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ORIGINAL PAPER

Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools

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Vox Sanguinis

Background and Objectives Microparticles (MPs) are small phospholipid vesicles of less than 1 μm, shed in blood flow by various cell types. These MPs are involved in several biological processes and diseases. MPs have also been detected in blood products; however, their role in transfused patients is unknown. The purpose of this study was to characterize those MPs in blood bank conditions.

Materials and Methods Qualitative and quantitative experiments using flow cytometry or proteomic techniques were performed on MPs derived from erythrocytes concentrates. In order to count MPs, they were either isolated by various centrifugation procedures or counted directly in erythrocyte concentrates.

Results A 20-fold increase after 50 days of storage at 4°C was observed (from 3370 ± 1180 MPs/μl at day 5 to 64 850 ± 37 800 MPs/μl at day 50). Proteomic analysis revealed changes of protein expression comparing MPs to erythrocyte membranes. Finally, the expression of Rh blood group antigens was shown on MPs generated during erythrocyte storage.

Conclusions Our work provides evidence that storage of red blood cell is associated with the generation of MPs characterized by particular proteomic profiles. These results contribute to fundamental knowledge of transfused blood products.

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Introduction

Millions of blood products are transfused worldwide every year; many lives are thus directly concerned by transfusion. Since the beginning of blood transfusion, numerous efforts have been made to secure blood products and gain knowledge about their molecular structures. The progress of proteomics allows re-examining important issues in blood research [1] and transfusion science [2] with the tools of large-scale biology [3].

The three main labile blood products used in transfusion are erythrocyte concentrates (ECs), platelet concentrates and fresh-frozen plasma. Each of these products has to be stored according to its particular components. However, during storage, modification or degradation of those components may occur and are known as storage lesions. Among these lesions, the generation of microparticles (MPs) has been identified [4–6].

Ageing erythrocytes in blood bank conditions differ from *in vivo*, therefore, it has been suggested that erythrocyte physiological ageing process may be accelerated by storage conditions [7]. Indeed, during storage, several biochemical and physiological changes occur in ECs, including an increase in the concentration of free haemoglobin, lipids, MPs and a pH reduction. Concerning red blood cells (RBCs), they lose adenosine triphosphate, 2,3-diphosphoglycerate or potassium and their membrane undergoes various modifications, such as more rigidity, disruption of phospholipids asymmetry, protein clustering, lipid raft rearrangement, loss

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of fragments or even release of MPs [8–10]. The exact effects of storage lesions on transfusion are still unknown.

Microparticles are small phospholipid vesicles of less than 1 μm in size, also known as microvesicles [11] or ectosomes [12]. They are released from a variety of cells, such as platelets, RBCs and white blood cells, or endothelial cells [13]. They contain a subset of proteins derived from their parent cells. However, MPs are heterogeneous and vary in size, phospholipid and protein composition. Release of MPs is a highly controlled process prompted by various stimuli, such as shear stress, complement attack, proapoptotic stimulation or damage [14].

Microparticles have long been considered as cell fragments or 'debris' without any biological function. Although their true biological function is still unknown, there are more and more indirect evidence that MPs are involved in a broad spectrum of biological activities, such as haemostasis [15], thrombosis [13], inflammation [15], transfer of surface proteins [16] or even angiogenesis [17].

An increase in the number of MPs in plasma has been demonstrated under various pathological conditions, such as heparin-induced thrombocytopenia [18], thrombotic thrombocytopenic purpura [19], diabetes [20,21], acute coronary syndromes [22], cardio vascular disease [23] or sepsis [24]. Despite their potential important activities, only few studies are available on MPs in blood products. It has been demonstrated that the number of MPs increases with the age of blood products [25] and that there is a link between the risk of transfusion complication and the age of the transfused blood products [26,27]. Thus, an appealing hypothesis is that a high number of MPs in ECs is linked to adverse transfusion reactions. In addition, Koch *et al*. recently demonstrated a link between the age of transfused RBCs and post-transfusion complications [28], indicating that progress in the knowledge of stored RBCs biology is urgently needed.

In order to have a better understanding of MPs in stored ECs, qualitative and quantitative experiments using flow cytometry or proteomic techniques were developed and performed. The goals of this study were: (i) to test centrifugation methods for the isolation of MPs from ECs; (ii) to count MPs directly in ECs and in their supernatant fraction; and (iii) to gain insight into the protein content of MPs as compared to RBC membranes, and (iv) to evaluate if erythrocyte-derived MPs also express Rhesus antigens on their surface.

