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Differences between calcium-stimulated and storage-induced erythrocyte-derived microvesicles

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ABSTRACT

Microvesicles (MVs), or microparticles, are a complex, dynamic and functional part of cells. Red blood cell (RBC)-derived MVs are naturally produced *in vivo* (during normal aging processes or in several diseases) as well as *ex vivo* during cold storage of RBCs, or *in vitro* by ATP depletion or treatment with Ca^{2+} and calcium ionophore. All these MVs are equivalently classified according to their size and/or surface markers. Nevertheless, their content in proteins can differ and a few differences in terms of lipid raft proteins, notably stomatin and flotillin-2, have been reported. Based on two-dimensional gel electrophoreses, the present study highlights the differences between MVs induced during storage of RBCs (storage-MVs) and MVs stimulated by Ca^{2+} entry (Ca-MVs). Upon treatment, Ca-MVs are formed following a clear recruitment of Ca^{2+}-binding proteins (sorcin, grancalcin, PDCD6) and particularly annexins (4 and 5). Therefore, it emerges that different molecular pathways are available to produce similar MVs by disturbing the membrane/cytoskeleton interactions. Interestingly, these differences provide non-negligible pieces of information on the parent cells, and the mechanisms and modes of actions involved in the formation of MVs. In addition to biophysical characterization, protein analysis is important to classify these cellular corpuscles and evaluate their potential impacts in diseases or transfusion medicine.

1. Introduction

Extracellular vesicles have emerged as a complex, dynamic and functional part of cells [1,2]. Their population is diverse in terms of shape and size, varying from 30 nm to a few micrometers [3], from exosomes to apoptotic bodies through microvesicles (MVs), and they are found in several disorders including autoimmune, cardiovascular, hematologic and neurodegenerative diseases, cancer, sepsis and so on [1,4]. In particular, elevated levels of MVs have been reported in sickle cell disease and thrombocytopenic purpura. *In vivo*, red blood cell (RBC) vesiculation occurs as a result of physiological aging process (accelerated by the spleen [5]) or eryptosis [6].

Hemoglobin and surface area losses by microvesiculation are also related to *in vitro* aging of RBCs and are one of the hallmarks of RBC storage lesions under blood banking conditions [7–12]. The number of MVs increases exponentially during storage [13,14], which serves to remove damaged materials (such as oxidized proteins [15], due to the presence of reactive oxygen species – ROS) and senescence molecules (e.g. phospatidylserine, PS, exposure at the RBC surface, IgG or neoantigen on band 3 [16]). This process postpones the cell removal and increases RBC shelf life [17]. The accumulation of MVs during storage in blood bags can lead to adverse effects on inflammation and hemostasis in transfused patients [4,18–22]. Hemostatic effects are a

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consequence of their pro-coagulant properties, which have been shown by reduced bleeding time and blood loss in thrombocytopenic rabbits and in Plavix-treated rats [23], or by the promotion of the thrombin generation [24,25].

The formation of MVs follows the disturbance of cytoskeleton–lipid bilayer interactions. The causes are diverse, as the rupture of the anchorage to the spectrin network that increases the compression and induces the formation of a bud that becomes a vesicle and releases the cell tension. This model proposed by Gov et al. is adapted for the rupture of band 3-ankyrin anchors as well as the rupture caused by the flip-flop of PS [26]. MVs are naturally shed during *in vivo* and *ex vivo* aging (formed during RBC storage, storage-MVs), and can also be mediated *in vitro* by calcium intake (Ca-MVs) [27–29] or ATP depletion [28,30]. All these MVs can slightly differ in terms of parent cells (indeed, the age of the parent cells that shed the MVs seems to be different according to Willekens et al. [5,31] and Greenwalt et al. [11,32]) and in terms of protein content as reported by Greenwalt in an excellent review [11].

Molecular analysis of both *in vivo* and *ex vivo* MVs has demonstrated a few proportions of spectrin, the presence of band 3 (where aggregated forms increased during storage), glucose transporter, proteins 4.1 and 4.2, actin as well as lipid raft proteins, and important quantity of hemoglobin, mostly in a soluble state [21,33–35]. In addition, they also bear blood group antigens [36,37]. In particular, stomatin, a member of lipid raft in RBCs [38], was enriched in MVs [33,39]. The analysis of Ca-MVs showed that the members of lipid rafts behave slightly differently. Whereas stomatin was enriched in MVs, flotillin-2 remained in RBC membranes, suggesting a lipid raft-based process [40]. In addition, this *in vitro* stimulation involved synexin (annexin 7) and sorcin. Storage-MVs and Ca-MVs were found similar in size and membrane protein composition by Salzer et al. [39]; the main differences were the amounts of stomatin and flotillin-2, which are 2-fold enriched in storage-MVs and up to 3-fold in Ca-MVs, respectively. However, no comment was made on either synexin or sorcin.

