Glycoproteins of GpIb α and GpIIb/IIIa on the Synthetic or Naturally Occurred Platelet-Derived Microparticles

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Abstract Infusible platelet membranes (IPM) prepared from new or outdated human platelets have been developed as an alternative to standard platelet concentrates (PCs), with the additional advantage of long shelf life and increased viral safety. Lack of gpIIb/IIIa has been reported as one of the properties of the IPM microparticles. In re-examining this issue, we studied the molecules of gpIIb/IIIa and gpIb α on the surface of IPM microparticles. These molecules could better evaluate the functional efficacy of these microparticles. In comparison, we also surveyed the expression of these molecules on the surface of the naturally occurred platelet-derived microparticles during 7 days storage of PC. Unlike the previous reports, the results of this study illustrated that the produced IPM retained gpIIb/IIIa molecules. Besides, gpIb α and gpIIb/IIIa were also present on the nPMPs and their levels were significantly decreased during 7 days storage of PCs (P value = 0.001).

Keywords Platelet microparticles · GpIb α · GpIIb/IIIa

Introduction

Platelet microparticles are formed spontaneously during the storage of platelets and have been found in platelet concentrates, fresh-frozen plasma, and cryoprecipitate. Platelet microparticles, like intact platelets, possess procoagulant activity; adhere to the vascular subendothelium; and

enhance platelet adhesion [1–3]. The level of these microvesicles has been studied in different pathological conditions. The relation between the deficiency of platelet microvesicle generation and bleeding disorders has been reported [4, 5]. Elevated levels of microparticles have also been described in different diseases like allergic [6] and autoimmune [7, 8] diseases. Because the naturally occurred platelet microparticles (nPMPs) possess properties similar to those of intact platelets, it appeared logical for investigators to examine their potential as a possible platelet substitute. The microparticles can be produced synthetically. A microparticulate human platelet preparation has been developed by Cypress Bioscience, San Diego, California. This company's infusible platelet membranes (IPM) are prepared from outdated platelet concentrates and have been shown to contain various procoagulant phospholipids. IPMs have been shown in vivo to shorten the prolonged ear bleeding time in thrombocytopenic rabbits for at least six hours post-infusion. However, by 24 h the hemostatic activity is no longer detectable [9–12].

Alternatively, nPMPs can express and transfer functional receptors from platelet membranes. More than 40 glycoproteins (GPs) are known to be present on the platelet surface. Many are routinely identified on nPMP, chiefly GP IIb/IIIa and GP Ib since they are widely used as markers to identify nPMP as such (platelet-derived). GP IIb/IIIa is modified on activated platelets to a form facilitating fibrinogen binding, and certain mAbs react preferentially with the activation-dependent epitopes. It was recently shown that nPMP can bind fibrinogen and participate in thrombus formation [13]. Additionally, the influence of nPMPs on the angiogenic activity of endothelial progenitor cells was recently shown [14].

Considering a little knowledge about the inducible platelet membranes, in this study, the expression of surface

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glycoproteins of these vesicles were re-assessed to distinguish more about the antigenic features of IPM. Additionally, nPMP were also considered in this regard to investigate the levels of these glycoproteins on nPMPs during storage for 7 days.

Materials and Methods

Preparation of the Samples

Thirty-five units of platelet concentrates (2–4 days old) prepared by the Iranian Blood Transfusion Organization were used to produce IPM after informed consent for the following studies was obtained from donors. Each time five units of platelet concentrates were used for the production of IPM. Preparation of the IPM microparticles was accomplished as mentioned before [15]. Briefly, the cells were lysed by three times by freezing and thawing. The platelets were then virally inactivated by wet heat at 60 °C for 20 h. Heating was carried out in the presence of different concentrations of sodium octanoate (0–0.6 M) as a stabilizer. Disrupted platelets were sonicated for 5 min with the Hielscher-Ultrasound Technology Sonicator (Hielscher, Teltow, Germany) at amplitude 80 % and cycle 0.5. IPM was separated using centrifugation and elimination of platelet larger fragments. nPMPs were also prepared from 30 PC units as we have illustrated in our previous report [16].