Materials and methods

Erythrocyte concentrates

Whole blood was collected and prepared at the Lausanne or Bern blood banks, according to standardized procedures. Only ECs that did not satisfy quality criteria for transfusion were used for this study, notably those collected from donors presenting with elevated levels of alanine transaminases (ALAT). Briefly, 450 ± 50 ml of whole blood was drawn by venipuncture and collected in blood bags (Fenwal, Lake Zurich, IL, USA) containing the anticoagulant solution (citrate–phosphate–dextrose). Leucocytes and platelets were then removed by filtration. After separation of plasma from erythrocytes by centrifugation, RBCs were finally suspended in 100 ml of preservative solution sodium–adenine–glucose–mannitol. For the experimental purpose of this study, ECs were stored up to 50 days at 4°C instead of 42 days, the usual expiration date for concentrates in sodium–adenine–glucose–mannitol solution.

Flow cytometry analysis and microparticles counts in erythrocyte concentrates

Samples were analysed on a FACScalibur flow cytometer with CellQuest pro software (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometer was daily calibrated with CaliBRIT™ 3 kit (BD Biosciences) containing different fluorescent beads. Size events were defined using flow cytometry size beads of 1–1·4 μm (Spherotech, Lake Forest, IL, USA). For the different windows used, the flow cytometer was set on a logarithmic scale.

Fluorescein isothiocyanate (FITC) anti-human CD47 (BD Pharmingen, San Diego, CA, USA) and phycoerythrin (PE) anti-human CD235a (or glycophorin A) (BD Pharmingen) were the two antibodies used to tag erythrocyte MPs. Moreover, experiments have been done with FITC human annexin V (BD Pharmingen) that tags negative phospholipids present on MPs surface.

Microparticles counts were determined in the supernatant of a stored EC at 4°C for 38 days, after various centrifugation conditions (870 ***g***, 1850 ***g***, 2550 ***g*** and 3250 ***g***). Four samples of 50 ml from the EC were spun down twice for 20 min, and 100 μl of supernatant was then mixed with 3 μl of FITC anti-human CD47 or 3 μl PE anti-human CD235a or both for double staining. After 20 min of incubation on an orbital shaker in the dark at 4°C, 400 μl of phosphate-buffered saline (PBS) was added and flow cytometry analysis was carried out within 1 h in a Trucount™ tube (BD Biosciences). Isotypic controls were performed with PE immunoglobulin G2b (IgG2b) or FITC IgG1 (both from Diaclone, Besançon, France). MPs were also determined in the supernatant of 13 different ECs stored from 2 to 50 days at 4°C after two centrifugations at 1850 ***g*** for 20 min. Measurements were done in triplicates. The flow cytometer settings for counting MPs in supernatants were as follows (detector: voltage): FSC: E00, SSC: 360, FL1: 500–600, FL2: 500–600 and FL3: 570.

Finally, to avoid pre-analytical variability due to centrifugation conditions, MPs counts were determined in ECs from seven different blood donors (without centrifugation). For each EC, six measurements were performed from day 2 to day

50 of storage at 4°C. Red blood cells were carefully mixed with the storage solution, and 5 μl of the RBC suspension was mixed with 4 μl of FITC anti-human CD47 for 5 seconds. The mixture was then incubated for 20 min on orbital shaker in the dark. Lastly, 4 μl of the solution was diluted to 1 ml with 0·9% NaCl in a Trucount™ tube, and was directly analysed by flow cytometry. Isotypic controls were performed with FITC IgG1 (Diaclone). The flow cytometer settings for counting MPs in concentrates were as follows (detector: voltage): FSC: E00, SSC: 300, FL1: 650, FL2: 520 and FL3: 600.

Proteomics

Proteomic analysis was done on MPs and erythrocyte membranes of the same 42-day stored ECs. MPs were obtained after three centrifugations (1850 *g* twice and 3200 *g* once, 20 min at 4°C) and the supernatant containing MPs was collected. Then, three ultracentrifugations at 120 000 *g* for 90 min at 4°C were done, each time pellets were suspended in PBS. To obtain erythrocyte membranes, RBCs were washed in PBS 10× and spun at 1850 *g* for 20 min at 4°C three times. Collected pellets were then washed in deionized water and after another centrifugation at 1850 *g* for 20 min at 4°C, pellets were collected and prepared for future analysis.

To determine the quantity of sample to load, protein concentration of each sample was measured according to the Bradford's method [29]. For sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), 30 μg of proteins was loaded onto a 4–12% NuPAGE Novex Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA, USA). The migration was carried out at constant voltage (200 V).

