Insulin-Like Growth Factor-1 Sustains Stem Cell–Mediated Renal Repair

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ABSTRACT

In mice with cisplatin-induced acute kidney injury, administration of bone marrow-derived mesenchymal stem cells (MSC) restores renal tubular structure and improves renal function, but the underlying mechanism is unclear. Here, we examined the process of kidney cell repair in co-culture experiments with MSC and cisplatin-injured proximal tubular epithelial cells (PTEC). Exposure of PTEC to cisplatin markedly reduced cell viability at 4 days, but co-culture with MSC provided a protective effect by promoting tubular cell proliferation. This effect was mediated by insulin-like growth factor-1 (IGF-1), highly expressed by MSC as mRNA and protein, since blocking the growth factor's function with a specific antibody attenuated cell proliferation of PTEC. Confirming this, knocking down IGF-1 expression in MSC by small interfering-RNA also resulted in a significant decrease in PTEC proliferation and increased apoptosis. Furthermore, in the murine model of cisplatin-induced kidney injury, administering IGF-1 gene-silenced MSC limited their protective effect on renal function and tubular structure. These findings indicate that MSC exert beneficial effects on tubular cell repair in acute kidney injury by producing the mitogenic and pro-survival factor IGF-1.

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Acute kidney injury (AKI)[1] is emerging as a public health problem worldwide. Its prevalence varies from 5% of all hospitalized patients to 30 to 50% in critical care units.[2–5] Despite major technical improvements in dialysis and intensive care, the mortality and morbidity among patients with severe AKI remain dismally high. Several pharmacologic therapies that can accelerate recovery and improve survival have been attempted and were efficacious in experimental models but failed to manifest any substantial beneficial effect in clinical practice.[6,7]

Acute tubular apoptosis and necrosis play a central role in the process underlying kidney failure after ischemic or nephrotoxic challenge.[3,8,9] Depending on the severity and the duration of the insult, tubular damage may recover, although a critical number of surviving cells is required to reconstitute structural integrity. For circumvention of such limitation, local supply of new cells to promote injured cell replacement represents an innovative strategy.

Several reports claimed a role of adult bone marrow (BM)-derived mesenchymal stem cells (MSC) or hematopoietic stem cells in the repopulation of tubules as a consequence of physiologic cell turnover or after AKI.[10–12] Mechanisms underlying their renoprotective effect are still controversial.

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Experimental findings revealed the plasticity of BM stem cells to differentiate into renal cell lineages,[12–16] but current data mainly support a paracrine action of BM stem cells in kidney tissue repair.[17] MSC represent a heterogeneous cell population[18] that is able to support hematopoiesis and differentiate into tissues of mesenchymal[19] and nonmesenchymal lineages.[20–22] We[16] previously showed that purified BM-derived MSC infused in mice with AKI induced by the nephrotoxic anticancer agent cisplatin restored renal function and minimized tubular injury by accelerating resident tubular cell proliferation and to a lesser extent acquiring tubular phenotype. Similarly, in a model of glycerol-induced AKI, MSC exerted renoprotective effect by inducing a marked proliferation of indigenous tubular cells and differentiating in tubular cells expressing cytokeratin.[23]

Studies in rats with ischemia-reperfusion injury have suggested that the renoprotective action of MSC is due to their ability to produce high levels of growth factors such as vascular endothelial growth factor, hepatocyte growth factor, and IGF-1 and inducing antiapoptotic signals.[17] IGF-1 was implicated as an important mediator of cardiac and kidney regeneration in models of AKI.[24–28]

Here, we developed an *in vitro* model of cisplatin-induced tubular cell damage to study the possible effect of MSC on tubular epithelial cell proliferation and apoptosis. The role of IGF-1 as a key mediator of MSC-induced tubular regeneration was explored both *in vitro* and *in vivo* in the mouse model of cisplatin-induced AKI.

RESULTS

MSC Protect Proximal Tubular Epithelial Cells from Cisplatin-Induced Toxicity

In the *in vitro* model of cisplatin-induced toxicity, mouse proximal tubular epithelial cells (PTEC) were exposed to 2.5 μM cisplatin for 6 h, and after drug withdrawal, cell count was performed at various time intervals. Viable PTEC at 1 d after cisplatin significantly decreased as compared with unstimulated cells ($P < 0.05$; Figure 1A). After 4 d, the number of cisplatin-treated cells that survived was further significantly reduced (56%) with respect to control ($P < 0.01$). A marked toxicity persisted at 7 d (Figure 1A).

