Platelet Storage Media Change the Expression Characteristics of the Platelet-Derived Microparticles

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Abstract Activated platelets shed microparticles in vivo and definitely in vitro upon aging under storage. Studies about the platelet-derived microparticles (PMPs) produced in different storage media of PC were very limited. The aim of this research was to compare some surface molecules of these microvesicles in dissimilar microenvironments; plasma and the candidate medium for the platelet concentrate, Composol. Thirty units of PCs were prepared from Iranian Blood Transfusion Organization. Each unit was divided into two portions. In one of the portions, plasma was replaced with Composol using a connecting device instrument. MPs were isolated from PC and the levels of PS exposure (the annexin-binding capacity) and binding to vWF were surveyed on their surface using ELISA and flow cytometry techniques. The levels of PS exposure were increased on MPs during 7 days storage in the both media but the differences were not significant (P value >0.05). In addition, binding of PMP to vWF was declined during storage. The binding capabilities of PMP were significantly higher in Composol than that of plasma at the day 4 or 7 of storage (P value = 001). It seemed that the binding of PMPs to vWF was affected from the storage media of PC (plasma and Composol) but PS exposure was not affected from the type of storage media.

Keywords Platelet microparticles · von Willebrand factor · Phosphatidylserine · Additive solution

Introduction

In 1967, Wolf described the membrane fragments that are shed from activated platelets as “platelet dust”, or “platelet vesicles” [1]. These are the particles now widely referred to as platelet-derived microparticles (PMPs) [2, 3]. The term microparticles (MPs) usually refer to particles larger than 100 nm in diameter that are derived from the plasma membrane among the various membrane vesicles that cells release. Cell membrane MPs circulate in the blood of healthy donors, and their elevated counts have been documented in various pathologies. MPs are released by various stimuli including shear stress, complement attack, or proapoptotic stimulation. All MPs harbor cell-surface proteins and contain cytoplasmic components of their original cells. Platelet MPs expose procoagulant anionic phospholipids such as phosphatidylserine (PS) in a similar fashion as activated platelets. In normal healthy cells PS is located in the inner leaflet of the plasma membrane. However, on activated platelets, early during the process of apoptosis and under specific circumstances, PS is actively externalized to the outer leaflet of the plasma membrane. Exposure of platelet membrane PS regulates blood coagulation [4–6].

Exposure of PS can be monitored for suspended cell types by flow cytometry or ELISA method using annexin, which is a Ca^{2+} -dependent, phospholipid-binding protein with high affinity for PS [7–9].

von Willebrand factor (vWF) is a multimeric plasma glycoprotein that supports platelet adhesion at sites of vascular injury [10]. vWF binds to platelets via the glycoprotein Ib and IIb/IIIa receptors. Under normal physiological conditions, very little vWF is associated with the surface of the platelet. In the event of vascular injury, however, it promotes hemostasis by mediating platelet adhesion to exposed vascular subendothelium. In certain

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bleeding disorders, vWF binds avidly to the platelets causing them to agglutinate [11, 12]. The GPIIb/IIIa platelet surface receptor on association with vWF can signal the binding event to the cell to activate the platelet, thereby activating other platelet surface receptors [13].

In spite of the abundant studies about the PMP in normal or some pathological conditions, studies about these microvesicles in platelet concentrate (PC) were very limited. Since PMP were one of the constituents of PC units in blood banks and some biological functions have been attributed to these vesicles, we studied some determinants on their surface. Besides, additive solutions [14–16] like Composol were salt solutions for replacement of plasma in PC units. So in this research, PS exposure and the binding capacity of PMP to vWF were surveyed during 7 days storage of PCs in the media of plasma and Composol.

Materials and Methods

Sample Preparation

Informed consent was obtained from the blood candidates by Iranian Blood Transfusion Organization (IBTO). Thirty single donor platelet concentrate bags (JMS Singapore Pte Ltd. contained CPDA-1 solution) were prepared from IBTO (24 h after PC preparation and completion of the viral safety tests).

The protocol for the division of PC into two portions and replacement with Composol was as below.

