EXTRACELLULAR VESICLES (EVs) DERIVED BY HUMAN ENDOTHELIAL PROGENITOR CELLS (EPCs) PROTECT HUMAN RENAL GLOMERULAR ENDOTHELIAL CELLS AND PODOCYTES FROM TUMOR NECROSIS FACTOR-α INJURY

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## Extracellular Vesicles (EVs)

<table>
<thead>
<tr>
<th></th>
<th>Exosomes</th>
<th>Microvesicles</th>
<th>Apoptotic bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Formation</strong></td>
<td>Endosomal pathway, internal budding, exocytosis</td>
<td>Budding off the plasma membrane</td>
<td>Cell fragmentation/blebbing</td>
</tr>
<tr>
<td><strong>Size</strong></td>
<td>30–100 nm</td>
<td>100–1,000 nm</td>
<td>1–5 μm</td>
</tr>
<tr>
<td><strong>Content</strong></td>
<td>Proteins, lipids, mRNA, miRNA and cytosol</td>
<td>Proteins, lipids, mRNA, miRNA and cytosol</td>
<td>Proteins, lipids, DNA, rRNA, organelles and cytosol</td>
</tr>
</tbody>
</table>

**Extracellular vesicles in renal disease**

D Karpman, A Ståhl & I Arvidsson

Exosomes are released by exocytosis through a mechanism dependent on cytoskeleton activation and under the regulation of p53 protein.

Microvesicles take place from the budding of small cytoplasmic protrusions followed by their detachment from the cell surface dependent on calcium influx, calpain and cytoskeleton reorganization.

**Exosomes/microvesicles as a mechanism of cell-to-cell communication.**

Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L. Kidney Int. 2010
Extracellular Vesicles (EVs)–Uptake and Biological Activity

EVs may mediate a cell-to-cell horizontal transfer of biological material after uptake by Pinocytosis, Membrane Fusion, Endocytosis, or Receptor-mediated Endocytosis.

Exosomes/microvesicles as a mechanism of cell-to-cell communication.
Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L. Kidney Int. 2010
Endothelial Progenitor Cells (EPCs)

EPCs are adult stem cells derived from the bone marrow that circulate in the peripheral blood.

EPCs play an important role in the regulation of vascular homeostasis and participate in the regeneration of injured endothelium and of different organs.

EPC-induced endothelial cell repair is mainly ascribed to the release of paracrine factors:

- Growth Factors (VEGF, HGF, etc.)
- Extracellular vesicles (EVs)
Endothelial progenitor cell–derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA

Maria Chiara Deregibus, Vincenzo Cantaluppi, Raffaele Calogero, Marco Lo Iacono, Ciro Tetta, Luigi Biancone, Stefania Bruno, Benedetta Bussolati, and Giovanni Camussi
Kidney
Analysis of biological effect of EPC-derived EVs on Proximal Tubular Cells and Peritubular Endothelial Cells.
EPC-derived EVs exerted functional and morphologic protection from renal Ischemia Reperfusion Injury

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Sham</th>
<th>IRI</th>
<th>IRI + EPC EV</th>
<th>IRI + EPC EV RNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casts (n/HPF)</td>
<td>0</td>
<td>0</td>
<td>2.6 ± 1.2</td>
<td>0.48 ± 0.21</td>
<td>2.93 ± 0.84</td>
</tr>
<tr>
<td>Tubular Apreosis (n/HPF)</td>
<td>1.6 ± 0.3</td>
<td>1.8 ± 0.6</td>
<td>17.6 ± 2.9</td>
<td>3.4 ± 1.3</td>
<td>14.8 ± 2.7</td>
</tr>
<tr>
<td>Infiltrating Granulocytes</td>
<td>2.2 ± 0.8</td>
<td>2.9 ± 1.2</td>
<td>29.6 ± 3.3</td>
<td>9.5 ± 2.2</td>
<td>23.8 ± 4.6</td>
</tr>
<tr>
<td>Infiltrating Monocytes</td>
<td>6.7 ± 1.1</td>
<td>6.5 ± 1.5</td>
<td>44.2 ± 0.6</td>
<td>12.3 ± 4.4</td>
<td>28.2 ± 3.8</td>
</tr>
</tbody>
</table>
EPC-derived EVs stimulate Angiogenesis on Peritubular Endothelial Cells in Hypoxia

EPC-derived EVs increase Resistance to Apoptosis on Tubular Epithelial Cells in Hypoxia
Characterization of EPC-derived EVs

**Nanosight analysis**
- Average size: 160 nm

**Bioanalyzer**

**FACS**

**160 microRNAs**

- let-7a, miR-106b, let-7c, miR-125a, let-7d, miR-125b, let-7e, miR-125a, miR-192
- let-7c, miR-191b, let-7d, miR-191d, let-7e, miR-195, let-7f
- miR-126, miR-128, miR-296, miR-378, miR-223, miR-210, miR-20a, miR-24
- miR-145, miR-15a, miR-15b, let-7e
- miR-21, miR-155, miR-375, miR-219a, miR-219b, miR-219c, miR-211, miR-200a, miR-200b, miR-200c, miR-200d

**Small RNAs**
- miR-126
- miR-296
- Control
Analysis of biological effect of EPC-derived EVs on Glomerular Mesangial Cells

Glomerular Mesangial Cells are involved in the mechanisms of filtration, basement membrane deposition and phagocytosis.