The capability of mouse MSC to induce the regeneration of tubular cells that are damaged by cisplatin was investigated using a co-culture system whereby cells established a direct contact. Addition of MSC to cisplatin-pretreated PTEC led to a significant increase in cell proliferation after 4 d of co-culture as compared with cisplatin-treated PTEC alone ($P < 0.01$; Figure 1B). The percentage of viable cells increased by 219% and was similar to control PTEC value. MSC seeded alone did not grow.

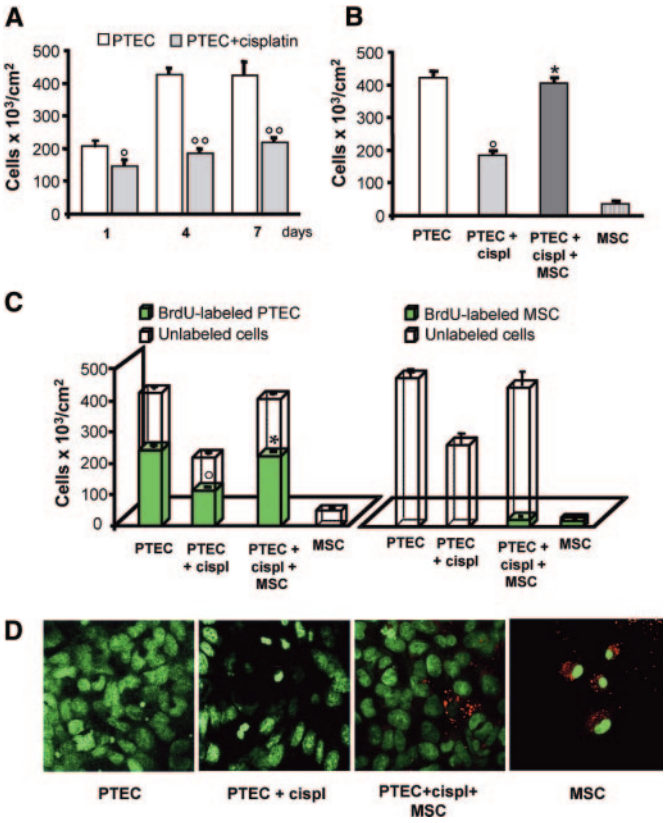


Figure 1. Effect of MSC co-cultured with PTEC that were damaged by cisplatin. (A) Cisplatin exerted cytotoxic effect on PTEC viability. PTEC were incubated with cisplatin (2.5 μM) for 6 h. After cisplatin removal, cells were maintained in medium alone, and viable cells were counted after 1, 4, or 7 d. °$P < 0.05$, °°$P < 0.01$ *versus* PTEC at the corresponding times ($n = 6$). (B) MSC protected PTEC from cisplatin-induced damage after 4 d of co-culture. PTEC were incubated with 2.5 μM cisplatin for 6 h and, after drug withdrawal, co-cultured for 4 d with MSC at the ratio of 1:1. At the end of incubation, viable cells were counted. °$P < 0.01$ *versus* PTEC, *$P < 0.01$ *versus* PTEC+cisplatin ($n = 10$). (C) MSC induced proliferation of cisplatin-treated PTEC. Identification of proliferating cells in the co-culture system was performed by BrdU incorporation. (Left) PTEC were prelabeled with BrdU for 4 h before cisplatin treatment (6 h). After drug removal, unlabeled MSC were added to BrdU-labeled PTEC in control medium for 4 d. Adherent cells were detached and counted, and BrdU-positive PTEC were evaluated by FACS. (Right) MSC that were prelabeled with BrdU were co-cultured with unlabeled PTEC that were previously treated with cisplatin. After 4 d, adherent cells were detached and counted, and BrdU-positive MSC were evaluated by FACS. °$P < 0.01$ *versus* PTEC; *$P < 0.01$ *versus* PTEC+cisplatin ($n = 3$). Data are means ± SEM. (D) Representative photomicrographs showing both cell populations alone or in co-culture system. Both PTEC and MSC were stained with the nuclear dye SYTOX Green (in green). In addition, MSC were labeled with the red fluorescence cell linker PKH-26. Magnification, ×630.

