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Biological functions of extracellular vesicles from mammalian cells

A B S T R A C T

Extracellular vesicles (EVs) are enclosed by a phospholipid bilayer and can be secreted by most types of cells. EVs deliver cargo from the secreting cell into the cytoplasm of recipient cells, influencing the function of the recipient cells. EVs are attracting increasing attention from a broad range of clinicians and scientists due to their ability to promote or inhibit various physiological pathways or pathological conditions. This special issue of *Biomedical Journal* contains articles describing the biogenesis and biodistribution of EVs and their role in the intercellular transfer of various molecules or viruses to target cells, in rejecting allogeneic transplants and maintaining immune tolerance of the allogeneic fetus, and in modulating innate and adaptive immunity. Characterization of the role of EVs in various pathological conditions and our ability to engineer modified EVs may lead to discovery of novel biomarkers and development of therapeutic strategies for treatment of disease.

1. Introduction

Extracellular vesicles (EVs) are small, membrane-bound particles released by cells under both normal and pathological conditions. EVs encapsulate lipids, proteins and nucleic acids such as mRNA, micro-RNA (mi-RNA) and mitochondrial DNA that mirror the physiological state of the cells that secrete them. The EVs can interact with neighboring cells in the immediate environment or travel longer distances in the body. Upon being internalized by host cells, the cargo carried by EVs can influence the function of the host cells. Three primary classes of EVs have been described, and they are categorized based on their size, content, and biogenesis: microvesicles (also called ectosomes), exosomes, and apoptotic bodies.

2. EVs and neurological disease

Neurological diseases such as multiple sclerosis, Alzheimer's disease and amyotrophic lateral sclerosis (ALS) represent the leading cause of disability and the second cause of mortality worldwide. The incidence of different types of dementia such as Alzheimer's disease is also increasing rapidly due to the aging global population.

Many risk factors have been identified for these diseases, but the exact trigger for each disease still remains largely unknown. Besides genetic and environmental factors associated with each disease, a role for EVs has recently been proposed.

A comprehensive review by Tang [1] describes first the biogenesis of exosomes and other EVs, and describes the proteins, lipids and nucleic acids encapsulated by exosomes [Fig. 1]. Tang acknowledges the difficulties in isolating one type of EV without co-isolating other EVs and accepts the consensus in the field endorsing the use of the generic term "EV", which Tang uses in the remainder of her article.

Cells in the central nervous system (CNS) release EVs that can spread within the CNS. In health, EVs can contribute to myelination and synaptic plasticity, and they can cross the blood brain barrier (BBB). In disease, EVs can carry damaging oxidative and inflammatory molecules that contribute to neuroinflammation and neurodegeneration. Tang summarizes evidence that different cells release EVs that play a role in development of multiple sclerosis and Alzheimer's disease, depending on the physiologic state of the secreting cell [1].

Conversely, EVs are also released from CNS cells and can cross the BBB carrying cell- and disease-specific molecules [1]. A small number of mi-RNAs have been identified in serum exosomes that could be used as biomarkers for multiple sclerosis and Alzheimer's disease. One could also envision using EVs loaded exogenously with therapeutic agents as a strategy for decreasing the risk of neurological disease.

3. EVs and viral infection

The review by Malnou and colleagues [2] considers the role that EVs play during viral infection. On the one hand, EVs can enhance replication of viruses and propagation of viral infection. On the other hand, EVs can also modulate viral infections. The review describes how viruses can subvert the biogenesis of EVs and compares the ways in which EVs can help or hinder viral infection [2].

One of the challenges in studying the effects of EVs on viral infection is the structural and biochemical similarity between EVs and viruses. For example, EVs and viruses have similar sizes and use the same cellular machinery for their biogenesis and secretion into the extracellular space. Nonetheless, Malnou and colleagues present clear evidence that viruses manipulate the pathway and content of EVs, with consequences for both

