Extracellular Vesicle-based Therapeutics

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Contributor: Elena Batrakova

This is a entry of recent developments of bio-inspired drug delivery systems based on extracellular vesicles (EVs). The main hurdles and limitations for therapeutic and clinical applications of EV-based formulations and various attempts to solve these problems are described.

Keywords: drug delivery ; extracellular vesicles ; exosomes ; clinical applications

1. Introduction

Extracellular vesicles (EVs) have emerged as a new class of nanocarriers, triggering significant interest and enthusiasm. Extraordinary efforts have been made to develop new techniques that would make it possible to manufacture EV-based drug formulations for the treatment of various diseases, including cardiovascular diseases [1][2][3][4][5][6], regenerative disorders [7][8], infectious diseases [9], cancer [10][11][12][13][14][15][16][17][18][19], as well as autoimmune [20] and neurological disorders [17][21]. EVs are short- and long-distance mediators of intercellular communication that offer distinct advantages, uniquely positioning them as highly effective drug nanocarriers. They comprise various types of nanovesicles, including exosomes (30–120 nm), microvesicles (MVs) (50 nm–1μm), and apoptotic bodies (500–1000 nm) [22][23][24]. Notably, EVs consist of cellular membranes with multiple adhesive proteins on their surface [25][26] that enable efficient cell entry and delivery of therapeutic cargo.

The unique properties of EVs can be attributed to their biogenesis. Exosomes are initially produced by invagination of the endosomal membrane to create multivesicular bodies (MVB) [27]. In contrast, exosomes' close relatives, MVs, are greater in size and bud directly from the plasma membrane. Therefore, exosomes and MVs originate from endosomal and plasma membranes, respectively. Apoptotic bodies form during the apoptotic process, when the cellular cytoskeleton breaks up, causing the membrane to bulge outward [28]. Different techniques have been developed for the characterization of EVs. Among them are nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS), that provide information about: count (NTA) and size distribution (NTA and DLS); flow cytometry, western blotting, and mass spectrometry (MS) that can be used to characterize biochemical content of EVs; and several microscopy techniques Atomic Force Microscopy (AFM) and Cryogenic Transmission Electron Microscopy (CryTEM) that make it possible to assess EV morphology [29]. The structure, biogenesis and composition of EVs have been extensively described in several excellent reviews [7][18][21][30][31][32][33][34][35][36].

Similar to artificial nanocarriers, EVs can improve the fundamental characteristics of a free drug, such as its stability and solubility, and protect the drug against degradation in the bloodstream [31]. Relatively tight lipid bilayers in EV membranes can provide a sustained and prolonged release of the incorporated drug. Furthermore, contrary to most synthetic nanocarriers, EVs can cross biological barriers, including the blood brain barrier (BBB), making them especially valuable for the treatment of neurodegenerative disorders. It has been shown that EVs can cross the BBB from the brain to the bloodstream [37][38], as well as from blood to the CNS in vitro [39][40] and in vivo [41][42][43] under pathological conditions. However, whether EVs cross the BBB in the absence of pathology is still debated. Furthermore, these natural nanocarriers have low immunogenicity (especially, autologous EVs) and low cytotoxicity, which are usually substantial impediments for conventional synthetic nanoparticles. Finally, some types of EVs exert tissue tropism that makes it possible to target their formulations to specific cell types or migration towards inflamed tissues [44][45]. It is worth mentioning that bioinspired nanocarriers may have unique biological activity which is reflective of their origin, i.e., parent cells, that provides additional therapeutic efficacy to the incorporated drug [43]. These attractive features have contributed to the growing interest in EVs and inspired numerous studies aimed at their introduction to the field of drug delivery.

Despite these advantages, the clinical translation of EVs has been greatly slowed down due to a number of drawbacks, including upscaling processes of isolation and purification, as well as the lack of a means of efficiently loading these natural nanovesicles with therapeutics. Reliability, reproducibility, and donor-donor variations of EV formulations are still of significant concern. Furthermore, EV functional heterogenicity and limited yields represent serious obstacles for their

future applications. Thus, depending on the mechanism of EV release, they may contain different proteins, active proteasomes, and even organelles (e.g., mitochondria) [46]. Inadequate targeting is another challenge for the clinical translation of different EV-based drug formulations. Herein, we will discuss how these hurdles can be overcome to introduce this unique biomimetic drug delivery system to the clinic.