For identification of the nature of proliferating cells in the co-culture system, PTEC and MSC were alternatively chased before co-culture with 5-bromo-2-deoxyuridine (BrdU), a nucleotide that incorporates in dividing cells. Cisplatin-treated

PTEC (BrdU labeled), co-cultured with unlabeled MSC, markedly proliferated with respect to cisplatin-treated PTEC alone (Figure 1C, left). By contrast, addition of BrdU-labeled MSC to cisplatin-treated unlabeled PTEC did not divide and accounted for 4% of the total cell number (Figure 1C, right). Similar data were obtained when MSC were labeled with the fluorescent dye PKH-26. PKH-26–labeled MSC co-cultured with cisplatin-treated PTEC appeared as single cells sparsely distributed within PTEC layer (Figure 1D). FACS analysis showed that fluorescent MSC did not grow when seeded alone or in co-culture with cisplatin-treated PTEC, and they represented 6.5 ± 0.2% of total cell number. In experiments in which PTEC and MSC were co-cultured but physically separated by a porous membrane (Transwell), we still observed an increased tubular cell proliferation (207 *versus* 100% in cisplatin-treated PTEC).

MSC Induce Proximal Tubular Cell Proliferation via IGF-1

The role of IGF-1, TGF-β, and IL-10 in MSC-induced tubular cell proliferation was investigated. PTEC expressed IGF-1 and TGF-β 1 receptors but negligible levels of IL-10 receptor (data not shown). Blocking of IGF-1 with a specific antibody (Ab) resulted in a complete inhibition ($P < 0.01$) of cell proliferation in the co-culture system (Figure 2A). Otherwise, incubation with Ab against TGF-β or IL-10 did not affect cell proliferation, as observed with irrelevant Ab.

MSC constitutively expressed 150-fold higher IGF-1 mRNA levels than unstimulated PTEC. For assessment of the direct role of IGF-1 in PTEC proliferation, RNA interference technique to block IGF-1 gene expression was used. We designed small interfering RNA (siRNA) for the two transcript variants of IGF-1 (si-IGF-1). Two days after MSC transfection, IGF-1 mRNA levels were inhibited by 76% with respect to cells that were transfected with the irrelevant siRNA (si-irrel; $P < 0.05$; Figure 2B, inset). Inhibition of IGF-1 expression was still present in transfected MSC that were added to cisplatin-treated PTEC after 4 d (Figure 2B). Constitutive IGF-1 expression in PTEC was very low and did not change after cisplatin exposure. The production of IGF-1 protein by MSC after 4 d of culture was 75-fold higher than that observed in PTEC that were unstimulated or treated with cisplatin. When MSC were transfected with si-IGF-1 RNA, they exhibited a marked reduction (63%) of IGF-1 protein as compared with cells that were transfected with si-irrel RNA after 4 d (si-irrel MSC 22.02 ± 3.9 *versus* si-IGF-1 MSC 8.07 ± 0.8 ng/10^6 cells).

Next, we studied the effect of IGF-1 blocking on the proliferation of cisplatin-treated PTEC co-cultured with si-IGF-1–transfected MSC. Silencing IGF-1 in MSC led to a strong reduction in proliferation of cisplatin-treated PTEC as compared with MSC that were transfected with si-irrel ($P < 0.05$; Figure 2C). Different concentrations of exogenous IGF-1 induced an increase in the growth of cisplatin-treated PTEC

