Extracellular Vesicles in CKD

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Over the last few years, preclinical and clinical studies have emphasized the role of extracellular vesicles (EVs) in human diseases. These particles are delimited by a lipid bilayer and are released by almost all cell types and in all organisms. EVs appear to have biological effects in various pathophysiological situations and especially in renal disease. In human organs, EVs can interact with cells and prompt the release of many different molecules, such as proteins, lipids and nucleic acids, that, in turn, regulate various cell signaling pathways. Moreover, EVs are present in the urine and the blood and therefore can be used as potential diagnostic biomarkers in human diseases, such as chronic kidney disease (CKD, also known as chronic renal failure).

extracellular vesicles chronic kidney disease uremic toxins

1. Introduction

1.1. Classification of EVs

In recent years, studies of the role of EVs in human disease have shown that these vesicles are involved in several physiological pathways. Firstly, EVs are involved in cell–cell communication because they deliver various bioactive molecules and components to recipient cells [1]. In fact, the vesicles carry proteins, lipids, nucleic acids and other metabolites that can have many different cellular and supracellular effects [1]. EVs are highly heterogeneous but can be divided into three main categories: exosomes (30–150 nm in diameter), microvesicles (also referred to as microparticles or ectosomes: 100–1000 nm) and apoptotic vesicles (50–5000 nm) [2]. The three subgroups differ with regard to their mode of biogenesis (Figure 1)

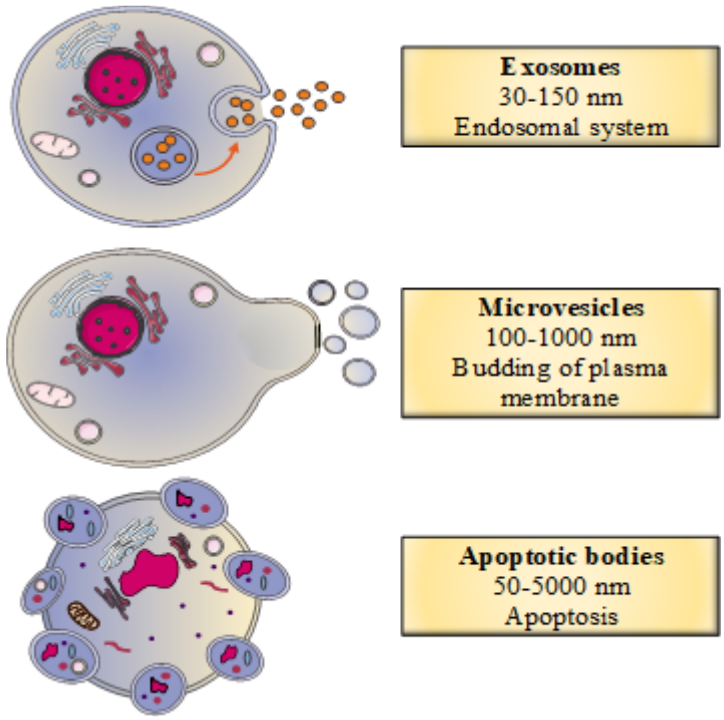

Figure 1. The classification of extracellular vesicles (EVs) as a function of their biogenesis.

1.2. Biogenesis of EVs

1.2.1. The Biogenesis of Exosomes

Exosomes are produced from a complex endosomal system involving two different pathways, one of which depends on a protein complex called the endosomal sorting complex, which is required for transport (ESCRT) [3]. Typically, the budding of late endosomes enables the production of intraluminal vesicles within multivesicular endosomes. These vesicles then fuse with the plasma membrane to give secreted exosomes [3].In the ESCRT-dependent pathway, four protein complexes (ESCRT-0, I, II, III) bind to the hexameric AAA ATPase Vps4 complex, which provides the energy for exosome biogenesis [3]. The intraluminal vesicles are formed because of the negative curvature of the endosomal membrane, which depends on recognition of ubiquitinated endosomal proteins by ESCRT-0 [4]. Next, ESCRT-I, II and III interact with ESCRT-0 to enable intraluminal vesicle formation in multivesicular endosomes [4]. Lastly, the AAA ATPase Vsp4 complex causes ESCRT-III to separate from the multivesicular endosomal membrane, and the ESCRT complex is recycled [4].Several studies have highlighted the existence of an ESCRT-independent pathway for exosome biogenesis, based on lipid raft microdomains located in the late endosome membrane [2]. These microdomains contain cone-shaped molecules (e.g., lysobisphosphatidic acid and ceramides) that can induce negative curvature of the endosomal membrane [4][5].