2. Implications Related to Biological Activity Inherited from EVs Origin

The biological activity of EVs released by various types of cells is vast and promising. Their ability to impact cells depends largely upon their protein markers and their cargo, which mimic the properties of their origin. Isolated EVs taken directly from specific types of cells, such as fibroblasts, neuronal cells, macrophages, and even cancer cells have a wide array of both pathogenic and therapeutic activities, largely depending their host cells. Therefore, one should pay special attention to the source of EVs and possible unwanted biological activity inherited from their parent cells. For example, EVs derived from diseased cells may contribute to the ability of a pathogen to spread throughout the body and evade the immune system [47]. Tumor-derived EVs are well-documented to express specific immune system markers such as MHC Class I and II molecules, death receptor ligands (FasL) and many others. The expression of these markers enables EVs to interact directly with prominent immune system cells such as T cells, B cells, and NK cells to encourage oncogenic activity and inhibit the immune system processes. Melanoma-derived EVs express FasL, which activates the Fas/FasL pathway to induce lymphocyte apoptosis, allowing tumors to evade cell-mediated cell death [47]. Next, EVs may contain prominent mediators that encourage angiogenic activity, metastasis, and mRNA transfer, leading to growth within the tumor microenvironment. Thus, gliomas, i.e., human brain and spinal cord tumors, express an oncogenic form of the epidermal growth factor receptor, EGFRvIII [48]. In mice, EVs containing EGFRvIII were shown to be released into the blood and fuse with tumor cells lacking EGFRvIII, conferring oncogenic activity upon previously benign cells. Moreover, cancer cell-derived EVs were shown to transport oncoproteins, including antigen MelanA/Mart-1 (melanoma), carcinoembryogenic antigen (CEA) (colon carcinoma), and HER2 (breast cancer) [49]. Finally, EVs can carry cancer-related miRNAs. Specifically, large amounts of small RNAs such as let-7, miR-1, miR-15, miR-16 and miR-375, which play an important role in cancer, were found in EVs [50]. Furthermore, Li at al. [51] studied the mechanism underlying the association between EVs and hypoxia during cancer progression. It was suggested that cancer cell-derived EVs mediate miRNA transfer and promote prometastatic behavior. Thus, oral squamous cell carcinoma (OSCC) cells secreted miR-21-rich EVs that ultimately contributed to the migration and invasion of OSCC cells [52]. In addition, miR-29a-3p carried by EVs from OSCC cells promoted M2-type macrophages polarization, and such macrophages enhanced the proliferation and migration of OSCC cells [53]. Hence, in many cases, it is preferable to use “clean” EVs without interior content that would not induce unwanted effects in patients. One approach to achieve this is to develop methods for the removal of the cargo of naive EVs without significant changes of the structure and content of their membranes. For example, Jang et al. [54] suggested using exosome-mimetic nanovesicles produced by the breakdown of monocytes via a serial extrusion through filters. These cell-derived nanovesicles should be depleted of their internal content inherited from parent cells.

Interestingly, EVs released by mesenchymal stem cells (MSCs) may deliver a bioactive cargo that inhibits or promotes tumor growth [55][56][57]. Thus, some studies indicated that MSC-derived EVs can play several roles in tumorigenesis, angiogenesis, and metastasis [42][58], although other studies showed tumor-suppressing effects [59][60][61][62]. These inconsistencies may be attributed to the source of parent MSCs, specifically, whether MSCs were obtained from cancer patients or healthy individuals [63]. Accordingly, nonmodified EVs may possess specific properties that would be beneficial to their therapeutic outcomes. For example, MSC-derived EVs have received much attention as potential therapeutic agents with regenerative properties [64][65][66][67][68][69][70], including protective effects in models of myocardial ischemia/reperfusion injury [65][66][71], pulmonary vascular disease [72], chronic myocardial infarction [73], and stroke [68][74][75][76]. Furthermore, EVs released by neural stem cells (NSCs) are known to promote neural tissue regeneration and functional recovery by releasing paracrine factors. In a recent report, Zhang et al. [77] demonstrated that the treatment of parent NSCs with interferon-gamma (IFN-γ) induced a generation of altered EVs that exerted improved therapeutic effects in an ischemic stroke rat model. Likewise, EVs derived from NSCs were shown to preserve and restore photoreceptors, decreasing apoptosis during retinal degeneration in rats [78]. Finally, EVs, particularly those produced by immune cells, are known to have immune-modulating, protective, and regenerative effects in conditions such as cardiovascular disease, atherosclerosis, and stroke [79]. Obviously, this additional biological activity may improve the therapeutic outcomes of drug-loaded formulations and should be considered when bio-inspired formulations are developed. For example, our earlier investigations demonstrated that naive EVs released by regenerative anti-inflammatory subtype of M2 macrophages produced synergistic neuroprotective effects in mouse models of Parkinson’s disease [43]. These effects were subtle but could be beneficial when added to the effects of incorporated therapeutics. Overall, these developments indicate that EVs can implement more than only inert carrier functions by being biological response modifiers. Further tailoring EVs may provide biologically active carriers that may be modified in accordance with the disease and produce,

