Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury

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Tögel F, Weiss K, Yang Y, Hu Z, Zhang P, Westenfelder C. Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. Am J Physiol Renal Physiol 292: F1626–F1635, 2007. First published January 9, 2007; doi:10.1152/ajprenal.00339.2006.—Acute kidney injury (AKI) is a major clinical problem in which a critical vascular, pathophysiological component is recognized. We demonstrated previously that mesenchymal stem cells (MSC), unlike fibroblasts, are significantly renoprotective after ischemia-reperfusion injury and concluded that this renoprotection is mediated primarily by paracrine mechanisms. In this study, we investigated whether MSC possess vasculoprotective activity that may contribute, at least in part, to an improved outcome after ischemia-reperfusion AKI. MSC-conditioned medium contains VEGF, HGF, and IGF-1 and augments aortic endothelial cell (EC) growth and survival, a response not observed with fibroblast-conditioned medium. MSC and EC share vasculotropic gene expression profiles, as both form capillary tubes in vitro on Matrigel alone or in cooperation without fusion. MSC undergo differentiation into an endothelial-like cell phenotype in culture and develop into vascular structures in vivo. Infused MSC were readily detected in the kidney early after reflow but were only rarely engrafted at 1 wk post-AKI. MSC attached in the renal microvascular circulation significantly decreased apoptosis of adjacent cells. Infusion of MSC immediately after reflow in severe ischemia-reperfusion AKI did not improve renal blood flow, renovascular resistance, or outer cortical blood flow. These data demonstrate that the unique vasculotropic, paracrine actions elicited by MSC play a significant renoprotective role after AKI, further demonstrating that cell therapy has promise as a novel intervention in AKI.

endothelial cells; angiogenesis; conditioned media; VEGF; renal blood flow; differentiation; apoptosis

STEM CELL-BASED APPROACHES have enormous potential for the development of future therapies (32). In recent years, a large number of publications have demonstrated the therapeutic capacity of different stem cell populations. Bone marrow-derived stem cells are among the most promising candidates for clinical applications because of already existing clinical experience with this cell type and their successful use in both autologous and allogeneic settings. The bone marrow harbors at least two types of stem cells: hematopoietic stem cells (HSC) and multipotential marrow stromal or mesenchymal stem cells (MSC). Furthermore, endothelial cells (EC) can be bone marrow derived (33). MSC have been of high interest for regenerative therapies because they are easy to harvest, can be readily expanded in culture, differentiate into a number of cell types in vitro, including EC (27), and are ideal vehicles for cellular gene transfer (29).

Although dogmas of lineage commitment of somatic stem cells, i.e., their ability to differentiate only into cell types of the same germ layer, have been challenged recently (19), the mechanism of action of stem cell therapy is unclear in most disease conditions. Very-low-level organ engraftment of circulating bone marrow-derived stem cells has been shown (25, 26) but was not corroborated by others (6, 22). The percentage of incorporated stem cells varies widely, but it is usually below 1% in a given organ, and, in addition, its magnitude depends on the studied disease model. Other mechanistic possibilities for the therapeutic effects of stem cells include fusion with resident organ cells (42), immunomodulation (1), and paracrine mechanisms elicited through trophic mediators (10, 12, 30, 37, 38) that result in the inhibition of fibrosis and apoptosis, enhancement of angiogenesis, stimulation of mitosis, and proliferation and differentiation of organ-intrinsic precursor or stem cells.

Acute kidney injury (AKI) is a multifactorial syndrome associated with high clinical mortality. However, disease-specific interventions beyond supportive therapy are currently not available (11, 40). Although there are a number of etiologies for clinical AKI, e.g., drug and radiocontrast agent toxicity and interstitial nephritis, ischemic damage is the most important cause for AKI in hospitalized patients (~50%). Prolonged periods of ischemic injury damage not only tubular cells in the most oxygen-sensitive S3 segment of the nephron but also microvascular EC. The great importance of vascular injury in the pathogenesis of AKI has only recently been fully appreciated, and the concept of an “extension phase” of ischemic AKI has been introduced (21, 35, 36). It is characterized by continued tissue hypoxia following an ischemic event as well as inflammatory and procoagulant responses, all triggered by EC damage. Because EC are located at the interface between blood and tubular cells, they fulfill an important regulatory function for inflammation and coagulation and are therefore crucial in the pathogenesis of AKI. Infusion of EC has been shown to be of therapeutic value in animal models of AKI (5, 9).