1.2.2. The Biogenesis of Microvesicles

In contrast to exosomes, microvesicles are generated by direct budding from the plasma membrane. During microvesicle biogenesis, the interaction between the cytoskeleton and the plasma membrane weakens and several proteins (such as calpains or lipid translocases) are activated [2]. This cytoskeletal remodeling has been linked to

an increase in the intracytosolic calcium concentration, which, in turn, induces phosphatidylserine externalization, bud formation and microvesicle secretion [6].

1.2.3. The Biogenesis of Apoptotic Bodies

Unlike exosomes and microvesicles (which are secreted during normal cellular processes), apoptotic bodies are only released during apoptosis [2]. First, the cell membrane buds. Next, externalization of phosphatidylserine (as observed during microvesicle biogenesis) leads to cytoskeletal remodeling and then the formation of apoptotic bodies [2].

1.3. Secretion of EVs

Several different molecules are involved in the secretion of EVs in general and exosomes in particular, since microvesicles and apoptotic bodies are generated directly by the budding of the plasma membrane.The Ras-related proteins in brain (Rab) family is composed of more than 60 GTPases [7]. Screening studies have identified a number of small GTPases proteins involved in exosome secretion in various cell-based models [7]. Rab GTPases are involved in (i) the movement of multivesicular endosomes from the cytoplasm to the plasma membrane and (ii) the fusion of the cell's plasma membrane with that of exosome-containing multivesicular endosomes [7].Other molecules are involved in exosome secretion [7]. Thus, soluble n-ethylmaleimide-sensitive-factor attachment protein receptor complexes are thought to be involved in exosome secretion in various cell types [7]. The interaction between soluble n-ethylmaleimide-sensitive factor attachment protein and its receptor leads to the fusion of the plasma membrane with the multivesicular endosome membrane [7]. Some studies have identified other molecules with a potential role in exosome secretion, including diacyl glycerol kinase α, which appears to downregulate multivesicular endosome formation [8]. Citron kinase (a RhoA effector) and the V0 subunit of V-ATPase appear to be also involved in exosome secretion [9][10].It is noteworthy that the exosome secretion pathway depends on the EVs' cellular origin.

1.4. Fate of EVs

The release of EVs from parental cells may interact with target cells and influence target cell behavior and phenotype behavior [11]. Indeed, EVs carry bioactive molecules such as proteins, lipids and nucleic acids, which have been shown to impact target cells via several mechanisms: (1) direct stimulation of the target cells upon binding to cell surface; (2) transfer of activated receptors to recipient cells; and (3) epigenetic reprogramming of recipient cells via the delivery of functional protein, lipids and RNA.After EVs are released, they can interact directly with recipient cells by binding to cell surface integrins, proteoglycans or extracellular matrix components, thus inducing different biological processes [1]. For instance, intracellular adhesion molecule-1 on exosomes can interact with lymphocyte function-associated antigen-1 on dendritic cells [12]. Similarly, milk fat globule-EGF factor 8 protein (a lactadherin precursor present on immune cells) can interact with the phosphatidylserine on exosomes [12]. These interactions allow exosome internalization, the presentation of exosome-derived peptides to T cells and T cell activation [12]. Several studies have identified other molecular interactions between EVs and recipient cells in various cellular models [7].The EV membrane can also merge with the plasma membrane of recipient cells, thus

releasing the EVs' contents (miRNAs, proteins, peptides, nucleic acids, etc.) into the recipient's cytoplasm [1]. EVs are particularly enriched with miRNAs loaded into EVs via RNA binding protein recruitment [13]. For instance, synaptotagmin-binding cytoplasmic RNA-interaction protein (SYNCRIP) can be associated with miR-3470a and miR-194-2-3p [13]. Other RNA binding proteins are implicated in the sorting process for miRNAs into EVs, such as argonaute2 protein (Ago2) or Y-box binding protein 1 (YBX-1) [13]. The uptake of EVs by the recipient cell occurs through a variety of processes, such as micropinocytosis, endocytosis and phagocytosis (for a review, see References [1][14]). After release, the EVs' contents can induce various biological processes [7]. For instance, some EVs are enriched in enzymes such as cyclooxygenase and thromboxane synthase, which can regulate platelet activation and aggregation by metabolizing arachidonic acid into thromboxane [15].After their release, EVs can also be found in various biological fluids, such as blood or urine. Although the half-life of EVs has not been determined, EVs appear to be stable in biological fluids for at least several hours [7]. Moreover, intravenously injected EVs can be found in many organs (such as the spleen, lung and liver) [7].

