Tissue Factor Expression on Platelet Surface during Preparation and Storage of Platelet Concentrates

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Summary

Background: Tissue factor (TF), the main activator of blood coagulation, is expressed on platelet surface and, together with procoagulant phospholipids, contributes to the global coagulation potential of these blood components. The present study evaluated, for the first time, the expression of TF on platelet surface during preparation and storage of platelet concentrates (PC) for transfusional use. **Methods:** Platelet TF was measured by flow cytometry in healthy donor whole blood (WB) and in pooled buffy-coat-derived PC on the day of preparation and up to 4 days of storage in parallel with classical markers of platelet activation, i.e., fibrinogen, P-selectin, and glycoprotein GPIIb. Data were analyzed according to donor age and blood ABO group. **Results:** TF was detected on whole blood platelets and was found highest in O donors. Compared to whole blood, platelet surface TF was higher upon PC preparation and further increased during storage. The rise in TF levels positively correlated with the elevations of the other platelet markers. **Conclusions:** Our findings show that platelet surface TF is maintained in PC obtained by the pooled buffy coat method. Further studies are warranted to investigate a possible correlation between TF levels and the hemostatic response of the platelet transfusion recipient.

Introduction

Thrombocytopenic patients need transfusions of high quantities of platelets to prevent or stop hemorrhages. These platelets are given in form of highly concentrated platelet suspensions, so-called platelet concentrates (PC), obtained either from single donors by apheresis or from pooling multiple random donor buffy coats or platelet-rich plasma [1]. Due to the risk of bacterial contamination and the phenomenon of so-called ‘platelet storage lesion’ [2, 3], it is recommended to transfuse PC as soon as possible; in Italy it is usually done within the 5th day from PC preparation.

Key to the success of PC transfusion is the availability of platelets endowed with a good hemostatic potential. Particularly, when PC is given to stop an ongoing bleeding event, platelet hemostatic effect has to be effective and immediate. Considerable data now support the evidence that platelets not only participate at primary hemostasis process by forming the platelet plug, but can also actively promote the propagation of the coagulation cascade by providing specific high-affinity receptors, proteases, zymogens, and cofactors, thus contributing to localize thrombin generation at the site of vascular injury [4].

With respect to coagulation receptors, several studies have demonstrated the presence of tissue factor (TF) on platelet surface [5]. TF, a membrane glycoprotein, is a high-affinity receptor for the plasma coagulation factor VII. The TF/FVIIa complex triggers the extrinsic pathway, ultimately leading to thrombin generation and fibrin formation [6]. Furthermore, it has been recently reported that platelets, under well-controlled experimental conditions, contain functionally active TF in the α -granules and in the canalicular system [7]. However, it is still debated whether this TF is actually of platelet origin [8] or is instead derived from leukocytes through a transfer mechanism mediated by microparticles [9].

Until now, no studies have evaluated the expression of TF on platelet surface in donor whole blood bags and in PC for transfusion use. In this setting, flow cytometry represents a powerful tool to study the pattern of the hemostatic proteins expressed on platelet surface and is able to reflect the changes induced by platelet activation [10]. Therefore, the present work aimed to study whether and to what extent the expression of platelet surface TF may change during PC preparation and storage, and whether this protein may be an indicator of platelet hemostatic potential. To calibrate this analysis, a number of well-known markers of platelet activation, i.e., P-selectin (CD62P), bound fibrinogen and GPIIb (CD41), were tested in parallel [10–12]. The possible influence of donor age and ABO blood group type on the expression of TF and other hemostatic markers was also analyzed both in donor whole blood and in PC over preparation and storage.

Material and Methods

Blood Collection and Sample Preparation

Whole blood (450 ± 50 ml) from 180 (155 M / 25 F; O group = 68, A group = 64, B group = 48) healthy blood donors of the Bergamo province (Italy) was collected in a triple bag system (Fenwal, Guibert, Belgium) containing 63 ml of citrate-phosphate-dextrose (CPD) as anticoagulant. Mean age of donors was 42.8 years (range 19–65 years), platelet count was $229.8 \pm 45.6 \cdot 10^9/l$ (mean $\pm$ SD).