Monitoring the Expression of gpIb α (CD42) and gp IIb/IIIa (CD41) on the IPM

3 μ l of mouse anti-human gpIb α (0.1 mg/ml, Gentaur Europe BVBA, Brussels, Belgium) or mouse anti-human CD41 (Chemicon International) were added to different tubes each included 100 μ l of 500 μ g/ml liquid-stored suspension of IPMs prepared in different concentrations of the stabilizer. The tubes were left 25 min at RT. Washing of the IPM and addition of 2 μ l of the FITC-conjugated goat anti-mouse IgG (Fab') 2 (Dako, Denmark) was then carried out. Again 20 min incubation at RT was chosen.

For all the experiments, nonspecific antibody background binding was determined with the FITC-labeled IgG1 mouse isotypic control. Additionally for each tube, one other control was also utilized which contained all reactants except the specific antibody, anti-gpIb α or anti-gpIIb/IIIa.

The Levels of gpIb α on the nPMP

2.5 μ l of mouse anti-human CD42b (0.1 mg/ml, Gentaur Europe BVBA, Brussels, Belgium) was added to the tubes each included 100 μ l of 140 μ g/ml of nPMPs. The tubes were left 40 min at RT. Washing of the microparticles

using centrifugation at 14,000 $\times$ g and addition of 2.5 μ l of the FITC-conjugated goat anti-mouse IgG (Fab') 2 (Dako, Denmark) was then carried out. Again 40 min incubation at RT was chosen.

For all the experiments, nonspecific binding was analyzed as it was explained.

The Levels of gpIIb/IIIa on the nPMP

In a one-step method, 7 μ l of FITC-conjugated mouse anti-CD41 (HIP8) (Abbiotec, LLC, San Diego) was added to the tubes each included 100 μ l of 140 μ g/ml of nPMPs. The tubes were left 40 min at RT. Washing of the IPM (centrifugation at 14,000 $\times$ g) was carried out before flow cytometry analysis.

Statistical Analysis

Statistical differences between the levels of glycoproteins on IPM were tested by Mann–Whitney test as two sets of data (without the stabilizer and with a defined concentration of the stabilizer). Besides, for nPMP the paired-samples *T* test was used to compare the results between the microparticles in different days of storage. A *P* value of <0.05 was considered significant.

Results

Retention of gpIIb/IIIa and gpIb α on the Prepared IPM

This study showed that gpIIb/IIIa and also gpIb α were present on the surface of IPM (Table 1). The expression of gpIIb/IIIa was lower in the higher concentration of the stabilizer (0.4 M) (Fig. 1). Therefore in the higher concentrations of the stabilizer, shedding of this molecule could be augmented. Additionally, some conformational changes may also take place which could affect the interaction of these antigens and the respective antibodies.

The Levels of gpIb α and gpIIb/IIIa on the nPMP During Storage

This study showed that both of the glycoproteins; gpIb α and gpIIb/IIIa were present on the surface of nPMP. The expression of gpIb α and gpIIb/IIIa were decreased on the nPMP during storage. The reduction may be related to the shedding of these molecules from the surface of microparticles by the passage of time. At the day 4 of storage, the level of these glycoproteins was significantly lower than that of day 2 (*P* value = 0.001). This was the case for the day 7 of storage in compare to the day 4 (*P* value = 0.001) (Table 2).