Each PC bag (A) was connected to two transfer bags (B and C) using a connecting device instrument (TSCD-II Terumo Sterile Tubing Welder). The transfer bags contained no anticoagulant solution. Half of the bag A constituent was transferred to the transfer bag B via transfer tube using a digital balance (Sartorius). The bag A was then separated from the B and C transfer bags by a tube sealer. After centrifugation of the A (as a control) and B bags, the plasma of the B was transferred into the C using an extractor. Then B and C bags were separated by a tube sealer. Finally, Composol solution (Fresenius Kabi) was added into the platelet pellets of B bag in the same weight of the removed plasma using a connecting device. Approximately, 20 % of plasma was left in the bag. In continuation, the suspended platelets of A and B were incubated and simultaneously agitated in a shaker-incubator that was adjusted at 22 °C. Sampling of the cords/bags was carried out at the days 2, 4 and 7 from the preparation time.

Assessment of PS Exposure on PMP Using ELISA Method

Briefly, microtiter plates (Greiner bio-one, Frickenhausen, Germany) were coated with 100 µl of a 5 µg/ml solution of

Annexin V (Abcam, UK) per well. The phosphate-buffered saline (PBS), pH 7.8 was used, and the plates were left 2 h at 37 °C. Blocking of the plates was performed using a 2 % solution of BSA in PBS (3 h, RT), followed by incubation with 100 µl of the platelets concentrate storage medium; plasma or Composol for 35 min at RT. Washing of the plates and addition of 100 µl of biotin-conjugated anti-human GPIIb/IIIa were then carried out. Again 35 min incubation at 37 °C was chosen. Subsequent to routine washing step, addition of streptavidin-HRP (Antigenix America, Huntington Station, NY, USA) was accomplished. Once more 30 min incubation at 37 °C was chosen. After routine washing step, the enzymatic activity was determined using tetramethyl-benzidine (Usb, USA) as substrate. The wells devoid of the coated antigen or the specific antibody to GPIIb/IIIa were used as negative controls. Beside, a pooled of the MPs obtained from PCs at the day 7 of storage was utilized as positive control.

Isolation of PMP from Platelet Concentrate

For preparing PMP from platelet concentrates, platelets were transferred into 15 ml falcon tubes. The tubes were centrifuged at 150×g for 15 min to remove remaining white and red blood cells. The supernatant were separated and then depleted from platelets using centrifugation at 1,200×g for 15 min. Finally PMP was isolated using centrifugation at 16,000×g for 30 min.

Evaluation of the Binding Properties of PMP to Annexin A5 Through PS Using Flow Cytometry Method

5 µl of Annexin-FITC was added to the microtubes each contained 100 µl of a 140 µg/ml PMP prepared from PC in the plasma medium. The tubes were left 40 min at 4 °C. Washing of the MPs was then carried out. For all the experiments, a positive and negative control was also used to evaluate the specificity of the results.

Assessment of the Binding of PMP to vWF

For studying the vWF binding properties of PMP, 100 µl of PMP (140 µg/ml) was added to the tubes containing 2 µl of 200 µg/ml concentration of purified human vWF (Biopur, Bubendorf, Switzerland) in the presence of 50 µg ristocetin (Helena Laboratories, Beaumont, TX, USA) as a modulator. After 45 min incubation at RT, the PMP was washed and incubated for 30 min with rabbit anti-human vWF (Abcam, Cambridge, UK). The PMP was washed again and incubated for 30 min with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma Chemical, St Louis, MO, USA). To be sure of the specificity of the results, nonspecific antibody background binding was determined with the appropriately

Table 1 The results of the mean $\pm$ SD of the ELISA (absorbance at 450 nm) and flow cytometry results (the fluorescent intensity) as an index for the evaluation of the annexin binding capacity of PMP obtained at the days 2, 4 and 7 of PC storage

