Ckj Review

Extracellular vesicles in the urine: markers and mediators of tissue damage and regeneration

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Abstract

As in several body fluids, urine is a rich reservoir of extracellular vesicles (EVs) directly originating from cells facing the urinary lumen, including differentiated tubular cells, progenitor cells and infiltrating inflammatory cells. Several markers of glomerular and tubular damage, such as WT-1, ATF3 and NGAL, as well as of renal regeneration, such as CD133, have been identified representing an incredible source of information for diagnostic purposes. In addition, urinary extracellular vesicles (uEVs) appear to be involved in the cell-to-cell communication along the nephron, although this aspect needs further elucidation. Finally, uEVs emerge as potential amplifying or limiting factors in renal damage. Vesicles from injured cells may favour fibrosis and disease progression whereas those from cells with regenerative potential appear to promote cell survival. Here, we will discuss the most recent findings of the literature, on the light of the role of EVs in diagnosis and therapy for damage and repair of the renal tissue.

Keywords: biomarkers; exosomes; kidney injury; progenitor cells; urine

Introduction

Extracellular vesicles (EVs) are small particles secreted by all types of cells under both physiological and pathological conditions. They are composed of a lipid bilayer, which encloses a broad variety of cytoplasmic proteins, lipids, as well as RNAs (mainly non-coding RNAs, microRNAs and small RNAs) that are representative to their cellular origin [1, 2].

According to the way they are being formed, EVs can be classified in three main groups: exosomes, microvesicles and apoptotic bodies [3]. Although the boundaries between these three groups have not been completely clarified yet, they seem to differ in diameter size, expressed biomarkers [3] as well as in their biological content [4]. In particular, exosomes are 30–120 nm in size and are released by fusion of the outer membrane of multivesicular bodies with the apical plasma membrane. Microvesicles are larger particles and directly originate from membrane budding. However, because of the lack of a distinctive characterization among them, the term EVs seems to be so far the most accurate one. Urinary extracellular vesicles (uEVs) express surface receptors of the cell of origin as well as selected patterns of proteins, mRNA and of microRNA [5]. After secretion, vesicles can act on their target cells through several mechanisms. They can stimulate target cells binding their surface receptors, or directly transfer receptors of their parental cells and finally they can deliver their biological cargo [2].

As renal cells along the nephron actively release EVs into the urine, variations in uEV number, origin or content may mirror the physiopathological state of the kidney, representing an interesting field of investigation in search of possible disease targets, markers or new therapeutic agents.

In the present review, we will discuss the recent data regarding the characteristics of EVs in the urine under normal and pathological conditions, and their possible exploitation as markers of disease and recovery. We will highlight the available information on the physio-(patho)logical function that EVs released by renal cells along the tubule may present and finally we will discuss the perspective therapeutic role EVs may exert.

Characterization of renal cell-derived EVs in the urine

As in several body fluids, urine is a rich reservoir of EVs directly originating from cells of different nephron segments or of the urinary tract as well as from infiltrating inflammatory cells [5]. Urine should be lacking plasma EVs, as they cannot pass through the glomerular filtration machinery, at least in physiological conditions. Several methods are currently being used to recover EVs from the urine, with ultracentrifugation remaining among the most common ones [6]. Addition of protease inhibitors and removal of whole