We have previously shown that MSC have renoprotective effects and enhance regeneration after AKI in an ischemia-reperfusion model in the rat (18, 30, 38). Because the positive therapeutic effect of MSC was prompt and virtually no MSC-derived tubular cells in the kidney were detected, we hypothesized that trophic factors play an important role in stimulation of renoprotection and renal repair through paracrine actions. Accordingly, the current study was designed to further inves-
tigate this hypothesis with a special emphasis on EC-MSC interactions. We show that MSC possess unique paracrine effects that beneficially target EC functions and thereby importantly contribute to overall renal repair after AKI.

MATERIALS AND METHODS

Animals and cells. All procedures involving animals were approved by the respective Institutional Animal Care and Use Committees of the University of Utah, Veterans Affairs Medical Center (Salt Lake City, UT), and Indiana University (Indianapolis, IN).

In vivo imaging studies were performed at the University of Indiana O’Brien Center, Division of Nephrology (directed by Dr. Bruce Molitoris). For delivery of cells, a PE-50 cannula was inserted into the carotid artery. Cells were labeled with carboxyfluorescein diacetate (CFDA; Molecular Probes, Eugene, OR). In vivo two-photon laser confocal microscopy was performed as described in detail previously (38).

EC were derived from rat aortic ring cultures (20), propagated in DMEM/F12 medium with 10% FCS (Hyclone, Logan, UT), and initially characterized for an endothelial phenotype by demonstrating Dil-acLDL uptake and absence of α-actin expression to exclude smooth muscle cell contamination.

MSC were generated by flushing the femurs of anesthetized rats with PBS and by centrifugation. Cell pellets were plated in culture flasks with DMEM/Ham’s F12 and 10% FCS. Nonadherent cells were removed after 24 h, and cells were expanded over several passages. An MSC phenotype was confirmed by the typical spindle-shaped appearance, by differentiation into osteocytes and adipocytes with specific differentiation media (34), as well as by the absence of CD45 expression on FACS analysis.

Endothelial phenotype induction. To assess the endothelial differentiation potential of MSC by a tube-formation assay, cells were seeded onto Matrigel-covered slide covers (BD Biosciences, San Jose, CA) and initially incubated for 4 h. After further incubation overnight, cells were released from the gel by dispase digestion (BD Biosciences), placed in flask culture, and processed for immunohistochemistry.

Assays to investigate cooperation or fusion of MSC with EC in tube formation were performed by seeding a mixture of equal numbers of green-fluorescent CFDA-stained MSC (Invitrogen) and red-fluorescent PKH-26-stained (Sigma) EC on Matrigel. Nuclei were counterstained with Hoechst 33342, and cooperation/fusion was assessed by fluorescence microscopy.

In vivo vasogenic potential of MSC was tested by Matrigel plug assays. Accordingly, MSC from human placental alkaline phosphatase (hPAP) transgenic F344 rats (17) (kindly provided by Dr. Eric Sandgren, University of Wisconsin, Madison, WI) were trypsinized and resuspended in 1 ml of Matrigel and injected subcutaneously into isogenic wild-type F344 rats. After 1 wk the Matrigel plug was excised from anesthetized animals and stained for hPAP-positive cells with an Envision kit (Dakocytomation, Carpinteria, CA).

Immunohistochemical staining of cells for CD31 and von Willebrand Factor (vWF) was carried out on coverslips with primary CD31 or vWF antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and secondary Cy5-conjugated antibodies directed against the species of the primary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA).

Blood flow studies. The effect of infused MSC or EC (1.5–2 × 10^9 each in 0.2 ml culture medium) on postreflow renal artery blood flow, arterial blood pressure, and renovascular resistance was examined in female Sprague-Dawley rats (Charles River) with severe AKI. Rats were anesthetized with isoflurane, and baseline renal artery blood flows and carotid blood pressures were determined with a Transonic TS240 perivascular flowmeter equipped with a rat flowprobe (Transonic, Ithaca, NY) and an arterial pressure transducer, respectively.

