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Antigenic composition of single nano-sized extracellular blood vesicles

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Abstract

Extracellular vesicles (EVs) are important in normal physiology and are altered in various pathologies. EVs produced by different cells are antigenically different. Since the majority of EVs are too small for routine flow cytometry, EV composition is studied predominantly in bulk, thus not addressing their antigenic heterogeneity. Here, we describe a nanoparticle-based technique for analyzing antigens on *single* nano-sized EVs. The technique consists of immuno-capturing of EVs with 15-nm magnetic nanoparticles, staining captured EVs with antibodies against their antigens, and separating them from unbound EVs and free antibodies in a magnetic field, followed by flow analysis. This technique allows us to characterize EVs populations according to their antigenic distribution, including minor EV fractions. We demonstrated that the individual blood EVs carry different sets of antigens, none being ubiquitous, and quantified their distribution. The physiological significance of antigenically different EVs and their correlation with different pathologies can now be directly addressed.

Keywords: Extracellular vesicles; Microvesicles; Exosomes; Flow cytometry; Antigenic make-up

Background

Extracellular vesicles (EVs): microvesicles, exosomes, and apoptotic bodies are released from cells through either direct membrane outward budding or the late endosomal–lysosomal pathway[1,2]. EVs play an important role in cell-to-cell communication because different proteins, lipids and RNAs are specifically incorporated into these vesicles, which can be targeted to remote cells through receptor–ligand interactions[1,3]. Release of EVs was reported to change in pathologies (reviewed in[4,5]) including cancer[6-9], neurological, hematological [9], cardiovascular[10,11], autoimmune and rheumatologic[12] diseases, and viral infection[13-15].

Since various cells supplying EVs express different antigens, EVs produced by these cells are antigenically different. For example, CD81, a member of the tetraspanin superfamily, is expressed on several cell types including hepatocytes and B lymphocytes[16]; CD63, another member of this family, is expressed on activated platelets, endothelium, fibroblasts, and macrophages[17,18]; CD41, an integrin alpha chain 2b, is a heterodimeric integral membrane protein expressed on platelets, megakaryocytes, and hematopoietic stem cells[19]; CD31, a platelet endothelial cell adhesion molecule, is expressed on vascular endothelial cells, platelets, naive T cells, monocytes, and neutrophils[20].

Analyses of blood EV composition, which have been performed predominantly in bulk, have revealed the presence of various cellular antigens in EVs[21] but could not reflect the distribution of these antigens on individual EVs although such distribution may report on physiological conditions of the donor[22,23].

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Conventional flow cytometry cannot be applied to analysis of small particles like EVs. Several attempts to overcome this limitation have been reported, including the use of single nanometric particle enumerators[24], microfluidics-based cytometers[25], and cytometers optimized to improve light scattering collection[26,27]. While these methods have confirmed the diversity of EV size and quantity[28,29], in most cases they failed to address the compositional diversity of EVs.

While EVs can become visible in flow cytometers upon their staining with fluorescent antibodies, it is difficult to distinguish them from free fluorescent antibodies. Recently it was reported that by using a BD Influx flow cytometer with wide-angle forward scatter it is possible to visualize small fluorescent particles, including EVs labeled with fluorescent antibodies[30,31].

Here, we report on the analysis of surface proteins on *single* nano-sized (<300 nm) EVs with a newly developed nanoparticle-based technique. We used a commercial flow cytometer and magnetic nanoparticles to isolate fluorescence-labeled EVs and to separate them from non-bound fluorescent antibodies. We demonstrate that the blood EVs are highly heterogeneous in surface proteins, with none of the analyzed antigens being ubiquitous. Our analysis revealed the distribution of several antigens and their combinations on *single* vesicles.

Methods

EV preparation and labeling

Microvesicles derived from the SUPT1-CCR5 CL.30 cell line were purified on sucrose gradients and non-specifically labeled with either Alexa Fluor 488 5C Maleimide (50 μM) or Alexa Fluor 633 5C Maleimide (38 μM) as described[32] (kindly provided by Dr. J. Lifson).

Normal blood plasma from the NIH blood bank was collected in several 8-ml tubes with sodium citrate (3.2%); the first tube was discarded to avoid collecting EVs released by platelets activated by venipuncture. Collection tubes were centrifuged at 3000*g* for 15 min to obtain platelet-poor plasma (PPP), followed by a thromboplastin treatment and by the isolation of EVs as described in the Exoquick protocol. Alternatively, we used PPP, enriched with EVs by centrifugation with 100 K MWCO (Amicon Millipore, Billerica, MA) concentrators (8-fold enrichment).