For mass spectrometry (MS) analysis, SDS-PAGE was run as previously described, but 300 μg of proteins was loaded. Upon electrophoresis completion, the gels were rinsed twice with deionized water and stained with colloidal Coomassie blue (National Diagnostics, Atlanta, GA, USA) overnight. The gels were destained with deionized water. Bands of interest were excised from the gels and transferred into an Eppendorf. In-gel proteolytic cleavage with sequencing-grade trypsin (Promega) was automatically performed in the robotic workstation Investigator ProGest (Perkin Elmer Life Sciences) according to the protocol of Shevchenko *et al.* [30]. Digests were evaporated to dryness and resuspended in 3 μl of α-cyano-hydroxycinnamic acid matrix [5 mg/ml in 60% (v/v) acetonitrile : water), of which 0·7 μl was deposited in duplicate on a target plate. Matrix-assisted laser desorption/ionization MS (MALDI-MS)/MS analysis was performed on a 4700 Proteomics Analyser (Applied Biosystems, Framingham, MA, USA). After MALDI – Time of Flight (TOF)/MS analysis, internal calibration on trypsin autolysis peaks and subtraction of matrix peaks, the 10 most intense ion signals were selected for MS/MS analysis. Non-interpreted peptide tandem mass spectra were used for direct interrogation of the Uniprot (Swissprot + TrEMBL) database using Mascot 2·0 (http://www.matrixscience.com). The mass tolerance for database searches was 50 p.p.m. MASCOT was set up to only report peptide matches with a score above 14. With the parameters used, the threshold for statistical significance ($P < 0{\cdot}05$) corresponded to a total (protein) MASCOT score of 33. Proteins scores above 80 were automatically considered valid, while all protein identifications with a total MASCOT score between 33 and 80 were manually validated. Validation included examination of the peptide root mean square mass error and of individual peptide matches. Peptide matches were validated only if at least an ion series of four consecutive y ions were matched, in addition to ions belonging to other series. Generally, only proteins matched by at least two peptides were accepted.

Western blotting

From 20 to 50 μg of proteins from the samples described before (MPs and erythrocyte membranes) were loaded onto a 4–12% NuPAGE Novex Bis-Tris polyacrylamide gel (Invitrogen). After migration, carried out at constant voltage (200 V), proteins were transferred to polyvinylidene fluoride membranes using a Novex blot module (Invitrogen) for 1 h 45 min at fixed voltage (30 V), according to the manufacturer's instruction. After transfer, blotted membranes were soaked overnight in blocking solution with PBS, 0·1% Tween-20 (v/v), 5% milk and 1% BSA (w/v). Four Western blots were done, each one with a different antibody, namely, anti-human CD235a (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human actin (Sigma, Saint Louis, MO, USA), anti-human stomatin (Proteintech Group, Chicago, IL, IL) and anti-human CD47 (from Santa Cruz or from AbCam, Cambridge, UK). All antibodies were used at a dilution of 1 : 500. The goat anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Dako, Baar, Switzerland) were both used at a dilution of 1 : 10 000. Subsequent visualization was performed using enhanced chemiluminescence (GE Healthcare, Uppsala, Sweden). The signal was finally captured using X-ray film.

Expression of Rhesus antigens

Flow cytometry was used to determine the presence of Rh antigens on MPs generated during storage. MPs were first isolated from the supernatant of an EC after two centrifugations at 1850 *g* at 4°C for 20 min. The supernatant was then spun down at 18 000 *g* for 5 min to pellet MPs. Pellet was dissolved in 100 μl of PBS. One μl of the primary antibody (anti-D, anti-C, anti-c, anti-E and anti-e, respectively) was later added and mixed on orbital shaker for 90 min. IgM anti-D were obtained from Orthobiotech (Bridgewater, NJ, USA), whereas IgM anti-C, IgM anti-c, IgM anti-E and IgM anti-e

were obtained from Biotest (Dreichen, Germany). One µl of the secondary antibody was then added, and after 1 h on orbital shaker in the dark, 400 µl of PBS was added, and samples were analysed by flow cytometry within an hour. Secondary antibody directed against primary IgM was FITC anti-human IgF(ab) from Chemicon (Melbourn, Australia). To demonstrate the presence of various Rh antigens on MPs, blood samples expressing different Rh phenotypes, such as DCCee, DccEE or dccee, were selected. The antithetical phenotype was used with each antibody as a negative control.

Results

Microparticle counts

Using flow cytometry, MPs clearly were distinguished from RBCs by their size as well as by the negatively charged phospholipids on their outer membrane detected by annexin V. Indeed, the great majority of MPs were annexin V-positive, while merely a few percentage of erythrocytes were positive. MPs were also identified using either anti-CD47, anti-CD235a or both antibodies, without any differences in their numbers according to the choice of the antibody (Figs 1 and 2).

In both methods used, an increase in the number of MPs during storage of ECs was observed (Figs 2 and 3). The number of MPs was clearly related to centrifugation conditions (Fig. 2). In the measurements performed directly in the concentrate, the number of MPs increases about 20-fold after 50 days of storage at 4°C and considerably varies among different samples; it starts from 3370 + 1180/µl after 6 days, up to 64 850 + 37 800/µl after 50 days of storage (Fig. 3). The intra-assay coefficient of variation was evaluated. With both methods, the coefficient was less than 15%, even after 50 days of storage (data not shown). Nonetheless, and without evident explanation, we observed a huge individual variation of the MPs counted among different donors.

