

Monitoring survival and function of transfused platelets in Glanzmann thrombasthenia by flow cytometry and thrombelastography

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Vox Sanguinis

Patients with Glanzmann thrombasthenia (GT) may form isoantibodies which induce refractoriness or inhibition of function of transfused platelets. We monitored the survival and function of transfused platelets by flow cytometry and thrombelastography in a patient with GT. Gating on CD42a⁺ allowed identification of even a few transfused platelets. Only by gating on these CD41⁺ CD42a⁺ cells were we able to demonstrate their capability to bind fibrinogen and PAC-1 upon activation. Platelets were rapidly cleared from the circulation as a result of boosted isoantibodies. The contribution of transfused platelets to clot formation was also demonstrated by thrombelastography by blocking their function with abciximab.

Key words: flow cytometry, Glanzmann thrombasthenia, isoantibodies, platelet transfusion, thrombelastography.

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Introduction

Glanzmann thrombasthenia (GT) is characterized by quantitative or qualitative abnormalities of the glycoprotein (GP)IIb/IIIa complex (integrin α IIb β 3). Its impaired function results in an abnormal platelet–platelet interaction, which is required at sites of vascular injury for the formation of a platelet plug. Platelet counts are within the normal range [1].

Transfusions of platelets are often necessary [2–4], which may induce isoantibodies. These react with a variety of epitopes of β 3 or the intact α IIb β 3 [2]. Thus, the active site of the receptor of transfused platelets can be blocked, and/or platelets are rapidly removed from the circulation, and the patient becomes refractory to further transfusions.

Physiological fluctuations of platelet counts and the measurement variability can mask refractoriness to platelet transfusions. Furthermore, the haemostatic competence of transfused platelets needs to be documented in patients with bleeding disorders.

The evaluation of platelet function by flow cytometry has been comprehensively reviewed [5]. In GT, quantification of GPIIb/IIIa can be used to monitor the survival of transfused platelets [6]. It is also important to assess the function of transfused platelets by the activation-dependent exposure of the fibrinogen-binding site.

The combined contributions of plasma and platelets to the viscoelastic properties during blood clot formation are estimated by thromboelastography (TEG) [7]. The platelet part can be separated by blocking platelet function [8].

We used flow cytometry to estimate the number and function of transfused platelets, and used TEG to assess platelet-dependent clot formation, in a patient with GT who underwent surgery.

Case report

An 18-year-old woman was found to have GT, owing to an A→G substitution of the splice acceptor site of exon 3 of GPIIIa (IVS2-2/A>G), by polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) (investigation by Vincent Jallu and Cecile Kaplan, Paris). Repeated platelet and red cell transfusions had induced waxing and waning platelet isoantibodies. Prior to laparoscopic surgery for endometriosis and bleeding, the patient received a single-donor apheresis

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platelet concentrate containing 4.2×10^{11} platelets. Platelet counts were $335 \times 10^9/l$ before transfusion and $320 \times 10^9/l$ on the following day.

Materials and methods

Flow cytometry investigations have been reported previously [5]. The concentrations of monoclonal antibodies (mAbs) and fibrinogen were determined by titration experiments with platelets from healthy controls. Data were expressed as the percentage of positive cells, or as mean fluorescence intensity (MFI).

Glycoprotein expression was assessed by staining diluted blood samples with anti-CD42a fluorescein isothiocyanate (FITC, GPIX complex, clone Beb1; Becton Dickinson (BD), San Jose, CA), anti-CD42b (GPIb α , clone SZ2; Immunotech, Marseille, France), anti-CD41 phycoerythrin (PE) (GPIIb, α IIB integrin, clone P2; Immunotech), and anti-CD61 peridinin chlorophyll protein (PerCP, GPIIIa, β 3 integrin, clone RUU-PL 7F12; BD) for 15 min.

For platelet function, 20 μ l aliquots were incubated for 10 min with anti-CD42a PerCP to avoid activation-induced internalization of the GPIb/IX/V complex. Then, 10 μ l of H-Ser-Phe-Leu-Leu-Arg-Asn-OH [thrombin receptor activator peptide 6 (TRAP-6); Bachem Biochemica, Bubendorf, Switzerland; 50 μ M or 150 μ M], or phosphate-buffered saline (PBS) was added and incubated for another 10 min. Platelets were then stained with mAb PAC-1 FITC (BD) and anti-P-selectin PE (clone CLB-Thromb/6; Immunotech) for 15 min. Alternatively, platelets were stained with anti-CD41 PE and PAC-1 FITC.

Fibrinogen binding was assessed by incubating 10 μ l of anticoagulated blood in 115 μ l of HEPES, followed by the addition of FITC-conjugated fibrinogen (WACK-Chemie, Bad Soden, Germany), anti-P-selectin PE and anti-CD42a PerCP

for 10 min, and the addition of 10 μ l of TRAP-6 or HEPES, for another 15 min.

