Circulating extracellular vesicles in patients with end stage Chronic Kidney Disease: new uremic toxins involved in inflammation, endothelial dysfunction and vascular calcification

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Background and aim

Inflammation, endothelial dysfunction and vascular calcifications are common features of patients with end stage chronic kidney disease (CKD). CKD is associated with high mortality rates due to multiple co-morbidities, particularly cardiovascular diseases (CVD).
The pathogenetic mechanisms of CKD-associated CVD are not only related to traditional factors such as hypertension and dyslipidemia, but also to endothelial dysfunction, circulating and tissue-released inflammatory mediators including water soluble and protein bound uremic toxins (UT).
Among these detrimental circulating mediators, extracellular vesicles (EV) are nanoparticles that represent a new concept of inter-cellular cross-talk through the transfer of proteins, mRNAs and microRNAs to different target cells.
Our main working hypothesis is that in the uremic milieu, EV may synergize with UT in the induction of CVD, altering the function of endothelial cells (EC), smooth muscle cells (SMC) and endothelial progenitor cells (EPC).

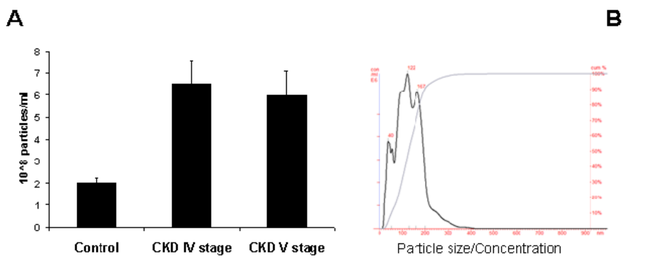

Fig. 1: Concentration of circulating EV in the different groups by Nanosight analysis (A).Example of Nanosight analysis of circulating EV in CKD patients (B).

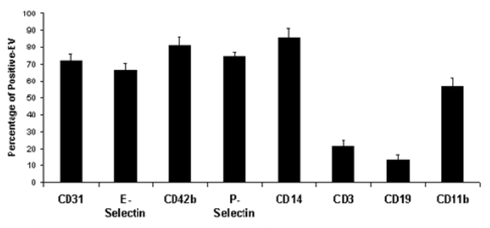

Fig. 2: FACS analysis of CKD EV for different cell markers.

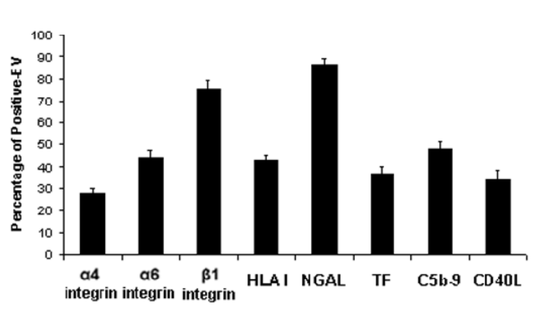

Fig. 3: FACS analysis of CKD EV for other surface molecules involved in inflammation, apoptosis, coagulation and complement activation.

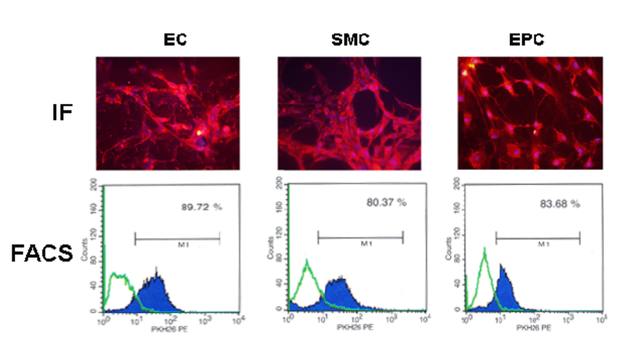

Fig. 4: Immunofluorescence analysis (IF) and FACS analysis of internalization of PKH26 red labeled-CKD EV within human endothelial cells (EC), smooth muscle cells (SMC) and endothelial-progenitor cells (EPC).

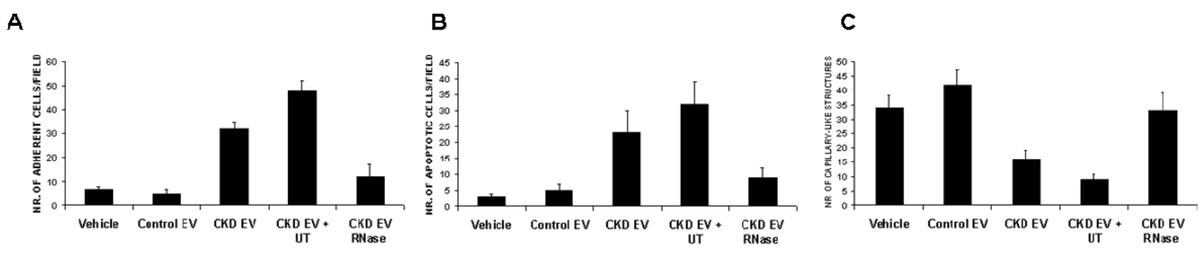

Fig. 5: Leukocyte adhesion (A), apoptosis (TUNEL assay in B) and in vitro angiogenesis on Matrigel-coated plates (C) of EC cultured in different experimental conditions.

Fig. 6: Analysis of NO production (DAF2 probe in A) and ROS generation (B) by EC cultured in different experimental conditions.

