [Shapiro et al., 2000].

Despite interspecies variation, mouse models have proven to be useful tools to investigate the role of PARs and thrombin signaling on platelet activation. In the mouse, however, PAR3 and PAR4 act coordinately as thrombin receptors on platelets. PAR3 itself does not mediate transmembrane signaling; rather, it functions as a co-factor for the cleavage and activation of PAR4 by thrombin [Coughlin, 2000].

**PLATELETS AND ANGIogenesis**

Angiogenesis is a pivotal process in all types of wound healing, and also plays a crucial role in tumour growth. It is modulated by both pro- and anti-angiogenic factors. Pro-angiogenic factors include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), platelet-derived endothelial growth factor (PDEGF), and epidermal growth factor (EGF). Anti-angiogenic factors include angio- statin and endostatin [Dhanabal et al., 1999a,b; Distler et al., 2002].

Platelet aggregation is a cardinal feature of vascular repair. A variety of potent angiogenic stimulators, including VEGF [Maloney et al., 1998], PDEGF [Miyazono et al., 1989], EGF [Hwang et al., 1992], and PDGF [Linder et al., 1979], are stored in platelets and released at the site of injury during the clotting process. Activation of platelets by α-thrombin [Fenton et al., 1991] stimulates angiogenesis in the chick chorioallantoic membrane [Tsopanoglou et al., 1993]. Platelets have also been shown to stimulate endothelial cell proliferation and capillary-like formation in vitro [Pipili-Synetos et al., 1998]. Of the many pro-angiogenic factors, VEGF is the most potent [Szabo and Vincze, 2000].

Endostatin appears to be among the most potent inhibitors of angiogenesis [O’Reilly et al., 1997]. Factors that influence the content of pro- vs. anti-angiogenic factors of platelets, or the release of these factors from platelets, have the potential, therefore, to profoundly affect angiogenesis and healing. This has been demonstrated recently in an animal model of gastric ulcer healing.

Platelets make an important contribution to ulcer healing, over-and-above their role in hemostasis. Thrombocytopenic rats exhibit retarded ulcer healing compared to healthy rats [Ma et al., 2001]. This delayed ulcer healing could be reversed by transfusion of platelets from a healthy rat [Ma et al., 2001]. Treatment of rats for one week with ticlopidine, an ADP receptor antagonist, resulted in a marked increase in platelet (and serum) levels of endostatin, but did not affect platelet VEGF levels. This effect was due to actions of ticlopidine at the level of the megakaryocyte. Treatment with ticlopidine also resulted in a marked delay in gastric ulcer healing, which occurred in a platelet-dependent manner. It was the elevated levels of platelet and serum endostatin that appeared to be responsible to the delayed ulcer healing. Thus, we proposed that the platelet acts as a delivery system for growth factors to sites of injury [Ma et al., 2001].

Endostatin release from platelets may be regulated by PARs. As shown in Figure 2, stimulation of
platelets with ADP or thrombin, at concentrations that induced aggregation, did not cause significant VEGF release in either group (vehicle or ticlopidine). Indeed, ADP-induced VEGF release was significantly reduced in the ticlopidine-treated group (Fig. 2), which is not unexpected given that ticlopidine is an ADP receptor antagonist. ADP stimulation did not cause significant release of endostatin from platelets of vehicle- or ticlopidine-treated rats. In contrast, stimulation with thrombin caused a marked increase in release of endostatin in the vehicle- and ticlopidine-treated groups (Fig. 2). While apyrase (ADP scavenger) could inhibit thrombin-induced aggregation, it did not influence endostatin release (Fig. 1). Moreover, as shown in Figure 1, endostatin release was triggered by thrombin at concentrations below the threshold for the induction of aggregation. Thus, release of endostatin occurred independently of the aggregation process. The site of storage of endostatin within platelets is not yet clear, although the fact that it can be released independent of VEGF release suggests that it is not contained within the alpha granules (where VEGF is stored).

Studies were performed in rats to determine if thrombin-induced endostatin release from platelets was mediated via effects on one or more PARs [Ma et al., 2001]. As shown in Figure 3, thrombin or the selective PAR4-AP (AYPGK-NH$_2$) caused significant release of endostatin. The release stimulated by either of these agonists was completely blocked by co-exposure of the platelets to a selective PAR4 antagonist (trans-cinnamoyl-YPGKF-NH$_2$). Thus, endostatin release from rat platelets appears to regulated via PAR4, and can occur independently of aggregation, and in an ADP-independent manner.

**SUMMARY**

The role of PARs in regulating platelet function remains only partially understood. There is no question that PARs mediate most of the actions of thrombin on platelet function, but there are very marked interspecies differences. What is also becoming clear is that PARs can regulate more than just aggregation. It would appear that PARs can selectively stimulate the release of some granular contents from platelets, and, in doing so, can modulate processes such as wound healing. Indeed, there is clear evidence from studies of rat platelets suggesting that PAR4 can cause selective release of endostatin, a powerful anti-angiogenic factor. Further studies are required to better characterize the roles of various PARs in regulating platelet secretion. Such studies will be greatly aided by availability of selective antagonists of the various PARs.

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