Modified TEG, using the Thrombelastograph D (Hemoskope, Skokie, IL), was performed as published previously [8]. Anticoagulated blood was incubated with 4 IU/ml heparinase (Hemoskope) and recalcified with 40 μ l of 0.645% CaCl₂. TEG tracings were performed on samples with and without the addition of 5 μ l of 2 mg/ml GPIIb/IIIa antibody-fragment abciximab (ReoPro; Centocor, Leiden, the Netherlands). We recorded the maximum amplitude (MA), representing the maximum strength of the clot, which depends on platelets and fibrinogen and is directly dependent on the dynamic properties of platelet-platelet binding via GPIIb/IIIa. The addition of abciximab abolishes the platelet contribution to clot formation and the MA then reflects only clot strength caused by fibrin formation. Thus, comparison of the MA without abciximab with that containing abciximab allows calculation of the platelet contribution to the clot formation: Δ MA = standard MA - abciximab-modified MA (normal value > 10 mm).

Platelet antibodies were determined by the monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay [9].

Results

There was no significant binding of anti-CD61 or anti-CD41 to the patient's autologous platelets (Table 1). The patient's serum contained GPIIb/IIIa antibodies reactive with all panel platelets (optical density (OD) = 234, normal < 75), but not with the autologous platelets. Antibodies were not detectable 2 days after transfusion, but were another 3 days later (OD = 756).

The MFI of fibrinogen increased only slightly after activation (Table 1). Platelets expressed, however, P-selectin after activation (before activation: MFI = 24, 12% P-selectin positive; after

Table 1 Platelet glycoprotein expression and 'activatability' of platelets from a patient with Glanzmann thrombasthenia, before and after platelet transfusion

	CD42a (% positive)	CD61 (% positive)	CD41 (% positive)	PAC-1 (MFI)	Fibrinogen (MFI)
Before transfusion, all platelets					
Without TRAP-6	98	< 1	< 1	8.2	41.4
TRAP-6 50 μ M	96	< 1	< 1	7.0	46.1
TRAP-6 150 μ M	98	< 1	< 1	7.1	51.8
After transfusion, all platelets					
Without TRAP-6	98	7.8	7.7	6.3	36.3
TRAP-6 50 μ M	98	NT	NT	7.8	41.0
TRAP-6 150 μ M	98	NT	NT	12.8	51.3
After transfusion, gating on CD41 ⁺					
Without TRAP-6	-	-	-	19.7	146.7
TRAP-6 50 μ M	-	-	-	38.7	199.3
TRAP-6 150 μ M	-	-	-	91.5	215.7

MFI, mean fluorescence intensity; NT, not tested; TRAP-6, thrombin receptor activator peptide 6.

activation: MFI = 313; 82% P-selectin positive). Only by using the alternate staining protocol did TRAP-6-induced PAC-1 expression become clearly discernible on the transfused platelets (CD41⁺ CD42a⁺, Table 1, Fig. 1a), while PAC-1 did not bind to CD41⁻ CD42a⁺ cells. CD41⁺ cells were not detectable another day later.

These findings were corroborated by TEG. The Δ MA, as a measure of platelet-dependent clot strength, was zero before transfusion but significantly increased after transfusion (Fig. 1b). Other TEG parameters were not responsive to transfused platelets. Also in agreement with the findings by flow cytometry, the Δ MA was low again on day 2.

