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Folman, C.C.

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Chapter 3

Analysis of the Kinetics of Thrombopoietin Uptake During Platelet Transfusion
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Claudia C Folman¹,², Shreyas CM de Jong¹, Masja de Haas², Albert EGKr von dem Borne¹,²

¹ Division of Internal Medicine and Dept. of Haematology, Academic Medical Centre, Amsterdam the Netherlands
² Dept. of Experimental Immunohaematology, CLB and the Laboratory of Experimental and Clinical Immunology, Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands

ABSTRACT

It has been demonstrated in several studies that platelets play a role in the removal of thrombopoietin (Tpo) from the circulation. For instance, in vitro studies have shown that platelets can bind and internalise Tpo, and transfusion studies have shown that the concentration of circulating Tpo decreases after platelet transfusion. In the current study, the in vivo kinetics of plasma Tpo levels and Tpo uptake by infused platelets was analysed in more detail. Serial blood samples from patients who received a platelet transfusion were analysed with respect to platelet count, plasma Tpo concentration and Tpo content per platelet. In addition, the capacity of infused platelets to bind Tpo in vitro was assessed.

Platelet counts increased directly after transfusion and subsequently started to decrease. Conversely, Tpo levels significantly decreased but were back at baseline level 44 hours after transfusion. Platelet count and plasma Tpo concentration were inversely correlated (r_p=-0.9; p<0.05). The decrease in Tpo concentration upon transfusion was accompanied by a significant increase in the platelet-associated Tpo concentration, providing evidence that platelets are indeed responsible for the clearance of Tpo from the circulation. After transfusion, platelets isolated from the patient still displayed functional Tpo receptors as indicated by their intact capacity to bind Tpo in vitro.

The current study shows that, also in vivo, platelets can bind and may degrade Tpo upon platelet transfusion.
INTRODUCTION

Tpo is the main regulator of thrombopoiesis. It is well established that platelets play a central role in the clearance of thrombopoietin (Tpo), from the circulation. Platelets express the Tpo receptor, Mpl, on their membrane via which they bind and internalise Tpo [1-5]. During chemotherapy, platelet counts and Tpo levels are inversely related, supporting the concept that platelets bind Tpo [6-15]. More direct evidence has been gathered from transfusion studies. In 1995, Kuter and Rosenberg showed that transfusion of platelets in thrombocytopenic rabbits with high plasma Tpo levels resulted in a decrease in the circulating Tpo concentration [16]. Similar findings were reported in mouse models [4]. In analogy, we and others have shown that also in humans with thrombocytopenia, the elevated plasma Tpo concentration decreases upon platelet transfusion [12,17,18]. In vitro studies, in which radio-labeled Tpo was presented to human platelets, have demonstrated that platelets did indeed bind Tpo. Upon binding, Tpo was internalised and degradation occurred [4,5]. Recently, direct in vivo evidence for the specificity and the role of the Tpo receptors in Tpo uptake by platelets was presented by Scheding and coworkers [18]. Thrombocytopenic patients who received a transfusion with unmanipulated or washed platelets showed a decrease in plasma Tpo concentration, whereas transfusion of recombinant Tpo-saturated platelets did not result in this decrease.

Although in all transfusion studies a decrement in plasma Tpo level was noted, the follow-up period was short and no direct evidence was provided showing that the content of Tpo in the transfused platelets increased in a Tpo-rich environment. Recently, we developed a method to measure the Tpo content of platelets. When platelets are lysed in the presence of a Tpo-receptor-agonist such as a Tpo-peptide mimetic, endogenous Tpo, both free and receptor bound, is released from the platelets [19].

The aim of the current study was to analyse the in vivo kinetics of plasma Tpo levels and Tpo uptake by infused platelets. Serial blood samples were taken from patients before and after they had received a platelet transfusion. In addition, the capacity of Tpo uptake in vitro by platelets already challenged with high Tpo levels in the patient in vivo, was analysed.