On donation day (day 0), 30 ml from each blood bag were sampled: of these, 500 μ l were immediately taken for antigen expression analysis on platelet surface by flow cytometry.

PC Preparation

PC were prepared at the Bergamo Hospital Immunohematology and Transfusion Medicine Center within 24 h from blood collection (day 1). Blood bags were centrifuged at 3,900 rpm for 10 min to separate buffy coat from platelet-poor plasma (PPP) and red cells. Four buffy coats from the same ABO group were joined together and added with 300 ml of platelet additive solution containing sodium citrate as anticoagulant ('T-sol', Baxter, Deerfield, IL, USA) to create pooled buffy-coat. This pooled buffy coat was subsequently centrifuged at 1,100 rpm for 10 min to separate white blood cells and residual erythrocytes and filtered to remove leukocytes.

50 ml from 45 (O group = 17, A group = 16, B group = 12) pooled buffy coat PC (from now on 'PC') were sampled in a pediatric bag, stored at 22 °C on lateral agitation and analyzed by flow cytometry on the same day (day 1) and after 3 (day 4) or 4 (day 5) days of storage.

Flow Cytometric Analysis

5 μ l of either donor whole blood or PC samples were added to 100 μ l of PBS and a saturating concentration of either TF-FITC (AbD Serotec, Oxford, UK), CD62P(P-selectin)-FITC (Biolegend, San Diego, CA, USA), fibrinogen-FITC (BioCytex, Marseille, France), CD41-FITC (Exbio, Praha, Czech Republic), or CD41-PE (BioCytex). As negative control an isotypic control IgG-FITC antibody (BioCytex) was used. All labelings were performed in flow cytometry test tubes (Becton Dickinson, Mountain View, CA, USA). After 20 min in the dark at room temperature, samples were diluted in 600 μ l of PBS and immediately analyzed by a FACScanto flow cytometer (Becton Dickinson). A gate around platelet population was defined by side-scatter characteristics and positivity to CD41-PE antibody. For each marker, the percentage of positive platelets on the total platelet population was recorded; data were also analyzed as

Fig. 1. TF expression on platelet surface in healthy donor whole blood. TF was measured by flow cytometry in whole blood samples from 180 donors. Data (mean $\pm$ SD) are presented as % of positive platelets found in all blood donor subjects group and according to ABO blood group. * = $p < 0.05$ vs. O; ** = $p < 0.01$ both.

mean fluorescence intensity (MFI) expressed as arbitrary units. The differences in the % of positive platelets or MFI between the values at either day 4 or day 5 and day 1 were also calculated.

Statistical Analysis

Student's t-test was employed to compare the differences between groups, according to the distribution of the test variable. Differences were considered statistically significant at a p value < 0.05 . Correlation and linear regression analysis was performed with the SPSS 19 statistical package (SPSS, Chicago, IL, USA) using Pearson's correlation and expressed as R coefficient.

Results

Donor Whole Blood

Figure 1 reports data of flow cytometry analysis of TF on platelets in whole blood, both in the overall donors and according to their ABO blood group. The mean % of TF-positive platelets was $10 \pm 6.7\%$ with an antigenic density of 796 ± 491 MFI units. Blood samples from O donors showed a significantly higher percentage of platelets positive for TF ($11.7 \pm 4.8\%$) compared to both A ($9 \pm 5.2\%$) and B ($8.4 \pm 5.3\%$) donors ($p < 0.05$ vs. A, $p < 0.01$ vs. B), while TF antigenic density levels were very similar across the groups (data not shown).

