Extracellular Vesicles(EVs)

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Extracellular vesicles (EVs), comprising large microvesicles (MVs) and exosomes (EXs), play a key role in intercellular communication, both in physiological and in a wide variety of pathological conditions. However, the education of EV target cells has so far mainly been investigated as a function of EX cargo, while few studies have focused on the characterization of EV surface membrane molecules and the mechanisms that mediate the addressability of specific EVs to different cell types and tissues. Identifying these mechanisms will help fulfill the diagnostic, prognostic, and therapeutic promises fueled by our growing knowledge of EVs.

extracellular vesicles exosomes click chemistry recipient cells glyco-proteomics

1. Introduction

in the last 20 years has revealed a new paradigm in intercellular communication. Originally regarded as mere garbage packaging, EVs rose to the privileged status of universal mechanism used by any cell type to exchange almost any molecule involved in cell physiology and pathology [1][2][3]. The basic principle underlying EV-dependent communication is quite simple, and resembles that of most hormones: EVs are first released by their donor cells into the extracellular space and then taken up by other close or distant recipient cells. The main purpose of this efficient communication system is the education of recipient cells by donor cells; to carry out this process, EVs exploit a variety of biological molecules (such as DNA, RNA, lipids, proteins and enzymes) packed as cargo.

The field of EVs has attracted a lot of interest from biomedical scientists in the last 10 years, leading to thousands of published experimental papers and reviews, covering almost every area of cell biology, physiology, and pathology. Although we understand in the literature alternative and more detailed EVs classifications have been proposed, for purpose of simplicity in this review we will distinguish two broad types of EVs based mainly on their mechanism of formation and their size: microvesicles (MVs) and exosomes (EXs).

Readers interested in a detailed account of EVs features, such as EVs release mechanisms, role, composition, and functional effects elicited by EV cargo, could refer to several of the comprehensive reviews that have appeared recently in the literature [4][5][6][7][8].

This hypothesis raises several intriguing questions: (a) is it possible to deduce the type of parent donor cells and the type of recipient cells by analyzing the molecules exposed on the outer membrane of EVs?; (b) do the donor and recipient delivery codes leverage their own different patterns of “sender” and “recipient” molecules to perform their duty? Cracking this presumed EV delivery code by leveraging the data from studies aimed at deciphering the

identity and functional role of “sender” and “recipient” molecules, would have profound implications for a wide range of crucial topics in cell biology. A variety of mechanisms is deployed in the formation and release of EV subtypes: small EVs such as EXs derive from endosomal compartments, while larger EVs are assembled via invagination of cell membranes, suggesting that EVs protein expression patterns should resemble those of their parent cells. The quest to find these indicators is especially relevant to cancer and metastasis studies, since the identification of EVs surface membrane molecules, especially glycoproteins, proteins, and lipids, would enable the inference of several features of their cancer parental cells [9].

One of the most promising fields of application, derived from deeper insights into the ability of EVs to target specific tissue or cells, deals with EVs as a potential tool for drug delivery [10][11]. EVs display important features and advantages over conventional drug delivery systems, (a) EVs are not immunogenic; (b) EVs can be loaded with drugs and other therapeutic agents; (c) EVs can cross almost any biological barrier; (d) EV surface membrane molecules can be engineered to modulate their uptake into recipient cells; and finally (e) EVs are highly suitable as a delivery tool for gene therapy or in small RNA-mediated interference applications. However, several important problems need to be addressed before these approaches can be approved by drug approval agencies.

The main problems are the homogeneous isolation and standardized large-scale production of EVs necessary to implement drug loading systems. EVs display a variable composition that depends upon several parameters such as parental cell type and cellular activation state, conditions of in vitro propagation and growth biogenesis pathway, which is in turn affected by intracellular cargo sorting routes [12][13].

Methods for loading EVs or modified EVs with drugs suitable for encapsulation [14] are being investigated but this strategy needs refining due to its low efficiency and the risk of toxicity.

Finally, the feature that most qualifies EVs as a promising vehicle for drug delivery is the potential to modify and engineer EV surface membrane molecules to enhance their selectivity and specificity toward target cells [15]. This feature has been fruitfully exploited in harnessing EXs as a precise and reliable system for delivering drugs to treat the brain: the bioavailability of such drugs is severely impaired by the blood brain barrier (BBB) [16].

Recent years have seen a rapid increase in our knowledge of the many features shared by viruses and EVs, such as size, packaging mechanisms and patterns of release [17]. Notably, the main difference between viruses and EVs is the content of their molecular cargo and the fact that EVs are not infectious, a key advantage for future applications (see the conclusion section). Perhaps the most important common functional property is that both viruses and EVs rely on the glycosylation of their surface membrane or envelope proteins to bind with specific surface membrane components of target cells [18]. This notion has been validated by a novel finding that highlights the crucial role of glycans in the interaction between virus glycoproteins and their targeted receptors, implicated in the mechanism of accession to cells.

