Mesenchymal Stem Cell-Derived Microvesicles Protect Against Acute Tubular Injury

Stefania Bruno,* Cristina Grange,* Maria Chiara Deregibus,* Raffaele A. Calogero,† Silvia Saviozzi,† Federica Collino,* Laura Morando,* Alessandro Busca,‡ Michele Falda,‡ Benedetta Bussolati,* Ciro Tetta,§ and Giovanni Camussi*

*Department of Internal Medicine, Research Center for Experimental Medicine and Center for Molecular Biotechnology, and †Department of Clinical and Biological Sciences, University of Torino, Torino, Italy; ‡Bone Marrow Transplant Unit, Haematological Division, San Giovanni Battista Hospital, Torino; §Fresenius Medical Care, Bad Homburg, Germany

ABSTRACT

Administration of mesenchymal stem cells (MSCs) improves the recovery from acute kidney injury (AKI). The mechanism may involve paracrine factors promoting proliferation of surviving intrinsic epithelial cells, but these factors remain unknown. In the current study, we found that microvesicles derived from human bone marrow MSCs stimulated proliferation in vitro and conferred resistance of tubular epithelial cells to apoptosis. The biologic action of microvesicles required their CD44- and β3-integrin-dependent incorporation into tubular cells. In vivo, microvesicles accelerated the morphologic and functional recovery of glycerol-induced AKI in SCID mice by inducing proliferation of tubular cells. The effect of microvesicles on the recovery of AKI was similar to the effect of human MSCs. RNase abolished the aforementioned effects of microvesicles in vitro and in vivo, suggesting RNA-dependent biologic effects. Microarray analysis and quantitative real time PCR of microvesicle-RNA extracts indicate that microvesicles shuttle a specific subset of cellular mRNA, such as mRNAs associated with the mesenchymal phenotype and with control of transcription, proliferation, and immunoregulation. These results suggest that microvesicles derived from MSCs may activate a proliferative program in surviving tubular cells after injury via a horizontal transfer of mRNA.


Several studies demonstrate that the administration of exogenous mesenchymal stem cells (MSCs) contributes to the recovery of tissue injury in several organs such as heart, liver, brain, and pancreas.1 Recently, the administration of in vitro expanded MSCs was shown to improve acute kidney injury (AKI).2–8 Indeed, the infusion of MSCs protected and accelerated the recovery from AKI induced by cis-platinum,3,4,9 glycerol,5,6 and ischemia-reperfusion injury.7,8 The mechanisms involved remain controversial. Some experiments based on bone marrow transplantation indicated that bone marrow-derived stem cells may contribute to repopulate the injured nephrons.10,11 MSCs were shown to localize within the injured kidneys when injected in mice with AKI.3,5,6,12 However, several reports indicate only a transient recruitment of MSCs in the renal vasculature without a direct incorporation within the regenerating tubules.7,8,13 It was suggested that the transient presence of MSCs within the injured kidney may provide a paracrine support to the repair, which is mainly sustained by intrinsic epithelial cells surviving injury.2,14 Recently Bi et al.9

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Correspondence: Dr. G. Camussi, Dipartimento di Medicina Interna, Ospedale Maggiore S. Giovanni Battista, Corso Dogliotti 14, 10126, Torino, Italy. Phone: +39-011-6336708; Fax: +39-011-6631184; E-mail: giovanni.camussi@unito.it

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showed that MSCs may protect the kidney from toxic injury by producing factors that limit apoptosis and enhance proliferation of the endogenous tubular cells, suggesting that the tubular engraftment of MSCs is not necessary for their beneficial effect.

Besides soluble factors, cell-derived microvesicles (MVs) were described as a new mechanism of cell-to-cell communication.15 MVs are released by various cell types15,16 including stem cells and progenitors.17,18 MVs may interact with target cells by surface-expressed ligands, transfer surface receptors, deliver proteins, mRNA, and bioactive lipids.15 Ratajczak et al.17 showed that MVs derived from embryonic stem cells may reprogram hematopoietic progenitors by an mRNA-dependent mechanism. Recently, we demonstrated that MVs derived from human endothelial progenitor cells (EPCs) are able to trigger angiogenesis in human vascular endothelial cells by a horizontal transfer of mRNA.18

In the present study, MVs obtained from human MSCs were characterized for the expression of surface molecules and for their mRNA content. Moreover, we evaluated whether administration of MSC-derived MVs in SCID mice with glycerol-induced AKI may favor functional and morphologic recovery. The effect of MVs was compared with that of MSC administration.

RESULTS
Characterization of MSC-Derived MVs
By cytofluorimetric analyses, MVs were detected mainly below the forward scatter signal corresponding to 1-μm beads (Figure 1A). When determined by Zetasizer, the size of MVs ranged from 80 nm to 1 μm, with a mean value of 135 nm. Transmission and scanning electron microscopy performed on purified MVs showed their spheroid morphology and confirmed their size (Figure 2A and B). When electron microscopy was performed on MSCs cultured overnight in serum-free condition, structures resembling MVs were found within larger vesicles in the cytoplasm or dismissed from the cell surface (Figure 2C, D, and E). Cytofluorimetric analyses showed the presence of several adhesion molecules known to be expressed on MSC plasma membrane such as CD44, CD29, α4- and α5 integrins, and CD73, but not α6-integrin (Figure 1A). In addition, MVs did not express HLA-class I at variance with the cells of origin.