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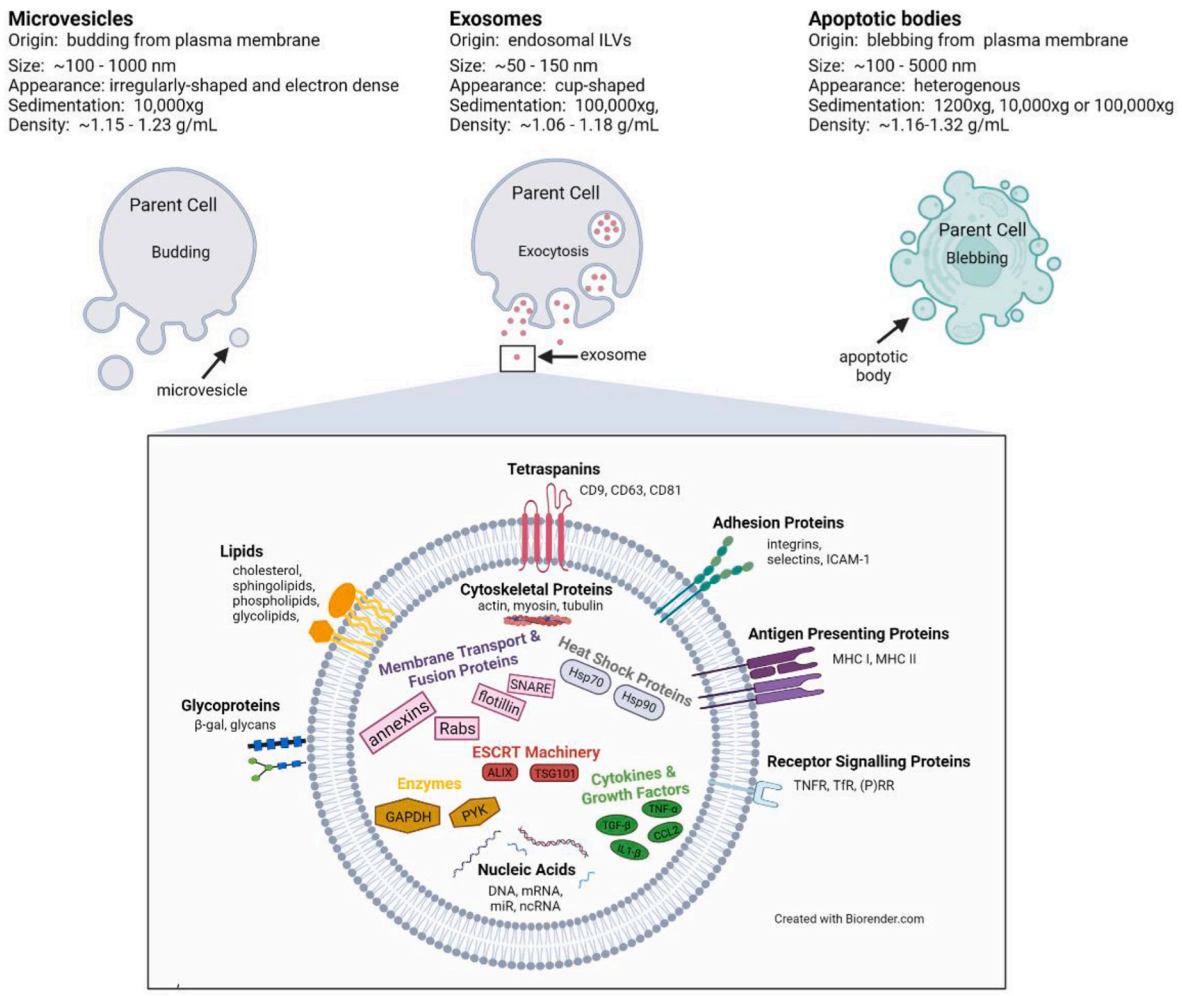

Fig. 1. Types of extracellular vesicles (EVs). The major classes of EVs are depicted based on their origin. Microvesicles and apoptotic bodies are generated via budding of the plasma membrane. Exosomes are generated via the endocytic pathway by exocytosis of multivesicular bodies that fuse with the plasma membrane. Figure from Ref. [1].

viral replication and the host immune response [2].