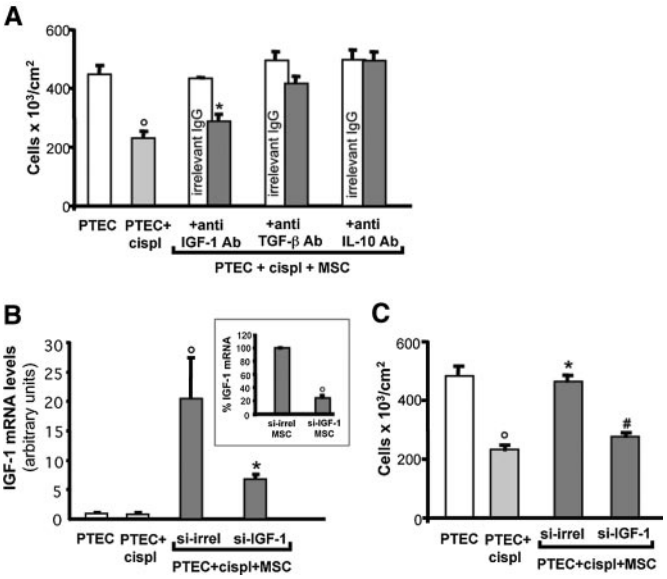


Figure 2. MSC exert their protective effect on cisplatin-treated PTEC *via* IGF-1. (A) Effect of anti–IGF-1, TGF-β, or IL-10 Ab on MSC-induced proliferation of cisplatin-treated PTEC. Three hours after the addition of MSC to cisplatin-pretreated PTEC, cells were exposed to specific functional blocking Ab against IGF-1, TGF-β, IL-10, or the corresponding irrelevant IgG. Cell count was evaluated after 4 d. °$P < 0.01$ *versus* PTEC and control IgG; *$P < 0.01$ *versus* corresponding irrelevant IgG ($n = 6$). (B) Expression of IGF-1 mRNA in PTEC alone, PTEC+cisplatin, and PTEC+cisplatin co-cultured with MSC that were transfected with si-irrel or si-IGF-1. (Inset) By real-time PCR, IGF-1 mRNA level (expressed as percentage of si-irrel MSC as 100%) was significantly reduced in MSC that were transfected with si-IGF-1 with respect to MSC that were transfected with si-irrel after 2 d. °$P < 0.05$ *versus* si-irrel MSC ($n = 3$). (Bottom) IGF-1 mRNA expression was evaluated in unstimulated PTEC and cisplatin-treated PTEC (2.5 μM) alone or in co-culture with si-irrel MSC or si-IGF-1 after 4 d. Data are means ± SEM. °$P < 0.01$ *versus* PTEC and PTEC +cisplatin; *$P < 0.05$ *versus* si-irrel MSC ($n = 4$). (C) Role of IGF-1 in MSC-induced tubular cell proliferation. Cell proliferation of cisplatin-treated PTEC alone or in co-culture for 4 d with si-irrel MSC or si-IGF-1 MSC was assessed. °$P < 0.01$ *versus* PTEC; *$P < 0.01$ *versus* PTEC+cisplatin; #$P < 0.05$ *versus* si-irrel MSC ($n = 6$). Data are means ± SEM.

starting from 1 ng ($P < 0.01$ *versus* cisplatin alone. IGF-1: 0.2 ng, 288 ± 2; 1 ng, 366 ± 1; 10 ng, 399 ± 10; 100 ng, 428 ± 1 *versus* cisplatin 258 ± 10 × 10^3 cells).

MSC Limit Cisplatin-Induced Apoptosis via IGF-1

The percentage of apoptotic PTEC—double positive for caspases 3 and 7 and propidium iodide—significantly ($P < 0.01$) increased at 4 d after cisplatin compared with untreated cells (Figure 3). Addition of MSC reduced ($P < 0.01$) the number of apoptotic cells to control values.

A role of mesenchymal-derived IGF-1 in limiting tubular cell apoptosis in response to cisplatin was documented by silencing the expression of IGF-1 in MSC. Results showed that transfection

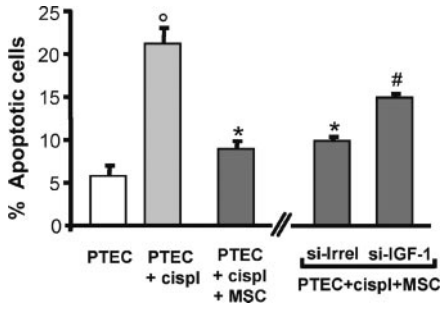


Figure 3. MSC reduce cisplatin-induced apoptosis on PTEC *via* IGF-1. Percentage of apoptotic cells (late apoptosis, caspases 3 and 7, and propidium iodide–positive cells) was analyzed by FACS in untreated PTEC and cisplatin-treated PTEC (2.5 μM, 6 h) alone or in co-culture for 4 d with MSC, si-irrel MSC, or si-IGF-1 MSC. °$P < 0.01$ *versus* PTEC; *$P < 0.01$ *versus* PTEC+cisplatin; #$P < 0.05$ *versus* si-irrel MSC and PTEC+cisplatin ($n = 3$). Data are means ± SEM.

of MSC with si-IGF-1 RNA and their addition to cisplatin-treated PTEC for 4 d caused a significant ($P < 0.05$) increase in the number of caspases 3– and 7–positive cells when compared with MSC that were transfected with si-irrel (Figure 3).