Gene Array Analyses of MSC-Derived MVs
MVs contained mRNA that was submitted to microarray analysis, not to define the amount of mRNA, but only to define which transcripts were present.18,19 A total of 239 transcripts were found with this procedure; 132 were associated to Entrez Gene identifiers20 by IPA 6.0 analysis (additional information: Table 1). This observation indicated that MVs shuttled a specific subset rather than a random sample of cellular mRNA. Several mRNA characteristic of mesenchymal cell lineages, such as neural, osteogenic, epithelial, and hematopoietic, were present (Table 1). Moreover, MVs contained mRNA related to several cell functions involved in the control of transcription, cell proliferation, and immune regulation (Table 2). Quantita-
hibited their incorporation in TECs, confirming the relevance of removal of surface molecules by trypsin treatment of MVs in-CD44 and CD29 is critical for their internalization. Moreover, prevent MV internalization, suggesting that expression of antibodies inhibited MV incorporation in TECs, whereas anti- soluble hyaluronic acid and anti-CD44 and -CD29 blocking copy and FACS analysis (Figure 3 A,B). MV treatment with tubular epithelial cells (TECs) as shown by confocal microscopy (white line = 100 nm). Images were obtained by secondary electron at a working distance of 15 to 25 mm and an accelerating voltage of 20 and 30 kV. Digital acquisition and analysis were performed using the Jeol T300 system. (B–E) Representative micrographs of transmission electron microscopy obtained on purified MVs (B) and on the MSC monolayer cultured over night in the medium used for collection of MVs (see Concise Methods). C shows the release of MVs from the surface of a MSC; D shows the presence of MVs within larger vesicles in the cell cytoplasm. E shows the extrusion of a MV from the surface of a MSC. Ultrathin sections, stained with led citrate were viewed by JEOL Jem 1010 electron microscope (black line = 500 nm).

tive real time PCR confirmed the presence in MVs of genes randomly chosen from those detected by Microarray (POLR2E, SENP2/SUMO1, RBL1, CXCR7, LTA4H).

Incorporation of MSC-Derived MVs in Tubular Cells

MV labeled with PKH26 dye were incorporated by cultured tubular epithelial cells (TECs) as shown by confocal microscopy and FACS analysis (Figure 3 A,B). MV treatment with soluble hyaluronic acid and anti-CD44 and -CD29 blocking antibodies inhibited MV incorporation in TECs, whereas anti-α4-integrin (Figure 3) and α5-integrin (not shown) did not prevent MV internalization, suggesting that expression of CD44 and CD29 is critical for their internalization. Moreover, removal of surface molecules by trypsin treatment of MVs inhibited their incorporation in TECs, confirming the relevance of surface molecules in MV internalization (Figure 3 A,B).

In Vitro Proliferative and Antiapoptotic Effects of MSC-Derived MVs

Incubation of TECs with different doses of MVs promoted cell proliferation compared with control cells incubated with vehicle alone (Figure 4A) and induced synthesis of hepatocyte growth factor (HGF) and macrophage-stimulating protein (MSP) (Figure 4B). In addition, incubation of TECs with MVs significantly inhibited apoptosis induced by serum deprivation (Figure 4C), vincristine, and cis-platinum (Figure 4D). MV treatment with soluble hyaluronic acid or trypsin, which inhibited MV incorporation, also inhibited the proliferative and antiapoptotic effects on TECs (Figure 4 A and B), suggesting that MV incorporation was required for their activity. However, when MVs were incubated with RNase that induced a complete degradation of the RNA shuttled by MVs, the proliferative and the anti-apoptotic effects elicited by MVs were significantly reduced (Figure 4 A, C, and D). Figure 1B shows by FACS analyses that size and expression of surface adhesion molecules did not change in RNase-treated MVs. Moreover, internalization of RNase-treated MVs in TECs did not differ form that of untreated MVs (Figure 3B). DNase treatment was ineffective (not shown). RNase treatment of MVs did not interfere per se with TEC proliferation induced by the EGF (Figure 4A). These results suggest that the MV biologic effects were mediated by the transfer of mRNA following MV internalization as described previously for EPC-derived MVs. MV derived from human fibroblasts did not stimulate TEC proliferation nor inhibited apoptosis (data not shown).

In Vitro Evidence of De Novo Human Protein Expression in Murine TECs by MV-Mediated Horizontal Transfer of mRNA

We used as reporter genes POLR2E and SUMO-1, which were present in MVs derived from human MSCs. Human POLR2E and SUMO-1 were detected by real time PCR (RT-PCR) after 1 and 3 h of MV incubation with TECs (Figure 5A). The primers used did not recognize murine mRNA, as seen by negative RT-PCR in RNA extracted from control murine TECs. De novo cytoplasmic expression of human POLR2E protein and cytoplasmic and nuclear expression of SUMO-1 protein were detected in murine TECs after 24 h incubation with MVs (Figure 5 B). Nuclear localization of both proteins was observed after 48 h (Figure 5B).