Perhaps the best-characterized common pathway used by both viruses and EVs is the Endosomal Sorting Complex Required for Transport (ESCRT) machinery. The ESCRT is involved in intracellular membrane remodeling, and different types of viruses have been observed to hijack this machinery for their own replication. The ESCRT can contribute to viral budding, but for some viruses, can also be used for viral genome replication. Other shared pathways are also described in the review article. Due to the overlapping pathways, there is competition between EVs and viruses for host-cell resources. This may lead to dysregulation of EV biogenesis, with functional consequences for the host cell and organism [2].

Many studies describe a stimulatory effect of EVs on viral infection. Thus, EVs secreted from infected cells can harbor viral particles, which protects viruses from the immune response and other environmental conditions and improves their transmission to new host cells, including cells that lack specific receptors for the viruses.

EVs secreted by infected cells can also interfere with the adaptive immune response. For example, the *Alphaherpesvirus* gB surface protein stimulates major histocompatibility complex (MHC) class II sorting to EVs, leading to a decrease of MHC II expression at the surface of immune cells. On the other hand, cells infected with human cytomegalovirus (HCMV) secrete EVs containing an Fc-γ receptor homolog, which divert neutralizing IgG antibodies from binding to the viral particles [2].

Conversely, EVs secreted by uninfected cells can also inhibit viral infection by interfering with the viral life cycle. For example, EVs in some body fluids such as saliva can protect host cells from infection by sequestering viral particles such as Zika virus and preventing their attachment to and internalization by host cells. This interaction has been proposed as a novel oral innate immune defense mechanism against some viral infections [2].

The review ends by citing examples of EVs that induce secretion of pro-inflammatory cytokines by the infected cells, thus enhancing adaptive immune responses [2]. These results suggest that EVs could also be exploited in antiviral therapy, by inhibiting viral infection directly or promoting innate and adaptive immunity. EVs are also potential candidates as biomarkers in liquid biopsies. Many studies are underway to identify novel biomarkers associated with EVs that could aid in diagnosis or treatment of viral infection.

4. EVs and trogocytosis

Trogocytosis refers to the transfer of plasma membrane fragments and cytoplasm from one cell to another and is dependent on direct cell-to-cell contact [3]. While this process is not exclusive to hematopoietic cells, it has been most extensively studied within the immune system. Both myeloid and lymphoid cells have been observed to engage in trogocytosis. The phenomenon of T cell-mediated trogocytosis was first identified when adoptively transferred murine T cells acquired and displayed allogeneic MHC class II molecules. Since murine T cells lack

endogenous MHC class II, the allogeneic MHC class II expression could only have originated from the recipient mice's cells and tissues [3].

An interesting article by Martinez-Martin and Alarcon [3] summarizes the steps that convert a trogocytotic T cell into an antigen presenting cell (APC). In brief, a trogocytotic vesicle fuses with intracellular endocytic compartments, forming multivesicular bodies (MVBs). In MVBs, trogocytotic vesicles can either stay intact or merge with the MVB. If these vesicles from APCs do not fuse, they are released when the MVB empties its contents into the extracellular space. This results in the formation of plasma membrane-associated exosomes that are enriched with acquired antigen/MHC complexes and other receptors originating from the APC [3]. As APCs, trogocytotic $CD4^+$ T cells play a role in shaping the differentiation of cytotoxic T cells and guiding other $CD4^+$ T cells towards becoming pro-inflammatory effector T cells. Additionally, the trogocytosis of antigen/MHC complexes drives the differentiation of these trogocytotic $CD4^+$ T cells into regulatory T cells and Th2 effector cells [3].

In T cells, the process of acquiring membrane fragments with antigen/MHC complexes from APCs through trogocytosis is an active mechanism that requires T cell receptor (TCR) signaling and the rearrangement of the actin cytoskeleton. The RAS family GTPase R-RAS2, which is directly recruited to the TCR, along with the Rho family GTPase RhoG, previously associated with the phagocytosis of apoptotic cells, serve as key mediators in TCR-triggered trogocytosis of membrane fragments from APCs [3].