Renal cortical blood flow was simultaneously monitored with a Laser-Doppler PeriFlux System 5000 (Perimed, Stockholm, Sweden). After baseline flows were obtained, both kidney pedicles were clamped for 60 min. After adequate reflow was confirmed visually, transcardiac infusion of MSC, EC, or serum-free culture medium as vehicle was given as above, and renal and cortical blood flows and carotid pressures were monitored for 60 min.

Gene expression. RNA was extracted with an RNasy kit (Qiagen, Valencia, CA) including a DNA digestion step. Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) for 60 min at 42°C. Real-time PCR with relative quantification of target gene copy numbers in relation to β-actin, 18S RNA, and RP-II transcripts (with a correction factor normalizing for these 3 housekeeping genes calculated by the REST program) was carried out as described in detail elsewhere (28, 39).

In vitro proliferation assay. EC were seeded onto 96-well plates (10,000 cells/well to achieve ~ 75% subconfluence) and incubated for 120 h with either serum-free medium, MSC-conditioned medium, MSC medium conditioned at a hypoxic oxygen pressure of 5%, fibroblast-conditioned medium (rat embryonic fibroblasts), or human VEGF (100 ng/ml, Sigma)-containing medium. Conditioned medium was generated by incubating serum-free medium (DMEM/Ham’s F12) on a confluent monolayer of MSC or fibroblasts for 24 h.

To determine cell numbers, wells were incubated with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylytetrazolium bromide] (MTT; 500 mg/l, Sigma, St. Louis, MO) for 60 min and dissolved in solvent (isopropanol/HCl) according to the manufacturer’s instructions. This was followed by optical density measurement with a plate reader (Tecan SpectraFluor plus, Tican, Durham, NC).

Cell survival assay. Aortic EC were seeded onto 96-well plates, and graded ATP depletion was induced by adding 20 μM antimycin A (Sigma) to serum-free DMEM/F12 for 45 min. Antimycin A at this concentration reduced cellular ATP levels by ~75%, as assayed by a plate reader-based ATP luciferase kit (Sigma). This decrease in ATP levels resulted in apoptotic changes of >30% EC at 1 h incubation, as assessed by nuclear condensation and formation of apoptotic bodies. After antimycin A incubation, cells were treated either by serum-free DMEM/F12, MSC-conditioned medium, MSC 5% oxygen-conditioned medium, DMEM/F12 plus VEGF (100 ng/ml), or DMEM/F12 plus 10% FCS. Cell survival after 24 h of recovery was determined by MTT assay as above.

Analysis of MSC-conditioned media. In six independent experiments, 10^6 MSC were placed on 96-well plates and allowed to attach overnight in DMEM/F12 culture medium with 10% FCS. Media were then replaced after three rinses with serum-free medium, and cells were incubated for 24 h in 1 ml DMEM/F12 only. At this time, 100 μl of media were assayed for VEGF by ELISA (Duoset, R&D Systems, Minneapolis, MN). FCS used initially was identically asayed. Media levels of VEGF are expressed as nanograms per milliliter per 24 hours. For HGF and IGF-1 measurements, conditioned medium was collected from 10^6 cells for 24 h and lyophilized. Lyophilized medium was resuspended in a defined volume and analyzed by ELISA (IGF-1: Diagnostic Systems Laboratories, Webster, TX; HGF: B-Bridge International, Mountain View, CA).

In vivo studies. Two-photon in vivo microscopy was conducted as described earlier (38). For analysis of apoptotic cells in kidney regions that do or do not contain MSC, we used a mouse model of AKI and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. C57/B16 mice were subjected to clamping of the left kidney for 30 min and intra-arterially infused with 10^5 MSC that were stained with the bright green fluorescent dye CFDA (Invitrogen). After 24 h, the animals were killed, arteries perfused with 2% paraformaldehyde, and frozen sections (10 μm) were stained with a TUNEL assay system (Roche, Indianapolis, IN). Right kidneys not subjected to clamping served as controls for methodology. For numerical analysis, 10 high-power fields (×40) that contained MSC...
were scored for the number of apoptotic cells and compared with 10 fields without MSC.