MSC-Derived MVs Protect Against Glycerol-Induced AKI

We compared the effect of human MSCs and MSC-derived MVs injected intravenously in glycerol-induced AKI in SCID mice (Figure 6). Three days after glycerol injection, we observed a significant rise in blood urea nitrogen (BUN) and creatinine (Figure 7A) associated with a marked tubular epithelial injury, whereas control mice injected with saline alone displayed no histologic alterations (not shown). At day 3, MSCs or MSC-derived MVs were injected intravenously at doses of 75,000 cells (an amount of cells releasing approximately 15 μg MVs overnight) or 15 μg MV proteins, respectively. Mice were sacrificed at 4, 5, 8, and 15 d after induction of AKI (Figure 6). The lesions observed in mice with AKI at days 4, 5, and 8 included tubular hyaline casts, vacuolization, and widespread necrosis of proximal and distal tubular epithelium (Figure 7B). Proximal tubules showed cytoplasmic vacuolization, swelling and disorganization of mitochondria, loss of brush border, and denudation of basal membrane (Figure 8 B and C). When mice were treated with MSCs or MVs, the tubular lesions were less severe at day 5 and almost absent at day 8 compared to those of mice treated with vehicle alone...
(Figure 7B). The quantitative evaluation of casts and tubular necrosis at day 5 showed a significant reduction in MV- and MSC-treated mice in parallel with the reduction of BUN (Table 3). The recovery was complete at day 15 (not shown). By electron microscopy, tubular cells in MV-treated AKI mice showed a marked increase of mitochondria at day 5 that was decreased at day 8. In addition, the brush border was already restored at day 5, and the ultrastructure of tubules was almost

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indistinguishable from that of control mice without AKI (Figure 8). In addition, MSC- and MV- treated mice showed a significant reduction of both BUN and creatinine (Figure 7A). There was no significant difference between the treatment with MSCs and that of MVs (Figure 7, Table 3). In addition, MVs treated with sHA or trypsin did not significantly improve functional and morphologic injury compared with untreated AKI (Table 3). The specificity of MSC-derived MVs was also indicated by the absence of protective effects of MVs derived from human fibroblasts (Table 3).

As shown in Figure 9, MV treatment of mice with AKI significantly enhanced tubular cell proliferation compared to treatment with vehicle alone, as detected by PCNA- and 5-bromo-2′-deoxy-uridine (BrdU)-positive cells. The enhanced proliferation was detected 24 h (day 4 of AKI) and 48 h (day 5 of AKI) after MV administration and found to decrease thereafter. MSC treatment induced a comparable enhancement of cell proliferation (Figure 9). The majority of PCNA- and BrdU-positive cells, seen in MV- and in MSC- injected animals, were located in distal and proximal tubules. On the contrary, in untreated mice with AKI, proliferation was delayed as the number of PCNA- and BrdU positive cells increased only at day 8 when no further proliferation was detected in mice with AKI treated with MVs or MSCs. sHA-treated MVs as well as fibroblast-derived MVs did not significantly enhance proliferation (Figure 9 A and B).

Evaluation of MV Localization After In Vivo Injection

When labeled MVs were injected, a significant accumulation in the kidney was observed at 6 h only in AKI mice (Figure 10A). After 1 h, MVs were detectable within the endothelial cells of large vessels and within the lumen of some injured tubules (Figure 10B). After 3 h, several tubular cells contained labeled MVs (Figure 10 C and D). The amount of tubular cells containing MVs was markedly enhanced at 6 h (Figure 10 E and F). When injected in normal control mice, the accumulation was significantly lower than in AKI, and MVs were not detected in tubular cells (Figure 10 A and H). In plasma, the concentration of MVs significantly decreased in AKI but not in normal controls (Figure 10A). Trypsin-treated MVs were not detected in the kidney of AKI mice and remained constant in plasma at any time (Figure 10A). MVs were minimally detected in the lung (Figure 10A) and as shown by confocal microscopy were mainly located in the endothelial cells of large vessels and not in the alveolar capillaries (Figure 10 J and K). In contrast, liver accumulation of MVs was detected both in normal controls and in AKI mice (Figure 10 A and L). Trypsin-treated MVs did not localize in any of the examined organs (Figure 10A).

RNA Shuttled by MV Mediates MV-Induced Recovery of AKI

As shown in Figure 7 and in Table 3, RNase treatment significantly reduced the recovery of BUN, creatinine, and tubular lesions that did not differ from those of untreated mice with AKI. Moreover at
day 8, in mice injected with MVs treated with RNase, the lesions persisted, with an increase in tubular casts and aspect of epithelial atrophy (Figure 7B). Tubular cell proliferation detected as BrdU- or PCNA-positive cells in mice injected with RNase-treated MVs did not differ from that of untreated mice with AKI (Figure 9).