2. Extracellular Vesicles in a Pathological Setting: A Focus on CKD

2.1. EVs and CKD

Although EVs appear to be associated with many diseases, their involvement in renal diseases (e.g., CKD) is especially strong. Furthermore, depending on their cell origin, EVs are linked to various pathophysiological processes in CKD.

Firstly, many clinical studies have shown that CKD patients have abnormally high levels of EVs in general and endothelial microvesicles in particular. Indeed, endothelial microvesicles are found to be higher in many cohorts of patients with end-stage renal disease (ESRD) [16][17][18][19][20][21]. Furthermore, it is known that plasma endothelial microvesicles are strong predictors of cardiovascular diseases. As such, they can be used as markers of endothelial dysfunction, atherosclerosis and arterial stiffness in CKD patients [16]. Moreover, the level of endothelial microvesicles in haemodialyzed patients is inversely correlated with laminar shear stress, which is a major determinant of plasma endothelial microvesicle levels in ESRD [22].

Other studies found that levels of platelet-derived microvesicles were also elevated in CKD patients [23] and that these EVs appear to have procoagulant and prothrombotic activity [24][25]. Circulating levels of microvesicles were also significantly elevated in CKD patients with coronary artery disease [26]. Furthermore, Benito-Martin et al. reported on elevated urine levels of exosomal proteins in CKD patients [27].

2.2. Effect of Haemodialysis on EVs

CKD progression leads to ESRD, which requires renal replacement therapy. Several dialysis techniques are currently used, including haemodialysis (HD), haemofiltration (HF), haemodiafiltration, and peritoneal dialysis (PD). HD is the most common renal replacement therapy for CKD patients. It effectively removes free small molecular

weight compounds but not protein-bound molecules or large molecules. The results of the few studies of the effects of HD on EVs are subject to debate. Indeed, some studies found that plasma EV levels are higher in haemodialyzed patients than in healthy subjects [18][20][28][29]. It is noteworthy that the types of altered microvesicle vary from one study to another. In contrast, some studies observed a fall in the level of EVs during HD [30][31]. By analyzing protein markers, Ruzicka et al. showed that the observed reduction in circulating EVs was due to adsorption of the EVs to the dialysis membrane rather than ultrafiltration [31]. Moreover, Cavallari et al. showed that online haemodiafiltration is better than HD because it decreased cardiomortality [32], inflammation [33] and miR223 expression in EVs, thus reducing VSMCs calcification and improving angiogenesis by human umbilical vein endothelial cells (HUVECs) [34]. In conclusion, the choice of the method for renal replacement therapy is critical for EVs.

2.3. Impact of Uremic Toxins on EVs

In addition to traditional cardiovascular risk factors, CKD patients also have non-traditional CKD-specific cardiovascular risk factors, such as the accumulation of uremic toxins. Indeed, as kidney function decreases, uremic toxins accumulate in the body fluids of CKD patients. Many studies have reported abnormally high concentrations of uremic toxins in the serum of CKD patients [35]. EVs appear to be linked to the physiopathology of CKD. Indeed, several studies have highlighted a link between the accumulation of uremic toxins in CKD patients and EV release [36]. Usually, EV secretion is elevated under uremic conditions. Thus, the level of platelet-derived microvesicles was higher in a cohort of 18 uremic patients than in a group of healthy subjects [37]. Gao et al. also reported that uremic patients had an elevated number of circulating microvesicles derived from peripheral blood cells and endothelial cells [38].