for example, the cytotoxic effects of EVs released by M1 macrophages for cancer treatment, or the neuroprotective effects of EVs released by M2 macrophages for the treatment of neurodegenerative disorders, and enhance the outcomes of their therapeutic cargo.

3. Improving Functional Heterogenicity and Yields of EVs Nanocarriers

EVs consist of various types of nanovesicles, namely exosomes, MVs, and apoptotic bodies [22][23][24]. It has shown to be difficult to separate EVs and MVs, mainly due to overlapping vesicle sizes and proteins expressed on their surface. Therefore, in most cases, a mixture of EVs and MVs is used to produce drug formulations [24]. It should also be noted that the absolute separation and definition of various EVs based on their size or biogenesis has yet to be established beyond doubt, and there is currently no consensus on markers that distinguish the origin of these vesicles once they have left the cell [80].

EVs can be isolated from conditioned cell culture media or bodily fluids by different methods, including differential centrifugation, filtration paired with centrifugation, concentration paired with ultracentrifugation, immunoaffinity chromatography, size exclusion chromatography, and polymer-based precipitation. Each isolation technique has advantages and disadvantages that should be considered in terms of being reproducible, specific, and feasible [81]. Differential ultracentrifugation combined with density gradient centrifugation are considered the “gold standard” for isolating EVs. This process involves applying a centrifugal force to a solution containing EVs, e.g., a conditioned cell culture media or biological fluids. It is worth noting that the type, quantity, and quality of EVs isolated by this method is sensitive to the *g* force, rotor type, angle of rotor sedimentation, radius of centrifugal force, pelleting efficiency, and solution viscosity. Gradient centrifugation requires extensive (62–90h) centrifugation time [82], but provides a more uncontaminated EV isolate than ultracentrifugation alone. Of note, ultracentrifugation is associated with morphological alterations and partial aggregation of vesicles. Immunoaffinity chromatography is a more efficient method for isolating EVs as compared to differential ultracentrifugation and density gradient ultracentrifugation [81]. It requires a single easy step without using harsh chemicals. However, this method provides a relatively low yield and can be used for small volumes only. In addition, because this method of EV isolation depends on antibody recognition of EV proteins, only a subset of all EVs (those expressing the antibody-recognized protein) can be captured. Size exclusion chromatography (SEC) preserves the integrity and biological activity of EVs using gravity flow when vesicle structure and integrity remain intact. This is a fast and easy procedure that requires a small sample volume. However, a low concentrated sample needs an additional process for enrichment. Finally, polymer precipitation is relatively easy to use and does not require specialized equipment or a lengthy run time. However, it has been shown that this method coprecipitates nonvesicular contaminants such as lipoproteins, as well as polymer material [83]. Thus, Patel et al. [84] compared four EV isolation techniques for yield and purity. The polymer-based precipitation method had the maximum yield, followed by size-exclusion chromatography and differential ultracentrifugation. The immunoaffinity-based isolation method yielded the fewest EVs. Importantly, a high yield of EVs was accompanied by contaminations with serum proteins and chemical impurities, including high salt concentration, Sodium Dodecyl Sulfate (SDS), or Polyethylene glycol (PEG) contaminations after polymer-based precipitation. These issues may be addressed by pre- and post- isolation steps. Pre-isolation involves the removal of subcellular particles such as lipoproteins. Post-isolation involves removal of the polymer, typically by using a Sephadex G-25 column [82]. Therefore, considering large-scale clinical manufacturing [85], a level of segregation EVs from copurifying components may influence the functionality and therapeutic activity of the final product.