cells and cell debris are required prior to processing the urine [6]. Vesicles in urine were first isolated by Pisitkun *et al.* in 2004, in a study that supported their characterization as exosomes due to their small size (35–40 nm) and the endocytic pathway of biogenesis [7]. However, later studies demonstrated the presence of different vesicles in urine varying in size and formation pathway, supporting the usage of the broader term of uEVs [5, 8]. The protein content of uEVs has been largely characterized. In 2004, through mass spectrometry, nearly 300 proteins were identified [7]. Later, through an improved mass analyser, more than 1100 proteins were shown to be associated to uEVs [9] leading to the creation of an open access online database for public research of specific proteic sequences, named Urinary Exosome Protein Database (http://dir.nhlbi.nih.gov/papers/lkem/exosome/). At present, the most complete characterization can list nearly 3280 proteins [10]. Among the described proteins, uEVs express typical exosomal markers, such as tetraspanins (CD9, CD63 CD81), flotillin-1, HSP70, apoptosis-linked gene-2-interacting protein X and tumour susceptibility gene [11]. In addition, uEVs express a variety of renal markers that indicate the prominent production by the epithelial cells of the different nephron segments. Besides CD24, a marker described for uEVs [12], they can express podocin and podocalyxin that sign a glomerular podocyte origin [13] or megalin, cubilin, aminopeptidase [14] and aquaporin-1 (AQP)-1 that indicate a proximal tubular cell origin. Moreover, vesicles carrying Tamm Horsfall protein, CD9 and type 2 Na-K-2Cl cotransporter (NKCC2) appear to derive from the thick ascending limb of the Henle's loop, whereas the presence of AQP-2 and mucin-1 reveals an origin from the collecting duct [7, 15]. Moreover, uEVs expressing the CD133 antigen, a marker of renal progenitor cells, have been described in normal urine [16]. The renal origin of vesicles present in the urine has been further demonstrated by the detection of donor-specific HLA-expressing EVs in the urine of transplanted patients in the first day after transplant [16].

Regarding the presence of RNA in the uEVs, a recent comprehensive analysis revealed that the large majority of RNA species belongs to ribosomal and non-coding RNAs. The remaining RNA encodes for proteins specific to the complete segments of the renal nephron and collecting duct as well as to the bladder and prostate, highlighting the origin of the uEVs from the entire genitourinary system. Of interest, as the non-coding RNA may have a role in cell regulation, the non-ribosomal RNA sequences contained in uEVs can potentially display a function within the kidney [17].

uEVs as biomarkers of glomerular and tubular damage

uEVs in acute kidney injury and kidney transplantation

Several studies indicate that uEVs may provide rapid markers of kidney injury that directly reflect the tubular damage (Table 1).

Using the two mice model of acute kidney injury (AKI), cisplatin and ischaemia/reperfusion injury, Zhou *et al.* demonstrated a significant increase of the levels of activating transcription factor 3 (AFT3) in uEVs but not in whole urine, after the induction of damage. Of note, the urinary vesicle marker AFT3 not only remained elevated for 24–48 h, but it increased before the raising of the serum creatinine [18], supporting the clinical interest for this

Table 1. Detection of uEV markers of glomerular and tubular damage and fibrosis in human renal diseases and animal models

Disease	Humans/animal models	uEVs marker	References
Tubular damage			
Acute kidney injury	I/R injury in rats	ATF3	[18]
		Fetuin-A	[6]
		AQP1	[19]
	Cisplatin-induced AKI in rats	ATF3	[18]
		Fetuin-A	[6]
	Humans	ATF3	[18]
		Fetuin-A	[6]
		AFT3 (mRNA)	[20]
Kidney transplant	Humans	AQP1	[19]
		NGAL	[21]
		NKCC2/NCC	[22]
Glomerular injury and chronic renal damage			
Focal segmental glomerulosclerosis	PAN-induced glomerulosclerosis in rats	WT-1	[18]
	Podocin/Vpr transgenic mice	WT-1	[18]
	Humans	WT-1	[18]
Diabetes nephropathy	Humans	WT-1	[23]
		AMBP, MLL3 VDAC	[24]
		miR-145	[25]
IgA nephropathy	Humans	α-1-antitrypsin ceruloplasmin	[14]
Chronic kidney disease	Humans	CD2AP (mRNA)	[26]
		miR-29	[26]
		OPG	[27]
Obstructive nephropathy	Humans	TGFβ	[28]

