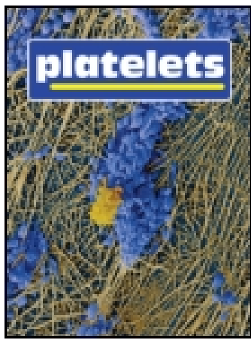

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ORIGINAL ARTICLE

Clearance of platelet microparticles in vivo

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Abstract
At present, little is known about the clearance of platelet-derived microparticles (PMP) in human blood, as due to ethical considerations infusion experiments with labeled microparticles are delicate. Therefore, we investigated the kinetics of PMP, which are abundantly present in apheresis platelet concentrates (PC), following platelet transfusion in severe thrombocytopenic patients ($n = 11$). PMP were double-stained with annexin V and cell-specific antibodies (anti-CD61, anti-CD63 or anti-CD62P, respectively) and detected by flow cytometry before and after transfusion of a single PC at fixed time intervals. Upon transfusion, the plasma levels of MP binding annexin V (2.5-fold), PMP (CD61+; 2.9-fold), and PMP from activated platelets (CD63+; 1.9-fold) or P-selectin (2.5-fold) increased immediately. The plasma levels of MP decreased with a half life of 5.8 hours (annexin V; 95% CI: 1.8–18.3) and 5.3 hours (CD61; 95% CI: 2.0–14.2). This is the first report in which the half life time of transfused PMP has been investigated in humans.

Keywords: *Microparticle, platelet transfusion, platelet concentrate, half-life time*

Introduction

Microparticles (MP) are submicron cellular membrane vesicles, sized between 0.1 and 1.0 μm present in the blood. They are highly thrombogenic [1], presenting a phospholipid surface with binding sites for coagulation factors [2–5]. Besides their role in coagulation, MP are involved in the transport and transfer of bioactive molecules, cell activation, and inflammatory processes [6–9]. Concentrations of MP are elevated in various pathological circumstances including vascular diseases, diabetes, severe trauma and several types of cancer [10–16].

At present, little is known about the clearance of microparticles. Rand et al. [17] demonstrated a rapid disappearance of infused biotinylated platelet-derived microparticles (PMP), generated from calcium ionophore-treated platelets, within minutes in a rabbit model. The half-life-time of PMP and their disappearance in humans, however, has never been analysed before.

Due to ethical considerations, infusion of biotin labeled microparticles in humans is problematic. Recently, high levels of platelet-derived MPs (PMP) in human apheresis platelet concentrates (PC) were described [18]. Since transfusion of PC is a routine method to prevent bleeding in severely thrombocytopenia, we compared the kinetics of transfused PMP and platelets in patients following platelet transfusion.

Methods

Study sample

In this study, 11 patients suffering from various haematological diseases were investigated (see Table I). All patients were in the aplastic period of hematopoietic stem cell transplantation and needed platelet transfusion for clinical reasons, as they were thrombocytopenic with platelet counts $<20 \times 10E9/l$. All patients were negative for HIV, hepatitis B and C, had no acute infection, and no

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Table I. Characteristics of the patients and the platelet concentrates under study.

Patient			*Platelet concentrate*		
Age (years)	*Gender*	*Diagnosis*	*Storage time (days)*	*Platelet count (×10E9/l)*	*Volume (ml)*
18	Female	Severe aplastic anemia	5	1213	239
43	Female	Acute lymphatic leukemia	5	1374	262
47	Male	Acute myeloic leukemia	5	1260	246
48	Male	Osteomyelofibrosis	5	1167	257
48	Female	Chronic myeloic leukemia	5	859	256
35	Male	Acute myeloic leukemia	4	1163	249
37	Female	Acute myeloic leukemia	4	1128	257
41	Male	Acute lymphatic leukemia	4	984	254
54	Female	Multiple myeloma	1	1102	245
59	Male	Acute myeloic leukemia	1	1218	238
68	Male	Acute myeloic leukemia	1	1185	253

history of platelet transfusion refractoriness. The Human Investigation Review Board of the Ludwig-Maximilians-University Munich approved the study. Signed informed consent was obtained from all patients.