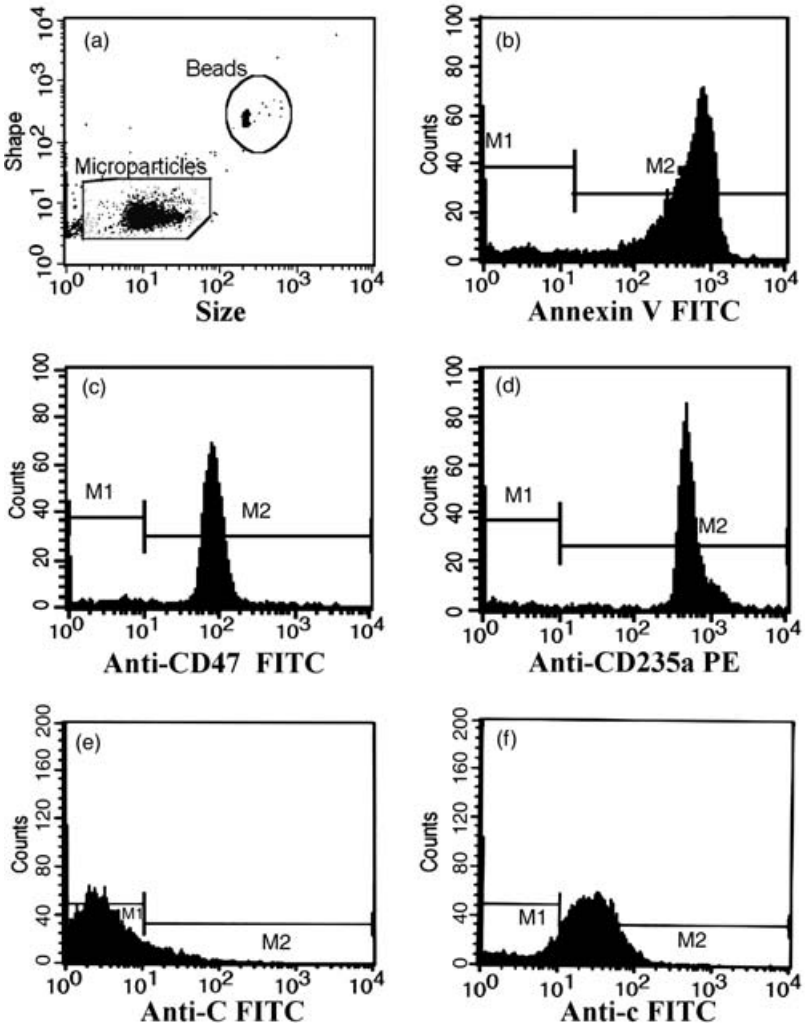

Fig. 1 Flow cytometry analysis of microparticles from a supernatant after centrifugation of erythrocyte concentrate. (a) Events that were sorted according to their size and shape. Two different regions were determined; microparticles and beads. A precise number of beads were used to determine the number of microparticles in each sample. Histograms represent events from the microparticle region (b–e) of (a) according to their fluorescence (due to an FITC- or PE-labelled antibody). M1 represents non-stained events whereas M2 shows stained event. (b) Microparticles stained with annexin V; (c) microparticles stained with anti-CD47 FITC; and (d) microparticles stained with anti-CD235a. FITC, fluorescein isothiocyanate; PE, phycoerythrin. In (e) and (f), microparticles were derived from an erythrocyte concentrate from a donor typing dccee. In this example, microparticles were negative for anti-C antibody (e), while they were positive for anti-c antibody (f).