**DISCUSSION**

In the present study, we demonstrated that MVs derived from human MSCs are able to stimulate *in vitro* proliferation and apoptosis resistance of TECs and to accelerate *in vivo* the recovery of glycerol-induced AKI in SCID mice. The effect of
administration of MVs was comparable to that of human bone marrow-derived MSCs. The RNase treatment of MVs abrogated both the in vitro and in vivo effects of MVs, suggesting that the mRNA shuttled by MVs is the final effector of their biologic effects.

Several studies demonstrated that the administration of bone marrow-derived MSCs may protect and reverse AKI in different experimental models.2–9 The low number of MSCs detectable within tubules after injury together with the increase in proliferating tubular cells throughout the kidney implies a trophic effect of MSCs on resident tubular cells that have survived injury, rather than a direct repopulation.7,8,14 It was suggested that MSCs may exert their effects by a paracrine action on resident cells.7,13,21,22 Recently, soluble factors were implicated in the MSC protective effect.23 Bi et al.9 showed that MSCs may protect the kidney from toxic injury by producing factors that limit apoptosis and enhance proliferation of the endogenous tubular cells, indicating that tubular engraftment of the MSCs is not necessary for their beneficial effect.

We demonstrated that intravenous administration of MVs derived from human MSCs has the same efficacy of MSCs on the functional and morphologic recovery of glycerol induced AKI in SCID mice. MVs are small vesicles released by cells that express the characteristic antigens of the cell from which they originate and carry membrane and cytoplasmic constituents.15,16 MVs may interact with target cells through specific receptor–ligand interactions and transfer receptors, proteins, and bioactive lipids.16 Moreover, several studies showed that MVs may shuttle selected patterns of mRNA and proposed this as a new mechanism of genetic exchange between cells.17–19,24

In the present study, we demonstrate that MVs derived from MSCs may induce in vitro proliferation and apoptosis resistance in TECs. It was previously reported that MVs may express surface molecules characteristic of originating cells.16–18,25–27 We found that MSC-derived MVs expressed several adhesion molecules of MSCs such as CD44, CD29 (β1-integrin), α4- and α5 integrins, and CD73, but not α6 integrin. Some of these molecules, namely CD44 and CD29, were found to be instrumental in MV internalization into TECs, as treatment with sHA or with anti-CD44 and anti-CD29 blocking antibodies prevented MV incorporation. Moreover, we found that the internalization of MVs was a requirement, but not the mechanism responsible, for their biologic activity. Indeed, RNase treatment almost completely abrogated the MV-induced in vitro proliferation and resistance to apoptosis of TECs.

The in vivo administration of MVs was found to accelerate the functional and morphologic recovery of AKI. These biologic
effects were specific for MSC-derived MVs as MVs obtained from fibroblasts were ineffective, and involved MV adhesion molecules as trypsin treatment abrogated MV accumulation in the kidney. MVs accumulated within the lumen of injured tubules, thus allowing an uptake from the apical part of tubular cells that survived injury. In addition, an uptake by endothelial cells was observed, suggesting that MVs may also reach the basolateral side of tubular cells via peritubular capillaries. Apart from inducing TEC proliferation, MVs may act by a mechanism of renal protection that limits the extent of injury. However, in the present experimental setting, MVs were administered at the peak of functional and morphologic alterations.5,6

As seen in vitro, pretreatment of MVs with RNase abrogated the protective effect of MVs. These results suggest that the transfer of small amounts of exogenous mRNA may stimulate tubular cell regeneration. Indeed, MVs derived from MSCs contained mRNA associated with the mesenchymal differentiative phenotype and with several cell functions involved in the control of transcription, proliferation, and cell immune regulation. Interestingly, as in EPC-derived MVs, MVs from MSCs carried a gene encoding the polymerase responsible for synthesizing mRNA in eukaryotes.28 In vitro evidence for an effective horizontal transfer of mRNA was obtained by the presence of the human-specific mRNA for POLR2E and SUMO-1 and by their de novo protein expression in MV-treated TECs. In vivo expression of human POLR2E and SUMO-1 proteins in tubular cells was also detected in mice with AKI treated with MVs. MV-mediated transfer of mRNA/proteins derived from stem cells may therefore induce de-differentiation of mature cells, triggering a proliferative program that may contribute to the repair of tissue injury. Moreover, stimulation of TECs with MVs induced synthesis of HGF and MSP, although their mRNA was not included in MVs, suggesting that the activation of cellular pathways that generate a cascade of multiple mediators that may be concurrent with recovery from acute tubular injury.29–31