Proteomics has revealed its potential in the analysis of living systems and recently in transfusion medicine [41]. In order to deeply analyze the differences at the protein level between storage-MVs and Ca-MVs, a proteomic approach was used based on two dimensional gel electrophoresis (2DE). In addition, the present work identifies proteins involved in Ca-MVs and points out the relevance of protein analysis in understanding the different mechanisms involved in MV generation.

2. Materials and methods

2.1. Chemicals

$CaCl_2{\cdot}2H_2O$, thiourea, silver nitrate, and calcium ionophore A23187 were purchased from Sigma-Aldrich (Steinheim, Germany); NaCl 0.9%, from Laboratorium Dr. G. Bichsel (Interlaken, Switzerland); urea and DTE from MP Biomedicals (Illkirch, France); Coomassie brilliant blue R250 from Fluka Chemie (Buchs, Switzerland); and Ficoll-Paque PLUS from GE Healthcare (Uppsala, Sweden). FITC mouse anti-human CD47 antibody and BD Trucount tubes were purchased from BD Biosciences (Franklin lakes, NJ, USA). Deionized water (>15 MΩ·cm) was prepared using an ELGA OPTION 4 apparatus (Omnilab AG, Mettmenstetten, Switzerland). Extraction buffer was made of 7 M Urea, 2 M thiourea, 2% CHAPS, 10 mM DTE and 0.5% v/v Pharmalytes pH 4.0–7.0 (or 3.0–10.0 for Off-gel electrophoresis).

2.2. Erythrocyte concentrates and microvesicles

Erythrocyte concentrates (ECs) were prepared from whole blood donations in CPD anticoagulant and component filtered [15]. RBCs were stored at 4 °C in SAG-M™ at a hematocrit of 0.5 ± 0.1. ECs that did not meet the quality criteria for blood transfusion, e.g. low hemoglobin content or a slightly too small volume, were used under the signed assent of blood donors.

2.2.1. Storage-MVs

Storage-MVs were collected from ECs older than 40 days (four independent ECs: 2 at 40, 1 at 50 and 1 at 56 days of storage). RBCs (10 mL of EC + 20 mL 0.9% NaCl) were centrifuged at 2000·*g*, 20 min and 4 °C, and the supernatant was centrifuged for the second time under the same conditions. Then, the supernatant was ultracentrifuged at 120,000·*g* for 60 min at 4 °C, and the pellet was washed two times in 40 mL of 0.9% NaCl. The final pellet of MVs was resuspended in 400 µL of 0.9% NaCl. Total protein amounts were measured according to Bradford assays and MVs were aliquoted at 600 µg of proteins (amounts required for off-gel and 2DE preparation) and centrifuged at 18,500·*g* for 15 min at 4 °C. Pellets were finally dissolved in 200 µL of extraction buffer and saved at −30 °C.

2.2.2. Ca-MVs

Ca^{2+}-ionophore-stimulated MVs were prepared from ECs fewer than 10 days of storage (four independent ECs). First, the storage-MVs were eliminated as follows. RBCs (10 mL of EC + 20 mL 0.9% NaCl) were loaded onto 10 mL of Ficoll and centrifuged at 500·*g* for 20 min at 4 °C. The pellet was washed with 50 mL of 0.9% NaCl and centrifuged for the second time under the same conditions. Remaining MVs were counted by flow cytometry [13]. Briefly, 95 µL of samples was mixed with 5 µL of FITC mouse anti-human CD47 antibody and incubated for 20 min at RT on a roller. Before analysis, 80 µL of labeled MVs was dissolved in 400 µL of 0.9% NaCl into BD Trucount tubes that contained a precise number of three-times labeled microbeads for quantitation.

Five milliliters of packed RBCs was diluted in 45 mL of 0.9% NaCl in the presence of 500 µL of $CaCl_2$ at 1 mM and 45 µL of calcium ionophore at 3.5 µM for stimulation of MVs. RBCs were incubated 1 h at 37 °C on a roller. Ca-MVs were harvested and processed as described above for storage-MVs.

2.3. Off-gel and 2D gel electrophoreses

MV extracts (600 µg in 1200 µL of extraction buffer) were loaded on IPGs (Immobiline Dry-Strip, pH 3–10, 13 cm, GE Healthcare) for Off-gel electrophoresis (to deplete in hemoglobin) and migration was run overnight as previously described [42]. Fifty to 100 µL of solution was collected per well and hemoglobin-free fractions (1–8) were pooled