Clearance and phenotype of extracellular vesicles after red blood cell transfusion in a human endotoxemia model

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ABSTRACT

Background: In the critically ill, extracellular vesicles (EV) from red blood cells (RBC) have been related to adverse effects of blood transfusion. Stored RBC units contain high concentrations of RBC- EVs, thereby increasing the concentration of EVs in the circulation after transfusion. The mechanisms underlying the clearance of donor RBC-EVs after transfusion are unknown. This study investigates whether membrane markers that are associated with clearance of RBCs are also implicated in clearance of RBC-EVs in human endotoxemic recipients of a transfusion.
Methods: Six volunteers were injected with *Escherichia coli* lipopolysaccharide, and after two hours transfused with an autologous RBC unit donated 35 days earlier. Samples were collected from the RBC unit and the volunteers before and after transfusion. RBC-EVs were labeled with (anti) glycophorin A, combined with (anti) CD44, CD47, CD55, CD59, CD147, or lactadherin to detect phosphatidylserine (PS) and analyzed on a A50 Micro flow cytometer.
Results: In the RBC unit, RBC-EVs solely exposed PS (7.8%). Before transfusion, circulating RBC-EVs mainly exposed PS (22%) and CD59 (9.1%), the expression of the other membrane markers was much lower. After transfusion, the concentration of RBC- EVs increased 2.4-fold in two hours. Thereafter, the EV concentration decreased towards baseline levels. The fraction of EVs positive for all tested membrane markers decreased after transfusion.
Conclusion: Besides a minor fraction of PS-exposing EVs, RBC-EVs produced during storage do not expose detectable levels of RBC membrane markers that are associated with clearance, which is in contrast to the EVs produced by the circulating RBCs.

1. Introduction

Although lifesaving, blood transfusion has been proposed to contribute to morbidity and mortality, especially in the critically ill [1]. The proposed mechanisms of these adverse effects include increased susceptibility to infection [2,3] and hypercoagulation [4,5]. The biological mediators in the blood product driving these adverse effects are unknown. Extracellular vesicles (EVs) derived from red blood cells (RBC) have been related to activation of coagulation [6–8], endothelial activation [9] and immunomodulation [10,11], and therefore may play a role in the observed adverse effects that occur after blood transfusion.

RBC-derived EVs are small membrane-enclosed vesicles with a diameter of less than 1 μm. The formation of RBC-derived EVs occurs when the asymmetry of the cell membrane is lost, and is triggered by different stimuli, including apoptosis and shear stress [12,13]. RBC-derived EVs are released from endogenous circulating RBCs, as well as during storage, the latter resulting in the accumulation of EVs in transfusion units [14].

During sepsis the amount of circulating RBC-derived EVs increases and also PS exposure increases [15]. During endotoxemia, injection of RBC-derived EVs in mice induced the production of proinflammatory cytokines, which did not occur in healthy mice [16]. Thereby, RBC-

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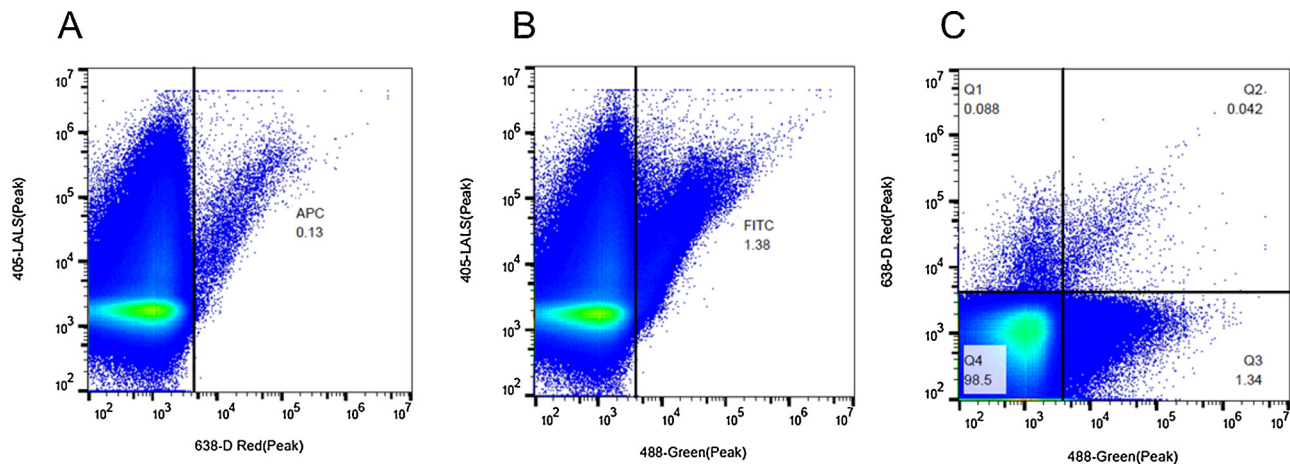[1] These authors contributed equally.