I/R, Ischaemia/reperfusion injury; AKI, acute kidney injury; ATF3, activating transcription factor 3; AQP1, aquaporin-1; NGAL, neutrophil gelatinase-associated lipocalin; NKCC2, Na-K-2Cl cotransporter in the loop of Henle; NCC, Na-Cl cotransporter; PAN, puromycin; AMBP, α-1-microglobulin/bikunin precursor; MLL3, isoform 1 of histone-lysine *N*-methyltransferase; VDAC1, voltage-dependent anion-selective channel protein 1.

biomarker. These results were subsequently demonstrated in four patients with AKI, where in one patient the increase of AFT3 in uEVs preceded the increase of the serum creatinine [18]. Further studies in uEVs from AKI patients showed that AFT3 also increased at the mRNA levels, being 60-fold higher as compared with normal controls [20]. Another uEV biomarker of AKI is fetuin-A. As observed for AFT3, the level of fetuin-A increased by 52.5-fold after damage and preceded the increase of serum creatinine both in animal models and in patients [29]. An opposite trend was observed for AQ1, as its content in the uEV rapidly declined both in a rat model of ischaemia/reperfusion injury and in patients immediately after kidney transplantation [19].

Analysing the uEVs collected from renal transplanted patients, Alvarez *et al.* demonstrated that neutrophil gelatinase-associated lipocalin (NGAL) protein, an emerging biomarker of AKI and of delay graft function [21], is abundant in the uEVs of all transplanted patients. High levels of NGAL protein were found in the isolated uEVs as compared with the cellular fraction and, in particular, they were elevated in the uEVs of patients with delayed graft function, suggesting that the exosomal NGAL might be a valid tool to evaluate the allograft damage [21]. In contrast, Peake *et al.* did not find any increase in the level of mRNA encoding for NGAL, interleukin-18, kidney injury molecule-1 and cystatin C in the uEVs of transplanted patients. As these markers of tubular damage are known to

increase at the mRNA level in the whole urine of AKI patients, the discrepancy with the finding in the uEVs may indicate variability in mRNA packaging in uEVs in different cell types of the nephron [30].

In order to detect possible markers of drug toxicity, uEVs have been also studied in transplanted patients treated with the calcineurin inhibitor cyclosporine. Esteva-Font *et al.*, evaluating the content of NKCC2 and Na-Cl co-transporter in the uEVs in kidney transplanted patients, found a significant increase of their level in the uEVs of cyclosporine-treated patients compared with the controls [22].

uEVs in glomerular damage and chronic kidney disease

uEVs were also shown to represent useful biomarkers of glomerular injury and chronic renal damage (Table 1). As vesicles deriving from glomeruli are constantly released into urine in their physiological turnover, the increase of a podocyte marker within uEVs has been regarded as a direct sign of injury, whereas its reduction may mark a general loss of the podocyte cell component in chronic injury. In particular, the increase in the podocyte marker Wilms' tumour 1 (WT-1) was successfully correlated with podocyte injury in both animal models as well as in patients affected by chronic glomerular pathologies [23, 31]. High levels of urinary vesicular WT-1 were also found in patients with diabetes mellitus type 1 with proteinuria compared with those without proteinuria and negatively correlated with the renal function. These data indicate that the increase of urinary WT-1 in EVs is a marker of podocyte malfunction [23]. Similarly, vesicles expressing the podocyte marker podocalyxin and originating from the tip vesiculation of glomerular podocyte microvilli were increased in patients with nephritic syndrome [32, 33]. The reduction and normalization of markers of podocyte damage, such as WT-1, in uEV can be useful to indicate the disease remission or correlate with the response to therapy in nephritic syndrome patients [31]. Unfortunately, this was not confirmed in paediatric patients with nephrotic syndrome in which WT-1 levels in uEVs did not vary according to the responsiveness to the steroid therapy [34].

