Pre-analytical influences on the population heterogeneity of human extracellular vesicles sourced for nanomedicine uses

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The ability to unambiguously distinguish different cell-derived extracellular vesicle (EV) types from one another is like trying to delineate blended colors in a rainbow. Nevertheless, defining EV types is useful to highlight the complexity associated with sourcing EVs for nanomedicine applications. It is generally accepted that the EV spectrum can be subdivided into two main classes, exosomes (EXOs) and shedding vesicles, depending on subcellular origin [1]. EXOs are released from cells when intracellular multivesicular bodies, containing EXOs, fuse with the plasma membrane and release their contents. Shedding vesicles are derived directly from plasma membrane budding and fission. The terms microvesicles (MVs), microparticles (MPs), ectosomes and oncosomes are often used to describe shedding vesicles. Apoptotic bodies (ABs), a specialized shedding vesicle, are formed by plasma membrane zeiosis during the late stage of apoptosis.

EXOs tend to have an average diameter of approximately 100 nm, depending on their cellular source [2], with size ranges reported from 30 to 200 nm [1]. MPs or MVs typically range in size from ~100 to 1000 nm in diameter, whereas ABs have reported diameters of ~50–5000 nm [3]. The size range of EXOs, MVs/MPs and ABs can potentially overlap. Separation of EVs by size alone does not often suffice to distinguish EV types. The use of differential centrifugation to separate EV types based on differences in sedimentation rate is traditionally employed as well.

Efforts to improve EV characterization, through the establishment of definitive phenotypic markers for EV types and subtypes, remain a work in progress. Recent studies demonstrate that the presence of CD63, CD9 or CD81 is indicative of endosome-derived EXOs [4]. Whereas, CD40 expression by EVs is a characteristic of shedding vesicles, and the association of histones with EVs can be used to identify ABs [5].

The phenotypic classifications used to define EV types and subtypes continue to increase in complexity, similar to the evolving definitions used to distinguish immune cell subsets. Within the immunology field, debate continues as to the extent to which immune cell type subpopulations possess fixed phenotypes and functions, versus exhibit a degree of plasticity, or ability to alter phenotype and function, depending on changes to their microenvironment [6]. It follows, that the rate and extent to which cells continuously adapt to ever-changing conditions in their microenvironment, will be conveyed by changes to the type, subtype, phenotype and function of the EVs they produce. Effectively, cellular plasticity, embodied by heterogeneous populations of EVs, prior to their ‘sourcing’ from biofluids, complicates EV-based nanomedicine manufacturing efforts.

Manufacturing EV-based nanomedicines

Sourcing human EVs from biofluids for nanomedicine applications, requires establishing good manufacturing practices (GMPs). Appropriate GMPs for cell-derived EV therapeutics might be developed similar to those for cell-based therapies as described by Smith *et al.* [7]. In general, EV-based GMPs should include rigorous EV biophysical and molecular content characterization, and possibly incorporate scalable production methods depending on the

therapeutic application. These objectives originate from a desire to ensure the production of maximally effective and minimally toxic drugs. However, while this approach is easily conceptualized and readily implemented for the development of synthetic drugs and nanomedicines, it is not entirely applicable to EV-based therapies. For example, EVs do not necessarily induce linear dose–response effects like synthetic drugs. Previously, we discovered that melanoma EXOs can elicit very different biphasic morphological responses by 3D cultured endothelial cells that cannot be explained by linear dosing [8]. They can also induce mixed M1 and M2 macrophage polarity, effectively imparting macrophages with a degree of plasticity or adaptability suitable for facilitating tumor survival [9]. Such complex cellular processes, mediated by EXOs, support the possibility that pharmacodynamic modeling of EV-based therapeutics is more likely to depend on biological ‘herbal-like’ versus allopathic medicine mechanisms. The efficacy and toxicity profiles of an EV-based biological therapy will be derived from the combined effect of many individual EV components working together. This remains true even when an EV is only used as the nanocarrier to deliver a drug. Loading an EV-based nanocarrier with drug cargo involves a plethora of complex interactions between innate EV components and the drug. The resulting configuration introduces an additional pharmacokinetic compartment (the nanocarrier), possessing multifaceted components and physiological interface properties, the permutations of which are unlikely to be fully detected, characterized and mathematically interpreted using traditional pharmacodynamic modeling.