Statistical analysis. Data are expressed as means ± SD. Primary data collection utilized Excel (Microsoft, Redmond, WA), and statistical analyses were carried out using Prism (GraphPad, San Diego, CA). ANOVA and t-tests were used to assess differences between data means as appropriate. A P value of <0.05 was considered significant.

RESULTS

Effects of MSC-conditioned medium on EC. MSC protect function and enhance renal recovery from AKI, as shown previously (18, 38), but do not significantly engraft into the kidney as differentiated tubular cells or EC, respectively. We hypothesized, therefore, that trophic mediators rather than replacement of lost renal cells by MSC both protect kidney cells and stimulate regenerative processes. Because vascular EC occupy a critical anatomic position between blood supply and parenchymal cells, and since their dysfunction and injury have been shown to be important in the pathophysiology of AKI, we examined whether MSC-conditioned medium exerts cytokine effects on EC in vitro.

Accordingly, serum-free medium was conditioned by MSC for 24 h under normoxic (21% oxygen) or hypoxic (5% oxygen) conditions. MTT cell viability assays showed a significant stimulatory effect of MSC-conditioned medium on cell oxygen conditions. MTT cell viability assays showed a significant stimulatory effect of MSC-conditioned medium on cell viability (Fig. 1A) and was specific for MSC, since fibroblast-conditioned medium did not have such an effect (Fig. 1C). Cell conditioning under hypoxia enhanced this effect further, but it did not reach statistical significance (P > 0.05, Bonferroni’s multiple comparison test). To test the effect of conditioned medium survival, we induced apoptotic cell death in ~25% of aortic EC by graded ATP depletion with antimycin A and assessed cell survival in the presence of serum-free control or serum-free 5% oxygen MSC-conditioned medium. MSC-conditioned medium significantly increased survival of EC as shown by increased cell numbers at 24 h compared with serum-free controls (Fig. 1B). VEGF in the media caused a small increase in cell survival, while 10% FCS was most potent in this regard. Since the addition of serum-free 5% oxygen-conditioned medium had no proliferative effect on EC at 24 h (not shown), the difference in cell numbers reflects primarily the number of cells rescued from cell death due to apoptosis.

Gene expression profiling and growth factor secretion. EC and MSC have both been shown to be renoprotective (9, 18, 38). Since we show here that MSC interact in vitro through paracrine mechanisms with EC (Fig. 1A and B), and since both cell types enter the renal vasculature after in vivo infusion, we hypothesized that they might share similar expression profiles for vasculotrophic genes. Comparative, real-time, quantitative RT-PCR for selected vasculotrophic factors shows expression ratios for both cell types that are close to 1 for VEGF-B, VEGF-C, bFGF, HB-EGF, and TGF-α, indicating that these genes are expressed at approximately the same level (Fig. 2) in both cell types. Expression levels of VEGF-A, VEGF-D, and IGF-I were, however, markedly higher in MSC. Measurement of secreted VEGF-A by ELISA in serum-free medium from 10^5 MSC cultured × 24 h showed a reproducible concentration of 4.1 ± 0.9 ng·mL⁻¹·24 h⁻¹, while neither DMEM/F12 nor FCS contained any detectable VEGF. Concentrations for IGF-1 and HGF, which are also renoprotective growth factors, are shown in Fig. 1D and were in the range reported by other groups (24).

MSC-EC interactions. To test the in vitro interactions of MSC and EC, they were labeled with different fluorescent dyes and seeded individually or together on Matrigel, an extracellular matrix product containing a number of growth factors. MSC undergo tubulization when grown on Matrigel in a fashion similar to that of EC (Fig. 3, a and b). To test whether cooperation or fusion of MSC with EC occurs in tube assays, green-labeled MSC and red-labeled EC were seeded in a 50:50 mixture on Matrigel. MSC and EC formed tubes together in a cooperative way, but fusion was not detected since there was no merging of the green and red fluorescent cell markers.

