SHORT COMMUNICATION

Specific activation, signalling and secretion profiles of human platelets following PAR-1 and PAR-4 stimulation

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Abstract

Blood platelets play a central haemostatic function; however, they also play a role in inflammation and are capable of secreting various cytokines, chemokines and related products. The purpose of this study was to identify subtle variations in platelet physiology using proteomics. We compared the levels of membrane proteins \( n = 3 \), \( \alpha \) and \( \delta \) granule proteins \( n = 18 \), and signalling proteins \( n = 30 \) from unstimulated platelets with those of protease-activated receptor (PAR)-1- and PAR-4-stimulated platelets \( n = 10 \). The vast majority of these proteins responded similarly to PAR-1 or PAR-4 engagement. However, differences were observed within membrane CD40L expressed, and \( \alpha \) granule GRO-\( \alpha \) and MDC secreted proteins.

Introduction

While essential to primary haemostasis, platelets play an important role in innate immunity and are important for pathogen and danger recognition; they are also involved in inflammatory processes and assist with adaptive immune responses [1, 2]. To participate in these processes, platelets display a range of receptor molecules such as Toll-like receptors, complement receptors, Ig receptors, semaphorins and siglec molecules [2–4]. Protease-activated receptors (PARs) modulate tissue inflammation and healing processes in altering on a variety of immune cells. Data obtained in PAR-deficient mice suggest that PAR is a cofactor-regulating immune function. The direct effect of PAR activation on immune cells appears to be proinflammatory in nature with a potential role in the innate immunity modulation [5]. Van Holten et al. described that the most abundant alpha-granule proteins are released in similar quantities from platelets after stimulation with either PAR-1 or PAR-4 [6]. Here, we investigated whether the platelet PAR-1 and PAR-4, which are major haemostatic function receptors [1, 6], function as innate immunity receptors. Evidence for the differential release of cytokines, chemokines and related products following PAR-1 and PAR-4 engagement has come from a study where a small number of target proteins were investigated [7]. In the present proteomics investigation, we detected a large \( (n = 51) \) number of factors shed from platelet membranes, or secreted from \( \alpha \) and \( \delta \) granules after PAR-1 or PAR-4 stimulation.

Methods

Peripheral blood samples from healthy subjects \( n = 10 \) were collected in endotoxin-free tubes containing 3.2% sodium citrate (Vacutainer®, Becton Dickinson, San Jose, CA) [8]. Informed consent was obtained from the blood donors prior to blood collection by the Regional Blood Service, according to current French legislation. Blood samples were centrifuged at 192 \( \times \) 10 minutes at room temperature to obtain platelet-rich plasma. Platelet suspensions were incubated with the appropriate monoclonal antibody for 30 minutes at room temperature in the dark, and washed once with phosphate-buffered saline (1 \( \times \)). Because all the platelets constitutively express CD41a, this marker (conjugated to fluorescein isothiocyanate) was used to define gates for the subsequent flow cytometry experiments. Activated platelets are characterized by their expression of CD40L, CD62P and CD63 (listed in the “Supplemental Material” section), among other markers. Allophycocyanin- or phycoerythrin-conjugated monoclonal antibodies against human CD40L, CD62P and CD63 were then used to define the cell population gates. Flow cytometry was performed using a FACSVantage SE device equipped with CellQuestS-Pro software (BD Biosciences, Le Pont de Claix, France) [8]. The \( \alpha \) granule protein (listed in the “Supplemental Material” section) content of platelet supernatants was quantified using Luminex® technology with the following panel of human cytokine/chemokine magnetic beads: HCYTOMAG-60K, HCYP2MAG-62K, HCYP3MAG-63K (Millipore, Molsheim, France). A canonical \( \delta \) granule molecule, i.e. serotonin (see “Supplemental Material” section) was quantified by an enzyme-linked immunosorbent assay – ELISA (IBL International, Hamburg, Germany). Absorbance at 450 nm (or 405 nm for serotonin) was measured using an ELISA plate reader (Magellan Software, Sunrise TM, Tecan Group Ltd.,

Keywords

Immunologic factors, inflammation, platelet, protease-activated receptors, signal transduction

History

Received 13 April 2015
Revised 4 May 2015
Accepted 8 May 2015
Published online 17 June 2015
Lyon, France). Results were normalized to 10⁹ platelets/ml [8]. For each condition, intracellular proteins from the platelets were extracted using a MILLIPLEX MAP EpiQuant Sample Preparation kit at 3 × 10⁷ platelets/ml of lysis buffer, according to the manufacturer’s instructions. The quantification of phosphorylated proteins was performed using MILLIPLEX MAP EpiQuant technology with the five following panels: MPEQMAG-100K, 102K, 103K, 104K and 110K (Millipore; see “Supplemental Material” section). The results were expressed in pM per 3 × 10⁷ platelets/ml. Platelet responsiveness tests were performed with PAR-1 (SFLLRN, 6 μM) or PAR-4 (AYPGKF, 200 μM) agonists (Sigma-Aldrich, Saint Quentin-Fallavier, France).