5. EVs and rejection or tolerance of allogeneic organs

The article by Benichou and Lancia [4] analyzes the different pathways of T lymphocyte allorecognition, leading to immune responses that induce graft rejection of allogeneic transplants. A brief description is given of the two well-known pathways of T lymphocyte allo-responses, the *direct* and *indirect* pathways. Various studies have shown that a third mechanism of T lymphocyte allorecognition exists, called "the *semi-direct* pathway". In the direct pathway, T cells react with well-folded donor MHC-peptide complexes present on donor cells while in the indirect pathway, T lymphocytes recognize donor MHC peptides bound to recipient MHC molecules present on the surface of host antigen-presenting cells (APCs). The main focus of this article analyzes the *semi-direct* pathway and how transfer of donor allogeneic MHC molecules to recipient APCs trigger T lymphocyte allo-responses leading to allograft rejection or tolerance.

In the first paragraph, the intercellular transfer of MHC molecules is described in different immunological settings. Initially, trogocytosis has been described as a mechanism by which T lymphocytes capture plasma membrane fragments from APCs through the specific binding of their TCR to its antigenic MHC-peptide. This active mechanism was thought to be antigen receptor-specific but later it was found that it could involve various molecules other than MHC-peptide complexes, such as immunoglobulins, co-stimulatory and adhesion molecules as well as chemokine and complement receptors. These exchanges occur between dendritic cells, endothelial cells and natural killer (NK) cells, and T and B lymphocytes. It is now clear that MCH class I- and class II- peptide complexes can be transferred from live APCs to other live cells such as other APCs but also to T and B lymphocytes. This transfer mechanism of well-folded MHC-antigenic peptide complexes to various cells is called *MHC-cross-dressing*.

Benichou and Lancia describe experiments showing that *in vitro* cross-dressing of DCs with allogeneic MHC class I and II molecules stimulate the proliferation of alloreactive T lymphocytes *in vitro*. *In vivo* adoptive transfer experiments in mice showed that DCs cross-dressed with allogeneic MHC class II molecules can stimulate $CD4^+$ T cell allo-responses. *In vivo* experiments showed that MHC class II molecules can be exchanged between donor and recipient cells in mice receiving heart or kidney allo-grafts. It was shown that recipient DCs cross-dressed with donor MHC class I peptides from the allograft can present them as well-folded complexes to $CD8^+$ T cells (semi-direct presentation). In addition, processed allo-peptides bound to recipient MHC class II molecules can activate $CD4^+$ T cells (indirect presentation).

The review summarizes results demonstrating a major role of EVs in donor MHC molecules cross-dressing leading to semi-direct allo-responses of T lymphocytes after grafting. Following skin, heart or pancreatic islets transplantation, there were very few or no donor lymphocytes in lymphoid organs of recipient mice bearing allogeneic grafts. However, a few days after the grafts, recipient cells with attached EVs bearing donor MHC class I and II complexes at their plasma membrane were detected. Interestingly, on days 7–10 following graft, EVs were no longer found on the cell surface but the cells presented recipient and donor MHC molecules on their plasma membrane. The number of recipient cells cross-dressed with donor MHC in the recipient draining lymphoid organs increased and reached significant numbers. The majority of cross-dressed cells were B lymphocytes with small numbers of DCs and T lymphocytes.

In the last paragraph on intercellular transfer of MHC molecules in allograft tolerance [4], Benichou and Lancia summarize the experimental evidence showing that allograft tolerance depends on antigen presentation to T cells. The nature and the differentiation status of APCs, their inability to deliver strong costimulatory signals, make T cells unresponsive to antigens and this paralysis is defined as *anergy*. T cell tolerance is also achieved when antigen presentation by APCs is given with coinhibitory signals. This leads to the activation of Foxp3 or other regulatory T cells (Tr1 and Th3 cells). Another tolerizing presentation occurs when antigen presentation is achieved in the absence of inflammatory and danger signals. They showed that MHC complexes carried by allogeneic exosomes released by allografts could bind to TCR on T cells but did not activate allospecific T cells *in vitro*. However, *in vivo* allogeneic exosomes could stimulate an alloresponse and induce allograft rejection if complete Freund's adjuvant is injected in mice, providing inflammatory signals.

In allotransplantation, it was found that allogeneic MHC class I and class II complexes are transferred from donor cells to recipient APCs by trogocytosis or transfer of EVs liberated by donor cells. The discovery of the semi-direct presentation in several transplantation models in mice and humans and the understanding of its role and function in allotransplantation have unveiled an exciting and innovative field.