The biological complexity of EVs requires that EV-based nanomedicine preparation approaches be different from synthetic drug production methods. An appropriate GMP for the production of any EV-based biological therapy, used as a therapeutic directly, or indirectly as a drug carrier, is to ensure that the formulation process is consistent, reproducible and specific to the application of interest. Maintaining consistency and reproducibility in the EV-based biotherapy formulation process requires that the heterogeneity of the EV population, sourced for therapeutic modification, exhibits minimal batch to batch variation.

While a number of studies have predominantly addressed the pros and cons of using different EV isolation, biophysical and molecular analysis methods to process EVs, pre-analytical influences on the diversity of EV populations, sourced from biofluids for EV-based nanomedicine applications, have not been appreciably examined. The following sections explore this topic. However, the scope of the discussion is limited to those influences generally relevant to the many therapeutic and diagnostic (biomarker) pursuits relying on EVs sourced from human cell culture or human blood-derived biofluids. Currently, these EV sources tend to be more accessible, and scalable for manufacturing human EV-based nanomedicines. A focused analysis of this issue will hopefully serve to highlight that similar pre-analytical influences are associated with sourcing EVs from other human biofluids including urine, ascites, seminal fluid and CSF, as well as animal, plant and milk products. Commonly encountered pre-analytical influences on the heterogeneity of human EV populations, sourced for nanomedicine applications, can be grouped into the following categories: human cellular nanofactories, anticoagulants and human blood-derived biofluid components.

Human cellular nanofactories

An early step in the EV-based nanomedicine production process is to select an EV source capable of achieving and/or delivering the desired downstream therapeutic effect when modified for nanocarrier use [10]. Having settled on an EV source, the key GMP goal is to ensure that the cell culture nanofactories [1] or bioreactors, used to construct the EV nanocarrier, remain metabolically stable to safeguard against fluctuating changes in the diversity of target therapeutic EV precursor populations.

In general, human EVs sourced from cell culture nanofactories should be produced using cell culture medium depleted of serum-associated fetal bovine or other EVs to prevent exogenous EV contamination in the EV-based nanomedicine manufacturing process. Two additional influences on the population heterogeneity of human cell culture-derived EV populations include mycoplasma contamination and antibiotics. Mycoplasma infection changes the content and function of cell-derived EXOs [11]. Antimycoplasma antibiotics might be added to culture to prevent growth and formation of mycoplasma EXOs and mycoplasma EVs in general. However, antibiotics can also influence EXO content resulting in production of EXOs carrying undesired chromosomal, mitochondrial and DNA-binding proteins on their surface [12]. To avoid these scenarios, EV source cells might be cultured in the absence of antibiotics using rigorous sterile techniques and periodically pathogen tested to ensure health. Similarly, routine authentication of cell lines used as EV nanofactories is warranted to prevent introduction of cell-line cross-contamination-derived EVs into the EV-based biotherapy manufacturing process.