Platelet concentrates

In all patients, transfusion of platelet concentrates (no HLA-match) was routinely performed when the platelet count was below 20×10^9/l. The PC were obtained from the peripheral blood of single donors with a Trima™ Accel® (Software 5.1, CaridianBCT; Lakewood, CO, USA) and stored at 22°C ± 2 on a shaker for a median time of 4 (1–5) days. The PC was given via a central line over a period of 15 minutes.

Blood sampling and processing

Blood samples were collected through a central venous catheter before receiving the PC, immediately after transfusion (0 h), 1 hour after transfusion, and 3 hours after transfusion. For MP analysis, platelet-poor plasma was prepared within 15 minutes after blood collection by centrifugation at 1550 g for 20 minutes. The plasma was snapfrozen in liquid nitrogen for 15 minutes and stored at −80°C until assayed.

Materials

Fluorescein isothiocyanate (FITC)-labelled annexin V (IQP-120F), phycoerythrin (PE)-labelled annexin V (IQP-120R), IgG_1-PE (IQP-191R) and IgG_1-FITC (A07795) were from Immuno Quality Products (Groningen, The Netherlands). Anti-CD61-PE (555754) was purchased from BD Biosciences (Heidelberg, Germany), and anti-P-selectin-PE (CD62p; PN IM 1759) and anti-CD63-PE (PN IM1914U) were from Immunotech (Marseille, France). All antibodies and annexin V were diluted with phosphate-buffered saline (PBS; 154 mmol/L NaCl, 1.4 mmol/L phosphate, pH 7.4). Final dilutions (v/v) used for labeling of MP 1 : 100 (annexin V-FITC 1 : 100, CD61-PE and anti-P-selectin-PE), 1 : 200 (annexin V-PE) and 1 : 20 (CD63-PE).

Isolation of PMP

Isolation and identification of MP was performed as described earlier [1]. In brief, frozen plasma (250 μl) was slowly thawed on melting ice for 1 hour. After centrifugation at 17,570 × g and 20°C for 30 minutes, 225 μl of MP-free supernatant was removed. The remaining MP pellet was diluted with 225 μl of PBS containing 10.9 mmol/l trisodium citrate (PBS/citrate buffer), resuspended and centrifuged again for 30 minutes at 17,570 g and 20°C. Subsequently, the MP-free supernatant (225 μl) was removed, 75 μl of PBS/citrate buffer was added to the MP, and the MP was resuspended. Five microliters of the MP suspension was diluted in 35 μl $CaCl_2$ (2.5 mmol/L) containing PBS. For MP staining, 5 μl PE-labeled annexin V was added plus 5 μl of a cell-specific monoclonal antibody (anti-CD61, anti-CD62P or anti-CD63, respectively) or isotype-matched control antibody. Samples were incubated in the dark for 15 minutes at room temperature. The reaction in all samples was stopped with 900 μl calcium buffer (2.5 mmol/l), except the annexin V control, to which citrate-containing PBS (900 μl) was added.

Flow cytometry

MP were analysed in a FACScan flow cytometer (Becton Dickinson) using Cell Quest Software (Becton Dickinson; San Jose, CA, USA). Forward scatter (FSC) and side scatter (SSC) were set at a

logarithmic gain. MP were identified on basis of their size and density and their ability to bind a cell-specific monoclonal antibody and annexin V. Cell-specific labeling with monoclonal antibodies was corrected for identical concentrations of isotype-matched control antibodies and annexin V measurements were corrected for autofluorescence. The concentration of MP/l plasma was estimated according to Berckmans [19].

Volume of distribution

The volume of distribution (V_d) was determined by using the following formula:

Vd (l) = (platelet count of the PC (×10E9/l) × PC volume (l))/(platelet count of the patient immediately after transfusion (×10E9/l)/platelet count of the patient immediately before transfusion (×10E9/l)).

The circulating plasma volume was estimated by assuming 44 (33–54) ml/kg body weight. This resulted in a median plasma volume of 3.1 l (2.2–3.8).

