Extracellular Vesicles and COVID-19-Related Thrombosis

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Extracellular vesicles (EVs) is a term used to describe a heterogeneous group of vesicles located in different types of tissues or biological fluids such as blood, urine, saliva, breast milk and the amniotic, cerebrospinal, synovial, seminal fluid and bronchial lavage.

The role of EVs in infectious diseases has been particularly controversial over the last years. It has been shown that they can influence the recipient cell activities by transporting viral proteins, RNA, DNA and receptors from infected cells to healthy cells and thus increasing the spread of virus infection. Thus, EVs may serve as potential predictors of COVID-19 severity. Importantly, due to their stability in the circulation, low immunogenicity, biocompatibility and biodegradation, the EVs are considered suitable for designing new therapeutic strategies or delivery systems for a vaccine against the SARS CoV-2 infection

Keywords: atherosclerosis ; thrombosis ; cardiovascular disease ; COVID-19 ; extracellular vesicles ; exosomes ; microvesicles

1. Introduction

Over the course of evolution, the communities of cells of all organisms have found means to converse and communicate their physiological or pathological state with each other, reminding us of the well-known promise, “for better for worse, … in sickness and in health, …till death us do part” (marriage vows in the Catholic church).

Among the various methods of communication, the most recently discovered common instruments are extracellular vesicles (EVs), an assembly of cell-derived vesicles of different origins and sizes encompassing endosome-derived exosomes, plasma membrane-derived microvesicles (ectosomes) and apoptotic bodies.

EVs are released from activated or apoptotic cells carrying biological molecules such as DNA, mRNA, microRNA (miRNA) and others as cargo. They play a particularly important role in cell–cell communication and signaling due to their ability to transport and transfer their cargo to recipient cells. In addition, EVs have been implicated in varied pathologies such as neurodegenerative diseases [1] and metastasis [2][3]. This review will focus on the role of EVs in atherosclerosis and thrombosis, the major causes of death in developed countries and major public health problems.

2. Extracellular Vesicles

Extracellular vesicles (EVs) is a term used to describe a heterogeneous group of vesicles located in different types of tissues or biological fluids such as blood, urine, saliva, breast milk and the amniotic, cerebrospinal, synovial, seminal fluid and bronchial lavage [4][5][6][7][8].

All cells, prokaryotes and eukaryotes produce and release EVs as a normal physiological process, and also in pathological conditions as a result of their activation or apoptosis. Based on their size, morphology and biogenesis, EVs have been classified as either exosomes and ectosomes, with the latter being called also microvesicles (MVs) or microparticles (MPs). In addition, apoptotic bodies are considered to be part of the EV family [9], although they differ in content and function.

Exosomes were defined in 1981 as “exfoliated membrane vesicles” [10]. Subsequently, an ultrastructural study revealed that vesicles measuring approximately 50 nm were exocytosed from multivesicular bodies (MVBs) [11]. Recent studies defined exosomes as EVs with a size range of about 40 to 160 nm (average ~100 nm) in diameter, and as being of endosomal origin [12].

As shown in Figure 1, exosomes are formed in a complex process originating from the early sorting endosome, which successively turn into late sorting endosome and MVBs. The latter contain intraluminal vesicles (ILVs) that, upon fusion with the plasma membrane, secrete ILVs as exosomes [12].

In the biogenesis of exosomes, the proteins involved include ESCRT (endosomal sorting complexes required for transport) proteins, Ras-related protein 2-interacting protein X), phospholipids, tetraspanins, ceramides, sphingomyelinases, SNARE proteins (SNAP Receptor) and others [13][14][15][16]. Further research is needed to understand the functions of these proteins in exosome biogenesis. The physiological role of exosome release is not completely understood, but it is assumed that they contribute to cellular homeostasis [17].

They are plasma-membrane-derived particles released into the extracellular space by the direct outward budding and fission of the plasmalemma taking within some of the cytosolic content and membrane receptors of the paternal cell [18] More specifically, the increase in the intracellular Ca2+ levels leads to the redistribution of plasma membrane phospholipids, causing phosphatidylserine (PS) exposure on the outer face of the membrane, disarrangement of the cytoskeletal proteins and finally, MV release [19]. The detachment of MVs from donor cells involves the contraction of cortical actin beneath the plasma membrane due to high levels of intracellular Ca2+ [20]. Also, it has been shown that MV release is regulated not only by membrane lipid microdomains, but also by regulatory proteins such as ADP-ribosylation factor 6 (ARF6) [21][22].