While careful evaluation of cell culture procedures might serve to minimize exogenous influences on EV population diversity, they do not address endogenous cellular influences. For example, suppose an EXO nanocarrier is conceived for the purpose of delivering therapeutic cargo in cancer patients. The EXOs are to be sourced from an immortal stem cell-based nanofactory, enabling a degree of standardized EXO production. One option might be to develop the EXO nanofactory from embryonic stem cells (ESCs). Alternatively, the factory might be derived from induced pluripotent stem cells (iPSCs) or mesenchymal stem cells. The decision to establish one EXO stem cell source over another might be guided by taking into consideration that cancer patients typically experience some degree of immune suppression, either from their cancer and/or chemotherapeutic regimens. Conceivably, certain types of EXO-based nanocarriers may exacerbate immune suppression in cancer patients resulting in tumor progression. While the ESC approach requires less initial cellular cultivation for production than the iPSC approach, tumorigenic potential is a concern. Human ESC cell lines can exhibit features of neoplastic progression including contact inhibition, accelerated growth rate, cytogenetic abnormalities and tumorigenicity *in vivo* [13]. Increasing mitochondrial dysfunction via long-term ESC culture is thought to be one driver for this process [14]. Mitochondrial components can also be exported by cells via EVs [15]. Presumably, normal or dysfunctional mitochondrial components might be relayed to local or distant tissue microenvironments. This raises the question of whether protumor processes might also be relayed by ESC-derived EXOs. Tumor EXOs have been demonstrated to promote immunosuppressive microenvironments supporting tumor growth and survival [3]. If ESC-derived EXOs possess similar innate tumor EXO-like properties, the end result could be that an ESC-derived nanocarrier may antagonize the benefits of its own therapeutic cargo by facilitating protumor processes. In such a scenario, use of iPSC or mesenchymal stem cell EXO sources would be preferable, since they would be expected to have a decreased likelihood of tumorigenic potential, coupled to increased biocompatibility, if sourced via autologous donation from the intended therapeutic recipient [16].

The degree of heterogeneity between EV types and subtypes within the circulatory compartment alone is impressive, as well as other body fluids. Recent studies have begun to explore the physiological significance of this diversity. Beit-Yannai *et al.* demonstrated that incoming EXOs can be intercepted by other EXOs released by target cells forming dimers [17]. This same process may occur during cellular EV production, whereby the generation of different EV subpopulations impedes efforts to establish a stable homogeneous EV population source. If such an EV source was processed for nanomedical applications, the result might be collection of EV dimers and/or aggregates composed of two or more different EV subpopulations.

EXO subpopulations, possessing different proteomes and transcriptomes, have been identified that exert differential effects on recipient cells [18]. It is likely that purified EV preparations always contain, to some degree, subtly different EV subpopulations possessing different biophysical, molecular and/or functional characteristics. Formulation of these mixed EV subpopulations into nanocarriers, would be expected to produce EV-based nanomedicine subpopulations, also possessing subtly different properties that could influence preclinical and clinical testing outcomes.

Anticoagulants

The downstream nanomedical application should also be taken into account when selecting the appropriate anticoagulant for sourcing EVs from blood-derived biofluids. Anticoagulants can influence EV population heterogeneity and EV-modification processes. Sourcing EVs from plasma is preferable to sourcing EVs from serum. The clotting process used to generate serum produces new contaminating EV populations [19]. Wisgrill *et al.* demonstrated that sodium citrate maintains the most stable MV count and function for MVs analyzed within 8 h of blood collection at room temperature or 4°C [20]. By 24 h, MV counts increase with the use of either sodium citrate or heparin. Heparin also induces the release of procoagulant tissue factor expressing MPs [21]. Hypothetically, the use of heparin also may impede procedures used to modify EVs during EV-based biotherapy manufacturing, since heparin can potentially block transfer of labeled EVs between donor and recipient cells [22]. In contrast, EDTA is able to maintain platelet and erythrocyte-derived MV counts for over 48 h, however it also increases the release of myeloid derived MVs [20]. Appreciating how anticoagulants influence EV composition is particularly important for developing EV-based biomarkers. Generation of new EVs, post sample collection, will change the collective EV proteome and transcriptome. This can potentially confound downstream results and interpretations.

While different anticoagulants exert different influences on EV population make-up, it is also important to consider their potential toxicity in the event they are retained throughout the EV-based nanomedicine manufacturing process. Given EDTA's cytotoxic and genotoxic properties [23], it should be avoided when used to source EVs

from biofluids to be therapeutically modified and administered to patients. Similarly, heparin should be used with caution. In apheresis procedures, relevant to autologous sourcing of EVs from blood, heparin can increase bleeding risk, and the potential for heparin-induced thrombocytopenia [24]. In contrast, sodium citrate has been used safely for apheresis procedures for decades with occasional calcium supplementation for mild cases of hypocalcemia [24]. Collectively, the studies within this section suggest that processing citrated whole blood samples into platelet-free plasma for EV isolation, as rapidly as possible, is conducive to reproducible EV-based nanomedicine manufacturing.