Figure 7. Effects of intravenous injection of microvesicles (MVs) or mesenchymal stem cells (MSCs) into acute kidney injury (AKI) mice. Mice were given intramuscular injection of 8 ml/kg of 50% glycerol on day 0, followed by intravenous injection of MVs or RNase-treated MVs or MSCs or vehicle as control on day 3. (A and B) Creatinine and blood urea nitrogen values at the beginning of the experiments and on day 3, 5, 8, and 15 after glycerol administration. ANOVA with Dunnet’s multicomparison test: *P < 0.05 MV- or MSC- treated AKI mice versus control AKI mice. (C) Representative micrographs of renal histology at day 5 and 8 after glycerol administration in control AKI mice, in AKI mice injected with 15 μg of MVs or RNase-MV or with 75,000 MSCs. Magnification: ×400.
Injection of 75,000 MSCs, of 15 µg of MSC-derived MVs with or without treatment with sHA or trypsin and 15 mg of fibroblast-derived MVs. Results are expressed as mean ± SD; ANOVA with Dunnet’s multicomparison test. *P < 0.05 treatments vs untreated AKI. AKI; AKI untreated with MVs; AKI; AKI treated with MSC-derived MVs; AKI + RNase; AKI treated with RNase inactivated MSC-derived MVs; AKI + MSC; AKI treated with MSCs; AKI + MV; AKI treated with MSC-derived MVs preincubated with sHA; AKI + F-MV; AKI treated with fibroblast-derived MVs.

Table 3. Effect of MSCs, MSC-derived MVs, and fibroblast-derived MVs on renal morphology and function at day 5 after AKI induction
Isolation of MVs

MVs were obtained from supernatants of MSCs and of fibroblasts cultured in RPMI deprived of FCS and supplemented with 0.5% of BSA (Sigma). The viability of cells incubated overnight without serum was > 99% for MSCs and 85% ± 4.3% for fibroblast as detected by trypan blue exclusion. No apoptotic cells were detected by TUNEL assay in MSCs, and 3.2% ± 1.3% apoptotic cell were detected for fibroblast. After centrifugation at 2000 g for 20 min to remove debris, cell-free supernatants were centrifuged at 100,000 g (Beckman Coulter Optima L-90K ultracentrifuge) for 1 h at 4 °C. MV pellets were suspended in medium 199, and the protein content was quantified by the Bradford method according to the manufacturer’s instruction (Charles River Laboratories, Inc., Wilmington, MA), and MV were stored at −80 °C. The morphologic analyses performed on MV suspension after staining with propidium iodide did not show the presence of apoptotic bodies.

In selected experiments, MVs from MSCs were treated with 1 U/ml RNase (Ambion Inc., Austin, TX) for 1 h at 37 °C, the reaction was stopped by addition of 10 U/ml RNase inhibitor (Ambion Inc.) and MVs were washed by ultracentrifugation. The effectiveness of RNase treatment was evaluated after RNA extraction using TRIZOL reagent (Invitrogen, Carlsbad, CA) by spectrophotometer analysis of total extracted RNA (untreated: 1.3 ± 0.2 µg RNA/mg protein MV; RNase treated: <0.2 µg RNA/mg protein MV). In addition, RNA extracted from RNase-treated and untreated MVs was labeled by oligo dT driven retrotranscription and analyzed on 0.6% agarose gel to show the complete degradation of RNA by RNase treatment, as described previously. As control, MVs were treated with 1 U/ml DNase (Ambion Inc.) for 1 h at 37 °C.

FACS Analysis of MVs

The size of MVs was determined by cytofluorimetric analyses. The instrument was rinsed with particle-free rinse solution for 15 min to eliminate the background. The beads of different sizes (1, 2, 4, and 6 µm, Molecular Probes, Invitrogen) were used as the size markers, and analysis was performed using a log scale for forward scatter and side scatter parameters. Moreover, the size of MV was evaluated by the Zetasizer Nano (Malvern Instruments, Malvern Worcestershire, UK) instrument, which permits discrimination of microparticles less than 1 µm in diameter. Cytofluorimetric analysis was performed as described previously, using the following FITC- or PE-conjugated antibodies: CD146, CD44 (Dako Cytomation), CD133, ICAM-1, v5-integrin, (Becton Dickinson), CD73, ICAM-1, α4-integrin (Becton Dickinson), αvβ3-inte-
Figure 10. Distribution of microvesicles (MVs) after in vivo injection. (A) Quantification by spectrofluorimetric analyses of the amount of PKH26-labeled MVs, treated or not with trypsin, in different organs of acute kidney injury (AKI) and healthy mice (control+MV) as described in Concise Methods. The amount of MV-PKH26 is expressed in μg/g of dry tissue or μg/μl of plasma. (B–M): Representative confocal micrographs of frozen tissue sections of mice injected with PKH26-labeled MVs (red) and stained with vWF (B and K) or laminin (C–J, L, M) antibodies (green staining). MVs were detectable, after 1 h, within the endothelial cells of a renal vessel stained with anti-vWF antibody and within the lumen of injured tubules (B); after 3 h several tubular cells contained red MVs (C, D); at 6 h (E, F) the number of tubular cells containing red MVs was enhanced. MVs treated with trypsin were not detected in the kidney (G). In a normal control mouse, red MVs were not detected in tubular cells (H). In the lung, red MVs were not detected in the alveolar capillaries (J), whereas they were located in the endothelial cells of a large vessel (K). Liver accumulation of red MVs was detected both in normal controls (not shown) and in AKI mice (L). No signal was observed in specimens of AKI not injected with labeled MVs (I = kidney; M = liver). Nuclei were counterstained with Hoechst dye. B, C, E, G-M, original magnifications: ×400; D and F, original magnifications: ×630. G, glomeruli. Per each group (control+MV, AKI+MV, AKI+trypsin treated MV) and each time points three animals were studied with similar results.