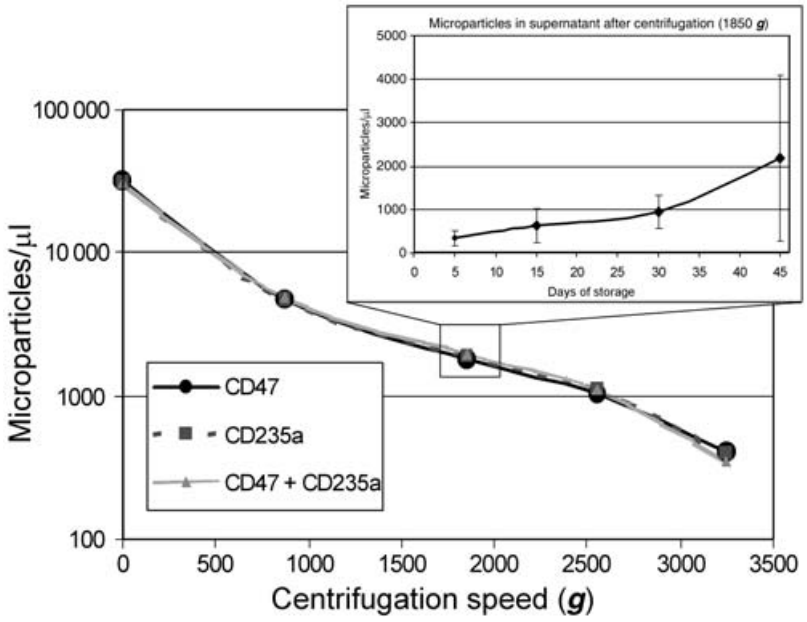

Fig. 2 Count of microparticles in supernatant of erythrocyte concentrates (ECs). The number of microparticles measured decreased with increasing centrifugation speed (test on one EC stored for 38 days). Note that the numbers measured were identical with the two antibodies used in this study (anti-human CD235a or CD47). The inset shows an increase in the number of microparticles in supernatant of ECs during storage (after centrifugation at 1850 ***g***, here anti-human CD235a was used).

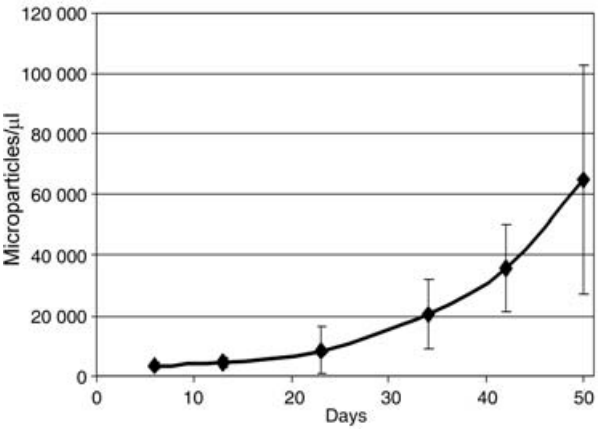

Fig. 3 Count of microparticles directly in erythrocyte concentrates during storage (without centrifugation). Data are expressed as the mean ± SD experiment (n = 7). At day 5, 3371 ± 1188 microparticles/µl were counted, whereas at day 50, their numbers were 64 858 ± 37 846 microparticles/µl. Anti-human CD47 was used to stain microparticles.

Proteomics

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis showed a number of large as well as discrete bands obtained after electrophoresis of RBC membranes (Fig. 4, lane A) and MPs (Fig. 4, lane B). A major difference was observed in the 25–35 kDa region; thus, in order to perform protein identification, bands of interest stained with Coomassie blue were excised and proteins were identified by MS. Table 1 lists the proteins identified in the three excised bands of lane A (erythrocyte membranes) and lane B (MPs). Not surprisingly, abundant proteins such as carbonic anhydrases or peroxiredoxins were identified both in RBC membranes and MPs with good sequence coverage. A set of 14-3-3 proteins was also identified in both samples: 14-3-3 proteins are abundant and ubiquitous proteins [31] that act as regulators of a number of processes, such as modulation of protein kinase activities, signal transduction [32]. Remarkably, 14-3-3ζ has been shown to be implicated in GPIb-IX-V translocation to the cytoskeleton during platelet activation [33]. It was thus not surprising to find 14-3-3 proteins in RBC ghosts. The gel band that appeared to be quantitatively the most different between lanes A and B was identified as stomatin, identified in MPs with a score of 1131, a sequence coverage of 76·4% from 46 peptides, whereas it was identified in RBC membranes with a score of 156, a sequence coverage of 14·9% from four peptides, indicating that stomatin was largely enriched in MPs compared to erythrocyte membranes.

Interestingly, some of the identified proteins did not have molecular weights that corresponded to their respective position on the gel. For example, haemoglobin subunits α and β, which have a molecular weight of about 15–16 kDa, were observed in the region corresponding to 25–35 kDa on the gel. However, it has been documented that denatured and cross-linked haemoglobin strongly binds to the cytoskeleton during RBC storage in blood banking conditions [34]. It is thus highly probable that the haemoglobin subunits identified were present as homogeneous or heterogeneous dimers. Additionally, Band 3 (a major membrane protein) as well as Rhesus protein were identified in MPs only, from three and two sequenced peptides, respectively. For Band 3, the identified peptides correspond to the cytoplamic domain of the protein (spanning the region 117–180), which means that the Band 3