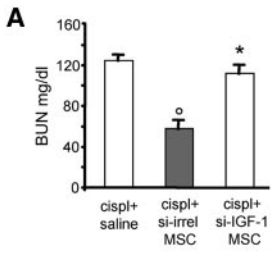
Role of Mesenchymal-Derived IGF-1 in Mice with Cisplatin-Induced AKI

For determination of whether MSC exert a protective effect *in vivo via* IGF-1, MSC that were transfected with si-irrel or si-IGF-1 RNA were intravenously injected into syngeneic mice 24 h after cisplatin administration. Renal function and tubular structure at 4 d[16] were impaired in cisplatin-treated mice that were given saline (Figure 4). MSC that were transfected with si-irrel significantly protected renal function in cisplatin-treated mice, as reflected by lower blood urea nitrogen (BUN) values with respect to vehicle ($P < 0.01$; Figure 4A). Blocking of IGF-1 reduced the beneficial effect of MSC on renal function ($P < 0.01$ *versus* si-irrel MSC; Figure 4A). MSC that were transfected with si-irrel ameliorated tubular cell morphology and reduced necrosis, whereas IGF-1 silencing in MSC resulted in a less protective effect on tubular injury (Figure 4, B and C).

Engraftment of PKH-26–labeled MSC, transfected with either si-irrel or si-IGF-1, was assessed in the kidney. MSC predominantly localized in peritubular areas and to a lesser extent within the tubular epithelium (Figure 5) in both groups. The number of PKH-26–positive cells did not differ significantly between the two groups (1.1 ± 0.7 and 0.5 ± 0.3 MSC/10^5 renal cells in mice that were administered an injection of si-irrel MSC or si-IGF-1 MSC, respectively). Scattered PKH-26–positive cells were found in glomeruli.

DISCUSSION

The capacity of MSC to ameliorate renal dysfunction and repair tubular injury in the cisplatin model of AKI has been attributed to MSC's ability to induce renal tubular cell proliferation and differentiate into tubular cells.[16] This latter phenomenon was limited in proportion and could not completely account for the observed reparative capacity of MSC; therefore, the paracrine mechanism that involves the production of growth factors and anti-inflammatory cytokines by MSC has been claimed as the most suitable one.[17] This study therefore explored the ability of MSC to stimulate directly cell proliferation of cisplatin-injured renal cells and the underlying mechanism. An *in vitro* model of cisplatin-induced toxicity in proximal tubular cells was instrumental to demonstrating that MSC co-cultured with cisplatin-treated PTEC induced a significant increase in cell proliferation. Tracking experiments with BrdU and PKH-26 indicated that tubular cells but not



B

		cispl + saline	cispl + si-irrel-MSC	cispl + si-IGF-1-MSC
Casts	(n/HPF)	8.3±1.6	1.5±0.3°	5.0±0.9*
Tubular necrosis	(n/HPF)	4.9±0.8	0.4±0.1°	2.6±0.6*

Data are mean values ± SE. HPF=high power field.