MATERIALS AND METHODS

Patient characteristics

After informed consent, 7 males and 1 female were enrolled in the study. Their ages ranged from 24 to 75 years. All patients were haemato-oncological patients with persistent thrombocytopenia after chemotherapy, with or without peripheral blood stem cell transplantation. All patients received 5 platelet units (300x10^9 platelets in 270 ml) administered within 30 to 90 minutes. For one patient, the administration took four hours due to a malfunctioning catheter. Citrate-anticoagulated blood was drawn at the following time points: immediately before platelet transfusion and 10 min, 1 hour, 3 hours, 20 hours and 44 hours after completion of the platelet transfusion.
**Blood processing**

Upon collection, blood was centrifuged at 850 g for 20 min. without braking. Platelet-rich plasma was collected and centrifuged at 1700g for 10 min. Plasma was isolated and stored for the analysis of the Tpo concentration. The platelet pellet was washed with a wash buffer (36 mM citric acid, 103 mM NaCl, 5 mM KCl, 5 mM EDTA, 5.6 mM D-glucose and 0.35% (w/v) bovine serum albumin; pH 6.5) to which 100 nM prostaglandin-E1 had been added to prevent platelet activation. Platelet number was counted (Technicon H3 RTXtm System; Bayer, Tarrytown, NY, USA) and platelets (on average 38 ± 27 x 10⁶) were resuspended in 100 μl of lysis buffer (1% (v/v) Nonidet P40 (NP40), 150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA) to which 10 ng/ml AF13948 [20], a Tpo-mimetic peptide, was added. Samples were left on ice for 30 minutes after which they were frozen at -20°C. Before determination of the Tpo concentration, platelet debris was removed by centrifugation.

**Analysis of Tpo uptake by platelets in vitro**

Platelets were isolated from 10 ml of citrate anticoagulated blood drawn at 20 hours after platelet transfusion. Subsequently, these platelets were incubated for 90 minutes at room temperature with 500 μl of plasma from the same patient, which had been harvested before platelet transfusion. After incubation, plasma was isolated and the platelets were lysed as described above.

**Tpo ELISA**

A previously described solid-phase sandwich ELISA was used to measure the Tpo concentrations in plasma and lysates [12]. For measurement of lysates, NP40 was added to the standards to obtain identical final NP40 concentrations in the standards and the samples. NP40 is known to enhance the ELISA signal in our assay (unpublished data). Normal plasma Tpo levels, as determined in 193 healthy individuals, ranged from 4-32 [2.5th-97.5th percentile] Arbitrary Units/ml (AU/ml). One A.U. equals 3.2 pg of Tpo when calibrated against rHuMGDF (MGDF-A), the full-length rHuTpo molecule, which was a generous gift from Amgen. When calibrated against the rhTpo standard from Research Diagnostics Inc. (Flanders, NJ, USA) 1 A.U. equals 9 pg of Tpo.

**Statistics**

The software package SPSS for windows, release 7.5 (SPSS Inc.Chicago, IL, USA) was used for statistical analysis. A p-value <0.05 was considered significant.

**RESULTS**

**Plasma Tpo concentration and platelet count**

Figure 1 shows the mean plasma Tpo concentration and the platelet counts before and at the various time points after platelet transfusion. Before transfusion, plasma Tpo levels were highly elevated in 6 out of seven patients. Only one patient, with acute myeloid leukaemia M2 that had progressed from myelodysplastic syndrome, had a plasma Tpo concentration within
Figure 1: Follow-up of platelet counts (A) and plasma Tpo levels (B) upon transfusion. Seven patients were evaluated. Mean and standard deviation of at least 5 measurements are shown. Asterisks indicate a significant difference compared to pretransfusion values. Paired T-test: p<0.05 (*); p<0.01 (**) 

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the normal range. Upon transfusion, a significant increment in platelet counts was seen. Subsequently, platelet counts declined again and at 44 hours, platelet counts were only slightly, but still significantly, above baseline level. The mean plasma Tpo levels of all patients inversely correlated with mean platelet counts in time ($r_p=-0.9$, $p<0.05$). Immediately following transfusion, plasma Tpo levels decreased significantly. Thereafter, no further decrease was noted. The average decrement at $t=10$ min. was $23 \pm 4\%$ (mean $\pm$ SD). No correlation was found between the extent of the change in platelet count versus the extent of the change in Tpo concentration. After 44 hours, plasma Tpo levels had returned to baseline values.

**Platelet-associated Tpo**

Previously, we have demonstrated that platelet-associated Tpo, both receptor bound and non-receptor bound, is released when platelets are disrupted in the presence of a Tpo-mimetic peptide [19]. This method was used to determine the Tpo content of platelets after transfusion. Figure 2 shows the follow-up of the Tpo content per $10^6$ platelets. Compared with donor platelets, the Tpo content of the patients’ own platelets was significantly elevated. Upon transfusion, the platelet Tpo content rose significantly, indicating that the donor platelets had
Tpo kinetics after platelet transfusion

Figure 2: Follow-up of the amount of platelet-associated Tpo upon platelet transfusion. Seven patients were evaluated. Mean and standard deviation of at least 5 measurements are shown. Asterisks indicate a significant difference compared to the Tpo content of the platelet donors. Paired T-test: p<0.05 (*).

bound, and possibly internalised, Tpo. The measurement at one hour post transfusion showed the highest Tpo content (fig 2). Thereafter, a decline was noted. At 44 hrs, platelet Tpo content was still significantly elevated compared to the baseline value of the donor platelets.