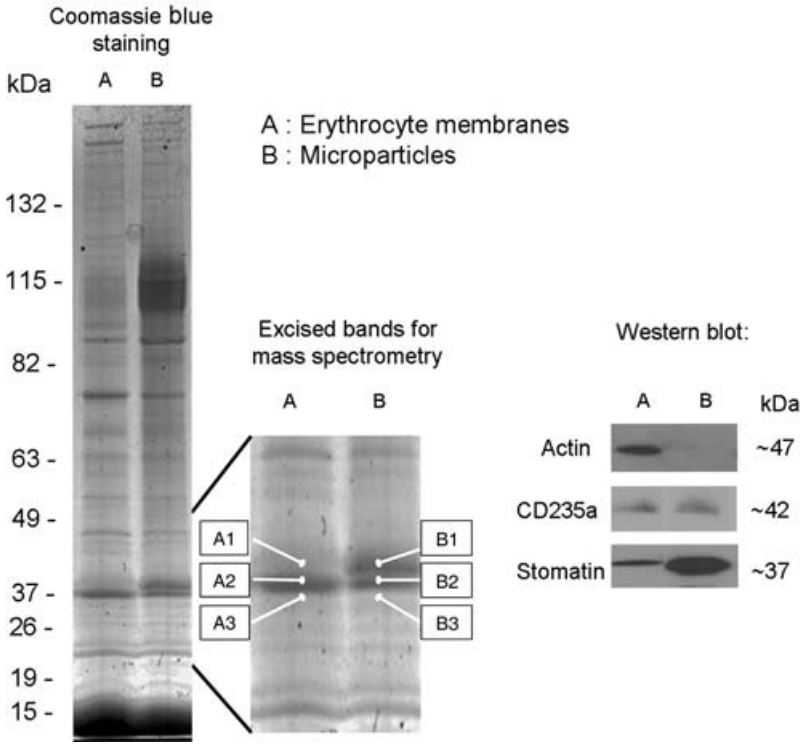

Fig. 4 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of erythrocyte membranes and microparticles, both were from a 42-day stored erythrocyte concentrate. The gel was stained with Coomassie blue (for better compatibility with mass spectrometry). Inset shows enlargement of Coomassie blue-stained gel used for the preparation of the bands submitted to mass spectrometry analysis. Western blot analysis points out the variation of protein expression. Thus, by comparing erythrocyte membranes and microparticles, a clear reduction of actin and an accumulation of stomatin were observed on microparticles. The staining of CD235a (glycophorin A) was similar in both gels.

fragment that appears on the gel in the 25–35 kDa region belongs to the cytoplasmic domain of the protein. The Rhesus protein was identified from two peptides covering the 17 last amino acids on the C terminus of the protein sequence. Of interest, Mascott allowed the identification of Rh peptides (Table 1; lane B of Fig. 4). However, it was not possible to discriminate between RhD and Rh(CE) proteins, a well-known problem in proteomics [35]. Nevertheless, the presence of various Rh antigens at the surface of MPs was confirmed by flow cytometry (see below).

Western blots

Western blotting confirmed the presence of CD235a, actin and stomatin on RBC membranes as well as on MPs, with a clear reduction of actin and an accumulation of stomatin on MPs (Fig. 4). However, using this technique and the antibodies available, neither CD47 nor Rh-proteins could be detected after SDP-PAGE. With blot techniques, no quantitative difference was observed between MPs samples from ECs stored for 5 or 42 days.

Rhesus systems

The presence of blood group antigens of Rhesus system on MPs surface was investigated by flow cytometry using specific antibodies. In each assay, positive and negative samples were selected for the corresponding antigen (Fig. 1e,f). By this approach, the presence of C, c, D, E or e antigens was observed on MPs when they were present on the RBCs from which they derived. However, it was not possible to exclude that a population of MPs was Rh negative.

Discussion

There is no standardized method to count MPs. Several approaches have been proposed in the literature, the majority dealing with platelet MPs from whole blood or platelet concentrates. Centrifugation speeds, for MPs characterization, varied from 200 ***g*** to 13 000 ***g*** [36]. In our hands, complete elimination of RBCs from supernatant at low centrifugation was obtained after two centrifugation at 1850 ***g*** for 20 min. Annexin V is frequently used to detect phosphatidylserine, a negatively charged phospholipid known to be present on the outer leaflet of apoptotic cells as well as on MPs of various origins [18]. In this study, MPs were counted using flow cytometry with different antibodies, notably anti-CD235a and anti-CD47. These antibodies were chosen because both are reactive towards RBC membrane molecules known to be present on MPs [14,29].