Days of storage of PC	2	4	7
Annexin binding in plasma (OD _{450 nm})	0.462 $\pm$ 0.182	0.811 $\pm$ 0.314	1.505 $\pm$ 0.523
Method: ELISA			
Annexin binding in Composol (OD _{450 nm})	–	0.781 $\pm$ 0.318	1.556 $\pm$ 0.606
Method: ELISA			
Annexin binding in plasma (intensity percent)	45.73 $\pm$ 13.41	67.04 $\pm$ 11.35	81.82 $\pm$ 11.94
Method: Flow cytometry

Fig. 1 Comparison of the annexin-binding of PMP at the days 2, 4 and 7 of storage in two storage media of plasma and Composol. ELISA method was used for this survey. It was noticeable that the day 2 was the transfer day for Composol so there was no measureable index in this day for the Composol medium**Table 2** The mean $\pm$ SD of the results of flow cytometry technique in the evaluation of the vWF binding capacity of PMP at the days 2, 4 and 7 of storage

Days of storage of PC	2	4	7
Binding of PMP to vWF	66.79 $\pm$ 15.60	51.18 $\pm$ 11.09	43.89 $\pm$ 9.62
Fluorescent intensity (%)			
Medium: plasma			
Annexin binding	^a	53.33 $\pm$ 12.25	45.15 $\pm$ 10.06
Fluorescent intensity (%)			
Medium: Composol			

^a The day 2 was the exchange day for plasma and Composol in PC units so there was no data for Composol in this day

labeled isotypic control immunoglobulin IgG. Additionally for each tube, a control was also utilized which contained all reactants except the specific antibody (rabbit anti-human vWF antibody). Finally, the analysis of the experiments was surveyed using flow cytometry technique.

Results

Evaluation of PS Exposure on PMP Using ELISA Method

PMP could bind to Annexin A5 and the extent of their binding was dependent on the levels of exposed PS and the length of storage time. In this experiment, a pool of PMP obtained from 10 PC units after 7 days storage was used as positive and a pool of PMP obtained from freshly prepared platelets was utilized as negative controls. The optical density for the positive and negative controls were obtained OD₄₅₀ = 2 & OD₄₅₀ = 0.3 respectively. Low background absorbance (0.15–0.2) was detected for the wells devoid of coated Annexin A5 or lacked the specific antibody (biotin-conjugated anti-human gplb α). In the assessment of the PMP binding to Annexin A5, the coefficient of variations (CV) for intra assays at days 2, 4 and 7 of storage in plasma were determined 0.3, 0.9 and 0.7 %, respectively. Beside, CV for intra assays at days 4 and 7 of storage in the Composol medium were determined 0.4 and 1.1 %, respectively.

On the other hand, the values of CV for inter assays at days 2, 4 and 7 of storage in plasma were determined 5.6, 3 and 2 %, respectively. Beside, CV for inter assays at days 4 and 7 of storage in Composol were determined 10.3 and 2 %, respectively.

Both methods of ELISA and flow cytometry showed consonant results and an increasing trend in PS exposure on the PMP during storage of PC (Table 1).

The results of ELISA method showed that there were no significant differences between the annexin-binding virtues of PMP produced from PC in the plasma and Composol at the day 4 of storage (P value = 0.138). It was also the same at the day 7 of storage (Fig. 1). Besides, at the 4th

Fig. 2 Flow cytometry plot. The vWF binding capacity of PMP at the days 2, 4 and 7 of PC storage in plasma. Binding to vWF was analysed during this time. **a** The microparticles were gated, **b** the isotypic control immunoglobulin IgG, **c** the binding levels in the negative control in the absence of specific antibodies to vWF, **d** the vWF binding levels of PMP at the day 2 of storage, **e** the vWF binding levels of PMP at the day 4 of storage, and **f** the vWF binding levels of PMP at the day 7 of storage

day of storage, the binding capacity of PMP to annexin was significantly higher compare to the 7th day in each of the plasma or Composol medium (P value = 0.001).

Assessment of the Binding of PMP to vWF in Plasma and Composol

This study demonstrated a capacity for PMP generated in plasma for binding to vWF. The binding capacity of PMP turned down during 7 days storage of PC at 22 °C (Table 2; Fig. 2).