On the other side, another podocyte marker, CD2AP, evaluated at the mRNA level, was reduced in uEVs of patients with chronic kidney disease (CKD) compared with the controls and correlated with the renal dysfunction, the amount of proteinuria and the degree of renal fibrosis [26], matching the decrease of the gene expression level observed in podocytes [35].

uEVs might also be useful as a diagnostic tool, as shown for the differential diagnosis between early IgA nephropathy and thin basement membrane nephropathy in paediatric and adult patients with isolated microscopic haematuria. In this regard, Moon *et al.* identified four different biomarkers differently expressed in uEVs of these patients: aminopeptidase N and vasorin precursor were higher in the thin basement membrane nephropathy group compared with the IgA nephropathy group, conversely α-1-antitrypsin and ceruloplasmin were high in the IgA group [14]. Comparing uEVs derived from healthy subjects and diabetic nephropathy patients, Zubiri *et al.* found 254 different proteins. Among those, they reported the increase of a histone methyltransferase, whose function is to methylate the Lys-4 of histone H3 [24]. As this protein is implicated in epigenetic transcriptional activation [36], it supports the possible involvement of uEVs in cell-to-cell communication.

In the evaluation of glomerular damage, uEVs appear to display several advantages as compared with proteinuria.

Although proteinuria represents an easy accessible and valid marker of the severity of the kidney damage and it may predict the decline of glomerular filtration rate [37], it fails to discriminate the type of the underlying renal diseases. The presence of a large quantity of information in uEVs (mRNA, microRNA, proteins and surface receptors) may provide useful markers able not only to obtain a more precise evaluation of the extent of glomerular damage, but possibly also to discriminate the type of glomerular injury, as reported above [14, 24]. Finally, the study of uEVs may also inform on the presence of regenerative, maladaptive or fibrotic responses occurring in the renal tissue as a consequence of the injury.

In search of biomarkers of renal fibrosis, several authors also investigate the content of microRNAs in the uEVs. Lv *et al.* [38] found that members of miR-29 and miR-200 family were significantly reduced in uEVs of patients with CKD compared with controls, correlated with renal function and with the degree of tubular-interstitial fibrosis. Barutta *et al.* [25] also assessed miRNA expression in the uEVs from diabetes mellitus type 1 with or without diabetic nephropathy and reported that uEVs derived from microalbuminuric patients were enriched in miR-130a and miR-145. The latter is known to be a glomerular marker of mesangial cells induced by transforming growth factor (TGF)-β1. They also found a decrease in uEVs of miRNA-155 and miRNA-424 that are expressed on podocytes and that negatively modulate the signalling of angiotensin II, TGF-β1 and vascular endothelial growth factor.

The evaluation of uEVs might be useful to assess the risk to develop renal dysfunction, as shown in young patients affected by posterior urethral valves. In these patients, uEVs contained high levels of E-cadherin, TGF-β1, N-cadherin and L1 cell adhesion molecule compared with the controls. Specifically, the level of the pro-fibrotic factor TGF-β1 in uEVs correlated with the glomerular filtration rate [28]. Finally, the inflammatory marker osteoprotegerin, a decoy receptor of the tumour necrosis factor superfamily pro-apoptotic cytokine, has been shown to increase in uEVs of CKD patients compared with the controls [27]. These studies altogether suggest that the vesicular content of both protein and miRNAs may mirror the existence of a pro-fibrotic and inflammatory renal environment.

uEVs as biomarkers of regeneration

Besides being markers of damage, as described above, EVs in the urine may provide information on the physiological state of the kidney and on the intrinsic mechanisms of its homeostasis and repair. Indeed, recent studies indicate that the kidney harbours a population of cells with progenitor characteristics involved in the continuous regeneration and renewal of kidney epithelia as well as in its repair after injury [39]. In the human kidney, a cell population with CD133 expression and progenitor characteristics has been identified [40–42] and its number was reported to increase in the cortex after acute renal damage, suggesting their role in renal repair after injury [43–45].

