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Quantitative proteomics analysis of platelet-derived microparticles reveals distinct protein signatures when stimulated by different physiological agonists

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ABSTRACT

Platelet-derived MPs (PMPs) are a heterogeneous population of microvesicles released from platelets upon activation and apoptosis. Different platelet activations may affect PMP protein profiles and roles in intercellular communication. Here, we performed a quantitative proteomics study to characterize the protein content of PMPs generated by four differentially activated platelet samples. We selected known physiological agonists for platelet activation such as ADP, thrombin and collagen. Thrombin, which is mostly used to generate PMPs *in vitro*, was set as control. Platelets were activated by following a known agonist strength scale in which ADP was the weakest activation and thrombin and collagen stimulations were the strongest ones. Our proteomic analysis allowed the quantification of 3383 proteins, of which 428 membrane and 131 soluble proteins were found as significantly different in at least one of the analyzed conditions. Activation with stronger agonists led to the enrichment of proteins related to platelet activation in PMPs. In addition, proteins involved in platelet degranulation and proteins from the electron transport chain were less abundant in PMPs when stronger activation was used. Collectively, our data describe the most detailed characterization of PMPs after platelet physiological activation. Furthermore, we show that PMP protein content is highly dependent on the type of physiological agonist involved in platelet stimulation.

Biological significance
Platelet-derived MPs (PMPs) are a population of vesicles generated upon platelet activation by various stimuli known to be involved in several physiological and pathological processes. This manuscript investigates the protein profile of PMPs obtained by performing four different activation protocols using mass spectrometry-based quantitative proteomics. By following a known physiological agonist strength scale our findings suggest a biological link between agonist strength and proteins associated to platelet mediated processes such

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as activation and degranulation. These data may provide new insights for understanding PMP biological role and formation.

1. Introduction

Platelet-derived MPs (PMPs), first identified by Wolf in 1967 as "platelet dust" [1], are submicrometer wide (0.1–1 μm) membrane vesicles, resulting from the reorganization of the platelet membrane in response to several conditions such as platelet activation and apoptosis [2]. In addition to microparticles (MPs), platelets are known to generate exosomes from the exocytosis of multivesicular bodies (MVBs) and alpha-granules [3]. MPs from different cell types can be found in human plasma, and PMPs represent the major population of circulating MPs [4,5]. It was recently shown that megakaryocytes, progenitor of platelets, also release MPs [6]. PMPs are crucial for several physiological functions, including regulation of hemostasis, and their importance has been demonstrated by the bleeding disposition with a diminished formation of PMPs in patients with Castaman disease [7]. Changes in the MP population have been detected in many pathological disorders such as cardiovascular (e.g. coronary heart disease and acute coronary syndrome [8]), infections (e.g. sepsis [9]), autoimmune diseases (e.g. multiple sclerosis [10] and rheumatoid arthritis [11]) and cancer (e.g. gastric and breast cancer [12,13]). However, little information is available about the mechanism of MP formation, in particular regarding the biological process that controls the distribution of proteins between the MPs that are being released and the remaining platelet [14]. During activation and apoptosis, signaling starts from specific receptors followed by common vesiculation processes such as Ca^{2+} entry, cytoskeletal remodeling, calpain/caspase activity and mitochondrial depolarization [15]. However, PMP formation-related events, such as phosphatidylserine (PS) exposure and precoagulant activity, are different in the case of activation and apoptosis. [16]. Platelet activation can be performed by both biochemical and mechanical stimuli. Disruption of the contact between platelet glycoprotein Ib beta chain and cytoskeleton is a key event in mechanical platelet stimulation [17] as well as integrin α_{IIb}-β_3 receptor activity in biochemical activation [18]. In particular, platelet activation promoted by biochemical agonist is also called sustained calcium induced platelet morphology (SCIP), due to the role of intracellular Ca^{2+} concentration for the MP generation.

Currently, the most common approaches to inspect MPs are flow cytometry, imaging by microscopy, ELISA and mass spectrometry (MS)-based proteomics [19]. The high mass accuracy, high speed and the possibility to perform high throughput analyses have made MS-based proteomics the preferred strategy to analyze complex protein mixtures in general [20–22]. This is also due to recent developments in the proteomics workflow, including sample preparation, liquid chromatography (LC) and bioinformatics tools for data processing. Recently, a shotgun proteomics approach was successfully applied to investigate the proteomic profile of ADP induced MPs [23]. By using gel filtration chromatography Dean et al. separated PMPs into four size classes ranging from greater than 500 nm to approximately 100 nm and analyzed each fraction by LC-MS/MS showing that PMP size classes contain proteins of different subcellular origin with mitochondrial proteins highly represented in the largest MPs and α-granule proteins predominating in the smallest fraction [24]. By using flow cytometry analysis Pérez-Pujol et al. revealed that the PMP composition is highly dependent on the activation mechanism, showing substantial differences between thrombin receptor activated peptide (TRAP) and calcium ionophore-stimulated MPs [25]. Comparative proteomics was also performed to characterize PMPs generated in response to biomechanical (high shear) and biochemical (thrombin) stimulations, revealing 26 differentially expressed proteins, 21 of which were part of a common network related to cell assembly, organization and cell morphology [26]. Recently, Aatonen et al. studied the protein content of extracellular vesicles generated from differentially activated platelet samples using both physiological and not-physiological agonist leading the identification of only 267 proteins in the MPs [27]. However, a comprehensive and detailed comparison between PMPs protein content, composition and abundance after platelet stimulation with different physiological agonists has not yet been performed.

In this work, we used MS-based quantitative proteomics to investigate changes in protein content and abundance in PMPs generated by differentially activated platelets. Two studies investigated the physiological strength of different agonists for platelet activation; i.e. C5b-9 > thrombin and collagen > thrombin > collagen > ADP > epinephrine [27,28]. Therefore, we selected for platelet stimulation ADP, collagen, thrombin and collagen/thrombin, where thrombin was used as control due to its frequent use to generate MPs *in vitro* [26]. Moreover, thrombin plays a key role in myocardial infarction [29], ischemic stroke [30] and peripheral arterial vascular disease [31]. Understanding how platelet interacts with thrombin has raised great interest also for the development of anti-platelet drugs [32]. In addition, we tested the action of thrombin and collagen together, in order to study a stronger platelet activation effect as compared to individual agonists [33]. Results highlighted a sustained increase of cytosolic Ca^{2+} concentration during thrombin and collagen co-stimulation [34]. After clustering and protein–protein interaction studies of the quantified proteins we found biological links between agonist strength and PMP composition such as platelet activation and degranulation proteins, energy related proteins important for the electron transport chain, glycolysis, pentose phosphate pathway and the proteasome.

2. Materials and methods

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise. Ultrapure water was from an ELGA Purelab Ultra water system (Bucks, UK).

2.1. Platelet isolation and MP generation

PMPs were obtained from fresh apheresis platelets provided by three healthy volunteers in accordance with the guidelines