Fig. 1. Example of flow cytometry analysis of vesicles stained with CD235a-APC (RBC-derived) and Lactadherin-FITC (Phosphatidylserine) before transfusion (t = 0). A) LALS vs. CD235a- APC B) LALS vs. Lactadherin-FITC C) Double positive vesicles for CD235a-APC and Lactadherin-FITC.

derived EVs might explains the higher incidence of adverse events of blood transfusions in the critically ill.

In a model of human endotoxemia, we showed that after transfusion of a stored RBC unit containing a high amount of RBC-derived EVs, the concentration of circulating RBC-derived EVs increases. The RBC-derived EVs are then cleared from the circulation within hours [14]. The mechanisms underlying their clearance however, are unknown. Knowledge on the mechanisms underlying the clearance may aid in designing interventions aimed at increasing the clearance rate, and thereby –possibly– decreasing transfusion-related adverse effects.

EVs expose proteins and phospholipids that also occur on their 'parental cells' [17]. Some of these are involved in the phagocytosis and clearance of RBCs, including CD44, CD47 (which is a marker of "self"), complement-regulating proteins CD55 and CD59, CD147, and phosphatidylserine (PS) [18–22]. In an animal model, the clearance of endogenous RBC-derived EVs was thought to be mediated by recognition of PS [23]. We therefore hypothesized that membrane markers involved in the clearance of RBCs may also be involved in the clearance of transfused RBC-derived EVs and are therefore present on the membrane of these vesicles.

The aim of this study is to investigate the presence of membrane markers that are associated with clearance of RBCs on RBC-derived EVs during endotoxemia. We compared the phenotype of RBC-derived EVs produced during storage of RBCs and RBC-derived EVs in the circulation before and after transfusion.

2. Materials and methods

All procedures are approved by the Amsterdam UMC medical ethical committee. All volunteers provided written informed consent before enrollment.

2.1. Transfusion model

This study is part of a larger study with healthy male volunteers, 18–35 years old, in which the aim was to investigate whether transfusion of stored RBCs induces lung damage [24]. In this study, we included the volunteers (n = 6) who received an autologous RBC unit after 35 days of storage since these units contained a high amount of RBC-derived EVs in contrast to the 2 days old RBC units.

35 days prior to the experiment blood was donated at the Dutch blood bank Sanquin (Amsterdam, The Netherlands) and processed according to their protocol to leukoreduced RBC units (< 1 × 10⁶ leukocytes/unit) containing saline, adenine, glucose and mannitol (SAGM) as storage medium. The RBC units were cooled to < 6 °C within 30 h and stored in polyvinylchloride-di-ethyl-hexyl-phthalate containers for

35 days.

Two hours before the autologous RBC transfusion, volunteers were infused with 2 ng *Escherichia coli* lipopolysaccharide (LPS) / kg body weight i.v. (National Institutes of Health Clinical Center, Bethesda, MD) to evoke an inflammatory response.