In glomerulonephritis, they are susceptible to immune-mediated and inflammatory damage.

Endothelial progenitor cell-derived extracellular vesicles protect from complement-mediated mesangial injury in experimental anti-Thy1.1 glomerulonephritis

Vincenzo Cantaluppi, Davide Medica, Claudio Mannari, Giulia Stiacci, Federico Figliolini, Sergio Dellepiane, Alessandro Domenico Quercia, Massimiliano Migliori, Vincenzo Panichi, Luca Giovannini, Stefania Bruno, Ciro Tetta, Luigi Biancone and Giovanni Camussi

Curr Opin Nephrol Hypertens. 2015 May; 24(3): 231–238.
EPC-derived EVs protect glomerular mesangium in experimental anti-Thy1.1 glomerulonephritis through inhibition complement cascade.

Proteinuria

BUN

A

TUNEL

SMA

Granulocytes

Monocytes

Apoptosis

Sclerosis

Leukocyte infiltration

Endothelial progenitor cell-derived extracellular vesicles protect from complement-mediated mesangial injury in experimental anti-Thy1.1 glomerulonephritis

Vincenzo Cantaluppi¹, Davide Medica¹, Claudio Mannari¹, Giulia Stiaccini², Federico Figliolini³, Sergio Delepine⁴, Alessandro Domenico Quercia⁴, Massimiliano Migliori⁴, Vincenzo Panichi⁵, Luca Giovannini⁵, Stefania Bruno⁶, Ciro Tetta⁶, Luigi Biancone⁶ and Giovanni Camussi⁷
Expression of complement inhibitors by EPCs and EPC-derived EVs
EPC-derived EVs significantly reduced C5b-9 deposition in Rat Mesangial Cells *in vitro*
Analysis of biological effect of EPC-derived EVs on Glomerular Endothelial Cells and Podocytes?
Glomerular Endothelial Cells (GECs), Basement membrane and Podocytes are part of the Glomerular Filtration Barrier (GFB)

Filtration of blood in pre urine in Bowman’s space

**Urinary lumen**
- **Bowman’s space**
- **Podocytes**
- **Basement membrane**
- **Endothelium**
- **Glyocalyx**

**Blood**
- Slit diaphragm: diameter = 30-40 nm
- Fenestrae: diameter = 60-80 nm

Complex cross-talk of growth factors between Glomerular Endothelial Cells (GECs) and Podocytes to maintain GFB integrity

- Angiogenesis
- Proliferation
- Apoptosis
- Differentiation
- Basement membrane production
EPC-derived EVs protect in Glomerular Endothelium and Podocytes in experimental anti-Thy1.1 glomerulonephritis
C5a Induces the Synthesis of IL-6 and TNF-α in Rat Glomerular Mesangial Cells through MAPK Signaling Pathways

Mingde Ji¹,², Yanlai Lu¹, Chenhui Zhao³, Wenxing Gao⁴, Fengxia He¹, Jing Zhang¹, Dan Zhao¹, Wen Qiu¹ *, Yingwei Wang¹
Tumor Necrosis Factor-α (TNF-α) is a key mediator of inflammation in kidney diseases, particularly in glomerulonephritis.

It acts on TNFR1 and TNFR2 receptors which are equally activated in glomerulonephritis in Podocytes and Glomerular Endothelial Cells. 

Tumor Necrosis Factor Receptors: Biology and Therapeutic Potential in Kidney Diseases
Peripheral blood of healthy volunteers

Isolation, characterization and maintenance of EPCs (CD133+/flk-1+)
in culture \textit{in vitro}

Ultracentrifugation of culture medium of EPCs after 2-3 passages

Isolation of EVs
Methods/2

RNase pre-treatment of EPC-derived EVs

EPC-derived EVs

Internalization on Cells with red fluorescent EPC-derived EVs stained by PKH26 dye

Glomerular endothelial cells (GECs)
- Angiogenesis
- Leukocyte adhesion
- Apoptosis
- Oxidative Stress