Because differentiation of MSC into EC is a possible renoprotective mechanism in AKI, we tested the capability of MSC to assume an endothelial phenotype both in vitro and in vivo. Under nonconfluent culture conditions, MSC do not take up DiI-acLDL (Fig. 4a). After incubation with VEGF for 7 days, MSC robustly take up DiI-acLDL and in a fashion comparable to that seen in EC (Fig. 4b). Confluent but not subconfluent MSC express VEGF receptors flt-1 and flk-4 as well as low levels of flk-1, explaining their response to VEGF induction (not shown). Furthermore, after induction overnight on Matrigel and release by dispase, MSC seeded on cover slides show de novo expression of EC-specific marker proteins vWF and platelet-endothelial cell adhesion molecule-1 (CD31) (Fig. 4, c–f).

We next assessed the in vivo vasculogenic capacity of donor hPAP-MSC (from Fisher 344 rats) suspended in Matrigel plugs and subcutaneously injected into wild-type Fisher 344 rats. Microscopic examination of hPAP-immunostained Matrigel plugs at 7 days showed hPAP-MSC forming vascular structures (Fig. 5). There was also a Matrigel plug-associated immune reaction resulting in leukocyte invasion, which is not unexpected since murine Matrigel is allogeneic in the rat.

MSC in vivo homing and engraftment. To determine whether infused MSC home to the kidney rather than have systemic effects, we used in vivo two-photon laser confocal microscopy of CFDA-stained MSC infused immediately or 24 h after AKI. Labeled MSC homed to kidney capillaries and stably attached to EC immediately after infusion (Fig. 6). We then analyzed kidney cortical regions in mice subjected to ischemia-reperfusion injury that still contained MSC at 24 h after ischemia-reperfusion and regions that did not contain MSC for apoptotic kidney cells by TUNEL assay. As shown before, at 24 h postinfusion most of the administered MSC have disappeared by mechanisms currently unknown, but significant numbers of MSC could still be found in glomeruli and peritubular capillaries. Analysis of high-power fields showed that areas surrounding MSC have significantly lower numbers of apoptotic kidney cells compared with areas that did not contain MSC (Fig. 6, C and D). This suggests that MSC secrete factors into their immediate area that protect cells from apoptosis after injury.

Having shown that MSC home to the injured kidney, we assessed the extent of engraftment of MSC as cells of endothelial phenotype into vascular structures at 24 h and 5 days after ischemia-reperfusion injury. In vivo incorporation into vascular structures was determined by immunostaining for...
Fig. 1. A: MTT assays on aortic endothelial cells (EC) with mesenchymal stem cell (MSC)-conditioned medium (CM). MSC-conditioned medium significantly stimulated the proliferation of aortic endothelial cells after 5 days compared with control serum-free medium. This effect was slightly but insignificantly (n.s.) enhanced by conditioning at 5% oxygen pressure. The proliferative effect of VEGF was significant but less robust than that of conditioned medium. B: cell survival assay of EC treated with hypoxic MSC-conditioned or control medium. Apoptosis was induced by ATP depletion with 20 μM antimycin A, and survival of EC was significantly enhanced by hypoxic MSC-conditioned medium. VEGF and 10% FCS served as positive controls. In ATP-replete EC, conditioned medium failed to stimulate cell proliferation at 24 h (not shown). SF, serum-free medium. C: growth stimulation is specific for MSC-conditioned medium. Fibroblast-conditioned medium did not stimulate the growth of EC. D: MSC-conditioned medium contains HGF, IGF-1, and VEGF (ELISA), factors known to have renoprotective actions after acute kidney injury (AKI).
fluorescein to detect infused CFDA-labeled cells. We detected essentially no engrafted MSC at 24 h postinduction of AKI (not shown), and only few MSC-derived cells engrafted into peritubular capillaries at 5 days after AKI (Fig. 7). However, the extent of differentiation and engraftment of administered MSC, on average, was not sufficient to explain the significant early renoprotection, i.e., at 24 h, that is obtained with the infusion of MSC.

Renal blood flow. Decreased renal blood flow after induction of ischemia-reperfusion AKI is a major pathophysiological mechanism that contributes to the subsequent decline in renal function. Enhancement of renal blood flow early after reperfusion is therefore a possible mechanism whereby cell therapy could improve the outcome. To investigate this possibility, we measured renal artery and cortical blood flows after reperfusion and infusion of MSC or EC. Infusion of either MSC or EC did not acutely improve early renal artery and cortical blood flows during the 60 min following ischemia-reperfusion (Fig. 8). In addition, since simultaneous carotid blood pressures did not change significantly, renovascular resistance remained unchanged as well.