A related article by Burlingham [5] discusses the role of EVs in fetal-maternal tolerance during mammalian pregnancy. Pregnancy in viviparous mammals presents the greatest challenge to the survival of such species: how to protect the mother and developing fetus against foreign pathogens and other external dangers, while maintaining maternal tolerance to the semi-allogeneic fetus. The author summarizes data showing that the local nature of immune tolerance at the fetal-maternal interface involves several types of regulatory T lymphocytes (T_{reg}) which produce immunosuppressive cytokines such as IL-35 and TGFβ which, in turn, are delivered as proteins bound to exosomes [5]. Foxp3 T_{reg} cells have been shown to deliver IL-35 and TGFβ to target lymphocytes, bystander or naïve T cells which acquire the capacity to suppress local immune T cell responses, after incorporation of the exosomes bearing these cytokines. Importantly, these bystander or naïve T cells become induced regulatory T (iT_{reg}) cells which express IL-35 and/or TGFβ and thus can spread tolerance to other T cells.

IL-35 is a heterodimer comprising two non-disulfide linked glycoproteins, the IL-12α chain associated with the Ebi3 subunit. TGFβ is secreted from T_{reg} or iT_{reg} cells in its latency-associated peptide (LAP) form but can be activated to become a systemic immunosuppressor. Interestingly, Burlingham and colleagues [5] have shown that IL-35 and TGFβ behave differently from IL-10. After centrifugation at 100.000*g*, IL-10 is soluble and remains in the supernatant, while IL-35 and TGFβ are found in the pellet. These two cytokines are present in exosomes associated with different *trans*-membrane proteins. In exosomes, IL-35 is associated with the tetraspannin CD81 while TGFβ is linked to a transmembrane Glycoprotein A Repetitions Predominant (GARP), present on

the surface of T_{reg} cells. Thus, naive target T lymphocytes capture these exosomes, present IL-35 and/or TGFβ on their plasma membrane and become iT_{reg} cells, suppressive of effector T cell responses. In addition, these newly induced T_{reg} cells have the ability to expand the tolerance signal to other T cells, a mechanism coined by Stephen Cobbold and Herman Waldmann as "infectious tolerance" in transplantation models [6].

Interestingly, an IL-35-independent immunosuppressive pathway has been identified. CD39 (extracellular ATPase) and CD73 (extracellular AMPase) are involved in the production of immunosuppressive adenosine and are found on exosomes released by T_{reg} cells. The recent detection of CD39/73 expressed on human amnion cells suggests that this mechanism plays an important role in fetal/maternal tolerance during human pregnancy.

Overall, these studies show an unexpected role for EVs in rejection of transplanted allogeneic tissues and organs and, conversely, maintenance of immune tolerance during pregnancy, and suggest that exogenous EVs may be designed to modulate the immune response to transplants.

6. Biodistribution of EVs

This special issue ends with an original report by Weng and colleagues [7] on EVs derived from umbilical cord-derived mesenchymal stem cells (UCMSC-EVs). These EVs are thought to promote tissue regeneration, regulate immunity, and exert anti-inflammatory effects. Some studies have reported the efficacy of UCMSC-EVs in wound healing and bone regeneration after local administration, but the biodistribution and pharmacokinetics of UCMSC-EVs in circulation was unclear [7].

In order to study the biodistribution of UCMSC-EVs, the authors radiolabeled these EVs with Technetium-99 m (^{99m}Tc-UCMSC-EVs) and visualized the distribution in mice using photon emission computed tomography (SPECT). SPECT images showed that most of the EVs were taken up by the liver and spleen, and to a lesser extent, by the thyroid and stomach. The EVs were stable *in vivo* at 24 hours. This noninvasive imaging technique is thus suitable for characterizing the biodistribution and stability of the EVs, which could provide useful information for dosage protocols and toxicity assessment in the future [7].

7. Concluding statement

EVs are attracting growing interest due to their role in intercellular communication, between cells and systemically, between organs. As summarized in this special issue, EVs are now acknowledged to mediate intercellular communication in both health and disease. In some cases, they contribute to disease, while they can also mitigate the symptoms of disease. EVs are being explored for their potential as biomarkers of disease and as potential vehicles for therapeutic interventions.

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