In this review, we will first illustrate the principal techniques used to analyze EV surface membrane molecules and then give an overview of experimental works that have attempted to deduce their functional effects. We conclude

by suggesting future trends and innovative approaches to improve current knowledge of EV-recipient cell interactions.

2. EV Targeting

The uptake of EVs by recipient cells mainly occurs through membrane fusion and endocytosis [13][19], which require the participation of distinct classes of molecules. The most abundant and best-characterized ligands implicated in EV-cell interactions are glycoproteins, lipids, and glycans, but the role of other surface membrane molecules such lectins, heparan sulfates proteoglycans and EGFR should not be dismissed [20].

Functional studies conducted so far have used one of two protocols: the former investigates the uptake of one EV population by distinct types of recipient cells, while the latter is based on a sort of reverse approach in which several EVs derived from different donor cell are tested for their intrinsic ability to internalize into one specific cell type. For example, EXs from oligodendroglia precursors are preferentially internalized by microglia but not by other brain cell types [21], and bone marrow dendritic cell (DC) EXs are internalized by splenic conventional DCs rather than by other immune cells [22]. In certain cases, binding and internalization mechanisms may differ between cell types; the kinetics and modality of uptake of microglia-derived MVs are different for microglia and astrocytes [23] and the same has been observed for EXs derived from leukemic cells toward recipient phagocytic cells [24]. Several in vivo experiments have confirmed targeted localization of EVs derived from distinct cell types toward diverse organs and tissues.

Another study broadened the functional role of astrocytes showing that upon activation by interleukins, these cells could affect neuronal uptake, differentiation, and firing via enrichment of surface membrane proteins such as integrins and the major histocompatibility complex [25]. That EVs have a differential affinity for specific target cells is supported by the finding that neuroblastoma-derived EXs interact with hippocampal neurons and glial cells but are preferentially taken up by glial cells whereas cortical neuron-derived EXs are endocytosed almost exclusively by hippocampal neurons [26]. This report suggests that at least in the central nervous system, EV targeting may entail both specific and non-specific mechanisms, probably reflecting the selective pattern of surface membrane proteins. However, although these studies indicate a promising direction for future in vivo and in vitro models, it should be remarked that few of them have clarified the type and composition of surface membrane molecules involved in EV-cell interactions.

Quite surprisingly, only two studies have investigated the molecular identity of conjectured sender or receiver biomolecules expressed on the outer membrane surface. In the first seminal report [27], the authors found that EXs derived from different tumor cell lines display organotropism toward specific tissues to prepare the pre-metastatic niche. , integrins α6β4 and α6β1 preferentially trigger lung metastasis, while integrin αvβ5 preferentially triggers liver metastasis. In addition, functional experiments have shown that integrin engagement with recipient cells activates the Src-dependent signaling pathway and induces S100 gene expression.

The pivotal role exerted by cellular integrins [28][29] and integrin expression in EVs in cancer-dependent mechanisms [30][31] has been further confirmed by a study which showed that in mice, blood or cancer-derived EVs modulate anchorage-independent growth of prostate cancer by recruiting β1 integrins [32]. In another study [33], heparan sulfate proteoglycans (HSPGs), localized on the recipient cells' outer membrane surface, acted as receptors for cancer cell-derived EXs. This result could have a critical impact on EV biology, because it strongly suggests that the pharmacological perturbation of the HSPG-dependent uptake route, together with identification of the EV binding partners of HSPG, could be a potential target for the modulation of EX-mediated tumor development. However, the results of these two studies should be taken with caution: those of the first report have not been fully reproduced and therefore require confirmation; while in the second study, the overexpression of HSPG in target cells may invalidate the conclusion, likewise necessitating validation in similar but more physiological experimental conditions.

EV biomarkers may be the key to classifying EVs subtypes in addition to being potentially reliable cancer biomarkers [34] that could improve the predictive performance of liquid biopsies in clinical oncology. In a similar report [35], in murine mast cell and human urine-derived EXs, tissue-specific common protein patterns involved in transport, signaling, and cytoskeletal proteins were detected. Proteomic analysis of EVs derived from human dendritic cells [36] has allowed the classification of EVs based on the expression pattern of five protein categories, thus allowing the authors to implement an EV immuno-separation method based on the use of the tetraspanins CD63, CD81, or CD9 as associated markers. In agreement with this finding, a recent MS-based proteomic approach defined markers in human plasma EVs that can distinguish between individuals with or without certain cancer types [37].

A targeted label-free proteomic strategy (SWATH-MS) demonstrated that certain common EV markers (CD9, CD63, ALIX, TSG101 and HSP70) were enriched in urinary EXs as compared to MVs and urinary free proteins [38], validating this approach for distinguishing between MV and EX protein expression patterns. In another application of proteomic analysis of EVs derived from human colon and lung cancer cell lines [39], the authors found that among nearly 30 cell line-specific markers, several proteins were involved in integrin-, Rap1-, and EGFR-dependent signaling pathways, further strengthening the idea that EV biomarkers may identify their parental tumor cells.