In vitro Isolation and Expansion of TECs

Kidneys were obtained from healthy female C57 mice. Kidneys were finely minced with scissors and then were forced through a 40-μm pore filter (Becton Dickinson, San Jose, CA); the glomerular part and aggregated remained on the surface of filter while tubular cells were collected. After 2 washings in PBS (Lonza), the cell suspension was plated in a T25 flask (Becton Dickinson) in the presence of DMEM (Sigma) and 10% FCS. We changed media after 5 d to eliminate non-living cells. TECs were characterized for their positive staining of cytokeratin, actin, alkaline phosphatase, aminopeptidase A, and megalin, and for negative staining for von Willebrand factor, CD45, nephrin, and desmin.

Incorporation of MVs in TECs

To study the capacity of MVs to incorporate into TECs, we incubated 50 μg/ml of MVs, labeled with PKH-26 dye, for 30 min at 37 °C. We studied the incorporation by FACS analyses and confocal microscopy.

To investigate the role of adhesion molecules expressed by MV surface in the incorporation in target cells, we pre-incubated MVs (15 min at 4 °C) with blocking antibodies (1 μg/ml) against the identified adhesion molecules anti-α4-integrin (BIO Legend), α5-integrin (BioLegend), CD29 (β1-integrin) (Becton Dickinson), CD44 (Becton Dickinson) or with sHA (100 μg/ml from Rooster comb; Sigma) before the incubation with the cells.

Immunofluorescence for Human Protein Expression in TECs

Indirect immunofluorescence was performed on TECs cultured on chamber slides (Nalgen Nunc International, Rochester, NY) and stimulated for 1 and 2 d in the presence of 30 μg of different preparations of MVs. The cells were fixed in 4% paraformaldehyde containing 2% sucrose and permeabilized with HEPES-Triton X100 buffer (Sigma). The following antibodies were used: rabbit anti-human POLR2E (Abcam, Cambridge Science Park, UK) and rabbit anti-human Sumo-1 (AbCam). Omission of the primary antibodies and substitution with nonimmune rabbit IgG were used as controls. Alexa
cor, Gaithersburg, MD) as described previously.38 As apoptotic stim-
nm. Apoptosis was evaluated using the TUNEL assay (ApopTag On-
soluble chromogenic substrate (Roche Applied Science, Mannheim,
an anti-BrdU peroxidase-conjugated antibody and visualized with a
digest the DNA. BrdU incorporated into the DNA was detected using
then fixed with 0.5 M ethanol/HCl and incubated with nuclease to
expression of BrdU into the cellular DNA after 48 h of culture. Cells were
TECs were seeded at 4000 cells/well into 96-well plates in DMEM
Cell Proliferation, Apoptosis Assays, and Release of
Hoechst 33258 dye (Sigma) was added for nuclear staining. Eight animals per groups were
Nuclei were counterstained with Hoechst dye. Original magnifi-
fluor 488 anti-rabbit (Molecular Probes) was used as secondary an-
to measure the SUMO-1 or POLR2E staining and the
Figure 11. Detection of human mRNA and human protein ex-
pression in kidneys of mice treated with human mesenchymal
stem cell (MSC)-derived microvesicles (MVs). (A) Representative
RT-PCR of acute kidney injury (AKI) mice untreated (AKI) or
treated with 15 μg of MSC-derived MVs (AKI+MV) and sacrificed
1 and 3 h after MV injection. Bands of PCR products specific for
human POLR2E of the expected size (90 bp) were detected in a
4% agarose gel electrophoresis in AKI treated with MVs but not in
untreated AKI or in normal murine kidney (Ctrl). As positive con-
trol the extract of human bone marrow-derived MSCs (BM-MSC)
was used. The asterisk indicates the control without cDNA. (B)
Representative confocal micrographs showing the nuclear expres-
sion of human POLR2E and SUMO-1 proteins in kidney sec-
sections of AKI mice treated or not with MVs and sacrificed 48 h later.
Nuclei were counterstained with Hoechst dye. Original magnifi-
cation: ×400. Arrows indicate positive nuclei. The right panels of
each show merge for the SUMO-1 or POLR2E staining and the
nuclear staining with Hoechst. Eight animals per groups were examined with similar results.
Fluor 488 anti-rabbit (Molecular Probes) was used as secondary an-
tibody. Confocal microscopy analysis was performed using a Zeiss
LSM 5 Pascal Model Confocal Microscope (Carl Zeiss International,
Germany). Hoechst 33258 dye (Sigma) was added for nuclear staining.