Stem/progenitor cells are known to act in a paracrine fashion to support the neighbouring cells [46] and, in analogy, the scattered CD133+ progenitor cells along the nephron may release CD133+ EVs with a functional effect along the renal tubules. Indeed, CD133+ expressing EVs have been previously described in normal human urine [9]. Once sorted, they were found to be positive for proximal tubule and glomerular markers [16], suggesting their

origin from the upper part of the nephron. It was recently shown that levels of urinary CD133^{+} EV are reduced in patients with end-stage renal disease, possibly indicating that these vesicles are only released by functioning renal tissue [16]. Indeed, in transplanted patients, CD133^{+} EVs were present at low levels the first day after transplantation, to increase thereafter. Of interest, no variation was reported for the CD24^{+} EVs, used as marker of uEVs, indicating that the composition of the vesicle population rather than its number may be a relevant marker. Moreover, the relative CD133^{+} EV levels did not vary in relation to urine concentration or glomerular filtration rate. It could be therefore speculated that the number of CD133^{+} EVs may reflect the activity of CD133^{+} cells in the kidney [16].

Regarding the possible significance of CD133^{+} uEVs shedding, they could be considered as a mechanism of cell differentiation and maturation. Similarly, the shedding of CD133^{+} EVs by both CD133+ neural stem cells and CD133^{+} hematopoietic stem cells was regarded as a mechanism of cell differentiation and specification [47, 48].

Function of uEVs in cell-to-cell communication and immunity

The biological role of EVs in the intracellular communication among cells is nowadays well established and supports the hypothesis that EVs present in the urinary lumen
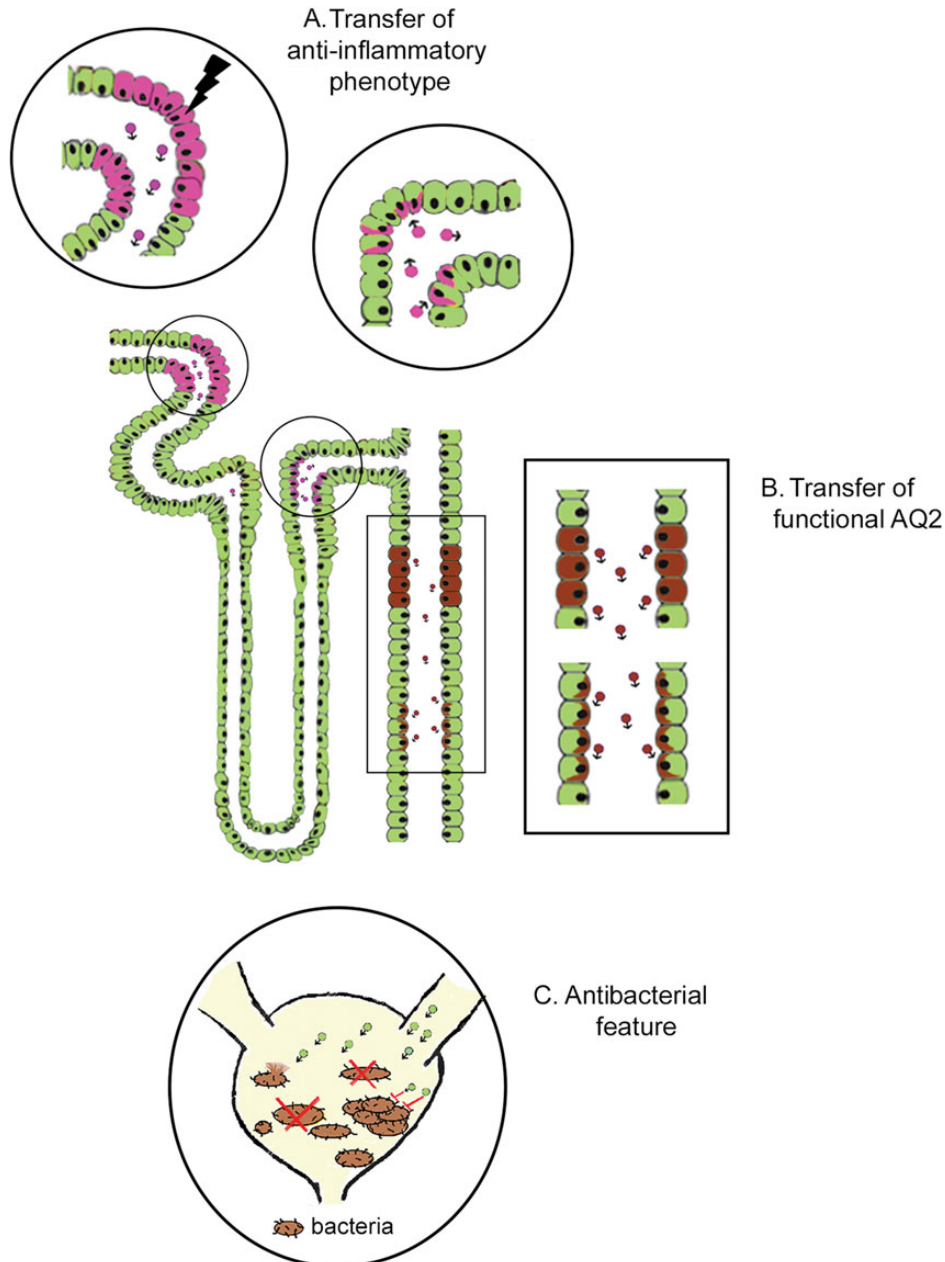