$10.6 \pm 6.8\%$ of platelet donors were positive for P-selectin, $17.3 \pm 8.8\%$ for fibrinogen and 100% CD41, while the antigenic densities were $1,228 \pm 986$ MFI units for P-selectin, $1,083 \pm 754$ MFI units for fibrinogen, and $8,996 \pm 2,736$ MFI units for CD41. The analysis according to donors' blood group showed that, similar to TF, O donors presented also the highest percentage of positive platelets for fibrinogen ($19 \pm 9.9\%$) compared to A ($15.9 \pm 7.9\%$; $p < 0.05$) and B ($16.4 \pm 7.6\%$; $p = \text{n.s.}$), again with no statistically significant differences in the protein density per platelet (data not shown).

Fig. 2. Expression of TF, fibrinogen, P-selectin, and CD41 on platelet surface in freshly prepared PC (black bars) is increased compared to whole blood (open bars). Data are presented as relative increments of the % of positive platelets (TF, fibrinogen, and P-selectin), or of the antigenic density for (CD41) compared to whole blood values considered as 100%. * = $p < 0.05$ versus whole blood.

Table 1. Flow cytometry analysis on platelets after PC preparation (day 1)^a

	All groups	Group O	Group A	Group B
Tissue factor				
%	14.8 ± 5.4	14.2 ± 6.5	14.4 ± 4	16.6 ± 7.5
MFI	652 ± 226	756 ± 267	561 ± 118*	652 ± 165
Fibrinogen				
%	23.9 ± 10.2	24.6 ± 11.3	23.5 ± 7.9	22.6 ± 12.4
MFI	816 ± 412	769 ± 306	795 ± 468	950 ± 330
P-selectin				
%	26.5 ± 13.0	25.2 ± 11.5	26.9 ± 14.9	26.5 ± 19.6
MFI	1,037 ± 426	1,027 ± 469	988 ± 350	1,129 ± 351
CD41				
%	100	100	100	100
MFI	9,920 ± 2,761	9,376 ± 3,277	9,670 ± 2,212	10,879 ± 1,747

^aData are reported as mean ± SD and expressed as % of positive platelets and as mean fluorescent intensity (MFI). * $p < 0.05$ versus 0.

On the opposite, among the groups analyzed no differences were found in the percentage of positive platelets for either P-selectin or CD41 (data not shown), however, O group donors presented the lowest antigenic density of both P-selectin (O: 990 ± 526 , A: $1,320 \pm 1,232$, B: $1,751 \pm 1,162$ MFI units; $p < 0.05$ O vs. A, $p < 0.01$ O vs. B) and CD41 (O: $8,230 \pm 3,055$, A: $9,269 \pm 2,452$, B: $10,112 \pm 2,049$ MFI units; $p < 0.05$ vs. A, $p < 0.01$ vs. B).

No statistically significant differences were found between A and B groups for any marker analyzed, neither as percentage of positive platelets nor as antigenic density.

The analysis of TF and the other hemostatic markers in the overall donor group revealed no statistically significant correlations with donor age (data not shown).

Effect of PC Preparation on Platelet TF

As illustrated in figure 2, the expression of TF and the other markers on platelets was found significantly ($p < 0.05$) higher in PC compared to donor whole blood. In particular, the calculation of the mean relative increments showed that in PC

TF increased by 48% (% positive platelets $14.8 \pm 5.4\%$), fibrinogen by 38.2% (% positive platelets $23.9 \pm 10.2\%$), P-selectin by 150% (% positive platelets $26.5 \pm 13\%$), and CD41 by 10.3% (MFI $9,920 \pm 2,761$ units). The increments of platelet positivity for TF, fibrinogen, and P-selectin in PC were accompanied by a concomitant reduction of the respective antigenic densities (data not shown) that was statistically significant for fibrinogen only (816 ± 412 MFI units; $p < 0.05$).

The analysis of PC according to ABO blood group (table 1) showed no statistically significant differences for any other marker evaluated, except for TF antigenic density that was lowest in the A group platelets ($p < 0.05$ vs. O; $p = \text{n.s.}$ vs. B).

