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Cellular origin of platelet-derived microparticles in vivo

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ABSTRACT

Introduction: Microparticles (MP), presumably of platelet origin, are the most abundant microparticles in blood. To which extent such MP may also directly originate from megakaryocytes, however, is unknown. During hematopoietic stem cell transplantation, patients undergo total body irradiation which leads to an irreversible destruction of hematopoiesis.
Material and Methods: We studied the levels of "platelet-derived" MP (PMP) in 13 patients before and after total body irradiation with 12 Gy (4 Gy for 3 days, dose rate 4.5 cGy/min). PMP were isolated and double-stained with annexin V and anti-CD61. In 6 patients, we additionally analyzed MP exposing P-selectin or CD63.
Results: PMP rapidly declined upon total body irradiation, which was 2.4-fold faster than platelet disappearance. In contrast, the kinetics of MP exposing P-selectin or CD63 was comparable to platelets.
Conclusions: Since CD61-positive MP disappear faster than platelets or MP exposing P-selectin or CD63, our data indicate that MP exposing P-selectin or CD63 are likely to originate from platelets, whereas at least a major fraction of CD61-exposing MP is likely to originate from megakaryocytes in vivo.

Introduction

Microparticles (MP) are cell-derived membrane vesicles released from the surface of a broad variety of cells such as platelets, erythrocytes, leukocytes and endothelial cells [1]. They circulate in blood and are involved in physiological functions associated with inflammation and coagulation [2]. Microparticles exposing typical platelet integrins or receptors such as integrin (or glycoprotein, GP) IIB-IIIa or GPIb represent about 90% of the microparticles in peripheral blood. They are thrombogenic [3–8] and are involved in transport and transfer of bioactive molecules, cell activation, and inflammatory processes [9–12]. The levels of platelet-derived microparticles (PMP) are elevated in a broad variety of pathologic states such as venous thrombosis [13], rheumatic disease [14], psoriasis [15,16], diabetes mellitus [17], cerebral infarction [18], acute coronary syndrome [19], recurrent spontaneous abortion [20], pre-eclampsia [21], breast cancer [22] and heparin-induced thrombocytopenia [23]. The before mentioned platelet integrins or receptors used to identify PMP are also exposed on megakaryocytes, however, and MP originating from residual megakaryocytes cannot be safely distinguished from PMP [24]. As megakaryocytes release platelets directly into the blood from cytoplasmic processes penetrating through the sinus wall into the sinus lumen [25], this may also result in the concurrent release of megakaryocyte-derived MP into the circulation. Thus, at present it is obscure to which extent the PMP originate from platelets in vivo, megakaryocytes or both.

Recently, in mice the MP exposing P-selectin were shown to originate from platelets, whereas the "PMP" not exposing P-selectin were demonstrated to originate from megakaryocytes [26]. Since the conditioning regimen preceding hematopoietic stem cell transplantation (HSCT) usually includes total body irradiation (TBI) that irreversibly destroys megakaryocytes of the patient, we hypothesized that analysis of circulating PMP and platelets in these patients may provide novel insights into the true cellular origin of circulating platelet-derived MP.

Material and Methods

Study sample

In this study, 13 patients receiving allogeneic hematopoietic stem cell grafts were included. Patients were admitted for a variety of hematological disorders, all of them in clinical remission (Table 1). All

Abbreviations: CD, cluster of differentiation; FITC, fluorescein isothiocyanate; GP, glycoprotein; TBI, total body irradiation; HSCT, hematopoietic stem cell transplantation; LD, lethal dose; MP, microparticle; PBS, phosphate buffered saline; PE, phycoerythrin; PMP, platelet-derived microparticle.

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Table 1
Demographic and transplantation characteristics of the patients under study and controls. If not stated otherwise, the numbers give cases.

	patients
total	13
gender *(male/female)*	9/4
age *(median years (interquartile range))*	33 (23-41)
underlying disease	
chronic myelogenous leukemia	3
acute myeloid leukemia	2
acute lymphoblastic leukemia	8

patients had a conditioning regimen starting with 12 Gy TBI (4 Gray for 3 days, dose rate 4.5 cGy/min) with no other therapy at least one month before start of the study. None of the patients had any bacterial, fungal or viral infection, and no transfusion of platelets or red blood cells within 2 month prior to the beginning and during the course of the study, nor did any have implanted stents, a history of anti-rheumatic medication, or active promyelocytic leukemia. All patients had a central line (Hickman catheter) inserted at least one week prior to the start of the study. Medication included only antiemetic drugs (Lorazepam 1 mg/day and granisetrone 3 mg/day during TBI). All patients had a normal platelet count before start of the study.

