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Review

Update on extracellular vesicles inside red blood cell storage units: Adjust the sails closer to the new wind

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ABSTRACT

Release of vesicles from cells is a universal biological system, an adaptive cellular response to endogenous or external physiological or stressful stimuli and a genius means for intercellular, inter-organ and even inter-organism communication. These secreted vesicles that are collectively designated extracellular vesicles (EVs) have increasingly attracted the interest of cell biologists due to their imaginable interactions with every piece of the known biological systems in both health and disease states. Although EVs isolation and characterization are challenges, owing to their particular physicochemical features and complex biology, recent technological innovation has offered better understanding and inevitably, driven the revision of previously established theories on them. However, a crucial question remains unsolved: the physiological relevance of EVs *in vivo*. Since membrane vesiculation is an integral part of red blood cell (RBC) aging and homeostatic machinery and a prominent feature of RBC storage lesion, the characterization of storage EVs and their probable clinical relevance with the therapeutic or adverse effects of transfusions are extremely important targets in the research fields of transfusion biology and medicine. The scientists involved should transfer nascent knowledge and state-of-the-art technological tools in the packed RBC unit in order to: (i) update the inventory of biochemical and biophysical features of storage EVs; (ii) gain insight into the molecular pathways/signals underlying their generation; and (iii) clarify their dependence on blood donor, storage strategies and analytical variations, in order to step forward on understanding their interactions with stored or recipient target cells.

Contents

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1. Introduction to EVs

Extracellular vesicles (EVs) by definition are submicrometer subcellular particles enclosed by a phospholipid membrane bilayer [1]. They are released constitutively by almost all cells in prokaryotes and eukaryotes or after various stimuli leading to cell activation, stress or apoptosis. In practice, EVs are found in the extracellular space, both in tissues and biofluids such as plasma, with a concentration of $>10^{10}$ particles per mL. They contain surface markers of their parent cells and intact or modified molecular components (nucleic acids, lipids, proteins, metabolites) that reflect their cellular origin, localization and mechanism of secretion [2]. The loss of structural and functional integrity of red blood cells (RBCs) during their storage in preservative/additive solutions, collectively referred to as RBC storage lesion (RSL), has been associated with the release of EVs.

Research on EVs is a fast-growing and exciting new field in biology and medicine, because of EVs' capacity to enclose, exocyte and traffic biological messengers from parent cells to a great variety of distant targets, acting as potent modulators of cell function/fate and as vectorial signalosomes working in an elegant intercellular communication system. In this transfer system, enclosing of bioactive molecules by a lipid bilayer effectively protects them from degradation by the soluble enzymes of the body fluids. Thus, EVs are involved in numerous physiological and pathological processes, ranging from removal of unwanted molecular material, salvage mechanism and maintenance of homeostasis to tissue remodeling, wound healing and disease evolving and progression [3].

The family of EVs consists of at least 3 species: tiny exosomes (EXs, <0.15 μm), microvesicles (MVs, also called shedding vesicles or microparticles) in the size range of 0.05–2 μm and larger apoptotic vesicles (also called apoptotic bodies, 0.05–5 μm) produced from cells undergoing cell death by apoptosis. Obviously, EXs overlap in size with viruses, MVs with bacteria and protein aggregates and apoptotic bodies with small platelets (PLTs) [1]. Dexosomes (dendritic cell-derived EXs), prostasomes (prostate-derived vesicles), matrix vesicles (found in bone, cartilage and atherosclerotic plaques), synaptic vesicles (from neurons) and retrovirus-like particles (90–100 nm non-infectious EVs that resemble retroviral vesicles and contain a subset of retroviral proteins) among others, are also included in the highly dynamic extracellular vesicular compartment [4]. These omnipresent biological particles of very low size and refractive index represent extremely heterogeneous structures in terms of biophysical properties, biochemical composition and molecular pathway of cellular release, eg, as byproducts of exocytosis of intraluminal vesicles contained in multivesicular bodies (MVB) upon their fusion with the plasma membrane (in the case of EXs) or directly from it (in the case of MVs) [4,5]. At present, there are no strict criteria and universal "specific" markers to distinguish the numerous subsets of EVs [1]; however, certain combinations of markers have been used for EXs (CD63 and CD9) and apoptotic bodies (thrombospondin and complement protein C3b). Markers of MVs are less well established, though a rho family member, GTP-binding protein ADP-ribosylation factor 6 and VCAMP3 have been proposed for tumor-derived EVs [4].

Despite increasing interest on EVs, their particular features and the biological complexity of the body fluids in both vesicular and non vesicular components render their isolation, detection and classification by the conventional detection methods rather challenging [3]. EVs' size, density, morphology, sedimentation, lipid composition, protein markers and subcellular origin are the currently used criteria for their classification, although a separation method based solely on size and density is not enough to distinguish the various sub-types of EVs. In the near future, novel criteria such as the refractive index and the zeta potential would be engaged [6]. For now, it is difficult to reliably quantify EVs recovery or contamination and to standardize the isolation protocols. The isolated material generally studied contains protein complexes and lipoproteins [7], and a mixture of EVs populations [8], arisen by the fact that the repertoire of EVs is dynamic and varies from cell to cell. Moreover, cells produce highly dynamic and versatile populations of heterogeneous EVs and even the same cell type may secrete different subgroups of EVs depending on cell age, stress, environmental factors and activating stimulus [9].

Consequently, both the isolation of biofluid and the isolation of EVs from it (when necessary) might lead to high pre-analytical variability and artificial generation of EVs. At low centrifugation forces used to remove cells and cellular debris, cell activation and loss of large EVs may occur. Limited data exist on pre-analytical variables in the case of EXs; however, the type of anticoagulant, the diameter of the needle used for the venipuncture, the time between blood collection and handling, the centrifugal speed to yield PLTs-free plasma (PFP), the storage and handling of collected plasma before EVs isolation, the storage and handling of isolated EVs (siliconized vessels are recommended to prevent adherence of EV to surfaces), the freeze/thaw cycles (there is evidence that small EVs are relatively insensitive to freeze/thaw cycles), the buffers used for the resuspension and the separation process may all affect the outcome of experiments in plasma MVs [10–12]. The extreme susceptibility of EVs to handling artifacts probably leads to collection of a vesicular material that does not reflect the natural population of EVs. It has been shown that high-speed centrifugation of plasma induces aggregation of PLT-derived EVs whereas the EVs of RBC origin have less tendency to aggregate [8,13]. A way to overcome these obstacles is the application of standardized protocols to whole biofluids.

2. Vesiculation of RBCs

Exosomes release is a selective cargo-disposal developmental mechanism for membrane remodelling that allows differentiation of reticulocytes into RBCs [14]. Mature RBC is probably the only type of cell for which release of EXs is not expected, as it lacks endosomal network. Nevertheless, (i) EVs indistinguishable from EXs can be released directly from the plasma membrane as shown in T-cells or erythroleukemia cell lines, (ii) diameters of EXs up to 250 nm have been reported and (iii) classic EX protein markers like