Several chemical engineering approaches have been proposed to enhance the ability of EVs to be taken up by recipient cells [40][41][42][43]. These modifications are mainly aimed at improving the selectivity of drug cargo delivery to reduce the adverse effects of drugs; and at synthesizing novel imaging reagents that will enable precise and early diagnosis of illnesses. The addition of the Arg-Gly-Asp (RGD) peptide on the EXs outer membrane surface improves targeting of blood vessels [16] thus enhancing not only the efficacy of therapeutic intervention against angiogenesis but also the performance of imaging procedures via click chemistry-dependent metabolic labeling. In another quite similar approach [44], the conjugation of the c(RGDyK) peptide to EX surface membrane proteins, via bio-orthogonal click chemistry, increased cRGDyK-EXs targeting in an in vivo mouse model of brain ischemia.

3. Analysis of Single Extracellular Vesicles

Although the intercellular heterogeneity of EVs has been clearly documented, their intracellular heterogeneity is still open to speculation due to the paucity of experimental data. Unfortunately, almost all studies so far published have carried out bulk analyses of EV proteins, masking individual EV expression profiles and leading to erroneous conclusions. Indeed, at present, the influence on cell culture models of parameters such as external stimuli, proliferation, motility, and cell density on the heterogeneity of released EVs is not known.

Recently, an innovative and extremely sensitive proximity-dependent barcoding assay, using antibody-DNA conjugates coupled with next-generation sequencing, was implemented to profile the surface membrane protein patterns of single EVs [45]. The authors reported that this assay was able to measure the expression of 38 different proteins in human body fluids or cell culture-derived EXs, thus strengthening the notion that the identification of EV surface membrane protein patterns is a reliable tool to distinguish EVs derived from different sources. When glioma cell-derived EVs were tested, the authors found that a considerable fraction of droplets were positive for only one marker (i.e., EGFR or EpCAM) but a discrete percentage (39%) of these EVs displayed positivity for both markers. Although in comparison with traditional massive MS analysis, these two approaches detect a smaller number of proteins and require DNA-barcoded antibodies coupled to microfluidic systems, their excellent sensitivity paves the way for the development of future platforms in single EV analysis.

A tool combining separation of EVs based on their size with individual surface membrane protein analysis by in situ fluorescence labeling, termed EV-Ident [46], found a difference in expression of certain markers between breast cancer and prostate cancer cell-derived EVs. Notably, this study allows the determination of EV markers in EVs from separate sources based on their size, thus expanding our knowledge of medium and large EVs, which are usually poorly investigated in other reports. Another approach based on localized fluorescent imaging, termed digital profiling of proteins on individual EV (DPPIE), uses an anti-CD9 antibody engineered biochip to capture EVs from clinical plasma samples and then a multiple DNA aptamer coupled to rolling circle amplification (RCA) to generate localized fluorescent signals [47]. Notably, this method was able to discriminate between EVs derived from lung adenocarcinoma and lung squamous carcinoma patients.

In another almost identical approach [48], EVs were immobilized using a biotin-avidin capture system and labeled with probes. This study revealed some interesting properties of examined EVs: (a) about half of EVs only expressed one or two of the 11 markers evaluated; (b) only a small percentage of EVs exhibited five or more markers; (c) four EV marker subtypes accounted for most of the total population and (d) cluster analysis identified 14 main populations based on marker expression. SEA analysis found that 366 proteins out of a total 2178 were consistently present at higher levels in EVs than in cells, and that EXs mostly express CD9, whereas 50% of large EVs are either CD9 or CD81 One major limitation of the SEA approach resides in the lack of a standardized method to establish a valid cutoff for a particular marker to differentiate between true negative EVs and false negative EVs expressing the same marker but below the assay threshold.

4. EVs and Vaccines

The SARS-CoV-2 virus uses the spike proteins sticking out of its surface to attach to and enter cells in the human body. These proteins are coated with glycans, which disguise parts of the viral proteins to the human immune system. Chemical analysis of the glycans coating the spike protein [49] will be critical in the development of new COVID-19 vaccines that rely on recombinant spike protein [50] triggering the immune response.

The similarities between viruses and EVs have prompted efforts by a drug company to use EXs in vaccine development [51]. Indeed, this company is exploiting the intriguing hypothesis that EXs could be a more efficient and safer delivery vehicle for mRNA than the currently used lipid nanoparticles (LNPs). This fast-growing field, still in its early infancy, has generated some interesting questions: (a) are EVs a better shuttle for mRNA delivery than LNP, ; (b) do cells infected with conventional vaccines release EVs bearing on their surface the exogenous protein codified by mRNA?; (c) is it possible to modify, chemically or by metabolic engineering, the composition of surface membrane EV glycoproteins to enhance their uptake in recipient cells?; and last but not least (d) could modified EVs bearing on their surface the protein(s) used by the virus to gain cell access, be used to trigger an immune response or compete with viruses to bind to cell entry receptors?

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