Coupling of monoclonal antibodies to magnetic nanoparticles

Carboxyl-terminated magnetic iron oxide nanoparticles (MNPs; 1 mg of 15-nm particles) (Ocean NanoTech, Springdale, AR) were coupled to mouse–anti-human monoclonal antibodies recognizing different EV antigens. The three antibodies used alternatively for coupling to MNPs were anti-CD81 (eBioscience, San Diego, CA), anti-CD63, and anti-CD31 (BioLegend, San Diego, CA), as previously described[33]. Briefly, 1 mg of MNPs was incubated in 200 μl of activation buffer supplemented with 1.7 mM 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and 0.76 mM *N*-hydroxysulfosuccinimide (Sulfo-NHS) for 10 min at room temperature. After activation, 500 μl of coupling buffer was added to the MNPs, followed by the addition of 1 mg of the purified antibody. After 2-hours in a thermo mixer at room temperature, the reaction was stopped with 10 μl of quenching solution, transferred to a 5 ml propylene round-bottom tube (12 × 75-mm, BD Falcon) and inserted into SuperMag Separator™ magnetic separator. We performed two washes using a SuperMAG-01 magnetic separator (Ocean NanoTech) at 4 °C. The coupled MNPs were suspended in 2 ml of storage buffer and stored at 4 °C at a concentration of 0.5 mg/ml of iron oxide (based on the initial iron oxide concentration provided by the manufacturer).

Capture and detection of EVs with nanoparticles

To visualize the anti-EV–antibody–MNP complexes, MNPs coupled to anti-EV antibody were incubated with 5 μg of Alexa Fluor 488-labeled goat anti-mouse IgG Fab fragments (Zenon anti-mouse IgG, Life Technologies, Carlsbad, CA). To separate free Fab fragments from the bound ones the preparation was washed twice with 300 μl of PBS on a 100-kD nanosep centrifugal device (Pall Corporation, Port Washington, NY) and recovered in its initial volume.

Next, a 10^6 excess of Zenon-labeled MNPs coupled to an antibody against an EV antigen were incubated with EVs 1 hour at 4 °C. Various combinations of monoclonal antibodies against other cellular antigens on EVs: anti-CD31–PE, anti-CD81–PE (BioLegend, San Diego, CA), and anti-CD41–APC (BD, Pharmigen, San Diego, CA) were added for 15 min at room temperature.

EV–MNPs–antibodies complexes were separated on μMACS magnetic columns (Miltenyi Biotech, Auburn, CA) in a high magnetic field generated by a OctoMacs magnet (Miltenyi Biotech). The columns were washed three times with a washing buffer consisting of PBS, 0.5% BSA, and 2 mM EDTA; the complexes were eluted off the magnet in 2 × 200 μl of PBS and fixed in 1% formaldehyde in PBS. To evaluate the efficiency of separation of unbound antibodies, to EV-MNPs complexes, we added fluorescent isotype control antibodies (which should not bind to EVs), and acquired this mixture on the flow cytometer and compared with the same preparation subjected to a magnetic column separation. There was less than 1% of non-specific fluorescent antibodies co-purified with the EVs (Figure S1).

Separated MNPs-EV complexes were subjected to flow analysis. AccuCheck beads (50 μl; Life Technologies) were added to each elution tube to evaluate the volume acquired for flow analysis. On the basis of this volumetric measurement, the number of events can be recalculated as EV concentrations.

iMFI, which reports on the integrated fluorescence intensity by combining the relative amount of positive events with the mean fluorescence intensity (MFI) of these events[34,35] was calculated as suggested iMFI = (MFI) × (*P*); where *P* is the fraction of positive events.

We used LSRII (BD Biosciences, San Jose, CA) flow cytometer equipped with 355-, 407-, 488-, 532- and 638-nm laser lines.

Compensation beads (BD) were used to perform compensation controls.

Detection of EVs by ELISA

Nunc MAXISORP plates (Nalgene- Nunc, Penfield, New York, USA) were coated with 50 μl of a 4 μg/ml solution of anti-CD81 antibody (clone 5A6, BioLegend) in PBS. The plate