2.2. Sample collection

A sample was collected from the RBC unit with a sterile take spike (Codan, Lensahn, Germany) and stored in an uncoated vacutainer tube (Becton, Dickinson and Company, Franklin lakes, New Jersey, USA). Blood samples of the volunteers were collected directly before transfusion of the RBC unit and at two, four and six hours after transfusion. The samples were collected in vacutainer tubes with 0.11 M Sodium Citrate (Becton, Dickinson and Company) from an indwelling arterial catheter. The blood samples were centrifuged (1500 g, 10 min, 20 °C) after which the supernatant was collected and centrifuged a second time (1550 g, 20 min, 20 °C). The supernatant was transferred to a new vial and stored at − 80 °C until analysis.

2.3. Extracellular vesicle characterization by flow cytometry

Before flow cytometric analysis, the samples were diluted 1:10 with phosphate buffered saline (PBS) containing 0.32% citrate to determine the dilution factor to achieve an event rate of 5000/sec to prevent "swarm" detection. Samples were diluted with this factor. The dilution factors of the antibodies were determined in a pilot experiment. Diluted and undiluted antibodies were centrifuged at 18.890 g for 5 min and transferred to a new tube to remove the aggregates. 20 μL of the diluted samples were incubated in the dark for 2 h with 2.5 μL CD235a – APC (2:1) (Miltenyi Biotec, Leiden, The Netherlands) to detect the RBC-derived EVs, combined with 2.5 μL CD44-FITC (1:1), CD47-FITC (1:1), CD55-FITC (undiluted), CD59-FITC (undiluted) (all eBioscience, Vienna, Austria), CD147-FITC (1:3) (Biolegend, Uithoorn, The Netherlands) or Lactadherin-FITC (Bio-Connect, Huissen, The Netherlands). The samples were measured on an A50 Micro with a detection limit < 100 nm (Apogee Flow Systems, Hemel Hempstead, United Kingdom) for 2 min at a flow rate of 3.01 μL/min triggered on large angle light scatter (LALS) and small angle light scatter (SALS). FlowJo (version 10, FlowJo LLC, Ashland, Oregon, USA) was used as flow cytometry analysis software (Fig. 1).

2.4. Statistical analysis

Variables are presented as medians with interquartile ranges. Comparisons between two paired groups were made using the Wilcoxon
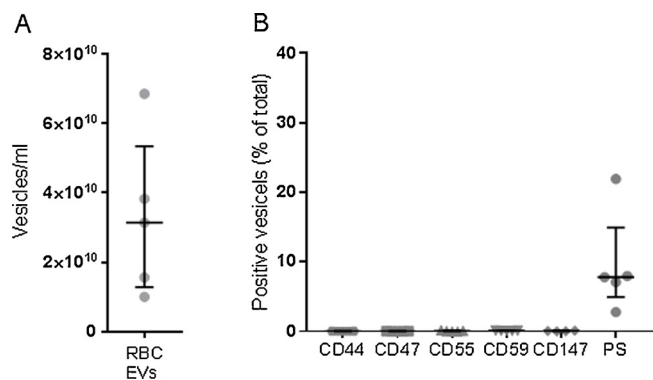
Fig. 2. RBC unit after 35 days of storage. A) Total red blood cell (RBC)-derived extracellular vesicle (EV) count B) percentage of RBC-EVs positive for CD44, CD47, CD55, CD59, CD147 or phosphatidylserine (PS). Bars show medians and interquartile ranges.

signed rank test. More than two paired groups were compared using the Friedman test. A p-value of less than 0.05 was considered statistically significant. Statistical analyses were performed with SPSS v24 (SPSS, Chicago, Illinois, USA). Figures were made using GraphPad Prism 7 (Graph-Pad software, San Diego, California, USA).

3. Results

3.1. RBC-derived extracellular vesicles in the transfusion unit

After 35 days of storage, the median RBC-derived EV concentration in the transfusion units was 3.1×10^{10}/mL (IQR 1.3×10^{10} – 5.3×10^{10}). Of the measured panel, PS was the only detectable membrane marker, present on 7.8% of the RBC-derived EVs (IQR 4.9–14.9). In contrast, CD44, CD47, CD55, CD59 and CD147 were absent or below the detection limit (Fig. 2). In line with this, the mean fluorescence intensity (MFI) of the markers was low (data not shown).