The translation of EV-based formulations into clinical practice requires compliance with existing regulatory frameworks [86]. EVs are a fairly heterogeneous population in terms of their biochemical composition, size, and the source [87]. Thus, the standardization and effective purification of large amounts of these nanovesicles is a critical, but still considerable, challenge. Specifically, the manufacturing of homogeneous drug nanoformulations, and production and quality control, are crucial requirements. Moleirinho et al. [88] developed a purification method using semicontinuous multicolumn chromatography, a robust, scalable and efficient tool for EV purification. Besides the higher recoveries obtained with the continuous system when comparing with batch chromatography, the EV properties were maintained during the purification process regarding their size and morphology. A fast and reliable method of isolating serum EVs was reported by Navajas et al. [89]. Using size-exclusion chromatography with qEV columns (Izon, Christchurch, New Zealand), a homogeneous population of EVs in terms of size, morphology, and protein composition was obtained.

Another challenge that has critical implications for the use of EV-based formulations is whether the sufficient number of these carriers can be generated [90]. Indeed, the EV yield per cell will impact the final production cost, as well as having clinical applications. In this respect, the choice of parent cells is very important. For example, MSCs are known to produce large numbers of EVs, suggesting that these cells may be efficient for EV production in a clinically applicable scale [65][91]. Several reports have indicated that specific treatments of EV producing cells could considerably increase the yield of

these natural nanocarriers. For example, culturing dendritic cells (DCs) for a prolonged time [92] or at low pH [93] increased EV production up to ten-fold. Furthermore, the addition of neutral and cationic-bare liposomes enhanced EV secretion in a dose-dependent manner [94]. However, the possible contamination of EVs with liposomes is a serious concern associated with this method. Gao et al. [95] reported high yield of EVs using nitrogen cavitation that instantly disrupted neutrophils to form nanosized membrane vesicles. The authors indicated that this approach made it possible to increase the manufacture of EVs by 16-fold. Another option is to break parent cells, for example, monocytes/macrophages with simultaneous loading with anticancer agents, followed by the isolation of EV-like nanoparticles [54]. In attempting to mimic the function of EVs with nanovesicles, Jang et al. [54] utilized human U937 monocytic cells to produce nanovesicles with the ability to carry large amounts of therapeutics. While maintaining the plasma membrane proteins of the targeted cells, the drug-loaded nanovesicles were able to efficiently induce tumor cell death and increase the production yield of chemotherapeutics in relation to naturally occurring EVs by 100-fold. Of note, one should consider that the alteration of cell culture conditions can certainly increase yield, but the impact on the biological effect of EVs has to be crucially assessed for biosafety reasons.

Next, the mass production of EVs by membrane fusion with lipid-based materials was suggested in several reports [96][97][98]. The manufacture of large quantities of drug nanocarriers was achieved via a membrane extrusion technique [96] that allowed up to a 43-fold increase in the numbers of vesicles postisolation. The production of hybrid EVs was also proposed by Rayamajhi et al. [97]. EVs from mouse macrophages were hybridized with synthetic liposomes that increased the yield and retention of the EV functional properties. The manufacture of hybrid EVs was also suggested by De La Peña et al. [98]. This group utilized coated liposomes as artificial EVs, and discovered that the obtained nanocarriers functioned as naturally occurring EVs and efficiently activated immune responses [98]. Chemically-induced membrane blebbing was suggested for the fast production of large numbers of EV-like vesicles [99]. Different chemical agents, for example, sulfhydryl, paraformaldehyde, or dithiothreitol, were shown to lock the cell in a fixed physiological state and promote the release of vesicles from a plasma membrane to the conditioned media.

A different approach for the upscaling production of EVs was reported in a study conducted by Li et al. [100]. Instead of manufacturing EVs from animal cells, the authors utilized biocompatible bovine milk EVs (mEVs) that can be obtained inexpensively in large quantities [100]. mEVs were loaded with doxorubicin (Dox) and decorated with hyaluronan (HA), in order to direct them to CD44-overexpressing tumor cells. HA is a CD44-specific ligand which ensures that the EVs are directed to the cell membrane of the specified tumor cells. mEVs were able to deliver chemotherapeutics to tumor-specific cells in vitro and trigger apoptosis [100]. Crashed grapes were also suggested as an abounded source for EV-like nanoparticles [101]. Thus, the oral administration of EV-like nanovesicles from grapes facilitated intestinal regeneration in a mouse model of colitis that was induced by exposing mice to dextran sodium sulfate in drinking water. The EV-like nanovesicles prevented the colitis-associated reduction of both intestinal length and villus height. As a result, mice treated with grape-derived EV-like nanoparticles lived twice as long as untreated mice.

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