3. Extracellular Vesicles as Biomarkers of CKD

Conventional biomarkers for CKD diagnosis include a low GFR and albuminuria [39]. Recent studies have focused on urinary EVs since they may be non-invasive diagnostic or prognostic biomarkers [40].EVs in urine are of great interest as renal disease markers because they reflect the nephron's physiopathological status [41][42][43][44][45][46][47][48]. Indeed, Pisitkun et al.'s proteomic analysis showed that most EVs in urine come from glomerular and tubular cells because circulating EVs cannot cross the filtration barrier under physiological conditions [41]. Lv et al. suggested that urinary exosomes containing the inflammatory chemokine CCL2 mRNA are biomarkers of CKD [49]. Indeed, elevated levels of these exosomes were observed in the kidney and urine of CKD rats and patients with IgA nephropathy [49]. Exosomes can transfer CCL2 mRNA from tubular epithelial cells to macrophages, promoting inflammatory kidney injury [49]. Furthermore, Lv et al. showed that mRNA encoding CD2AP in urinary exosomes was correlated with kidney function and renal fibrosis [50].Clinical and preclinical studies have highlighted the value of miRs (transported by EVs) as CKD biomarkers [51][52][53][54][55][56][57][58][59][60][61][62]. Indeed, exosomes of patients with kidney diseases contain high levels of miR [55]. Liu et al. have shown that urinary miR-126 (probably secreted by exosomes) is more strongly expressed in patients with diabetic nephropathy, whereas treated patients have lower expression levels of this miR [57]. A specific urinary exosomal miR profile was found in patients with focal segmental glomerulosclerosis, a disease that can lead to CKD [59]. Furthermore, miR29 and miR200 are

downregulated in urinary exosomes from CKD patients [62]. The miR-451-5p and miR-16 found in urinary exosomes from diabetic rats also appear to protect the kidney tissue [61].Some researchers provide technical information about urinary EVs and miR transported by these EVs as non-invasive biomarkers for kidney diseases such as CKD [55][56]. However, it is important to note that EVs cannot always be handled easily and that the creation of standardized protocols is required if EVs are to be used as biomarkers in the clinic.

4. The Therapeutic Potential of Extracellular Vesicles in CKD

As described above, EVs are involved in the physiopathology of CKD, VC and uraemic conditions. Therefore, EVs are potential therapeutic targets that could be inhibited by various pharmacological agents [63][64]. EVs could also be used as drug delivery systems because of their involvement in cell–cell communication [65].

4.1. Inhibition of EVs

Several in vitro studies have demonstrated that various molecules can be used to block EV release or the uptake of EVs by recipient cells, as reviewed elsewhere [63][64].The complexity and heterogeneity of EV biogenesis complicates the development of drugs that inhibit EVs. In principle, inhibitors could act at several different stages of EV biogenesis. The Rab family is involved in exosome secretion [7]. It has been shown that the inhibition of Rab proteins (Rab27A and Rab27B) leads to a decrease in exosome secretion [66]. Several other inhibitors (including calpeptin, Y27632 and manumycin A) affect EV trafficking [64]. The best studied compound is calpeptin, a reversible, semi-synthetic peptidomimetic aldehyde inhibitor of calpains [64]. Calpains is a family of calcium-dependent cytosolic proteases the action of which on cytoskeletal remodeling promotes microvesicle shedding [2]. Therefore, the inhibition of calpains leads to a decrease in microvesicle release by cells [67].EV release can also be inhibited by blocking lipid metabolism [64]. As described above, lipid metabolism in general and ceramide metabolism in particular are involved in EV biogenesis [7]. One such inhibitor is panthetine. It inhibits cholesterol synthesis by changing the membrane fluidity and thus blocking the translocation of phosphatidylserine to the outer leaflet, an essential process in microvesicle production [64]. Sphingomyelinases are important enzymes for exosome and microvesicle formation and therefore constitute potential therapeutic targets [64]. Thus, treatment with imipramine or GW4869 (inhibitors of acid sphingomyelinase and membrane neutral sphingomyelinase, respectively) leads to a reduction in EV secretion [64]. Other inhibitors (such as antiplatelet molecules, antioxidants, statins, calcium-channel blockers and proton pump inhibitors) also reduce EV biogenesis (for detailed reviews, see References [63][64]).Another way of counteracting the EVs' effects is to reduce their uptake by recipient cells [63]. Even though the inhibition of EV uptake increases the levels of circulating EVs, it will still reduce the harmful effects in recipient cells. This inhibition can be either achieved with antibodies targeting various molecules (e.g., Annexin V and integrin αvβ3) or with drugs that modify, for example, microfilament formation [63].These inhibitors must be used with caution, however. Special attention should be paid to their mechanism of action and side effects to ensure that these drugs do not reduce EV biogenesis in or uptake by healthy cells.