Effect of PC Storage on Platelet TF

During PC storage the levels of TF as well as those of the other platelet markers continuously increased from day 1 to day 5 (fig. 3). In particular, at day 5, the percentage of positive platelets raised (mean increase) by 32% ($p < 0.05$ vs. day 1) for TF, by 31% ($p < 0.05$) for fibrinogen, and by 148% for P-selectin. At this time point, the elevation in P-selectin-positive

Fig. 3. Effect of PC storage on platelet hemostatic markers. The expression of TF and the other markers is here depicted at PC preparation (D1, open bars), and after 4 (D4, grey bars) and 5 (D5, black bars) days of storage. Data (mean $\pm$ SD) are presented as percentage of positive platelets for TF, fibrinogen and P-selectin, and as antigenic density MFI arbitrary units for CD41. * = $p < 0.05$ versus D1. ** = $p < 0.01$ versus D1.

Fig. 4. Correlations between the expression of platelet TF and other hemostatic markers in PC. Platelet TF expression is compared with the expression of P-selectin (upper panels) and bound fibrinogen (lower panels), at PC preparation (D1) or during storage (D4/D5). Data of all markers are expressed as % of positive platelets.

platelets was associated also with a 58% increase in the mean density of this protein (day 1 vs. day 5: $1,037 \pm 426$ vs. $1,634 \pm 486$ MFI units; $p < 0.05$). The percentage of positive platelets for CD41 remained 100% during storage but it was associated with a significant rise (32%) in the mean quantity of proteins per platelet ($p < 0.05$ vs. day 1, fig. 3).

As shown in figure 4, after PC preparation (day 1) TF levels significantly correlated with both P-selectin ($R = 0.739$, $p < 0.001$) and bound fibrinogen ($R = 0.785$, $p < 0.001$) expression. These correlations were maintained also after 4 and 5 days of storage (TF vs. P-selectin: $R = 0.757$, $p < 0.001$; TF vs. fibrinogen: $R = 0.867$, $p < 0.001$). Moreover, positive statisti-

Fig. 5. ABO group analysis of platelet surface markers during PC storage. The expression of TF and the other markers is depicted at PC preparation (open bars), and after 4 (grey bars) and 5 (black bars) days of storage. Data (mean $\pm$ SD) are presented as percentage of positive platelets for TF, fibrinogen and P-selectin, or as antigenic density MFI arbitrary units for CD41. * = $p < 0.05$ vs. day 1.

cally significant correlations (all $p < 0.01$) were found when the relative changes of markers during storage were analyzed (data not shown). In particular, the variations in % of TF-positive platelets correlated with variations in both fibrinogen- ($R = 0.743$) and P-selectin-positive ($R = 0.691$) platelets, while the antigenic density of bound fibrinogen correlated with CD41 MFI ($R = 0.487$, $p < 0.01$).

The analysis of data according to ABO blood group did not show any statistically significant differences between the three blood groups analyzed (fig. 5) neither for TF nor for the other markers.

Discussion

This paper analyzed for the first time the expression of TF on platelets prepared for transfusion use. Our results show that TF is present on the platelet surface of platelets derived from donor whole blood bags and its expression is preserved during PC preparation and storage.

TF is the main initiator of the blood coagulation cascade. After an injury to the vessel wall, TF is exposed and binds to plasma FVII/FVIIa; then the newly formed TF/FVIIa complex activates FX coagulation factor, which in turn elicits the generation of thrombin that converts fibrinogen to fibrin [6].

In blood, TF can be detected in a soluble form, associated to microparticles, or carried by other blood cells, mainly monocytes and platelets [5]. There is a still ongoing debate on whether platelet-associated TF is actually of platelet origin or is uptaken by platelets from the circulation [13]. As documented in the recent years, platelets have indeed the capacity to uptake TF from circulating TF-positive microparticles [14, 15].

Regardless of the source of platelet TF, its presence, together with procoagulant phospholipid expression, significantly contributes to the role of platelets in the propagation of coagulation by enhancing local thrombin generation. Furthermore, it has been recently demonstrated, in an in vitro system that platelets containing TF are more reactive to thrombogenic surfaces, thus promoting an increase in their adhesive and aggregating properties [16]. This underlines the importance of preserving the expression of TF in PC, and indeed our study indicates that TF present on platelet surface is not removed in PC prepared by the pooled buffy coat technique.