3.2. Presence of membrane markers associated with clearance on RBC-derived EVs before and after transfusion

Before transfusion, the median RBC-derived EV concentration in the circulation was 1.0×10^8/mL (IQR 5.6×10^7– 1.5×10^8). Two hours after transfusion, the RBC-derived EV concentration increased 2.4-fold compared to baseline. Four hours after transfusion, the RBC-derived EV concentration had decreased with 27% and was further decreased at 6 h after transfusion (Fig. 3).

Before transfusion, 22.0% (IQR 15.2–24.6) of the endogenous RBC-derived EVs exposed PS, 9.1% (IQR 8.2–11.6) of the EVs were positive for CD59 and a low but detectable fraction of EVs were positive for CD44, CD47, CD55 and CD147 (Fig. 3). After transfusion, the levels of EVs positive for CD44, CD47, CD55, CD59 and CD147 remained constant (Friedman test p > 0.05) (Fig. 2). The level of EVs with PS exposure increased after transfusion (p = 0.028), however the fraction of the EVs positive for PS decreased, likely due to the transfusion of high numbers of EVs of which only a small part exposed PS (Figs. 3 and 4).

In line with the amount of positive vesicles, the MFI of all markers (except PS), was low and remained low after transfusion. For PS, the MFI was high and significantly decreased after transfusion (data not shown).

4. Discussion/conclusion

In this study, we found that markers associated with RBC clearance were not detectable on the RBC-derived EVs in stored RBC units, except for PS exposure, which was present on a limited fraction of the EVs. In the circulation, a low but detectable fraction of the RBC-derived EVs do
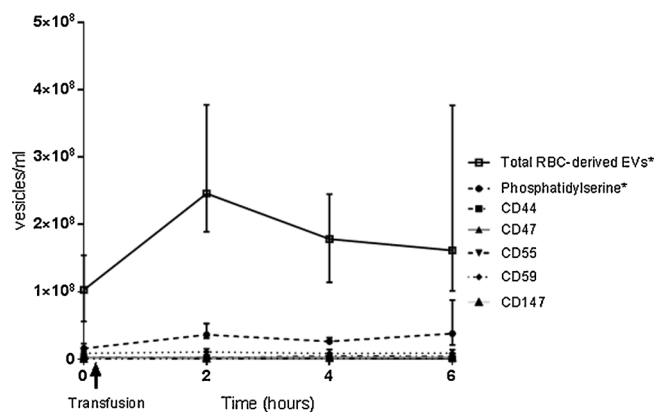

Fig. 3. Red blood cell (RBC)-derived extracellular vesicle (EV) count and EVs positive for membrane markers that are associated with phagocytosis (CD44, CD47, CD55, CD59, CD147 or phosphatidylserine (PS)) in blood before (T = 0) and 2, 4 and 6 h after transfusion. Figure shows medians with interquartile ranges. *p < 0.05 for differences between time points.
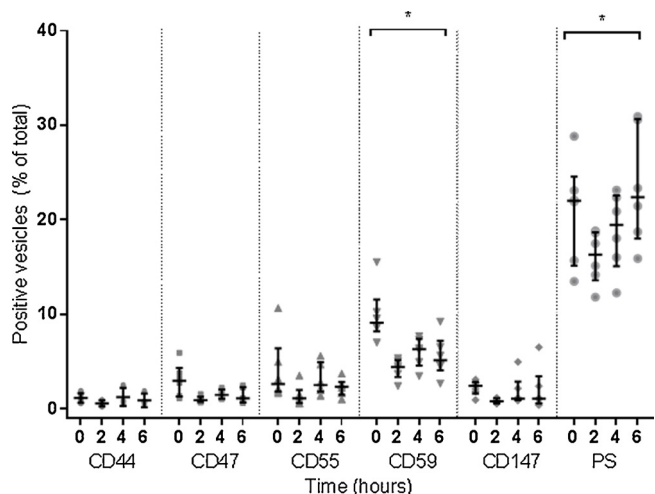

Fig. 4. Percentage of total red blood cell-derived extracellular vesicles count positive for CD44, CD47, CD55, CD59, CD147 or phosphatidylserine (PS), before (T = 0) and 2, 4 and 6 h after transfusion. Figure shows medians with interquartile ranges. * p < 0.05 for differences between timepoints.

express these markers. Thus, RBC-derived EVs generated during storage of an RBC product contained lower levels of EVs that expressed markers associated with clearance when compared to endogenous RBC-derived EVs.