Cell Proliferation, Apoptosis Assays, and Release of
HGF and MSP
TECs were seeded at 4000 cells/well into 96-well plates in DMEM
(Sigma) deprived of FCS. DNA synthesis was detected as incorpora-
tion of BrdU into the cellular DNA after 48 h of culture. Cells were
then fixed with 0.5 M ethanol/HCl and incubated with nuclease to
digest the DNA. BrdU incorporated into the DNA was detected using
an anti-BrdU peroxidase-conjugated antibody and visualized with a
soluble chromogenic substrate (Roche Applied Science, Mannheim,
Germany). Optical density was measured with an ELISA reader at 405
nm. Apoptosis was evaluated using the TUNEL assay (ApopTag On-
cor, Gaithersburg, MD) as described previously.38 As apoptotic stim-
uli, we used serum deprivation or stimulation with 100 ng/ml of vin-
cristine (Sigma) and 5 μg/ml of cis-platinum (Sigma) in DMEM plus
3% FCS.

To evaluate the production of HGF and MSP, 1 × 10⁶ TECs were
cultured with or without MV (30 μg/ml) and, after 24 and 48 h of
incubation, the supernatants were recovered and MSP-1 and HGF
production measured by ELISA (Raybiotech, Norcross GA) in accor-
dance with manufacturer’s instruction.

Gene Array Analysis
RNA extraction, Samples Labeling and Hybridization
on BeadChips
RNA was extracted from MVs using TRIZOL reagent (Invitrogen)
following the procedure suggested by the manufacturer. Total RNA
was quantified spectrophotometrically (Nanodrop ND-1000, Wil-
mington DE). cRNA was synthesized using three different quantities of
total RNA (0.25 μg, 0.5 μg, and 1 μg). cRNA synthesis and labeling
was done using Illumina RNA Amplification Kit (Ambion) following
the procedure suggested by manufacturer. Sentrix Human-6 Expres-
sion BeadChip hybridization, washing, and staining were also done as
suggested by the manufacturer. Arrays were scanned on Illumina
BeadStation 500 (Illumina, San Diego, CA). Transcripts present in the
MVs were defined as those characterized by a positive linear relation
between the transcript signal detected by the microarray analysis and
the amount of total RNA hybridized.18

Microarray Data Analysis
The analysis was done hybridizing arrays with labeled-cRNA pro-
duced using three different concentrations of total RNA extracted from
two independent preparations of MVs. BeadChip array data
quality control was performed using Illumina BeadStudio software,
version 1.3.1.5. Transcript average intensity signals were calculated
with BeadStudio without background correction. Raw data were an-
alyzed using Bioconductor.39 Average transcript intensities were log₂
transformed and normalized by the Loess method.30

A simple statistical linear model15 was used to identify transcript
signals linearly correlated to the increment of total RNA concentra-
tion used to prepare cRNA. In equation (1), ỹᵢⱼ is the observed expres-
sion level for transcript i in sample j (j = 1, . . . , 6), μ is the average
expression level of transcript I, and βRNA represents the effect of total
RNA concentration on the expression level of transcript i. ɛᵢ represents
random error for transcript i and sample j, and it is assumed to be
independent for each transcript and sample, and normally distributed
with mean 0 and variance ɛ².

\[
ỹᵢⱼ = μ + βRNA + ɛᵢⱼ \tag{1}
\]

Transcripts characterized by a model with P ≤ 0.05, r² ≥ 0.8, and a
positive slope were selected (239).

Transcripts annotation and data mining were performed using IPA 4.0 software (www.ingenuity.com). Microarray data were depos-
as geo accession GSE12243.
Quantitative Real Time PCR
Quantitative real-time PCR was performed as described previously. Quantitative real-time PCR was performed on total RNA extracted from cells used to produce MVs and from an MV preparation different from those used for microarray analysis. Cell cDNA was used to evaluate primers efficiency. The primers used for real time PCR are shown in Table 4. First-strand cDNA was produced from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Briefly, 200 to 400 ng mRNA, 2 μl RT buffer, 0.8 μl dNTP mixture, 2 μl RT random primers, 1 μl MultiScribe reverse transcriptase, and 4.2 μl nuclease-free water were used for each cDNA synthesis. After the reverse transcription, cDNA was stored at −20 °C. Twenty microliters of RT-PCR mix, containing 1X SYBR GREEN PCR Master Mix (Applied Biosystems), 100 nM of each primer, and 0 μl, 1 μl, and 2 μl of MV cDNA, were assembled using a 48-well StepOne Real Time System (Applied Biosystems). Negative cDNA controls (no cDNA) were cycled in parallel with each run.

Reverse Transcriptase PCR (RT-PCR)
Total RNA extracted from TECs or from kidneys of SCID mice was submitted to RT-PCR using the primer for human POLR2E and SUMO-1 reported in Table 4. Bands of the expected size (90 bp) were detected in a 4% agarose gel after electrophoresis. cDNA from a preparation of human bone marrow MSC was used as positive control.