The Human Investigation Review Board of the Ludwig-Maximilians-University Munich approved the study. Signed written informed consent was obtained from all patients allowing analysis of all clinical and laboratory data mentioned in this paper.

Blood sampling and processing

Blood samples were collected through a central venous catheter the evening before and after TBI (day 0 and day 3). In a subgroup of 6 patients, additional samples were taken on a daily base (evening day 0 – day 3). 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 snap frozen 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, 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 labelling 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 [3]. In brief, frozen plasma (250 µl) was slowly thawed on melting ice for one hour. After centrifugation at 17.570×*g* and 20 °C for 30 minutes, 225 µl of MP-free supernatant was taken off. 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 µl of the MP suspension was diluted in 35 µl $CaCl_2$ (2.5 mmol/L)-containing PBS. For MP staining, 5 µl PE-labelled annexin V was added plus 5 µl of a cell-specific monoclonal antibody (anti-CD61, CD62P or CD63) 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 analyzed in a FACScan flow cytometer (Becton Dickinson; Heidelberg, Germany) using Cell Quest Software (Becton Dickinson; San Jose, CA). 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 labelling with monoclonal antibodies was corrected for identical concentrations of isotype-matched control antibodies and annexin V measurements were corrected for auto-fluorescence. The concentration of MP/l plasma was estimated according to Berckmans [27].

Statistical analysis

Results are reported as median (interquartile range), except when stated otherwise. Independent variables were analyzed by the Mann Whitney-U-test and Fishers's exact test, dependent variables by the Wilcoxon test. Statistical analysis was performed with the Statistical Package for the Social Sciences SPSS for Windows 17.0 (SPSS Inc., Chicago, IL, 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

Study population

During the course of the study, no platelet or red blood cell transfusion was necessary, nor did any patient develop infection or other complications.

Decay of platelets

Platelet count declined slightly during TBI from 185 G/l before TBI to 168 G/l ($p<0.028$) after 12 Gy (see Fig. 1). If we assume a half life of 7 days for platelets, a level of 90% (4 Gy), 82% (8 Gy) and 74% (12 Gy) is expected, which is significantly lower than observed ($p<0.033$ for 12 Gy), indicating a partially ongoing platelet production by megakaryocytes.

Decay of MP

Irradiation reduced the numbers of circulating CD61-exposing MP from 413.6 G/l before TBI to 10.1 G/l (12 Gy; $p=0.004$). This disappearance was 2.4 fold faster (1.9-9.6; $p=0.028$) than platelets or the PMP exposing P-selectin or CD63 (see Fig. 1). Based on the half life time of CD61-exposing MP in rabbits of 10 minutes (28) and humans up to 5 hours (Rank et al., submitted), a relatively fast disappearance is expected after impairment of their production. As after 12 Gy the platelet count had dropped by only 10% but the level of CD61-exposing MP by 33%, at least 23% of the MP exposing CD61 do not originate from circulating platelets. If we take 50% vital megakaryocytes after 12 Gy (30), megakarycytes as a source may explain 46% of the CD61 + MP level. In contrast, the levels of MP exposing P-selectin decreased slowly and their disappearance or removal paralleled the decay of platelets (see Fig. 1). The CD63-exposing MP showed a modest increase. In contrast to the decay of MP