Although research on EVs is constantly evolving, the classification of EVs is still as either exosomes and ectosomes or MVs. However, it is important to note that many publications include apoptotic bodies (ApoBDs) or apoptosomes in the EV group.

ApoBDs are larger vesicles released from dying cells with sizes ranging from 1000 nm to 2000 nm that, under specific conditions, can be more abundant than exosomes or MVs [9].

ApoBDs are released during the early stages of apoptosis upon rearrangement of membrane lipids that induce PS translocation from the inner to the outer leaflet and a subsequent release of ApoBDs into the extracellular space [23] (Figure 1). The external translocated PS binds to Annexin V, which is recognized by phagocytes, and These apoptosis-derived large cellular fragments, that are taken-up by neighboring cells (macrophages, parenchymal cells or neoplastic cells), are degraded within phagolysosomes or recycled; therefore, they cannot be regarded as EVs which are involved in intercellular communication.

Depending on, or because of, the different cellular origin of EVs, differences in the protein and lipid composition exist between exosomes and MVs, on the basis of which they exert specific biological functions.

Exosomes have several cell membrane proteins on their surface, such as tetraspanins, integrins and immunomodulatory proteins, and as cargo, have cell cytoplasmic proteins, i.e., RNA, DNA, amino acids and metabolites. As depicted in Figure 1, the most common proteins used as biomarkers for exosomes are tetraspanins (CD63, CD81, CD82, CD9 and CD37), heat-shock proteins (Hsp60, Hsp70, Hsp90 and Hsp20), tumor susceptibility gene (TSG101), annexin, flotillin and apoptosis-linked gene 2-interacting protein X (ALIX) [9]. Exosomes may contain and transport DNA, mRNAs, miRNAs, pre-miRNAs and other noncoding RNAs that are transferred to recipient cells and tissues [24][25], and thus, function in intercellular communication and signaling [26][27][28].

In addition, specific transmembrane proteins are present on the exosome surface, i.e., epithelial cell adhesion molecule (EpCAM), epidermal growth factor receptors (EGFRs), lymphocyte function-associated antigen 1 (LFA-1) integrin, intercellular adhesion molecule-1 (ICAM-1, known as CD54), L1 cell adhesion molecule (L1CAM) and endoglin (CD105). The latter is a transforming growth factor β (TGF-β) receptor and integrin ligand which plays a key role in vascular pathology, angiogenesis, inflammation and hemostasis [29]. in hereditary hemorrhagic telangiectasia (HHT), a disease caused by mutations in the endoglin gene, patients infected with SARS-CoV-2 suffer milder symptoms with lower clinical impact than the general population [30].

The presence of the above molecules point to the cellular origin of exosomes, and may explain their capacity to adhere and fuse with the plasma membrane of recipient cells [31].

As a result of this molecular composition, several pathways have been proposed for exosomes interaction with target cells: (1) direct fusion with the cell plasmalemma; (2) adhesion to the cell surface by receptor-ligand interaction; (3) paracrine signaling as a result of the release of the exosome content generated by the destabilization of their membrane under low pH conditions, and (4) endocytic uptake [32][33] (Figure 1).

Microvesicles (MVs) express numerous features of the donor cell including specific surface antigens and receptors [34]. Thus, endothelial cell-derived microvesicles (EMVs) express the speciflc protein CD144 on their external leaflet, platelet-derived microvesicles (PMVs) released from activated platelets express specific protein CD41 [35], and leukocyte-derived microvesicles (LMVs) All types of MVs have PS on the outer face of plasmalemma, as a common specific marker. Thus, the specific molecular signature of MVs consists of several surface proteins which are specific to paternal cells and PS as a key identifier [36][19] (Figure 1).