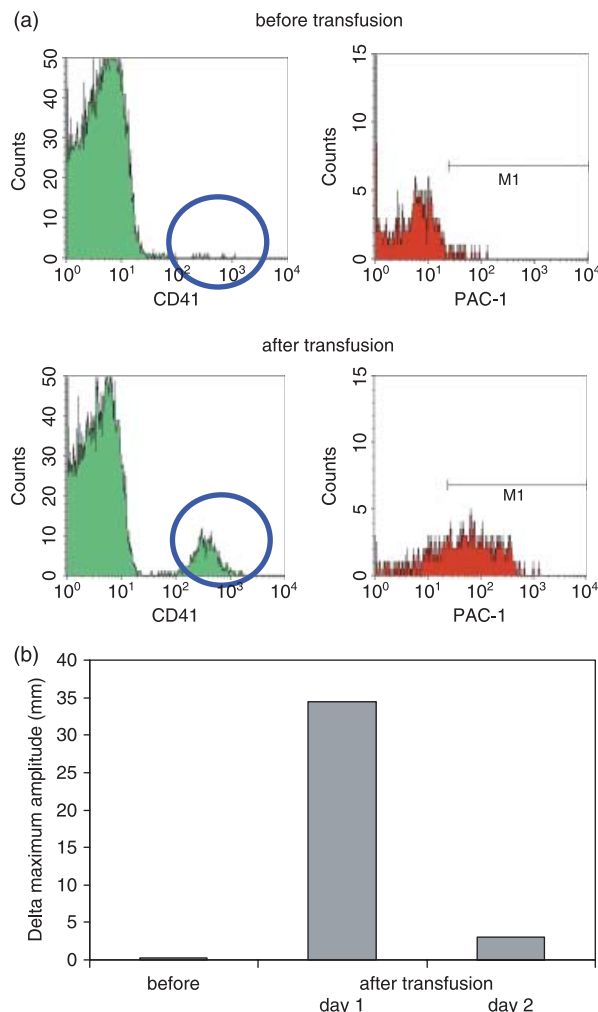


Fig. 1 (a) Detection of CD41⁺ platelets by gating on CD42a-positive events before and after platelet transfusion. By further gating on the CD42a⁺ CD41⁺ cells, we estimated their capability to bind the monoclonal antibody (mAb), PAC-1, after activation with thrombin receptor activator peptide 6 (TRAP-6). (b) Thrombelastography (TEG), before and after platelet transfusion. Determination of the Δ maximum amplitude (MA) (i.e. total MA – MA after incubation with abciximab) was performed as described in the Materials and methods.

Discussion

The identification of transfused platelets by their GPIIb/IIIa expression enabled them to be followed, although their number was small compared with the patient's autologous platelets. In a previous report, about 40% of the total platelet population was estimated to come from the transfusion [6]. Our patient received 4.2×10^{11} platelets, which was expected to raise the platelet count by 25–30%. Unfortunately we did not measure the platelet count immediately after the transfusion, and the assessments of platelet function by flow cytometry and TEG were only performed on the next day. We therefore may have missed the peak of improvement. Surgery and the presence of platelet antibodies may be responsible for the low number of donor platelets on the next day and the failure to detect these by flow cytometry or the modified TEG 2 days later, when the isoantibodies were at a higher titre than before. The GPIIb/IIIa isoantibodies seemed to have been absorbed by the transfused platelets as they were transiently undetectable, but then boosted.

Because of the wax and wane of platelet isoantibodies, it was necessary to evaluate not only survival but also competence of the fibrinogen receptor of the transfused platelets. By gating on these cells, we demonstrated specifically their TRAP-6-induced PAC-1 and fibrinogen binding. Likewise, we were able to estimate the contribution of the transfused platelets to clot formation by TEG by blocking GPIIb/IIIa with abciximab. In correspondence with our *in vitro* findings, the patient underwent an uneventful operation.

In summary, we demonstrate successful monitoring of survival and function of transfused platelets in a GT patient with isoantibodies.

References

- 1 Nurden AT: Qualitative disorders of platelets and megakaryocytes. *J Thromb Haemost* 2005; 3:1773–1782
- 2 White GC II: Congenital and acquired platelet disorders: current dilemmas and treatment strategies. *Semin Hematol* 2006; 43:S37–S41
- 3 Coppola A, Tufano A, Cimino E, Agangi A, Maruotti GM, Martinelli P, Di Minno G: Recombinant factor VIIa in a patient with Glanzmann's thrombasthenia undergoing gynecological surgery: open issues in light of successful treatment. *Thromb Haemost* 2004; 92:1450–1452
- 4 Laurian Y, Tisseron-Maury B, Bibi Triki T, Kaplan C, Gaudelus J: Red blood cell transfusion in patients with type 1 Glanzmann's thrombasthenia. *J Thromb Haemost* 2005; 3:2346–2347
- 5 Panzer S, Hocker L, Koren D: Agonists-induced platelet activation varies considerably in healthy male individuals: studies by flow cytometry. *Ann Hematol* 2006; 85:121–125
- 6 Nurden A, Combrie R, Nurden P: Detection of transfused platelets in a patient with Glanzmann thrombasthenia. *Thromb Haemost* 2002; 87:543–544
- 7 Monte S, Lyons G: Peripartum management of a patient with

- Glanzmann's thrombasthenia using Thrombelastograph^R. *Br J Anaesth* 2002; **88**:734–738
- 8 Kettner SC, Panzer OP, Kozek SA, Seibt FA, Stoiser B, Kofler J, Locker GJ, Zimpfer M: Use of abciximab-modified thrombelastography in patients undergoing cardiac surgery. *Anesth Analg* 1999; **89**:580–584
- 9 Mandelbaum M, Koren D, Eichelberger B, Auerbach L, Panzer S: Frequencies of maternal platelet alloantibodies and autoantibodies in suspected fetal/neonatal alloimmune thrombocytopenia, with emphasis on human platelet antigen-15 alloimmunization. *Vox Sang* 2005; **89**:39–43