DISCUSSION

Stem cell-based therapies are promising new treatment approaches for a large number of diseases. However, controversy remains as to the mechanisms whereby stem cells mediate the obtained therapeutic actions. While the predominant belief was that improvement in outcome following organ injury and stem cell administration was the result of replacing damaged cells by differentiated stem cells, we and other groups have shown that paracrine mechanisms are primarily responsible for the organ-protective actions of administered stem cells (12, 15, 16, 38). Indeed, differentiation of stem cells into tissues different from their “natural” commitment is a rare phenomenon in healthy tissues (41), while it occurs in damaged tissues at widely varying rates of <0.1 to almost 20% (14). However, the functional contribution of this process to recovery and tissue regeneration has actually not been conclusively demonstrated.

We show in the present study that MSC and EC have multiple in vitro interactions with each other as well as in vivo interactions with cells in the injured kidney. These paracrine effects appear specific for MSC since they cannot be elicited by fibroblasts. The elicited responses suggest that identified mechanisms mediate, at least in part, the renoprotective and regenerative actions of cell therapy after AKI. Since we excluded significant early incorporation of MSC into the vasculature and

Fig. 2. Comparative gene expression profiles between EC and MSC for growth factor- and EC-specific genes show that MSC share the following angiogenic factors with EC: VEGF, bFGF, HGF, and TGF-α. Other growth factors, like IGF-1, are exclusively expressed by MSC. A value >1 means higher expression in MSC, and a value <1 higher expression in EC. At a ratio of 1, gene expression is comparable in both cell types.

Fig. 3. a: MSC undergo tubulogenesis on Matrigel (×40), as do control EC (×20) in b. c and d: When grown together on Matrigel-coated cover slides, PKH26-labeled EC (red) and carboxyfluorescein diacetate (CFDA)-labeled MSC (green) cooperate to form tubes without fusion. Nuclei are stained blue with Hoechst 33342. Original magnification, ×20 and ×40, respectively.
since renal blood flow did not change immediately after infusion, our data indicate that renoprotection is mediated by paracrine and endocrine effects. Although the exact nature of these mechanisms and factors remains to be elucidated in further studies, we measured several candidate factors that are secreted by MSC and that are known to exert renoprotective actions. Importantly, kidney cells in the vicinity of administered MSC were protected from apoptosis, which is further indication for the paracrine nature of the observed cytoprotection. These effects include secretion of growth factors, VEGF, HGF, and IGF-I, i.e., renotropic factors that are known to both decrease apoptosis of endothelial and tubular cells and to stimulate proliferation of surviving cells.

Our present data are in strong support of the notion that therapeutic targeting of the endothelial damage early in the course of ischemic AKI contributes to the protection of renal function. This can be accomplished by the administration of MSC that possess, as we demonstrate here, complex vasculo-protective actions that are also shared by EC. The EC is at the center of regulating coagulation, leukocyte adhesion, microvascular blood flow, and regulation of intra-/extravascular fluid distribution. These regulatory processes are disrupted during the early “extension phase” of AKI (21), where cell swelling leads to reduction in regional blood flow, vasocongestion, and vasoconstriction, accompanied by increased leukocyte adhesion, fluid extravasation, and coagulation (7). Targeting this extension phase by treatment with EC or MSC is thought to provide secondary protection of tubular cells and acceleration of subsequent recovery from AKI. This has been shown previously by Brodsky et al. (9) in a rat model of AKI, where the infusion of human umbilical cord blood cells into athymic, nude rats improved renal function. The importance of the
microcirculation of the medulla and the highly oxygen-sensitive corticomedullary junction in the pathophysiology of AKI has been well recognized (8). Although we did not observe a rise in total renal and outer cortical blood flows, and renovascular resistance did not fall after MSC or EC infusion, it is possible that improvement in microvascular flow and function in the deeper corticomedullary region does occur, which may have resulted in better regional perfusion of injured areas and secondary limitation of parenchymal damage. However, if such redistribution of cortical blood flow to the inner cortex and outer medulla had occurred, this should have resulted in a further fall in outer cortical blood flow, which was not observed (data not shown).