Table 1 The results of the mean $\pm$ SD of the percent of the GpIb α and gpIIb/IIIa expression on the IPM prepared in different concentrations of sodium octanoate

Concentrations of sodium octanoate	0	0.1 M	0.25 M	0.4 M
gpIb α on the IPM (%)	59.1 $\pm$ 3.8	54 $\pm$ 3.1	26.6 $\pm$ 1.6	8 $\pm$ 1.3
gpIIb/IIIa on the IPM (%)	43.8 $\pm$ 2.4	30.4 $\pm$ 2.13	12.2 $\pm$ 1.73	4.3 $\pm$ 1.6

Fig. 1 Flow cytometry plot. IPM was prepared in different concentration of sodium octanoate and liquid-stored in 4 °C. Expression of gpIIb/IIIa was analysed. **a** The microparticles were gated, **b** represented the gpIIb/IIIa levels in the prepared IPM in the absence of heat stabilizer, **c** represented the gpIIb/IIIa levels in the surface of IPM in 0.25 M of heat stabilizer, **d** represented the gpIIb/IIIa levels in 0.4 M of the heat stabilizer. **e** the isotypic control immunoglobulin IgG, **f** negative control lacking the specific antibody (anti-gpIIb/IIIa)

Discussion

Membrane glycoproteins play an important role in the main functions of platelets. Platelet microparticles play a role as a synergistic agonist for platelets. It has been elucidated that release of thrombin-induced platelet

microparticles into circulation would improve blood coagulation and platelet aggregation [17]. It seems that the main role of nPMP could be attributable to the presence of the glycoproteins like gpIb and gpIIb/IIIa. For example nPMP can bind fibrinogen and participate in thrombus formation [13].

Table 2 The results of the mean $\pm$ SD of the percent of the GpIb α and gpIIb/IIIa expression on the nPMPs at the days 2, 4 and 7 of storage

Days of storage	2	4	7
gpIb α on the nPMPs (%)	71.87 $\pm$ 3.12	64.18 $\pm$ 2.11	59.45 $\pm$ 4.14
gpIIb/IIIa on the nPMPs (%)	81.82 $\pm$ 11.94	67.04 $\pm$ 11.35	45.73 $\pm$ 13.41

In this research, the retention of gpIIb/IIIa and gpIb α molecules was demonstrated on the surface of platelet-derived IPM microparticles using flow cytometry that is the usual method for the study of microparticles [18]. The obtained results had conflict with the previous report related to the absence of gpIIb/IIIa molecules on the surface of produced IPM [10]. The described difference may be related to the age dissimilarity in the platelets that were used for the production of IPM. We used 3–5 days-old PCs whereas Chao et al. [10] had employed 10–19 days-old PCs. Alternatively, the main cause of the difference may be related to the preparation method as its effect was elucidated in our study using different concentrations of the heat stabilizer but the use of heat protection method were not explained in the work of Chao. Our previous study showed that during the heating step of IPM preparation the usage of a heat stabilizer (sodium octanoate) was necessary to obtain the maximum vWF binding activity of IPM [15].

The expression of gpIIb/IIIa in this study could not be attributed to the platelets remained in the IPM preparation because the size of prepared IPM microparticles was determined to be in the range of 84.7–291 nm (Z-average = 174 nm) [15] that had no correlation with the size of platelets.

Besides, in this study, the reduction of gpIb α and gpIIb/IIIa molecules was detected on the surface of nPMP over time for 7 days at 22 °C. Horstman and Ahn [13] showed that many of the platelet-specific glycoproteins are routinely identified on PMP, chiefly gpIIb/IIIa and gpIb. Sandgren et al. [19] reported that no significant difference in gpIIb/IIIa expression was detected over time whereas the percentage of platelets expressing gpIb α gradually decreased at 22 °C. In our study, the significantly decrease in the levels of both gpIb α and gpIIb/IIIa was demonstrated on the nPMP during 7 days storage of PCs (P value = 0.001). This could imply the shedding of these surface glycoproteins from nPMP during storage. We didn't find any similar study related to the survey on the glycoproteins of the naturally occurred platelet-derived microparticles during storage.

Research on the kinetic of the shedding of glycoproteins could be useful in the evaluation of their specific functions.

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Conflict of Interest None of the authors have any conflicts of interest to declare.

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