Interestingly, we found that the percentage of TF-positive platelets increased during PC storage. Since this transfusion product does not contain other blood cell types, unless as residual contaminants, we can hypothesize different mechanisms for the observed phenomenon. First TF-negative platelets could mobilize TF from inner compartments to the surface upon activation. Some evidences show that TF is stored in platelets, particularly in the open canalicular system and in the α -granules; in case of platelet activation, this preformed TF may be mobilized to the surface membrane [17]. A second mechanism is based on the production of 'new' protein. This is well supported by a work that demonstrated that human platelets do contain TF mRNA, which is translated into de novo synthesized protein upon activation and then translocated to the plasma membrane [8]. The last hypothesis is that TF-negative platelets could uptake TF from microparticles contained in residual plasma present in the PC.

A measurement of the expression of classical markers of platelet activation, i.e., P-selectin, bound fibrinogen and CD41, was performed to evaluate possible correlations with TF expression. Our data showed a significant increase in the expression of the markers both during PC preparation and storage, which is in agreement with previous reports [18].

Among the markers analyzed, P-selectin, which is considered the most sensitive platelet activation marker [19, 20], presented the highest relative increments both during processing from whole blood to PC and during storage. At the end of PC storage, the percentage of P-selectin-positive platelets was increased by approximately 500% compared to whole blood, amounting to a rate of 60% positive platelets. This was associated with a concomitant increase of the mean antigenic density.

Expression of fibrinogen on platelet surface followed the same pattern observed with TF and P-selectin. Fibrinogen binds to GPIIb/IIIa (CD41/CD61) only when the complex is in its active conformation [12, 21]. Binding of fibrinogen to GPIIb/IIIa results in conformational changes in both counterparts, leading to the generation of either ligand- or receptor-induced binding sites and thus favoring platelet aggregation [12, 21]. During PC storage, the increase of fibrinogen bound to platelets might be caused by platelet activation, as demonstrated by the rise in P-selectin expression, leading to a higher proportion of active GPIIb/IIIa complexes on platelet membrane. However, we cannot exclude that this could be in part related to the observed concomitant increase in the overall number of GPIIb molecules expressed by platelets.

The present work also evaluated the influence of ABO blood group on platelet hemostatic parameters, a field not yet explored. It is known since the early 1960s [22], and widely demonstrated thereafter [23, 24], that ABO blood group legacy has a significant influence on von Willebrand factor (vWF) and coagulation factor VIII in that the circulating levels of these two markers are lower in O group subjects compared to non-O group subjects. A study that evaluated whether these differences in vWF levels in healthy subjects may actually reflect changes of nonvascular primary hemostasis found small but statistically significant differences in O group subjects who presented with a lower platelet aggregation potential compared to non-O group subjects [25]. However, it is not known

whether the blood group may influence platelet hemostatic marker expression as well. We observed that platelets from O donors present the highest positivity for TF, fibrinogen, and CD62P, which, however, is accompanied by the lowest antigenic density of the same parameters. On the contrary, B platelets presented a lower positivity for TF, CD62P, and fibrinogen compared to O, and for TF and CD62P compared to A, but at the same time showed the highest antigenic density for all parameters evaluated. The differences in platelet hemostatic surface markers observed between ABO blood groups in whole blood samples, disappeared upon PC preparation. Indeed, we no longer found statistically significant differences between the blood groups at the day-1 time point. This is likely due to the fact that the assembly of PC induces an overall activation of platelets, and this may mask the differences found in healthy donor whole blood.

In conclusion, our data show for the first time that TF expression is preserved on platelet surface through the process of PC preparation and until the end of storage period. Our findings pave the way for further studies. Whether this may have a clinically relevant impact on the hemostatic balance of the recipient subjects in the transfusion practice warrants further investigations.

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Disclosure Statement

The authors declare no conflicts of interest in relation to the present paper.

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