After transfusion of a RBC unit containing high amounts of *in vitro* generated RBC-derived EVs into a recipient, the total amount of EVs increased but the levels of EVs positive for the tested markers in the circulation remained constant. The increase of EVs two hours after the transfusion is almost certainly caused by the transfusion. An increase in production of RBC-derived EVs due to the injection of LPS is not likely since it is shown that this increase of EVs only occurs after transfusion of a RBC unit with a high EV amount and not after transfusion of RBC unit with a low amount of EVs [14]. The change in composition of the vesicles after transfusion (a decrease in the fraction EVs that was positive for CD59 and PS was seen) also suggests that the EVs measured are partly originating from the RBC unit. Possibly, the donor RBCs also produce EVs after transfusion. The stable concentration of EVs positive for the tested markers suggests that in that case these newly formed EVs also do not express the studied markers on their membrane, since this would have increased the levels of positive EVs, assuming that the production of EVs by the circulating RBCs stayed the same before and after transfusion.

The complement regulatory proteins CD55 and CD59 are expressed on EVs in the circulation, but not on EVs in the RBC unit. As both CD55 and CD59 protect EVs from lysis and likely clearance, the absence of these markers possibly plays a role in the rapid clearance of transfused RBC-derived EVs. However, as complement deposition on the EVs was not investigated in this study, the role of these complement regulatory proteins could not be confirmed. Since CD44, CD47, CD147 and PS induce clearance instead of protecting against clearance, the low presence of these membrane markers suggests that these markers are not involved in the clearance of the EVs. It is possible that other membrane-anchored markers or bridging molecules are involved in the clearance of transfused EVs. Further research is needed to elucidate the clearance mechanism of transfused EVs.

This research is the first to study the phenotype of EVs following RBC transfusion. The few studies that are performed on the clearance of RBC-derived EVs investigated the clearance of vesicles that were isolated from a blood sample and re-injected [22,23]. However, for transfusion medicine knowledge on EVs originating from RBC units is important. Our study shows that the presence of the studied membrane markers *in vitro* is different from *in vivo* generated EVs. During sepsis the total amount of vesicles and the amount of PS positive RBC derived vesicles increases [15]. Also, the impact of the injection of RBC-derived EVs is different during endotoxemia [16]. We used an endotoxemia model to increase comparability with critically ill patients. Another strong point of our research is that autologous blood was used, thereby we were able to look solely at the effect of storage and endotoxemia. However, this can also be seen as a limitation since it lacks comparability with clinical practice.

Our present study has limitations, including a small sample size. Another limitation is that the first blood sample was drawn two hours after transfusion. Assuming that the volunteers had a circulating volume of 5–6 L and 120 mL of supernatant was given with the transfusion, a three times higher number of circulating EVs was expected shortly after the transfusion. This suggests that a substantial part of the vesicles is already cleared at the first time point after transfusion. Also, we were not able to separate the EVs produced by the donor RBCs from the EVs produced by the RBCs that were already in the circulation. It is very likely that the EVs we measured at the different time points after transfusion were partly derived from the RBC unit, however we cannot conclude this with certainty from our study.

Given the implication of EVs in the adverse events of RBC transfusions [6–11], knowledge about the physiology of EVs produced during storage is important. Knowledge on the clearance mechanisms of these EVs can help designing intervention strategies to increase the clearance rate of these EVs and thereby hopefully decrease adverse events.

In conclusion, RBC-derived EVs produced during storage do not have detectable levels of membrane markers associated with clearance on their membrane, except for PS which was exposed in a low amount by a small fraction of the EVs. This in contrast with the EVs produced by the circulating RBCs. RBC transfusion results in a decrease of the fraction of EVs positive for membrane markers associated with clearance in volunteers with endotoxemia.

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