PMP produced in the Composol medium was also able for binding to vWF. The binding capacity of PMP also declined during 7 days storage of PC at 22 °C (Table 2).

The binding capacity of PMP to vWF was significantly higher in the Composol medium than that of plasma at the

fourth day of storage (P value = 0.007). It was also true at the 7th day of storage (P value = 0.01).

Discussion

It has long been thought that 70–90 % of MPs in plasma are derived from activated platelets in comparison to other MP generating cells which contribute to the total pool. PMP play a role as a synergistic agonist for platelets. It has been elucidated that release of thrombin-induced platelet MPs into circulation would improve blood coagulation and platelet aggregation [17]. PMP can bind fibrinogen and participate in thrombus formation [3]. They also contribute to vascular remodeling and angiogenesis [18].

Since PMP constitute one part of the blood product (platelet concentrate) and was simultaneously co-injected with platelets to patients, studies about these microvesicles was of specific importance. In this research, we affirmed that PS exposure and vWF binding were occurred on PMP originated during the storage of platelet concentrate. We showed that PS was exposed on PMP and had elevated levels during storage of PC. The difference between PS exposures on PMP was not significant in the studied media at the 4th or 7th day of study.

The expression of PS was demonstrated on outer membrane of activated platelets and its role was discussed by researchers [19, 20]. For example Bevers et al. [21] showed that in resting human platelets, PS was distributed across the platelet plasma membrane to the cytoplasmic face and after activation, platelets revealed an increased exposure of PS at their outer surface. Beside, Lai et al. [22] showed that PS elevated on the surface of human platelets during storage. In another survey, Comfurius et al. [23] pointed that the vesicles shed by activated platelets have PS exposed on their surface. In correlation with Comfurius, we showed PS on the surface of PMPs. Additionally, we followed PS exposure on PMP during storage of platelet concentrate in two different medium and surveyed the differences. The results of Lai et al. [22] and ours could demonstrate that PS exposure had similar pattern in platelets and the PMPs during storage of PC.

There were no studies found in which PS exposure was surveyed on PMP in platelet concentrate. Additionally, no similar studies were found in any additive solution. So this study could be important in this regard.

There are numerous studies related to the binding of platelets to vWF [10–13] but the studies about vWF binding virtue of PMP was not previously reported. In our study, the significantly higher binding capacity of PMP to vWF was demonstrated in the Composol medium at the fourth and seventh day of storage. This finding could confirm higher functional potency of MPs in the Composol medium than plasma.

Platelet MPs were one of the constituents of platelet concentrate and the additive solutions were salt replacement solutions for plasma.

This study showed that with replacing the storage media with Composol, the functional potential (vWF binding) of PMP was increased. PMP may have a synergistic role with platelets and act as a surrogate factor for platelets in hemostasis. Matijevic et al. showed that decline in platelet MPs contribute to reduced hemostatic potential of stored plasma. They also demonstrated that removal of PMP by filtration resulted in reduced thrombin generation, while the PMP replacement restored it. Additionally, the presence of platelet MPs in freshly thawed plasma may be beneficial and protective in the initial treatment of hemorrhage [24].

Because PMPs expose anionic phospholipids such as PS in a similar fashion as activated platelets [4–6], similar results for PS exposure on PMP in two storage media could imply the suitability of Composol for replacing plasma regarding the similar activation/apoptosis induction potential.

This study could be included among different experiments for the comparing of platelet indexes in plasma and Composol. Further studies may clear other advantages of Composol as storage medium for platelet concentrate regarding MP formation.

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Conflict of interest None.