Fig. 1. uEVs mediate cell-to-cell communication and immunological functions within the nephron. The schematic picture shows the possible effects of uEVs in the intra-nephron communication. uEVs may provide an antioxidant effect in distal tubular cells when derived from tubular cells stimulated with anti-inflammatory mediators; or they may mediate the transfer of functional molecules, such as AQP2 to the recipient cells. In the bladder, uEVs may exert bacteriostatic and bacteriolytic effects by inhibiting bacteria adhesion to the bladder cells, blocking their growth or inducing their lysis.

of the nephron could act in a 'urocrine' manner [49]. The intercommunication of EVs among cells of the nephron was first shown by the ability of distal tubule and collecting duct cell lines to uptake EVs released by proximal tubular cells [50]. EVs from proximal tubular cells were shown to accumulate into the multivesicular body of the recipient cells. In contrast, in polycystic kidney disease, the EVs were shown to mainly interact with the primary cilia of recipient cells both *in vivo* and *in vitro* [49]. In addition, EVs from renal cells were shown to modulate the function of the recipient cells. For instance, *in vitro* experiments showed that the transfer of AQP2 via EVs isolated from kidney collecting duct cells was functional and increased the water flow of recipient cells [51]. Another example of functional transfer comes from experiments using EVs from proximal tubular cells treated with a dopamine receptor agonist, known to induce a decrease in cell radical production. These EVs were able to modulate the levels of radical production of the recipient distal tubular cells, transferring an anti-inflammatory message [50]. All these data support the notion of an inter-nephron communication throughout the whole kidney via EV release (Figure 1).

Recently, EVs of the urine have gained a novel role as 'innate immune effectors' of the renal tract, due to their unexpected antibacterial function (Figure. 1). uEVs were shown to contain proteins connected to innate immune response such as bacterial receptors, to functionally inhibit bacterial growth and to induce bacterial lysis [52].