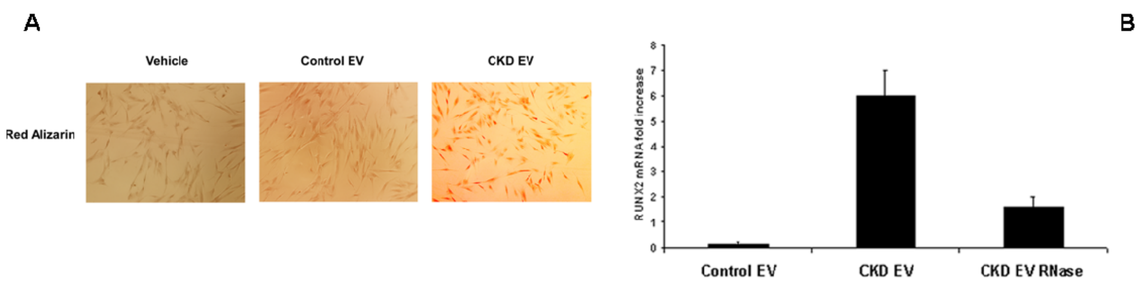

Fig. 7: Red alizarin staining (calcification assay in A) and Runx2 mRNA expression (B) by SMC cultured in different experimental conditions.

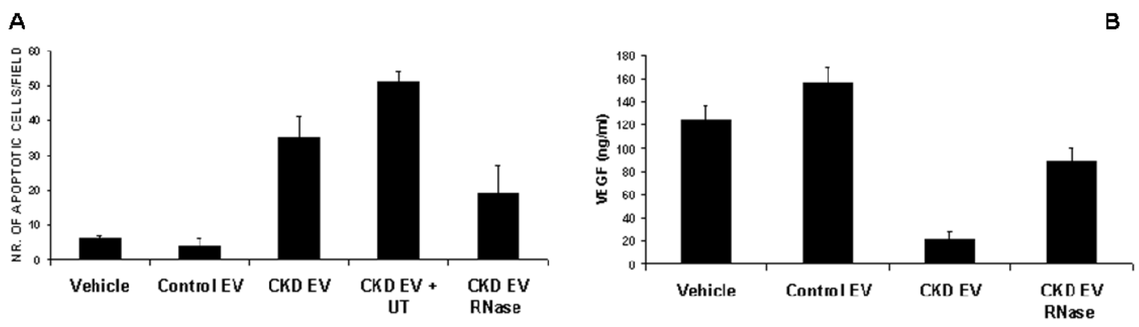

Fig. 8: Apoptosis (TUNEL assay in A) and VEGF release (ELISA in B) by EPC cultured in different experimental conditions.

Methods

We analyzed plasma drawn from 65 end stage CKD patients (stage IV-V). Plasma EV were evaluated by Nanotrack (Nanosight technology) to detect concentration and size and by GUAVA FACS analysis to detect surface molecule expression. Circulating CD34+CD133+flk-1+ EPC were also evaluated by FACS on patients' peripheral blood.
EV isolated from CKD patients' plasma by ultracentrifugation were added to cultured human EC, SMC and EPC evaluating apoptosis (TUNEL assay), angiogenesis (culture on Matrigel-coated plates), NO/VEGF release and calcification capacity (red alizarin staining, Runx2 mRNA expression).

Results

In comparison to healthy subjects, CKD patients (both stage IV and V) showed a significant increased concentration of plasma EV ($p<0.05$) (Fig. 1). FACS analysis revealed that plasma EV originated from different cell types including endothelial cells, monocytes, neutrophils, T and B lymphocytes and platelets (Fig. 2). In addition, EV expressed on their surface molecules involved in inflammation, apoptosis, coagulation and complement activation such as Tissue Factor (TF), the terminal complement factor C5b-9, CD40-ligand, Fas-Ligand, NGAL, class I HLA and different proteins of the integrin and of the selectin families (Fig. 3). RNA profiling of EV is currently under investigation.
Plasma EV inversely correlated with the percentage of circulating CD34+CD133+flk-1+ EPC. By contrast, EV directly correlated with plasma levels of homocysteine, ADMA and C reactive protein, markers of inflammation and endothelial injury (data not shown).
***In vitro*, FACS and microscopy analysis showed that plasma EV were internalized in isolated human EC, SMC and EPC (Fig. 4).**
In EC, plasma EV increased leukocyte adhesion (Fig. 5A), triggered apoptosis (Fig. 5B) and reduced their pro-angiogenic properties on Matrigel-coated plates (Fig. 5C).
These effects may be related to EV-induced decrease of eNOS expression and consequent NO production (DAF2-DA probe staining in Fig. 6A) with a concomitant increased generation of ROS (Fig. 6B).
In SMC, plasma EV increased calcification capacity as assessed by alizarin red staining (Fig. 7A) and qRT-PCR for Runx2 mRNA expression (Fig. 7B).
In EPC, plasma EV induced apoptosis (Fig. 8A) and reduced VEGF release (Fig. 8B). All the described detrimental effects induced by EV on EC, SMC and EPC were enhanced by co-incubation with protein bound UT such as p-cresyl sulfate and indoxyl sulfate (Fig. 5-8). By contrast, the biological activities of EV were significantly decreased by their pre-treatment with RNase, the enzyme able to destroy mRNA and microRNA within EV (Fig. 5-8).

Conclusions

Plasma EV concentrations are significantly increased in end stage CKD patients. EV originate from different cell types and may synergize with protein bound UT in the development of EC, SMC and EPC injury and functional alterations.

The transfer of specific mRNA and microRNA from EV to target cells may have a key role in inflammation, endothelial dysfunction, vascular calcifications and CVD in patients with end stage CKD, providing new diagnostic and therapeutic targets.

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H) Clinical Nephrology, CKD. H5) Pathophysiology and clinical studies in CKD 1-5.
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