All the statistical analyses were done with Prism Software (GraphPad Software, San Diego, CA). We set the non-activated sample 100% and calculated the difference resulting from agonist-induced activation. Samples were compared with non-parametric Wilcoxon tests (n = 10); a p value < 0.05 was considered statistically significant. A correlation between two variables was assessed by Pearson’s coefficient, and p values were calculated to test the null hypothesis that Pearson’s coefficient was not significantly different from zero. Correlations were considered significantly different from zero when p values were < 0.05 with α = 0.05 risk.

Results

The optimal agonist concentration for stimulation of PAR-1 or PAR-4 was predetermined by examining CD62P expression in platelets (data not shown). Among the 51 proteins quantified (Table S1), 11 were released from or expressed by platelets in a comparable range after stimulation with either of the PAR-1 (or PAR-4 agonists (R² correlation of 0.9609, Figure 1, panel A).

PAR-1- or PAR-4-stimulation of platelets was followed by a significant release of the following α granule proteins: RANTES (Figure 1, panel B), GRO-α (Figure 1, panel C), and d granules, intracellular signalling and activation membrane marker profiles following PAR-1- or PAR-4-specific platelet stimulation.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** α and δ granules, intracellular signalling and activation membrane marker profiles following PAR-1- or PAR-4-specific platelet stimulation. (A) Correlation between the protein ratios obtained for the significantly modulated proteins of interest upon PAR-1- and PAR-4-platelet stimulation (R² = 0.9609). Flow cytometric investigation of platelet proteins and/or Luminex® bioassays of α granule the constituents from 10 individuals: (B) RANTES, (C) GRO-α, (D) sCD40L, (E) MDC and (F) sCD62P; (G) IFN-γ; and (H) δ granule (serotonin). Intracellular proteins: (I) SyK (pY629), (J) IKKα and (K) Axl (pY859). Membrane markers: (L) CD63, (M) CD62P and (N) CD40L after stimulation with PAR-1 (SFLLRN, 6 μM) and PAR-4 (AYPGKF, 200 μM)*. We set the non-activated sample 100% and calculated the difference resulting from agonist induced activation. Samples were compared with non-parametric Wilcoxon tests (n = 10), a p value < 0.05 was considered statistically significant. Changes in the platelet proteins of interest are shown following specific PAR-1 or PAR-4 stimulation (controls: resting, unstimulated platelets). Blood donations, n = 10.
sCD40L (Figure 1, panel D), MDC (Figure 1, panel E), sCD62P (Figure 1, panel F) and IFN-γ (Figure 1, panel G). Additionally, the δ granule compartment was represented by serotonin (Figure 1, panel H). Similar results were observed after activation of intracellular and signalling proteins: SyK (pY629) (Figure 1, panel I), IKKa (Figure 1, panel J) and phosphorylated Axl (pY859) (Figure 1, panel K); platelet surface membrane markers CD63 (Figure 1, panel L), CD62P (Figure 1, panel M) and CD40L (Figure 1, panel N) moved to increase (MFI). Next, we examined intracellular, membrane and secreted proteins from the platelets of 10 healthy individuals after stimulation with PAR-1 (SFLRN, 6 μM) and PAR-4 (AYPGKF, 200 μM). PAR-1 mediates activation of human platelets at low thrombin concentrations. In contrast, PAR-4 mediates platelet activation only at high thrombin concentrations, according to what is usually described [9, 10].