MSC have been previously shown to have vasculotropic properties (2, 4) that may be of utility in regenerative medicine. Infused MSCs have been shown to contribute, via differentia-

Fig. 6. A and B: in vivo homing of CFDA-stained MSC at 2 and 30 min after injection into a rat with AKI at 24 h after ischemia-reperfusion injury. MSC are stained bright green, yellow-green autofluorescence is derived from kidney tubules, and the microvasculature is shown by infusion of red (tetramethylrhodamine albumin). Two-photon laser confocal microscopy was used. C: assessment of apoptotic scores at 24 h [terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, green nuclei] in kidney containing MSC and those without. Regions where MSC are still present (circles; CFDA-stained MSC can readily be identified by the bright green cytoplasmic stain) do not contain significant numbers of apoptotic cells (green nuclei, arrowheads), thereby suggesting that these regions are protected. Two representative regions containing MSC (bottom) and regions without MSC (top) are shown. D: numerical analysis of apoptosis of TUNEL-stained kidney high-power fields (×40) shows a significantly lower number of apoptotic cells at 24 h after ischemia-reperfusion injury in fields containing MSC compared with fields that do not (P < 0.0001).
tion and engraftment, to the cells of many organs, including the normal kidney (3). However, these engraftments are in general very rare events that cannot explain the prompt protective and regenerative responses MSC elicit in significantly injured organs. Other investigators in the cardiovascular field have found that MSC improve cardiac function after myocardial infarction or dilated cardiomyopathy by supporting angiogenesis, myogenesis, and significantly through VEGF production (2, 23, 24). Furthermore, human adipose stromal cells, cell type that is very similar to MSC, secrete angiogenic and antiapoptotic factors that elicit cardiovascular protection (31). Ziegelhoeffer et al. (43) have reported that bone marrow-derived cells do not incorporate into the injured vasculature but may function as supporting cells. Gnegi et al. (13) as well showed that

Fig. 7. MSC (labeled with CFDA and immunostained for fluorescein) incorporate into renal vascular endothelium 5 days after AKI [top (left and right) and bottom left], although this is a very rare event (<1 positive event/section on average). Negative control, bottom right.

Fig. 8. MSC and EC do not increase renal artery blood flow and renovascular resistance (mmHg·ml·min⁻¹) after ischemia-reperfusion injury. Baseline measurements were conducted, and renal pedicles were clamped. After reflow, either vehicle, MSC, or EC were infused (arrows) and blood flow was measured every 10 min for 1 h. Cortical blood flow changed in the same fashion (data not shown).
protection from apoptosis by paracrine mechanisms contributes to the cardiac protection elicited by Akt-transduced MSC. Together, these and our studies support the hypothesis that paracrine actions are of major importance in mediating the protective and regenerative effects of administered MSC after organ injury.

The fact that the tested gene expression patterns for several members of the VEGF family, bFGF, HB-EGF, and TGF-α, were similar in MSC and EC may suggest that this mix of growth factors collectively mediates, at least in part, the vasculotoxic effects of either cell type in AKI.

Cell therapy is a new strategy in the treatment of complex disorders and has potentially more value than single-agent drug therapy due to the highly versatile response of cells to their environment both in situ and systemically. These cells are able to secrete a number of factors within the injured organ, intervening at different pathophysiological manifestations, e.g., inflammation, proliferation, apoptosis, and necrosis, and are therefore potentially more effective than drug or growth factor therapies primarily directed at only a few pathological processes, while potentially exerting adverse systemic effects. It is finally obvious that the great therapeutic promise of cell therapies makes it mandatory that the mechanisms of action and the long-term safety of this form of therapy are clearly defined. A systematic analysis of the individual and collective significance of identified growth factors as mediators of renoprotection is underway using siRNA technology.

In conclusion, our data show that MSC and EC interact and that these interactions are likely responsible, at least in part, for the kidney-protective effects of MSC in AKI, mediated by complex paracrine actions that are able to significantly protect and regenerate the damaged vasculature in AKI.

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