EVs as amplifying or limiting factors in renal damage

It is well known that the content of EV and the subsequent effect on the recipient cell may depend *in primis* on the cell type of origin of EVs, but it may also vary according to the cell patho-physiological state and stimulation. During renal tissue damage, EVs present in the urinary microenvironment may exert positive or negative effects, amplifying or limiting the damage through modulation of the recipient cells (Figure. 2). This was clearly shown for EVs deriving from hypoxic tubular cells that induced the expression of TGF-β1, α-smooth cell actin and F-actin in fibroblasts, promoting their subsequent activation. This effect was lacking when EVs were obtained from normoxic tubular cells, implicating a role for EVs from damaged cells of the nephron in amplifying tissue damage [53]. On the other side, EVs from renal CD133^{+} progenitor cells may exert a protective effect during damage. When co-incubated with
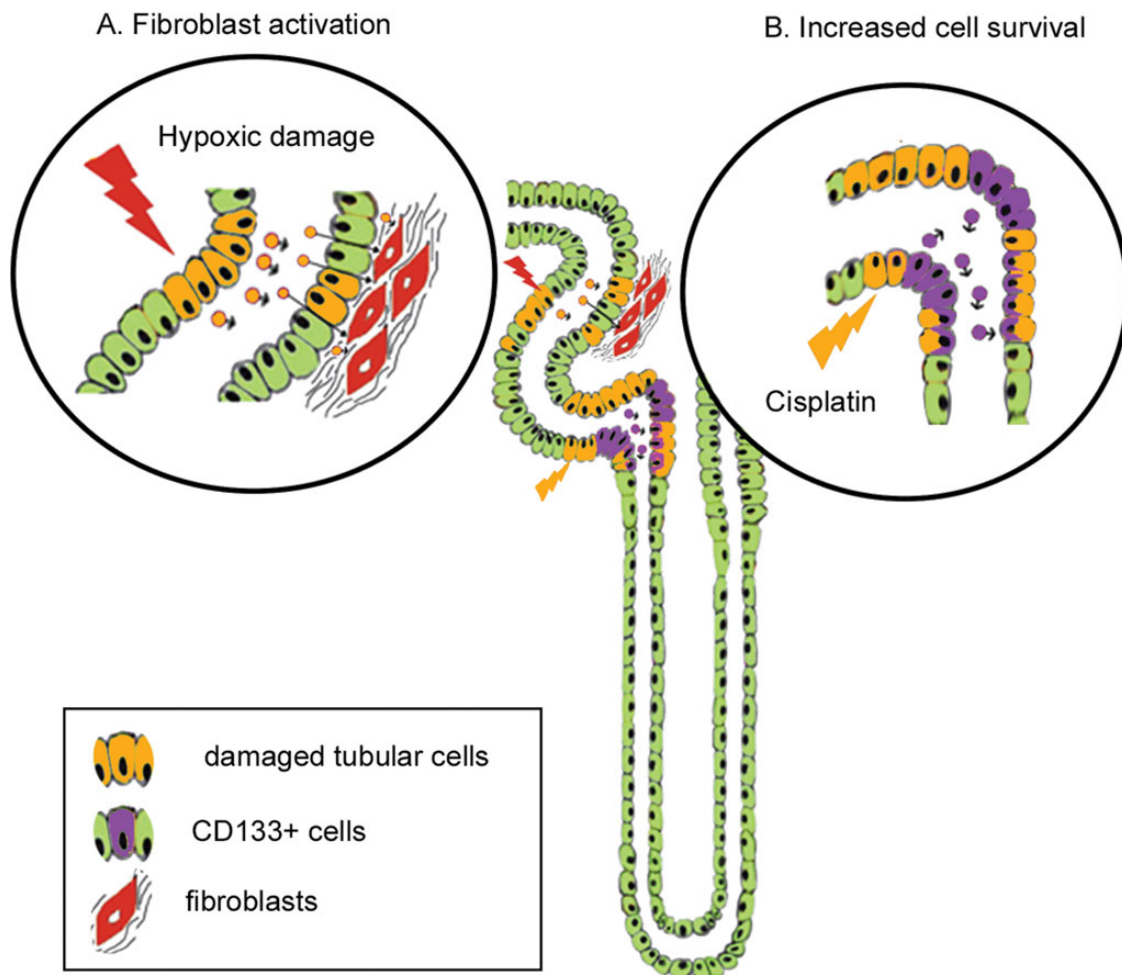