Human blood-derived biofluid components

EV population diversity differs between blood products, and within individual blood donors, depending on their current state of health. The presence of viruses, or noninfectious retrovirus-like particles in particular, can impede reliable and consistent sourcing and processing of therapeutic EV precursors from blood-derived biofluids [25]. This is especially true for enveloped viruses such as HIV which have been shown to influence EXO content [26], and/or EV population heterogeneity [25].

Further, blood-derived biological constituents, such as albumin, immunoglobulins and high-density lipoprotein (HDL) may not entirely be removed by many EV isolation methods [19]. Their presence complicates purification, and accurate biophysical characterization of EV populations. HDL density (1.063–1.21 g/ml) can overlap that of EXOs [27]. However, albumin, immunoglobulin and HDL dimensions being approximately <10 nm [28–30], tend to be sufficiently smaller than most EVs to enable their depletion from biofluid samples via size-exclusion methods. Size-exclusion chromatography, using a 60 nm cutoff, has been shown to remove >99 and >95% of soluble plasma proteins and HDL [19].

In contrast to HDL, the dimensions of low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and chylomicrons overlap those of EVs [30]. Of these, recent evidence demonstrates that an order of magnitude more LDL particles are present in blood compared with EVs [30]. While LDL density ($\sim$ <1.06 g/ml), is less than that reported for EXOs ($\sim$ >1.08 g/ml), the high biofluid concentration of LDL particles, coupled to the influence of their size and spherical shape on sedimentation rate, drive their co-isolation with EXOs post ultracentrifugation [30]. The nature of the association between LDL and EVs and its relevance to EV-based nanomedicine efficacy *in vivo* requires further investigation. Variations in individual LDL particle and aggregate size can potentially influence the accuracy and reliability of EV population analyses [27], and thus the consistency of downstream EV-based biotherapy formulation methods.

To decrease the potential for lipoprotein contamination of EV preparations, it is important to take into account patient fasting state prior to blood collection. Increased chylomicron and VLDL production occur postprandial [30]. Similar to HDL [19], size-exclusion chromatography approaches can be used to minimize chylomicron and VLDL co-elution with EVs [30]. Lipoprotein contamination may be encountered with the use of EV precipitation reagents as well, which also promote co-isolation of albumin and/or immunoglobulins. Assessment of chylomicron, HDL and LDL levels during EV-based nanomedicine processing might be incorporated into EV-based GMPs depending on the therapeutic application. ApoB48, ApoA1 and ApoB100 are useful markers to quantify chylomicron, HDL and LDL [19]. To minimize lipoprotein co-isolation during initial EV processing, Simonsen recommends a two-step approach for purification of blood-derived EVs. Samples are first fractionated by size alone, and then isolated by density [30].

Summary

The difficulty in definitively classifying EVs is derived in part from the fact that cells can continuously, depending on microenvironmental conditions, produce multiple EV types and subpopulations simultaneously and in various proportions. The innate biological complexity of EVs requires that EV-based nanomedicine GMPs be different from that of synthetic therapeutics. An appropriate GMP for the production of any EV-based biotherapy, used as a therapeutic directly, or indirectly as a drug carrier, is to ensure that the formulation process is reliable, reproducible and specific to the application of interest. Ensuring consistency and reproducibility in the EV-based nanomedicine formulation process requires that the diversity of the EV population, sourced for biotherapy conversion, does not vary between batches. Pre-analytical influences on the population heterogeneity of human EVs sourced for nanomedicine applications include human cellular nanofactories, anticoagulants and human blood-derived biofluid components.

Final thoughts

Beyond the controllable influences on EV population diversity discussed throughout, a key question concerns the extent to which fractionation of inherently heterogeneous populations of EVs, into increasingly purer homogeneous subpopulations, is necessary for their use as biological therapies. It could be that there is an ‘irreducible heterogeneity’ limit for a given EV population, beyond which a diverse EV population loses its collective functional and/or desired medicinal potential. In the end, perhaps a degree of EV-based nanomedicine plasticity, made possible by a spectrum of subtly different EV types and subpopulations working in concert, is necessary to achieve desired therapeutic outcomes.

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