References

1. Wolf P (1967) The nature and significance of platelet products in human plasma. *Br J Haematol* 13:269–288
2. Simak J, Gelderman MP (2006) Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers. *Transfus Med Rev* 20(1):1–26
3. Horstman LL, Ahn YS (1999) Platelet microparticles: a wide-angle perspective. *Crit Rev Oncol Hematol* 30(2):111–142
4. van Engeland M, Ramaekers FC, Schutte B et al (1996) A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. *Cytometry* 24(2):131–139
5. Lentz BR (2003) Exposure of platelet membrane phosphatidylserine regulates blood coagulation. *Prog Lipid Res* 42(5):423–438
6. Krishnaswamy S, Church WR, Nesheim ME et al (1987) Activation of human prothrombin by human prothrombinase. Influence of factor Va on the reaction mechanism. *J Biol Chem* 262(7):3291–3299
7. van Engeland M, Nieland LJ, Ramaekers FC et al (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 31(1):1–9
8. Dasgupta SK, Guchhait P, Thiagarajan P (2006) Lactadherin binding and phosphatidylserine expression on cell surface-comparison with annexin A5. *Transl Res* 148(1):19–25
9. Mobarrez F, Antovic J, Egberg N et al (2010) A multicolor flow cytometric assay for measurement of platelet-derived microparticles. *Thromb Res* 125(3):e110–e116
10. Doggett TA, Girdhar G, Lawshé A et al (2002) Selectin-like kinetics and biomechanics promote rapid platelet adhesion in flow: the GPIb (alpha)-vWF tether bond. *Biophys J* 83(1):194–205
11. Stewart MW, Etches WS, Gordon PA (1988) Analysis of vWF binding to platelets by flow cytometry. *Thromb Res* 50(3):455–460
12. Kang M, Wilson L, Kermod JC (2008) Evidence from limited proteolysis of a ristocetin-induced conformational change in human von Willebrand factor that promotes its binding to platelet glycoprotein Ib-IX-V. *Blood Cells Mol Dis* 40(3):433–443
13. Mody NA, King MR (2008) Platelet adhesive dynamics. Part II: High shear-induced transient aggregation via GPIIb/IIIa-vWF-GPIIb/IIIa bridging. *Biophys J* 95(5):2556–2574
14. Van der Meer PF (2007) Platelets additive solutions: a future perspective. *Transfus Clin Biol* 14(6):522–525

15. Gyongyossy-Issa MI, Zhang JG, Culibrk B et al (2009) Novel system for storage of buffy-coat-derived platelet concentrates in a glucose-based platelet additive solution: parameters and metabolism during storage and comparison to plasma. *Vox Sang* 97(2):102–109
16. Sandgren P, Hansson M, Gulliksson H et al (2007) Storage of buffy-coat-derived platelets in additive solutions at 4 degrees C and 22 degrees C: flow cytometry analysis of platelet glycoprotein expression. *Vox Sang* 93(1):27–36
17. Xiao H, Jepkorir CJ, Harvey K et al (2002) Thrombin induced platelet microparticles improved the aggregability of cryopreserved platelets. *Cryobiology* 44(2):179–188
18. Morel O, Toti F, Hugel B et al (2006) Procoagulant microparticles: disrupting the vascular homeostasis equation? *Arterioscler Thromb Vasc Biol* 26:2594–2604
19. Sinauridze EI, Kireev DA, Popenko NY et al (2007) Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. *Thromb Haemost* 97:425–434
20. Comfurius P, Smeets EF, Willems GM et al (1994) Assembly of the prothrombinase complex on lipid vesicles depends on the stereochemical configuration of the polar headgroup of phosphatidylserine. *Biochemistry* 33(34):10319–10324
21. Bevers EM, Comfurius P, Zwaal RF (1983) Changes in membrane phospholipid distribution during platelet activation. *Biochim Biophys Acta* 736(1):57–66
22. Lai M, Rumi C, D'Onofrio G et al (2002) Phosphatidylserine exposure in platelet concentrates during the storage period: differences between the platelets collected with different cell separators. *Transfus Apher Sci* 273:239–245
23. Comfurius P, Senden JM, Tilly RH et al (1990) Loss of membrane phospholipid asymmetry in platelets and red cells may be associated with calcium-induced shedding of plasma membrane and inhibition of aminophospholipid translocase. *Biochim Biophys Acta* 1026:153–160
24. Matijevic N, Wang YW, Kostousov V et al (2011) Decline in platelet microparticles contributes to reduced hemostatic potential of stored plasma. *Thromb Res* 128(1):35–41