CD40, as well as cholesterol, sphingomyelin and ceramides [32] In addition, they carry proteins derived from the cytoplasm of the cells of origin: von Willebrand factor (vWF), monocyte chemoattractant protein-1 (MCP-1), matrix metallopeptidases (MMP2, MMP9), vascular endothelial growth factor (VEGF), DNA, mRNAs, miRNAs, noncoding RNAs and peroxisome proliferator-activated receptor gamma [31][37][38][39][40][41]

MVs released into biological fluids or tissues have the ability to interact with target cells and transfer their abundant and complex biological content, potentially affecting their function. The most significant changes induced by MVs on recipient cells are those caused by the release of the miRNAs, mRNAs The genetic material transferred into target cells regulates the gene expression and protein synthesis of the recipient cells, influencing their biological characteristics. MV-target cell interaction occurs through several pathways: (1) specific receptor–ligand interactions affect different intracellular signaling pathways; (2) direct fusion to the cell plasma membrane, and (3) endocytic uptake [42][43] (Figure 1).

Apoptotic bodies or apoptosomes are variable in size, structure, composition, and their specific identification marker is PS. They differ from MVs by the presence of caspases 3 and 7 and their substrates (e.g., Pannexin1 (PANX1), ROCK1), and of Annexin V, thrombospondin and complement protein C3b [44][45] (Figure 1).

In summary, due to their variable size, diverse cellular origin and varied biological content, EVs (exosomes and ectosomes) can be regarded as a highly heterogeneous population with multiple functionalities which are able to mediate complex cell-to-cell communication over short or long distances.

Importantly, in various diseases, the detection of EVs in body fluids (liquid biopsies) offers a window into altered cellular or tissue states, and provides a multicomponent diagnostic readout. The trafficking and efficient exchange of cellular components through EVs has led to their use in the design of EV-based therapeutics.

3. COVID-19-Associated Thrombosis and Extracellular Vesicles

COVID-19 (Corona-Virus Disease 19) is a new infectious disease that grew into a major human health problem with catastrophic global impact. It is caused by the severe acute respiratory syndrome-coronavirus-2 (SARS CoV-2), a positive-sense, single-stranded RNA virus that exhibits membrane proteins, spike proteins, nucleocapsid proteins and envelope proteins. The virus uses the cellular angiotensin-converting enzyme 2 receptor (ACE2) for internalization, aided by transmembrane protease, serine 2 (TMPRSS2 protease) [46].

The role of EVs in infectious diseases has been particularly controversial over the last years. It has been shown that they can influence the recipient cell activities by transporting viral proteins, RNA, DNA and receptors from infected cells to healthy cells and thus increasing the spread of virus infection. For example, it has been reported that EVs during human papillomavirus deliver miRNAs to unaffected cells thus contributing to progression of cervical inflammation [136]. Other researchers hypothesized that EVs may negatively regulate the virus infection through cytokine secretion that could induce immune system responses against viral pathogens [137].

Identification of specific biomarkers is vital for the prevention, evolution and clinical decisions in the COVID-19 patients. As mentioned above, EVs contain numerous and diverse biomolecules and their cargoes can be modified by microenvironmental stimuli including viral infection. EVs exhibit surface molecules, such as CD9 and ACE2 [138]. Thus, Fujita et al., hypothesized that serum EVs may serve as potential predictors of COVID-19 severity. They analysed EV proteins, including coagulation-related markers and antiviral response-related EV proteins with the potential to serve as early predictive biomarkers for COVID-19 severity. Analysis of proteome profile by liquid chromatography mass spectrometry of EVs collected from 31 SARS-COV-2 infected patients and 10 healthy donors the authors identified significant differences in the EV-cargo. They found that fibrinogen gamma chain (FGG), CD147, calpain 2 (CAPN2), extracellular matrix protein 1 (ECM1), coat complex subunit beta 2 (COPB2), KRAS proto-oncogene (KRAS), protein kinase C beta (PRKCB), ras homolog family member C (RhoC), are significantly more abundant in SARS-COV-2 infected patients that in healthy donors. This group of markers can distinguish between severe and mild cases of COVID-19, and among them, COPB2 has the best predictive value [1139].