Fig. 2. EVs may amplify or limit renal damage. The schematic picture shows the cellular communication through uEVs in tubular cell damage. Ischaemic damage may induce fibroblast activation through the release of EVs from proximal tubular cells. Alternatively, upon cisplatin damage sensing, CD133^{+} progenitor cells may release EVs in order to protect the neighbouring tubular cells from damage.

cisplatin-damaged renal epithelial cells *in vitro*, CD133^{+} progenitors were shown to release EVs overexpressing molecules implicated in cell cycle regulation and survival [54]. In particular, the protective effect of EVs was ascribed to cyclin D1 and decorin mRNA shuttle from the renal progenitors to the damaged tubular cells. Interestingly, no evidence of repair was shown when progenitor cells had not been in culture with the injured cells, suggesting the importance of damage sensing in the released vesicles [54].

Finally, an increasing field of the literature is devoted to investigate the therapeutic use of EVs administered *in vivo* in acute and chronic renal pathology. The most promising cell source is the use of mesenchymal stem cell from different origin [55–57]. The major mechanisms involved in the effect of these EVs are as follows: a pro-survival effect exerted by stimulating the resistance to apoptosis of healthy tubular epithelial cells and by accelerating the recovery in glycerol-induced AKI damaged mice [55], an anti-inflammatory function due to the down-regulation of genes involved in the inflammatory response, such as CARD6, in cisplatin-damaged tubular epithelial cells [56] and finally a regenerative effect by transfer of growth factors/mRNAs such as IGF-1 [57]. Indeed, a recent study demonstrated the potential use of EVs derived from mesenchymal stem cells obtained by the kidney itself that exhibited a protective role in a model of ischaemic injury [58]. Moreover, a recent report showed for the first time that mesenchymal-derived EVs could have a protective effect also for genetic diseases as they can transfer vesicle-associated wild-type molecules. In particular, EVs containing wild-type cystinosin protein and mRNA reduced *in vitro* the pathologic cystine accumulation in proximal tubular cells isolated from cystinosis patients [59]. The major mechanisms involved in the effect of these EVs are anti-inflammatory functions, pro-survival effect and finally the transfer of regenerative factors/mRNAs such as IGF-1 and HGF [55–57]. In this regard, a recent study demonstrated the potential use of EVs derived from mesenchymal stem cells obtained by the kidney itself that exhibited a protective role in a model of ischaemic injury [58]. As EVs maintain several characteristics of the cell of origin, it can be prospected that EVs obtained by renal stem cells or from stem cells during renal differentiation may display renal-specific regenerating properties. Another possible approach in search for therapeutic EVs could be the isolation of specific EV populations from the urinary pool. These EVs may possibly specific applications in nephrology and may help to understand the physiological role of EVs within the nephron.

Conclusions and future prospects

In conclusion, as depicted in this review, EVs released from cells of the nephron and present in the urine represent an incredible source of information for diagnostic purposes, and possibly for future therapeutic application. Several markers of glomerular and tubular damage, such as WT-1, ATF3 and NGAL [60], as well as of renal regeneration, such as CD133 [16], have been identified and could possibly be combined to gain information about tissue damage and regeneration (Table 1).

Several methodological hurdles limit at the moment the clinical application of uEVs, being the major limit the lack of a standard and easy-handling laboratory technique for routinary screening. Several efforts are now in progress on the possibility to freeze down urine and to isolate EV without ultracentrifugation. In addition, methods applicable to large sample number, such as ELISA or cytofluorimetric analysis, should be developed. Normalization for EV number and urine dilution is still to be improved [61].

In addition, the knowledge on the function of EVs in intra-nephron communication appears of incredible interest to understand renal physiology (Figures. 1 and 2) and possibly new mechanisms involved in renal acute and chronic damage and its progression to organ failure. Finally, the therapeutic role of EVs appears to be a new frontier in regenerative medicine. The identification of the most suitable cell type and of the best cell stimulation may lead to therapeutic tools, possibly with renal-specific characteristics.

Conflict of interest statement. The authors of this manuscript have no conflicts of interest to disclose.

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