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Microvesicles Derived From Endothelial Progenitor Cells Protect From Antibody- And Complement-Mediated Endothelial Injury Through Transfer Of Specific mRNAs and microRNAs

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BACKGROUND

Antibody-mediated rejection (ABMR) is one the main causes of acute injury in kidney transplantation with consequent graft loss. ABMR is due to complement-mediated endothelial activation and injury following the binding of anti-HLA antibodies, including donor specific antibodies (DSA).

Endothelial progenitor cells (EPCs) are bone marrow-derived stem cells known to promote angiogenesis and tissue repair by paracrine mechanisms including the release of growth factors and microvesicles (MVs), biologically active small particles able to induce epigenetic reprogramming of target cells through mRNA and microRNA (miRNA) transfer.

Stem cell-mediated inhibition of complement-mediated injury may lead to new therapeutic options to limit ABMR. Indeed, different complement inhibitors such as Factor H, CD55 (DAF) and CD59 have been explored as potential therapeutic tools to limit ABMR.

AIMS OF STUDY

<u>The aim of this study were:</u>

- to isolate and characterize MVs released from EPCs;
- to evaluate *in vitro* the protective effect of MVs on antibody- and complement-mediated endothelial injury in 2 different models:

a) human glomerular-derived endothelial cells (HGECs) in presence of anti-HLA antibodies including DSA;

b) porcine aortic endothelial cells (PAECs) in presence of anti-Gal antibodies.

METHODS

EPCs were isolated from peripheral blood of healthy volunteers. MVs were collected from supernatants of cultured EPCs by ultracentrifugation (100000g) for 1 hr at 4°C and characterized for protein and RNA content.

The biological effects of MVs were evaluated on HGECs and on PAECs cultured in presence of plasma drawn from DSA-positive kidney transplanted patients or of anti-Gal antibodies, respectively (pro-inflammatory molecules expression, adhesion assay, apoptosis assay, angiogenesis on Matrigel and permeability assay).

In selected experiments, in order to evaluate the effect of mRNA/microRNAs shuttled by MVs, we pre-treated MVs with RNase (1U/ml) or we used MVs obtained from supernatants of EPCs engineered to knock-down Dicer, the intracellular enzyme essential for miRNA synthesis.

In selected experiments, MVs were also labeled with the red fluorescent dye PKH26 and evalated by confocal microscopy to study MV internalization in both cell types.

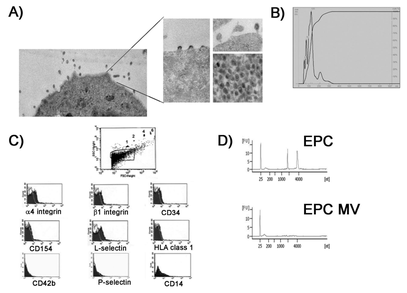

Figure 1: A) Transmission electron microscope analysis of MVs showing a spheroid shape, B) Nanosight analysis of EPC MVs, C) FACS analysis of EPC MV surface molecules D) Bionalyzer profile of RNA subtypes present in EPC and EPC MVs showing an enrichment for small RNA in MVs.

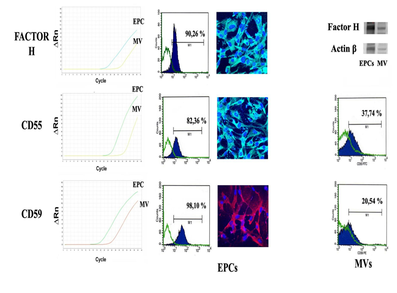

Figure 2: qRT-PCR, FACS , immunofluorescence and Western blot analysis of Factor H, CD55 and CD59 expression in EPC and EPC MVs.

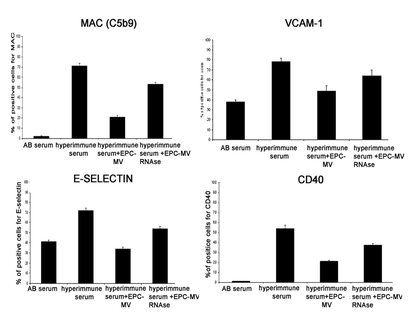

Figure 3: Representative histograms of FACS quantification of MAC, VCAM-1, E-selectin, CD40 protein expression in HGECs stimulated with AB sera, hyperimmune sera in presence or absence of EPC MVs pre-treated or not with RNAse.

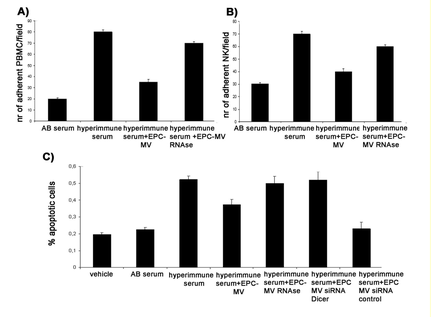

Figure 4: In vitro adhesion assay of PBMCs /A) and NKs (B) on HGECs stimulated with AB sera, hyperimmune sera in presence or absence of EPC MVs pre-treated or not with RNAse. C) TUNEL assay performed on HGECs stimulated with vehicle alone, AB sera, hyperimmune sera in presence or absence of EPC MVs and MVs derived from EPCs transduced with a control siRNA or siRNA Dicer.