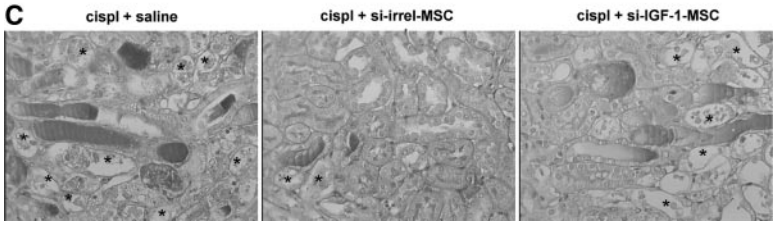


Figure 4. MSC exert their protective effect on mice with cisplatin-induced AKI *via* IGF-1. (A) Silencing mesenchymal IGF-1 gene expression limited the protective effect of MSC on renal function. BUN was evaluated at 4 d in cisplatin-treated mice (12.7 mg/kg subcutaneously) that were administered an intravenous injection of saline, si-irrel MSC (2×10^5 cells), or si-IGF-1 MSC (2×10^5 cells). Data are means ± SEM ($n = 12$). °$P <$ 0.01 *versus* saline; *$P < 0.01$ *versus* si-irrel MSC. (B) Renal histology (casts and necrotic tubuli, quantified as numbers per high-power field) in cisplatin-treated mice at 4 d after receiving saline, si-irrel MSC, or si-IGF-1 MSC. Knocking down mesenchymal IGF-1 expression was associated with as high numbers of casts and necrotic cells as in kidneys of mice receiving saline. Data are means ± SEM. °$P < 0.01$ *versus* saline; *$P < 0.01$ *versus* si-irrel MSC. (C) Light micrographs of kidney sections of C57BL6/J cisplatin-treated mice that were administered an injection of saline, si-irrel MSC, or si-IGF-1 MSC and killed at 4 d. The kidney of a cisplatin-treated mouse receiving saline shows tubular epithelial cell degeneration and necrosis and casts (*). Treatment with si-irrel MSC resulted in less severe tubular damage with occasional luminal casts. Lesions in kidney of si-IGF-1 MSC cisplatin-injected mouse were more pronounced and almost comparable to those found in mice that were given saline. Magnification, ×400.

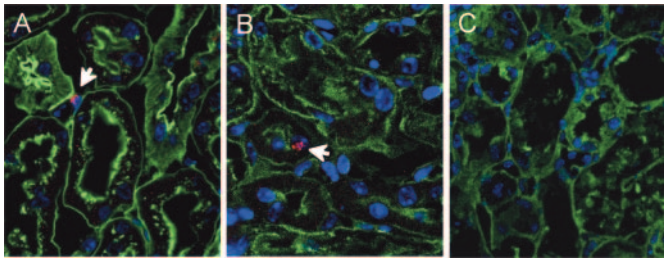


Figure 5. Detection of specific fluorescence for PKH-26–labeled MSC in kidney sections of cisplatin-treated mice. PKH-26 cells (red fluorescence, arrows) were localized both in the peritubular interstitium (A) and in the context of the tubular epithelium (B). (C) Kidney section of cisplatin-treated mice that were given saline. Sections were incubated with DAPI (nuclear staining, blue) followed by WGA Lectin (green). Magnification, ×630.

MSC were the very population that proliferated. Increased mitosis of tubular cells occurred also when the two cell types were physically separated by a porous membrane, suggesting that soluble growth factors could be responsible for the phenomenon. MSC produce a variety of cytokines and growth factors[17,29,30] that could account for the increased tubular cell proliferation. Among potential candidates, we focused on IGF-1, which possesses mitogenic and antiapoptotic properties[27,31]; TGF-β, which is involved in tubulogenesis and in the dedifferentiation of adult tubular cells[32,33]; and IL-10, a cytokine with anti-inflammatory activities.[34] By using specific functional blocking antibodies, we evidenced a determinant role of IGF-1 in PTEC proliferation induced by MSC. MSC constitutively expressed IGF-1 mRNA and protein, as already described,[17,35] with protein levels remarkably higher than those detected in control or cisplatin-treated PTEC. Direct blocking of IGF-1 expression in MSC by siRNA confirmed that the proliferation of cisplatin-damaged PTEC was mediated by mesenchymal-derived IGF-1. That IGF-1 directly induces tubular cell proliferation was indicated by evidence that PTEC highly expressed IGF-1 receptor mRNA, and exogenous IGF-1 at the concentration comparable to that produced by cultured MSC stimulated cisplatin-treated PTEC to proliferate. Our findings are in line with previous studies[36,37] showing that *in vitro* IGF-1 stimulated the proliferation of cultured rabbit and rat proximal tubular cells.

These results also established an antiapoptotic activity of IGF-1. In our experimental setting, cisplatin induced consistent apoptosis of PTEC, which was reduced by co-culture with MSC. Data that silencing mesenchymal IGF-1 expression resulted in increased percentage of apoptotic cells with respect to MSC that were transfected with si-irrel suggested that the mechanism by which MSC inhibit apoptosis is, at least in part, mediated by IGF-1. In this context, the observation that recombinant human IGF-1 reduced apoptotic cell death *in vitro* in rat proximal tubular cells that were subjected to anoxia-reoxygenation is interesting.[31]

A major finding of this study is the evidence that *in vivo* IGF-1 produced by MSC is a key mediator in the process of tubular repair in AKI. In fact, MSC silenced for IGF-1 failed to exert any protective effect when injected in mice with cisplatin-induced AKI. At variance, MSC that were transfected with si-irrel improved renal function and tubular damage, in agreement with our previous observations.[16] The use of siRNA carries off-target effects as a result of unintentional silencing of genes containing sequences with imperfect complementarity of the target gene. For limitation of unintended mRNA changes, siRNA was designed as having a high degree of similarity to the IGF-1 mRNA after BLAST search.