Interestingly, the levels of circulating EVs and the associated TF expression (EV-TF) are significantly higher in patients with COVID-19 compared to controls. The TF expression is tightly implicated in the activation of coagulation and thrombosis. There are several mechanisms underlying the increase in TF expression during viral infection. In a clinical study on 100 patients with COVID-19, it was found that EV-TF activity correlates positively with D-dimer, prothrombin time (PT), international normalized ratio (INR) calculated based on the PT test result (PT/INR), prothrombin, fibrinogen, and antithrombin. Therefore, the circulating EV levels and EV-TF activity can be used as prognostic biomarkers in patients with COVID-19 [140].

Recently, in a cohort of 111 hospitalized patients with COVID-19, the EV-TF activity, correlated positively with the inflammatory state, the disease severity, and the thrombotic events. This study recommends systematic preventive anticoagulation in hospitalized patients with COVID-19 and potential intensification of anticoagulation in patients with severe disease [141]. A significant higher level of circulating platelet-derived MVs in COVID-19 patients in comparison with healthy subjects was reported [142]. Interestingly, although the concentration of platelet-derived MVs was increased, the number of circulating platelets has not been changed between the two cohorts. This is indicative that the circulating platelet-derived MVs are not only significantly correlated with Sars-Cov-2 infection, but they may be used as diagnostic biomarker of the viral infection. Reportedly, they contain elevated levels of coagulation factors and immune mediators that can induce platelet aggregation, mediate the coagulation pathway and activate enzymes such as cyclooxygenase-1 and 12-lipoxygenase [143]. Although, these studies provided candidate biomarkers, future investigations should focus on elucidating their pathogenic role in venous thrombosis in COVID-19 patients.

Regarding the viral transmission, it was shown that EVs from patients infected with COVID-19 transfer to target cells pro-thrombotic and endothelial injury factors such as: TF, t-PA, vWF, proteins associated with cardiovascular pathology (MB, PRSS8, REN, HGF), cytokines (TNF-α, IL-6), chemokines (MCP-1, CXCL16), proteases and peptidases including cathepsin L1, an enzyme involved in tissue remodelling [144]. Moreover, using an in vitro approach, the authors showed that EVs from patients infected with COVID-19 can contribute to increased caspase 3/7 activity, leading to apoptosis of pulmonary endothelium. Their experiments on human pulmonary microvascular EC revealed that EVs isolated from the plasma of severe diseased patient play an essential role in the pathogenesis of COVID-19.

4. Conclusions and Future Directions

Accumulated evidence, including our own, has led to the perception that EVs function as effective vectors of biological material, protagonists of intercellular communication, signaling mediators, and promising prognostic biomarkers and therapeutic agents in various diseases, including atherosclerosis and COVID-19-related thrombosis.

Cells use EVs as biological tools to communicate and transfer matter from donor cells to recipient cells. Upon transfer, cargo molecules (DNA, mRNA, miRNA and others) affect and influence the physiology or/and pathophysiology of the receiver cell.

In atherosclerosis, and even more so in COVID-19 disease, EVs could have either a harmful or a protective effect. Thus, depending on the cargo and the microenvironment, EVs may promote the propagation of atherosclerotic plaque or COVID-19-related thrombosis. Alternatively, EVs could be a reliable biomarker and signal disease progression. Consequently, EVs could be employed as a therapeutic agent. In this respect, engineered EVs might represent a novel therapeutic tool in cardiovascular medicine and regenerative therapy. Although important advances have been made in EV science, there is yet much to be discovered. More data are needed on their capacity to select biological content, the mechanisms of interaction with target cells and the implications in physiological and pathophysiological processes. We have to clarify the capacity of cells to produce EVs of different sizes and compositions. An explanation is needed for the selection by which EVs, during their biogenesis, selectively encapsulate either DNA, RNAs, proteins or lipids, characteristics that are part of the EV molecular signature. Since the cargo is usually in small quantities, there is a question about whether this is sufficient to explain its biological effects. More experiments may reveal how EVs recognize target (recipient) cells and how the cargo is processed upon cell endocytosis. Also, we hope to find whether a connection exists between the size, the content and the cargo of EVs and the stage of the disease. Moreover, there is an urgent need to validate results obtained in cultured cells in vivo.

There are still a great number of questions to be answered. But this is the beauty of science; the more one discovers, the more questions arise.

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