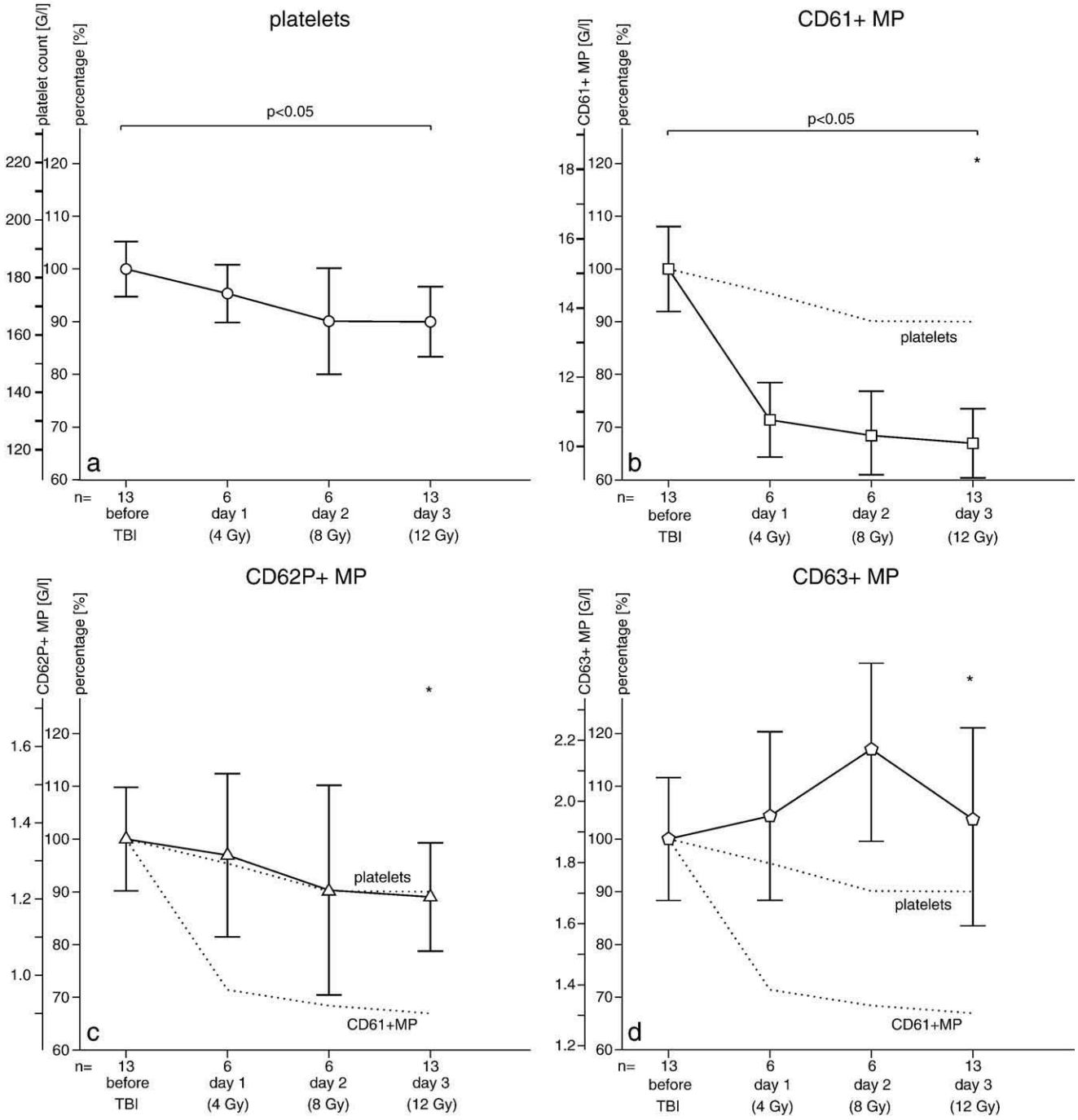

Fig. 1. During 3 days of TBI with a total dose of 12 Gy the platelets decay only slightly (a), whereas CD61 + MP decrease 2.4-fold more rapidly (b). The decay of the CD62P+ (c) and CD63 + MP (d) is similar to that of the platelets. The CD63 + MP seem to rise slightly on day 1 and day 2. However, this increase is not significantly different from the platelet curve. The two Y-axis show both the absolute level and percentage of decay in relation to the start level before TBI. Data are presented as mean ± SD. * = $p<0.05$ to CD61 + MP.

exposing CD61, the changes in both MP exposing P-selectin or CD63 were insignificant (both $p = 0.63$).