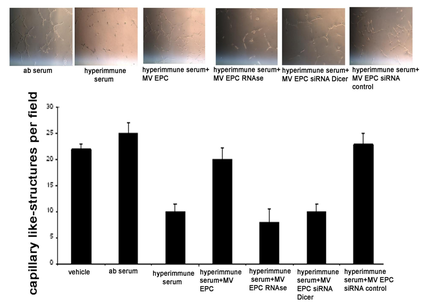

Figure 5: Representative micrographs and count of capillary like-structures formed by HGECs seeded on Matrigel-coated plates and stimulated with vehicle alone, AB sera, hyperimmune sera in presence or absence of EPC MVs pre-treated or not with RNAse or with MVs derived from EPCs transduced with a control siRNA or siRNA Dicer.

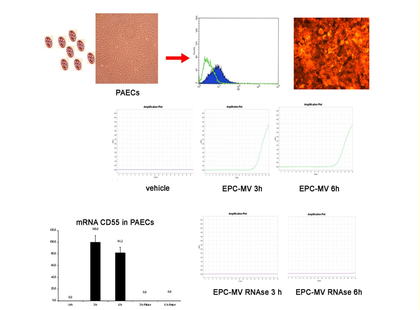

Figure 6: Upper panels: FACS analysis and confocal microscopy of red labelled EPC MVs internalized in P Lower panels: qRT-PCR and relative quantification of CD55 mRNA shuttled by EPC MVs in PAECs.

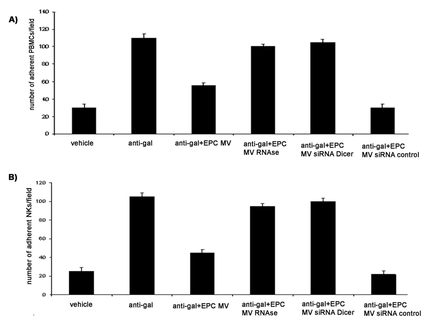

Figure 7: In vitro adhesion assay of PBMCs (A) and NKs (B) on PAECs stimulated with vehicle alone or anti-gal antibodies in presence/absence of MVs pre-treated or not with RNAse.

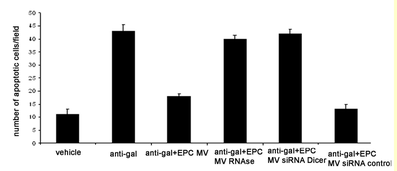

Figure 8: TUNEL assay performed on PAECs stimulated with vehicle alone or anti-gal antibodies in presence/absence of MVs pre-treated or not with RNAse.

RESULTS

MVs were spheroid-shaped, sized 60-130 nm and were released from EPC by a membrane-sorting process (Fig. 1A-B). MVs expressed surface adhesion molecules of the integrin and selectin families (Fig. 1C) and carried different types of mRNAs and miRNAs, small non coding RNAs able to modulate gene transduction (Fig. 1D). We identified within MVs 146 miRNAs involved in cell proliferation, resistance to apoptosis and angiogenesis such as miR-126 and miR-296 (not shown). Moreover, we identified within both EPCs and EPC-derived MVs mRNAs coding for the complement inhibitors Factor H, CD55 and CD59. FACS and western blot analysis confirmed the presence of all complement inhibitors at protein level in both EPCs and MVs (Fig. 2).

After internalization in HGECs treated with DSA-positive plasma, MVs reduced the expression of the terminal component of the complement cascade MAC (C5b9), VCAM-1, E-selectin and CD40 (Fig. 3). In addition, MVs decreased lymphocyte/NK cell adhesion (Fig. 4A-B) and apoptosis (Fig. 4C) and promoted angiogenesis on Matrigel-coated plates (Fig. 5) of HGECs incubated with DSA-positive plasma. The protective effects of MVs were not observed after their treatment with RNase or using MVs released from EPCs engineered to knock-down Dicer, the intracellular enzyme essential for miRNA synthesis (Fig. 4 and Fig. 5).

After internalization in PAECs, MVs horizontally transferred mRNAs coding for human CD55, CD59 and Factor H into porcine cells, thus overcoming the cross-species barrier (Fig. 6). As observed in HGECs, MVs also reduced in PAECs PBMC (Fig. 7A) and NK (Fig. 7B) adhesion and apoptosis (Fig. 8). These results were not observed when MVs were treated with RNase or using MVs derived from EPCs subjected to knock-down Dicer by specific siRNA (Fig. 7 and Fig. 8).

CONCLUSIONS

EPC-derived MVs protected from antibody- and complement-mediated endothelial injury by delivering their RNA content. The mRNA/microRNA cargo of MVs was shown to contribute to the reprogramming of injured endothelial cells toward a regenerative program associated with triggering of angiogenesis and inhibition of complement-mediated injury.

Indeed, the complement inhibitors (CD55, CD59, Factor H) shuttled by MVs may prevent the generation of the terminal complex MAC (C5b-9), thus limiting endothelial cell injury.