In vitro uptake of Tpo by platelets isolated from the patient
To investigate whether platelets that have been challenged with high Tpo levels in vivo in patients, still have the capacity to bind exogenously added Tpo, blood was drawn 20 hours after transfusion. Platelets were isolated, washed and divided into two samples. One platelet sample was lysed directly. The other sample was incubated in pre-transfusion plasma from the same patient. Figure 3 shows for five patients, the plasma Tpo level and the Tpo content per 10^6 platelets directly after isolation and after plasma incubation. Plasma Tpo levels declined in four out of five experiments (fig 3a). However, this did not reach statistical significance. The Tpo content of the incubated platelets increased significantly (fig 3b). On average, Tpo content increased 2.0 fold (±0.9).

DISCUSSION
In the current study it was shown, in accordance with previous findings, that upon platelet transfusion, the plasma Tpo concentration declines [16-18]. Similar as in patients undergoing chemotherapy, who have fluctuating platelet counts, an inverse correlation was found between platelet count and Tpo concentration. However, the extent of the change in platelet count did not correlate with the change in plasma Tpo concentration. A similar finding was reported by Moller and coworkers [17]. The inverse relationship they found between the change in Tpo level and the corrected count platelet increment (CCI) only reached borderline significance. Possibly, the apparent lack of a strong relationship is due to differences in the
transfused product, i.e. the inter-individual variation in receptor expression/occupancy of the donor material, storage time of the platelet concentrates, etc.

All patients, except one, had highly elevated plasma Tpo concentrations, which was in agreement with their haematological status. All patients with elevated Tpo levels suffered from an impaired megakaryocytopenia as a result of malignant bone marrow infiltration and/or chemotherapy. An impaired platelet production results in a diminished clearance of Tpo, which in turn leads to Tpo accumulation. The one patient with a normal Tpo concentration suffered from acute myeloid leukaemia (AML), which had progressed from myelodysplastic syndrome. Possibly, the Tpo levels in this patient were low due to clearance of Tpo by Mpl expressing malignant blast cells. It has been reported that blast cells in 50% of patients with AML express c-Mpl mRNA [21].

In agreement with in vitro experiments showing that platelets can bind and internalise Tpo [3-5], the concentration of platelet-associated Tpo increased in the donor platelets upon exposure to the high Tpo levels in vivo. Before transfusion, the Tpo content of donor and patient platelets was different with the Tpo content of the patient platelets being higher. This may be explained by a higher Tpo uptake per platelet, resulting from the continuous exposure to high Tpo levels in vivo. The increase in platelet-associated Tpo upon transfusion showed that the
donor platelets still had the capacity to bind Tpo. Indeed, after transfusion, the platelet Tpo content had already increased 10 minutes after infusion. Subsequently, a decrease in Tpo content was noted and after 3 hours post transfusion the Tpo content seemed to remain stable. The decrease in and stabilisation of platelet-associated Tpo concentration suggest that Tpo can be degraded in the platelets and that a balance between Tpo uptake and degradation is reached in time. Alternatively, selection of the circulating platelets may have occurred, i.e. platelets with a high Tpo content may have been cleared from the circulation more rapidly.

In order to examine whether the transfused and Tpo-exposed platelets still expressed intact and functional Tpo receptors on their surface, platelets were isolated 20 hours post transfusion, and were challenged in vitro with pre-treatment plasma. In vitro, platelets still bound Tpo, since the amount of platelet-associated Tpo increased after incubation in plasma. The plasma Tpo levels only slightly decreased. Similar to the in vivo data, there was no relation between the extent of the decrease in plasma Tpo concentration and the extent of the rise in Tpo content per platelet. The in vitro experiment showed that platelets, isolated from the patient, still expressed intact Tpo receptors capable of binding Tpo. However, in vivo, these platelets did not seem to increase their Tpo uptake. Instead, it seemed that an equilibrium between the concentration of circulating Tpo and the platelet-mediated Tpo uptake and degradation was reached. Possibly, the increased Tpo uptake in vitro was caused by manipulation of the platelets during the experimental procedure (e.g. extra wash step).

In conclusion, the current study showed that, in vivo, platelets can bind and internalise and also may degrade Tpo upon platelet transfusion.

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REFERENCES