Statistical analysis

Results are reported as median (interquartile range), except when stated otherwise. If percentages are given, these were analysed for every sample before calculating the median. Independent variables were analysed by the Mann Whitney-U-test and Fishers's exact test, dependent variables by the Wilcoxon test. Time trends were modeled by means of linear mixed models, using the natural logarithm of the measurements as dependent variable and specifying a random intercept for each patient. Half life was estimated from the slopes b as (log 0.5)/b. Limits for the 95% CI of the half life time were estimated based on the standard errors of the slopes using the delta method. Statistical analysis was performed with the Statistical Package for the Social Sciences SPSS for Windows 17.0 (SPSS Inc., Chicago, IL, USA) and the Statistical Analysis System SAS for Windows 9.2 (SAS Institute, Cary, NC, USA). All p-values are given as two-sided values, a p-value below 0.05 being regarded as significant. Due to the exploratory nature of our analyses no adjustment for multiple testing was undertaken.

Results

Platelet concentrates

The PMP levels in the PCs were several fold higher than the MP level in the plasma collected from patients immediately before transfusion (annexin V-positive MP: 8751 × 10E6/l (6563–14304) and 691 × 10E6/l (405–796); PMP (CD61+: 8342 × 10E6/l (5761–13531)) and 490 × 10E6/l (379–647); P-selectin-exposing PMP: 398 × 10E6/l (263–830) vs. 21 × 10E6/l (5–46) and CD63-exposing MP: 258 × 10E6/l (139–350) vs. 37 × 10E6/l (13–51); all $p < 0.05$).

The fraction of annexin V-binding MP differed between PCs and patients before transfusion. In the PC 93% (90–95) of the annexin V-positive MPs were CD61+, i.e. PMP, whereas this fraction was 70% (56–90; $p < 0.05$) in the patient plasma before transfusion. The percentage of PMP exposing P-selectin was equal in the PC (4.8% (3.2–7.7)) than in the patient plasma (3.9% (2.0–9.1); n.s.), whereas the percentage of CD63-exposing PMP was slightly higher (2.6% (2.0–4.0)) vs. 7.6 (6.1–9.2); $p < 0.05$). Taken together, the concentration of PMP was higher in the PC than in the peripheral plasma of the patients before transfusion, but no signs of apparent platelet activation were present in either PC or patients.

Patients after transfusion

After transfusion, all patients showed an increase of MP binding annexin V, as of MP exposing CD61, CD62P or CD63 (see Figure 1). The MP then declined with a half life of 5.8 hours and 5.3 hours for annexin V and CD61 (95% CI: 1.8–18.3 and 95% CI: 2.0–14.2, respectively). In contrast, the platelet count dropped with a half life time of 24.0 hours (95% CI: 3.6–176.3). The half-life time of PMP exposing either P-selectin or CD63 did not differ from that of the CD61-positive MP. The composition of MPs (percentage of annexin V, CD61, CD62P and CD63-exposing MP) did not change for any of the MP subgroups and the percentage of CD62P/CD63-exposing PMP stayed invariant. Three hours after transfusion, the level of PMP was significantly lower than the platelet count (Figure 2). Thus, the clearance or removal of platelets from the blood after transfusion of the PC was slower than the clearance or removal of PMPs.

Volume of distribution

The V_d did not differ between the tested subgroups of PMP (CD61: 10 l (3–45); CD63: 2.6 l (0.6–10.2); P-selectin: 4.8 l (2.2–45.8); CD62E: 3.8 l (0–19)). The V_d for all annexin V-positive MP was 9 l (4.5–42). The V_d of all annexin V-positive MP and of the MP-subgroups equalled the estimated circulating plasma volume of the patients (3.1 l (2.2–3.8)).

Discussion

This study describes the kinetics of infused PMP in humans. We observed an immediate increase in circulating PMP in patients after transfusion, which is explained by the infused PMP that were present in the PC before transfusion. Our present data exclude a