Discussion

In this study, we describe differences in the disappearance of PMP from peripheral blood following lethal irradiation. Since TBI destroys megakaryocytes but not platelets, and our present finding that the kinetics of platelet disappearance (slow) and "platelet" MP (fast) differ markedly, we hypothesize that a large fraction of the CD61-exposing MP present in peripheral blood is of non-platelet origin, likely megakaryocytes, whereas the MP exposing P-selectin or CD63 may originate from activated (degranulated) platelets, since in our study these MP showed the same rate of disappearance as platelets. The PMP profile before start of TBI resembles published data and does not differ from disease free subjects.

The MP exposing CD61 decreased 2.4-fold more rapidly than the platelets, indicating a concurrent interruption of the production of both PMP and platelets. Due to the much shorter half-life time of PMP [28; Rank et al, submitted] they are expected to disappear faster than platelets, indicating that these (P)MP may not (only) originate from platelets that are still present within the circulation. Indeed, in mice it was shown that PMP without activation markers originate largely from megakaryocytes [26]. Our present data show that this may also

be true in humans, as in our patients a minimum of 23% of the CD61 + MP are certainly not from platelets.

Due to the experimental limitations in man, however, several uncertainties remain. Following irradiation with 12 Gy, we observed a slow decrease of platelets which is due to impaired thrombopoiesis. The decay of platelets, however, is slower than expected. At an assumed half life time of 7 days, 48 hours after start of TBI a level of 82% is anticipated. The significant higher fraction we observed (90%; $p<0.05$) suggests that after the first TBI the platelet production is not stopped abruptly. A dose between 3 and 5 Gy is thought to be the LD50 for human pluripotent hematopoietic stem cells [29], although more diffentiated megakaryocytes need higher doses and disappear slower than stem cells. Indeed, three days after a lethal single-shot TBI about 50% of the megakaryocytes are still present within bone marrow [30]. This is in line with our present data, which indicate a surviving fraction of megakaryocytes with ongoing residual platelet production.

If megakaryocytes are actually the main source for CD61-exposing MP in vivo, a persistent fraction of megakaryocytes would also explain that the decay of the PMP is slower than expected. If we assume a 50% rest activity of megakaryocytes on day three [30], this would implicate that 46% of "platelet" (CD61+) MP may originate directly from megakaryocytes. Even this may be an underestimation, as TBI is given fractionated in our patients, which may result in an even higher survival rate of megakaryocytes on day three. As a consequence, we expect this percentage to be only a lower limit.

Different clearance kinetic between the three types of MP might explain our observations. However, these have not described yet. In contrast, recently lactadherin has been described as the central mediator for MP clearance [31]. As this mechanism seems to be an universal clearing mechanism for apoptotic cells and MP [32], differences between different types of MP seem quite unlikely.

From our data we cannot exclude that TBI may have a direct toxic effect on the circulating platelets, thereby affecting the mechanisms underlying the release of PMP, or that the release of PMP is affected by platelet age. MP are mainly released under well-controlled, stimulus-dependent process [33], which requires proteolysis of components of the cytoskeleton [34,35]. Therefore, one may postulate a direct damaging effect of irradiation on circulating platelets. Accumulating evidence has shown that extra-nuclear radiation induces biological effects [36], which may affect the release of PMP. Based on our data this explanation seems unlikely, however, since similar effects on MP exposing P-selectin or CD63 are expected.

Another effect, which must be taken into account, is platelet ageing. As a consequence of the terminated thrombopoiesis, circulating platelets age continuously before being removed from the circulation. A reduced production of PMP by aging platelets has not been shown so far, although young platelets demonstrate increased aggregation and release of dense- and α-granules compared to aged platelets [37,38]. Since aging would similarly affect the production of MP exposing CD61, P-selectin or CD63, also this explanation seems unlikely.

Taken together, we conclude that -similar to mice- also in humans a large fraction of CD61-exposing MP may originate from megakaryocytes rather than circulating platelets in vivo. In light of our study, the numerous observations of elevated "platelet-derived" MP levels in various diseases such as cancer may have to be re-evaluated.

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Conflict of interest statement

The authors state no conflict of interest.

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