Several studies have shown a critical role for the immune receptors SyK [11], Axl [12] and IkappaB kinase [13] in platelet activation following treatment with a PAR-4 agonist; hence, we sought to examine whether PAR-1 and PAR-4 stimulation could mobilize these signalling pathway targets in a similar manner. As shown here, SyK (pY629) (Figure 1, panel I), IKKa (Figure 1, panel J) and phosphorylated Axl (pY859) (Figure 1, panel K) could be similarly mobilized by PAR-1 and PAR-4 engagement, as were the membrane proteins [CD63 (Figure 1, panel L) and CD62P (Figure 1, panel M)], and δ proteins (serotonin (Figure 1, panel H) and some α proteins [RANTES (Figure 1, panel B), sCD40L (Figure 1, panel D), sCD62P (Figure 1, panel F) and IFN-γ (Figure 1, panel G)].

However, membrane expression of CD40L (Figure 1, panel N) and GRO-α (Figure 1, panel C) and MDC (Figure 1, panel E) secretion differed, significantly following PAR-1 or PAR-4 engagement with the reference peptides SFLRN and AYPGKF, respectively. PAR-4 stimulation mobilized CD40L membrane expression in preference to PAR-1 and, conversely, PAR-1 stimulation mobilized GRO-α and MDC release in preference to PAR-4 at different doses of SFLRN and AYPGKF (data not shown).

**Discussion**

Using thrombin receptor PAR-1 (SFLRN) and PAR-4 (AYPGKF) activating peptides, Van Holten et al. measured protein release by platelets using an original approach (i.e. use of mass spectrometry with angiogenic factor proteins, growth factors, b-thromboglobulin, platelet-factor-4, thrombospondin, PDGF-A/B, RANTES/CCL5, endostatin, CXCL12 and VEGF) [6]. The authors concluded that PAR-1 and PAR-4 signalling did not differ in terms of their ability to mobilize cytokine and related factors [6]. Although this was a quite large proteomics study, it was performed using only three samples of normal human platelets [6]. The conclusions presented by Van Holten et al. were surprising considering the popularity of previous publications on inflammation and platelet release of cytokines, chemokines and similar molecules (collectively termed Biological Response Modifiers, BRMs), and the general behaviour of platelets upon stimulation, especially upon haemostatic stimulation. Here, we investigated whether the platelet PAR-1 and PAR-4 play a major role concerning the modulation of platelet function as innate immunity receptors. Indeed, distinct signals engage differential activation and signalling pathways to induce secretion of haemostatic and BRM factor profiles [14–16]; our own conclusions differ from Van Holten et al. [6], as we propose that platelets sense differentially PAR stimuli as they sense differentially microbial and other danger signals, adjusting response to stimulus variation [14, 17–19].

Some researchers have postulated that pro- and anti-angiogenic factors are present in distinct α granules and that stimulation of platelets with a PAR-1-agonist (or adenosine diphosphate – ADP) or PAR-4-agonist (or TXA2) results in the selective secretion of pro-angiogenic factor (VEGF) or anti-angiogenic factor (endostatin), respectively [1, 20]. It has even been proposed that factors with opposing functions are stored in distinct α granule sub-organizations, and that platelet secretion is contextually thematic in response to specific agonists [7, 21]. However, these studies differ in terms of the agonist types, agonist concentrations, duration of platelet stimulation and readouts (markers tested). Despite overall evidence that the vast majority of proteins (or at least those tested to date) seem to be similarly produced in response to PAR-1 or PAR-4 engagement, our findings provide evidence that even differences slight stimuli, lead to differences in membrane expression (CD40L) or BRM release (GRO-α and MDC). Our findings support the hypothesis that PAR-1 and PAR-4 platelet stimulation trigger differential protein mobilization; however, further studies exploring physiological conditions are needed to draw firmer conclusions about this. Indeed, this conjecture is made irrespective of the compensation mechanisms that can be hypothesized regarding platelet physiology at the intracellular level. While all the studies conducted thus far have investigated normal platelet responses to thrombin peptide stimuli, none have investigated the additional alterations that might occur in the context of viral, parasite or bacterial infections, all of which have been consistently shown to interfere with haemostasis [2].

**Acknowledgements**

We thank Drs Catherine Argaud, Alain Lefebvre and Patricia Chavarin, and Ms Marie-Ange Eyraud and Mr Charles-Antoine Arthaud (EFS Auvergne-Loire, France) for their help in obtaining and preparing the human blood cells.

**Declaration of interest**

The authors declare no competing financial interests and no conflicts of interest regarding this study. This study was supported by the Etablissement Français du Sang – EFS, the French National Agency for Drug Safety and Health Products (ANSM – AAP-2012-011), the “Amis de Rémi” Association, and Agence Nationale de la Recherche (ANR), reference ANR-12-JSV1-0012-01.

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Supplementary material available online.