Counting MPs directly within the homogenized ECs appeared as the simplest approach, avoiding handling, centrifugation and washing. The drawbacks were due to the presence of a great number of RBCs, the need of quite large

Table 1 Proteins identified in the region 25–35 kDa of RBC membranes (lane A of Fig. 4) and microparticles (lane B of Fig. 4). Reported are the sequence coverage (%) and the number of sequenced peptides

AC	Entry name	Protein name	MW (Da)	RBC (sequence coverage %)	RBC (identified peptide)	MPs (sequence coverage %)	MPs (identified peptide)
P31946	1433B_HUMAN	14-3-3 protein β/α	28 082	25·2	7	16·7	4
P62258	1433E_HUMAN	14-3-3 protein epsilon	29 174	34·9	11	NO	NO
P61981	1433G_HUMAN	14-3-3 protein γ	28 303	NO	NO	13·4	4
P27348	1433T_HUMAN	14-3-3 protein θ	27 764	14·3	5	NO	NO
P63104	1433Z_HUMAN	14-3-3 protein ζ/δ	27 745	44·5	11	8·2	2
P02730	B3AT_HUMAN	Band 3 anion transport protein	101 792	NO	NO	6·8	3
Q4TWB7	Q4TWB7_HUMAN	β-Globin chain (Fragment)	11 487	93·3	15	NO	NO
P07738	PMGE_HUMAN	Bisphosphoglycerate mutase	30 005	45·9	13	37·8	10
P00915	CAH1_HUMAN	Carbonic anhydrase 1	28 870	72·8	27	65·5	20
P00918	CAH2_HUMAN	Carbonic anhydrase 2	29 246	72·7	25	71·9	15
P07451	CAH3_HUMAN	Carbonic anhydrase 3	29 557	36·2	8	17·7	4
A0N071	A0N071_HUMAN	δ-Globin chain (haemoglobin δ)	16 055	55·1	10	55·1	9
P27105	STOM_HUMAN	Erythrocyte band 7 integral membrane protein	31 731	14·9	4	76·4	43
P17931	LEG3_HUMAN	Galectin-3	26 188	11·6	3	NO	NO
P78417	GSTO1_HUMAN	Glutathione transferase ω-1	27 566	33·2	10	7·9	2
P69905	HBA_HUMAN	Haemoglobin subunit α	15 258	71·1	9	71·1	9
P68871	HBB_HUMAN	Haemoglobin subunit β	15 998	83·0	16	93·9	15
Q16775	GLO2_HUMAN	Hydroxyacylglutathione hydrolase	28 860	8·8	2	NO	NO
P30041	PRDX6_HUMAN	Peroxiredoxin-6	25 035	25·9	6	16·1	4
P18669	PGAM1_HUMAN	Phosphoglycerate mutase 1	28 804	8·3	2	NO	NO
Q06323	PSME1_HUMAN	Proteasome activator complex subunit 1	28 723	16·5	4	NO	NO
P25788	PSA3_HUMAN	Proteasome subunit α type 3	28 433	6·7	2	NO	NO
P25789	PSA4_HUMAN	Proteasome subunit α type 4	29 484	12·3	4	NO	NO
P60900	PSA6_HUMAN	Proteasome subunit α type 6	27 399	9·3	2	NO	NO
O14818	PSA7_HUMAN	Proteasome subunit α type 7	27 887	21·4	4	NO	NO
P00491	PNPH_HUMAN	Purine nucleoside phosphorylase	32 118	59·5	16	17·6	4
Q0KG01	Q0KG01_HUMAN	RhD protein	45 052	NO	NO	4·1	2

AC, accession number; MW, molecular weight in Da; NO, not observed.

amount of antibodies and an intra-sample variability in the number of MPs counted. In addition, samples cannot be stored, contrasting with supernatants containing MPs, which can be kept at 4°C or even be frozen before being evaluated by flow cytometry. Disadvantages of working with supernatants were related to the handling procedures and, more importantly, to the influence of centrifugation conditions. After centrifugation, residual RBCs were eliminated. However, a number of MPs appeared to be pelleted together with RBCs (Fig. 2). In any case, an increase in the number of MPs in ECs during storage was observed, even if the number of MPs counted differs according to the method. From our results, it is really clear that the number of MPs counted in EC was dependent on the centrifugation protocols.

Whereas it cannot be excluded that MPs from platelets, white blood cells or endothelial cells be present in the starting EC, this increase in total of MPs count can be attributed only to the shedding of MPs from RBCs present in the concentrate. Noteworthy, the increase varied quite importantly from donor to donor. The reason of such a variation is unknown, but factors like ABO blood group, age, fasting or sex of blood donor may have a role and should be investigated. Finally, the most important parameter associated with the number of MPs in ECs was the duration of storage at 4°C.

A set of experiment with three ECs satisfying quality criteria for transfusion (normal ALAT level) was done and gave very similar, if not identical MPs count when compared to ECs with elevated ALAT level (data not shown).