4.2. The Therapeutic Potential of MSC- and EPC-Derived EVs

Given that MSCs have beneficial effects in human diseases like CKD [68], MSC-derived EVs have attracted great interest for their therapeutic potential in CKD. Thus, a protective role for MSC EVs was found in patients with CKD stage 3–4, as evidenced by a higher estimated GFR and lower values for blood urea, serum creatinine and the urine albumin to creatinine ratio [69]. These effects were accompanied by an increase in the plasma levels of anti-inflammatory cytokines (such as TGF-β1 and interleukin-10) and a decrease in the plasma level of TNF-α [69]. Furthermore, MSC-derived microvesicles have been shown to protect rats against CKD and inhibit renal fibrosis [70]. Another in vivo study in mice with CKD induced by subtotal (5/6) nephrectomy showed that MSC-derived EVs were able to decrease not only proteinuria, serum creatinine and uric acid but also fibrosis, interstitial lymphocyte infiltration and tubular atrophy [71]. Kidney MSC microvesicles exert antifibrotic effects by decreasing the endothelial-to-mesenchymal transition and increasing TGF-β-induced HUVEC proliferation [72]. In vivo, the researchers obtained the same results in unilateral ureteral obstruction (UUO) mice and evidenced the inhibition of inflammatory cell infiltration and tubulointerstitial fibrosis [72]. The MSC microvesicles' protective role was also observed in vitro for proximal tubular epithelial cells, with enhanced E-cadherin expression and lower α-smooth muscle actin (α-SMA) secretion; these results were confirmed in vivo in UUO mice [73]. Furthermore, MSC-derived exosomes containing miR-let7c induce antifibrotic effects, especially with a decrease in expression levels of collagen IVα1, α-SMA and TGF-β1 receptor in vitro [74]. MSC-derived EVs can also reduce renal inflammation and fibrosis and increase medullary oxygenation in pigs with metabolic syndrome and renal artery stenosis [75]. Interestingly, van Koppen et al. have shown that MSC-derived exosomes had no effect on CKD progression, systolic blood pressure and renal damage in 5/6-nephrectomized rats but did stimulate angiogenesis [76]. However, the conditioned, exosome-containing medium in the latter study had protective effects on induced CKD, hypertension and glomerular injury, suggesting that, along with exosomes, other molecules in the conditioned medium were needed to induce protective effects [76].Circulating endothelial progenitor cells (EPC) play an important role in the maintenance of vascular integrity and the regeneration of the vascular system. The number of circulating EPC in CKD patients is 30% lower than in healthy controls [77][78][79]. Surdacki et al. showed that EPC number is in fact inversely correlated to the degree of kidney dysfunction [80]. The low EPC count [81] and dysfunction [82] may contribute to an increased risk of CVD in CKD patients [83][84]. EPC therapy has been shown to decrease inflammation and proteinuria and to preserve kidney function [85]. This beneficial effect is thought to be mediated in part in a paracrine manner, and recent studies have shown that EPC-EVs may be responsible for this effect by transferring miRNA. Thus, EPC-EVs activate angiogenesis by the transfer of mRNA, especially miR126 and miR296, which have been shown to modulate proliferation, angiogenesis, apoptosis and inflammation [86][87][88]. EPC-EVs also protect against CKD progression after ischemia-reperfusion injury I by inhibiting, in particular, tubule interstitial fibrosis [88].

4.3. EVs as Drug Carriers

Thanks to their small size and low immunogenicity, EVs are able to transfer endogenous and exogenous drug compounds (such as siRNA [89], protein drugs or pharmaceutical drugs) into recipient cells. Moreover, the EVs' lipid bilayer of membrane protects their contents from degradation. The advantages of EVs include their non-toxicity, their long half-life and, importantly, their ability to cross barriers such as the blood-brain barrier [90]. Specific binding of EVs to the target cells is important for effectiveness. To this end, donor cells can be genetically engineered so

that a specific ligand is expressed on the EVs they release [91] and can be specifically recognized by the target recipient cells. Most studies have been performed in cell-based or animal models. Only a few drug-carrying EVs have been tested in the clinic [65].Despite the EVs' potential as delivery systems, some important issues need to be addressed for clinical use; these include the culture conditions, EV purification/quantification and the choice of the donor cells.

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