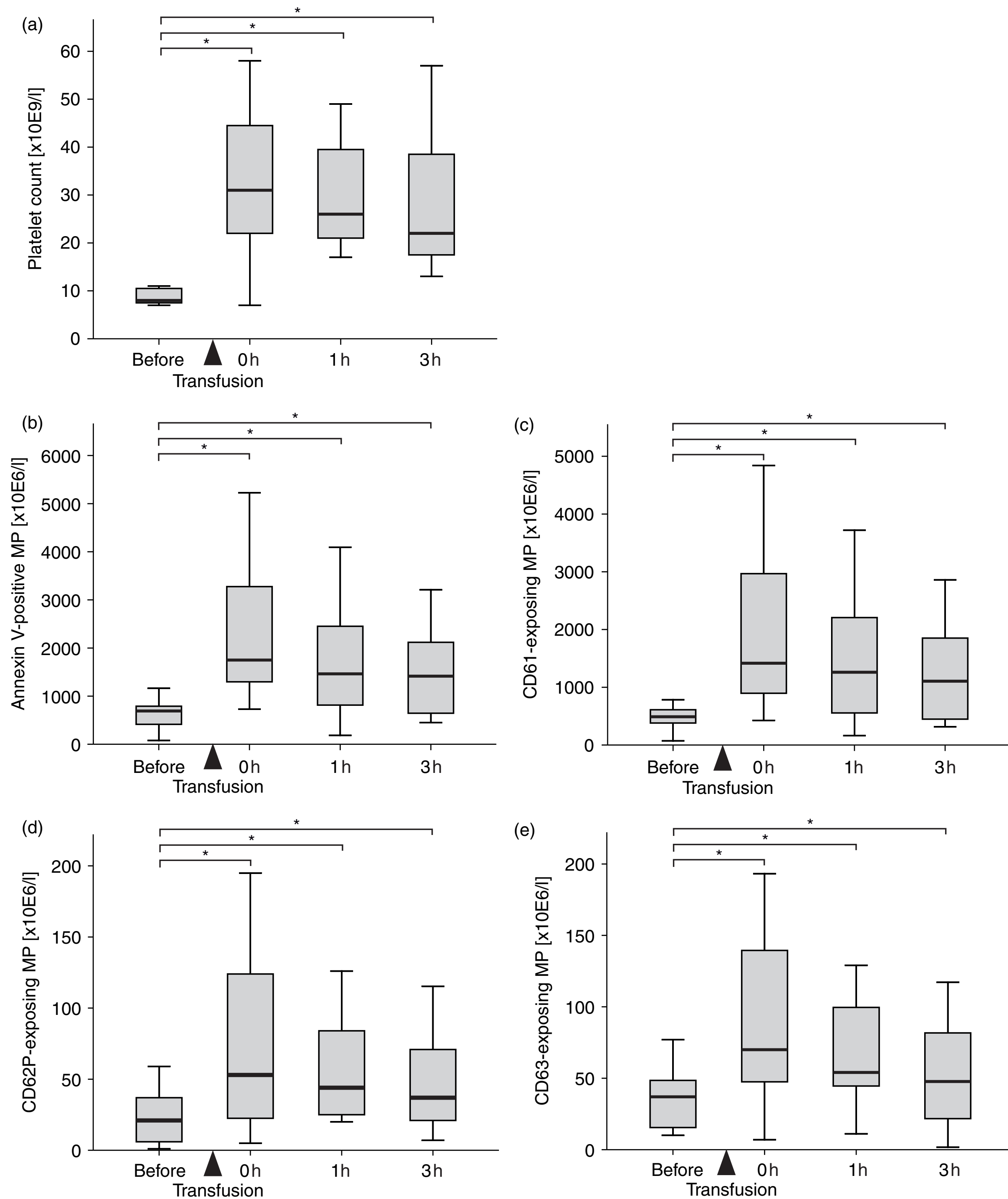

Figure 1. Platelet transfusion increases the plasma levels of platelets (a), annexin V-positive MP (b), CD61-exposing platelet-derived MP (PMP) (c), CD62P-exposing PMP (d) and CD63-exposing PMP (e) in patients immediately after transfusion (0 h) with a continuous decay afterwards. $* = p < 0.05$.

contribution of de novo generation of PMP immediately after transfusion due to platelet activation. Since the volume of distribution of the annexin V-binding MP, the PMP (CD61+) and the PMP from activated platelets (CD62P+ or CD63+) equalled the circulating plasma volume and the percentage of CD62P/CD63-positive PMP did not change after transfusion, a secondary effect of transfusion such as activation of infused or circulating platelets seems unlikely.