Microparticle tend to aggregate at high concentration, either related to the methods used for their isolation or to their 'intrinsic' adhesion properties, which have been already evidenced with platelet-derived MPs [16]. We observed heaps of erythrocyte MPs by electron microscopy (data not shown), thus erythrocyte MPs may also have adhesion properties. This observation is important for the quantitative results, because MPs counts are evaluated according to their sizes

using flow cytometry. Thus, in samples with high concentrations of MPs, their number are probably underestimated due to the fact that MPs tend to form more heaps and flow cytometry does not distinguish between big MPs or aggregated MPs, even if the technique is the method of choice to study MPs [36]. So, the counting approach of MPs presented in this study (as well as in other published studies) should be considered as semiquantitative.

According to our proteomic and Western blot studies, MPs from stored RBCs appeared to be enriched with stomatin. Remarkably, the enrichment in stomatin, depletion in actin and stability of glycophorin A (as compared to erythrocyte membranes) were the same at day 7 and day 40. In this respect, MPs generated after a few days of storage or at the end of storage appear equivalent. Those results are well in line with previous reports [37,38]. Stomatin is a membrane protein involved in regulation of monovalent cation transport through lipid membranes [39]. Interestingly, stomatin (which has a structure similar to caveolin) is a major lipid-raft component of erythrocytes [40]. Precise reasons of stomatin enrichment in MPs are not well known and are still subject of investigation, but may have a role in membrane microdomains modulation leading to membrane budding and MPs release [38]. The cell membrane plays a key role in the formation of MPs. Indeed, following a stimulus, increase in intracellular Ca^{++} occurs and activates proteases that cleave cytoskeleton proteins (actin and spectrin). Membrane is thus less rigid and can bud until formation of MPs. Furthermore, the asymmetry between the neutral phospholipids on the outer membrane and the negatively charged phospholipids on the inner membrane held by translocases is broken [37]. Consequently, phosphatidylserine, a negatively charged phospholipid, is also located on the outer side of MP membrane. Using annexin V, flow cytometry confirmed that phosphatidylserine was present on MPs derived from erythrocytes but essentially lacked from fresh RBCs and was externalized in only a small fraction of old RBCs. Finally, as shown by Western blots, actin was not a dominant protein of MPs when compared to RBC membranes.

The precise reasons for the huge increase in MP counts during storage of EC observed in this study are unknown. Although controversial, it has been speculated that MPs could be a means for erythrocytes to prevent a premature removal from circulation when they are still functional or when lesions are reversible [41]. According to this hypothesis, MPs would allow erythrocytes to clear away non-functional molecules that would trigger an apoptosis-like pathway, or to get rid of autologous IgG binding senescent erythrocytes for removal by spleen macrophages [8,10,42]. Indeed, Willekens *et al.* have recently shown that MPs contain erythrocyte removal proteins such as bound IgG and altered Band 3, and thus concluded that microvesiculation serves as a removal pathway for damaged proteins [42]. Complementarily, a detailed proteomic investigation of RBCs and MPs generated during storage led Bosman *et al.* to hypothesize that there are two possible mechanisms at work in MPs generation: first, immunoglobulins could bind to senescent surface proteins, thereby triggering microvesiculation. Alternatively, oxidatively damaged proteins could bind to or disrupt normal interactions within the cytoskeleton, thereby altering the tight balance between the cytoskeleton pressure and the membrane bending stress [10]. These two passive mechanisms could well be only one part of the picture, because activation of protein kinases has been demonstrated to trigger phosphatidylserine exposure in erythrocytes [43], as well as tight concomitant regulation of microvesiculation and Band 3 phosphorylation/dephosphorylation [44]. Lastly, lysophosphatidic acid, an important lipid mediator, has been shown to be able to trigger phosphatidylserine exposure and microvesiculation in erythrocytes [45]. These studies show that MP generation can be triggered by various processes, including senescence or protein alteration, external or internal exposure to lipid mediators, and that phosphorylation plays a role in microvesiculation control.

In this study, we also showed evidence that Rhesus blood group antigens are located on erythrocyte MPs. The presence of theses proteins was evidenced by determination of Rh peptides by MS and by flow cytometry, indicating that the antigenic parts of the Rh proteins are located outside MPs membranes. The presence of the Rh complex is also reinforced by the expression of CD47 (shown by flow cytometry), which is a member of the complex within the RBC membrane. Those blood group antigens present on MPs are likely immunogenic, and thus may play a potential role in RBC alloimmunization after transfusion.

As recently shown by Koch *et al.*, there is a link between duration of RBC storage and complications after cardiac surgery [28]. According to the result of this research, transfusion of erythrocytes that have been stored for more than 14 days in patients undergoing cardiac surgery significantly increases the risk of postoperative complications and reduces survival time. Reasons for such complications remain unclear; however, storage lesions may be a possible mechanism. Physicochemical changes occurring during storage of ECs are indeed known to affect RBCs function and viability. Our results confirm that important changes occur during storage of RBCs and that storage techniques allowing a better conservation of the integrity of the membrane should be thus developed in the future.

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