SCID Mice Model of AKI
Studies were approved and conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. As described previously, a model of rhabdomyolysis-induced AKI was performed in male SCID mice (7 to 8 wk old) (Charles River Laboratories), by intramuscular injection with hypertonic glycerol (8 ml/kg body weight of 50% glycerol solution) into the anterior hind limbs (Figure 6). Intramuscular injection of glycerol induces myolysis and hemolysis causing toxic and ischemic tubular injury. On day 3 after glycerol administration, mice received an intravenous injection into the tail vein of 15 g MVs from MSCs treated with sHA, or trypsin or RNase-MVs, or 15 g MVs alone. The following groups were studied: group 1, AKI group; group 2, AKI plus 15 μg MVs injected 3 d after induction of renal injury; group 3, AKI plus 15 μg RNase-treated MVs; group 4, AKI plus 75,000 human MSCs; group 5, AKI plus 15 μg of MVs from fibroblasts; group 6, AKI plus 15 μg MVs from MSCs treated with sHA; group 7, AKI plus 15 μg MVs from MSCs treated with trypsin. The amount of 15 μg MVs was chosen because it corresponds to the amount released overnight by 75,000 MSCs. For in vivo detection of proliferation, mice were administrated an injection of BrdU (100 μg/kg) intraperitoneally for 2 successive days before being killed. In each group, mice were killed at different time intervals (day 3 [n = 10], day 4 [n = 8 per group], day 5 [n = 8 per group], day 8 [n = 8 per group] and day 15 [n = 6 per group] after glycerol administration) and kidneys and samples for BUN and creatinine determination were collected.

Kidney tissues were processed for histology, immunohistochemistry, immunofluorescence and transmission electron microscopy.

Morphologic Studies
For renal histology 5-μm-thick paraffin kidney sections were routinely stained with hematoxylin and eosin (Merck, Darmstadt, Germany). Luminal hyaline casts and cell lose (denudation of tubular basement membrane) were assessed in nonoverlapping fields (up to 28 for each section) using a 40x objective (high power filed, HPF). Number of casts and tubular profiles showing necrosis were recorded in a single-blind fashion.

Transmission electron microscopy was performed on Kar

Table 4. Human primers for real-time quantitative PCR

| POLR2E   | 5'-GCTCTGAAAATCCGCAGCAAG-3' | 5'-TCCTCCAGGCCTGTCGTCGAAG-3' |
| SENP2/SUMO1 | 5'-AAATAAGATCGACACATTGCAAGTG-3' | 5'-TCAAGTTCCAGAGTAAGTACTA-3' |
| RBL1     | 5'-GAGAAGCCTGGCTTATTGACTTTCTT-3' | 5'-TGCAGTTCCAGAGTAGATTAC-3' |
| CXCR7    | 5'-TGGTTTACCAAGAGCTGCCAATCA-3' | 5'-TGGCTTGTGCTGGCTGCT-3' |
| LTA4H    | 5'-CCTATCGCTTTGAGCAAATCA-3' | 5'-GTGTCTCTTCCCAGAAAGTCTGTC-3' |
Plasma BUN and Creatinine

Blood samples for measurement of BUN and plasma creatinine were collected before and 3, 4, 5, 8 and 15 d after glycerol-induced AKI. Creatinine concentrations were determined using a Beckman Creatinine Analyzer II (Beckman Instruments, Inc., Fullerton, CA). Creatinine levels that exceeded 0.3 mg/dl were considered abnormal (normal range in our laboratory: 0.1 to 0.3 mg/dl). BUN was assessed in heparinized blood using a Beckman Synchrotron CX9 automated chemistry analyzer (Beckman).

Spectrofluorimetric Detection of MVs in Different Tissues after In Vivo Injection

To evaluate the amount of MVs in different murine organs, we injected intravenously AKI or healthy mice with 100 µg of PKH-26 labeled MVs treated or not with trypsin. Mice were sacrificed after 15 min, 1, 3, and 6 h and liver, lungs, kidneys, and blood were recovered. The content of PKH-26 in different samples was measured after lipid extraction with chloroform-isopropyl alcohol (1:1 vol/vol) and 0.125% SDS (Sigma) of homogenized tissues (Tissue Ruptor, Qiagen) or plasma. The fluorescence intensity of PKH-26-containing lipid extracts were measured with Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon, Edison NJ). Excitation wavelength was positioned at 550 nm; emission wavelength was set at 567 nm. Calibration values for maximum and minimum were obtained using 20 µg/ml and decreasing concentration until 1 µg/ml of lipid extracts of PKH26-labeled MV. Each lipid sample (from different organs and plasma) was analyzed in PBS (Sigma) containing 0.1% triton X100 (Sigma). As negative control, we measured fluorescence intensity in lipids extracted from untreated data. Were expressed as micrograms per gram of dry tissue or micrograms per microliter plasma.

Statistical Analysis

Statistical analysis was performed by using the t test, ANOVA with Newman-Keuls, or ANOVA with Dunnet’s multicomparison tests, as appropriate. A p value of <0.05 was considered significant.

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DISCLOSURES

M. C. D. and G. C. are named inventors on related patent applications.

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