Podocytes
- Viability
- Apoptosis
- Nephrin expression

Cytokines
- TNF-α
- IL-6
- C5a
EPC-derived EVs

TNF-α
IL-6
C5a

Glomerular endothelial cells (GECs)

Monolayer integrity and permeability

Podocytes

Glomerular endothelial cells (GECs)
Aims of the study

1) Description of the internalization mechanisms in Glomerular Endothelial Cells (GECs) and Podocytes
2) To evaluate the pro-angiogenic properties of EPC-derived EVs on GECs
3) To evaluate the protective effects of EPC-derived EVs in a inflammation model with TNF-α and other cytokines (CK) in GECs and Podocytes
4) Analysis of the effect of EPC-derived EVs in a co-culture model of GECs and Podocytes
Internalization of EPC-derived EVs in Glomerular Endothelial Cells (GECs) and Podocytes

Glomerular Endothelial Cells

Podocytes

**Figure:**

- **Positive control**
  - L-selectin
  - α4-integrin
  - α6-integrin
  - β1-integrin
  - αvβ3-integrin

- **Bar graph:**
  - Glomerular endothelial cells
  - Podocytes

- **Comparative analysis:**
  - 1 μg/mL PKH26 EV:
    - Glomerular endothelial cells: 19.47%
    - Podocytes: 9.52%
  - 10 μg/mL PKH26 EV:
    - Glomerular endothelial cells: 64.66%
    - Podocytes: 23.01%
  - 25 μg/mL PKH26 EV:
    - Glomerular endothelial cells: 84.15%
    - Podocytes: 57.00%
EPC-derived EV effect on Angiogenesis of GECs

**Angiogenesis on Matrigel**

A

- FBS
- Vehicle
- EV
- EV RNase

**Count of Angiogenesis on Matrigel**

B

- Nr of capillary-like structures/field

- FBS
- Vehicle
- EV
- EV RNase

**Proliferation assay (BrdU)**

C

- Average O.D.

- FBS
- Vehicle
- EV
- EV RNase

**Migration assay**

D

- Average speed (mm/hr)

- 0 3h 6h 9h 12h

*$^{x}$ $^{x}$ $^{x}$ $^{x}$ $^{x}$
EPC-derived EVs increase expression of proteins involved in GEC angiogenesis
EPC-derived EVs modulate gene expression of GECs

- Angiogenesis
- Anti-angiogenetic genes
- Growth factors for podocytes (cross-talk)
- Cell Migration
- Glomerular basement membrane
EPC-derived EVs protect GECs in a inflammatory model with TNF-\(\alpha\) and other cytokines (CK)

**Cytotoxicity assay (XTT)**

- **A**
  - Vehicle
  - CK
  - CK + EV
  - CK + EV RNase

**Apoptosis assay (TUNEL)**

- **B**
  - Vehicle
  - CK
  - CK + EV
  - CK + EV RNase

**Reactive Oxygen Species expression**

- **C**
  - Vehicle
  - CK
  - CK + EV
  - CK + EV RNase

**D**

- Vehicle
- CK
- CK + EV
- CK + EV RNase
EPC-derived EVs inhibit Leukocyte adhesion on GECs in a inflammatory model with TNF-\(\alpha\) and other cytokines (CK)
EPC-derived EVs protect Podocytes in an inflammatory model with TNF-α and other cytokines (CK)

**Cytotoxicity assay (XTT)**

- Vehicle
- CK
- CK + EV
- CK + EV RNase

**Apoptosis assay (TUNEL)**

- Vehicle
- CK
- CK + EV
- CK + EV RNase

**Nephrin expression**

IF images showing nephrin expression levels:
- Vehicle: 67.02%
- CK: 31.91%
- CK + EV: 64.29%
- CK + EV RNase: 34.99%
Biological effect of EPC-derived EVs on a co-culture model of GECs and Podocytes

Cytotoxicity assay (XTT)

Trans-Epithelial Electrical Resistance

Permeability to Albumin
Conclusions

1) EPC-derived EVs internalize in Glomerular Endothelial Cells (GECs) and Podocytes effectively through mechanisms mediated by various integrins (L-selectin)

2) EPC-derived EVs trigger an angiogenic program in GECs that regulates proliferation, migration, and remodeling of the Glomerular Basement Membrane

3) In an experimental model of inflammatory injury from TNF-α and other cytokines *in vitro*, EPC-derived EVs protect GECs and Podocytes

4) EPC-derived EVs stimulate GECs to release growth factors able to maintain the vitality and functionality of the Podocytes in inflammatory conditions

5) The role of mRNAs/microRNAs is confirmed by experiments using RNase-treated EVs.

6) EPC-derived EVs could be exploited as potential therapeutic approach in glomerular injury without the risks associated with whole stem cell transplantation (maldifferentiation and tumorigenesis).
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