The clearance of platelets in our study was in accordance with former studies. Our data show that the disappearance of annexin V-binding MP or PMP

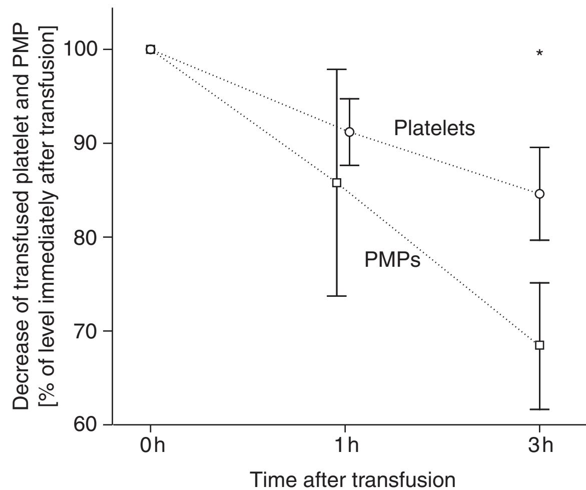

Figure 2. After transfusion, disappearance of platelets and PMP show different kinetics. The numbers give the percentage (median; interquartile range) of the start level immediately after transfusion (0 h). *= $p < 0.05$ between groups.

(CD61+) was faster than disappearance of platelets from the circulation. This difference in kinetics in removal from the circulation suggests that clearance of PMP and platelets may occur via different mechanisms in vivo. To date, very little knowledge is available on the underlying mechanism with regard to the clearance of MP from the circulation. It has been proposed that macrophages remove MP by phagocytosis since disappearance of bacteria by macrophages shows similar kinetics [20]. For erythrocyte-derived MP, Willenkens et al. [21] suspected a role for Kupfer cells from the liver and the mononuclear phagocytes system in the clearance of MP. Whether or not these cell types play a role in the removal of PMP from the circulation remains to be investigated. As the leucocyte count is nearly 0 in our patients and the platelet count is quite low, this may have influenced our data resulting in a prolongation of the half life time of MP. Recently, however, lactadherin was shown to be the central mediator for the clearance of PMP [22], which largely excludes an important impact of either leucocytes or platelets in the removal of PMP from the circulation. Alternatively, MP, especially those exposing cell-specific adhesion receptors such as P-selectin or their ligands, may adhere to their counter ligands or receptors exposed on the plasma membranes of other cells that are present within the blood or to endothelial cells

The half life time of MP in our study is markedly longer than that observed by Rand et al. in rabbits [17]. Our data, however, are difficult to compare to their data, since they infused in vitro activated platelets in rabbits, which most likely exposed ligands for adhesion receptors and therefore may have been more susceptible to clearance. With regard to our data, only 4% of the PMP exposed either P-selectin or CD63, illustrating that only a minor fraction of PMP originates from activated platelets. The half life time of all MP, PMP without and PMP with activation markers were comparable in our study. However, due to ethical limitations the observation time was rather short, and we cannot completely exclude differences in the kinetics of different MP subgroups.

From our present data we cannot exclude that transfused platelets continuously produce low level PMP. In addition, there might be a concern that our data could miss rapid initial clearance of microparticles, which is followed by a slower clearance of residual microparticles. If so, the half life time of PMP as measured in our present study may be overestimated, and the real half life time of PMP may be shorter in vivo. In addition, the general conclusion is limited as the clearance of transfused platelets and microparticles is analysed in patients with severe thrombocytopenia, which might relevantly differ from the clearance of circulating platelets and microparticles in individuals with physiological platelet counts. However, in spite of these methodical concerns, which are due to the ethical limitations in humans, our data are useful, as they for the first time give an upper limit of the MP half life time in humans.

To which extent PMP in vivo originate from platelets, megakaryocytes or both, is obscure. Our data may give some new insights, since our observation that PMP disappear faster from the circulation than platelets excludes that PMP are only produced by the transfused platelets in vivo. To which extent the behavior of transfused and non-transfused platelets is comparable in this aspect is unknown, but we suspect that our present findings may also occur under physiological conditions. As MP released from residual megakaryocytes cannot be safely distinguished from PMP [23], we suspect that megakaryocytes in healthy humans may produce at least a part of "platelet-derived" MP. The latter source, however, is absent in our patients due to the severe stem cell toxic conditioning therapy, which all of our patients had undergone before transfusion of platelets and PMP. Indeed, in mice, MP exposing CD61 were shown to originate mainly from megakaryocytes, whereas CD62P-positive PMP originated from platelets [24]. This may also be true in humans.

In conclusion, our data show the half life time of PMP after platelet transfusion and, more indirectly, point to a role for megakaryocytes in production of "PMP" in humans.

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Declaration of interests: The authors report no declarations of interest.

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