Here, the preferential localization of MSC to peritubular interstitial areas rather than within tubular epithelium in both groups of mice with AKI indicates that MSC acted by exerting paracrine actions. Of interest, limited numbers of engrafted MSC were found in the kidney at 4 d, despite the remarkable beneficial effect of MSC administration on the kidney. This is consistent with previous findings that 2 h after MSC infusion, administered cells were observed in the kidney, liver, lung, BM, and spleen in ischemia-reperfusion model. By 24 h, occasional cells were still present in lung, whereas very few MSC were found in the kidney and were no longer detectable at 72 h.[17]

The use of IGF-1 as pharmacologic approach for the treatment of AKI is controversial. In some experimental studies, IGF-1 administered systemically was found to increase renal blood flow and GFR,[38,39] accelerate the recovery of renal function, and enhance the healing of injured nephrons.[26–28,31] A more recent report instead described that in rats with ischemia-reperfusion injury, IGF-1 treatment enhanced the inflammatory response and was associated with higher risk for death.[40] Likewise, two clinical double-blind studies showed that injection of recombinant human IGF-1 did not improve GFR or accelerate the rate of recovery of renal function in patients with AKI and substantial comorbidity.[41,42] It is conceivable that such lack of efficacy is primarily a consequence of limitations to a therapeutic trial imposed by AKI in the intensive care unit setting.[41] Another plausible explanation is that IGF-1 has high affinity for circulating IGF-1–binding proteins that would affect the delivery of the growth factor to the target organs,[41,43] limiting its effect on renal tubuli. Local supply of IGF-1 to injured tissues may overcome IGF-1 clearance by binding proteins, as shown by reports in models of ischemic heart failure. In rats with myocardial infarction, local delivery of IGF-1 with peptide nanofibers increased the survival of cardiac cells that were injected into the site of damage, improving the growth of transplanted cells and cardiac function.[25] In addition, cardiac-restricted overexpression of a transgene encoding an IGF-1 isoform protected myocytes against apoptosis and ventricular dilation after infarction.[24] Another study[44] highlighted the concept that skeletal muscle–restricted IGF-1 overexpression increased local tissue repair either by acting on resident progenitor cells or by mediating the recruitment of BM-derived stem cells to the site of injury in mice after toxin-induced muscle damage. In our setting as well, local release of IGF-1 by MSC that are recruited to the sites of tubular damage might act as a powerful regenerative agent by inducing prolif-

eration and inhibiting apoptosis of endogenous tubular cells. Mesenchymal IGF-1 is instrumental to recruitment of circulating-derived or resident progenitor/stem cells that contribute to the regenerative processes of the kidney.

Our data suggest that MSC represent a powerful form of treatment for AKI by virtue of their tropism for damaged kidney and their ability to provide a local environment supporting the growth of injured endogenous tubular cells through the release of IGF-1. Given the longstanding lack of progress either in survival or in recovery of renal function in patients with acute renal failure, the potential therapeutic implications of these findings are highly valuable.

CONCISE METHODS

Cell Culture and Incubation

SV40-transformed murine PTEC line was provided by Dr. Eric G. Neilson (Vanderbilt University, Nashville, TN). PTEC were grown as described previously.[45]

MSC were obtained from BM of 2-mo-old male C57BL6/J mice[16] by their tendency to adhere tightly to plastic culture dishes.[46] Cells were plated at the density of $1 \times 10^6/cm^2$ in DMEM plus 10% FCS (Life Technologies-Invitrogen, S.Giuliano Milanese, Italy) and antibiotics[47]; after 6 h, unadhered cells were removed. At 2 wk, cells were detached by trypsin 0.25%/1 mM EDTA at 37°C for 2 min.[48] Subconfluent MSC were immunodepleted of $CD45^+$ cells and characterized.[16] For *in vitro* studies, PTEC were seeded at 46×10^3 cells/cm^2 and 24 h later incubated with DMEM plus 2% FCS (test medium) alone or in the presence of 2.5 μM cis-platinum (II)-diamine dichloride (cisplatin; Sigma-Aldrich, St. Louis, MO) for 6 h. After cisplatin removal, viable cells were counted by trypan blue dye exclusion (Sigma).

To study the effect of MSC on tubular cell proliferation in co-culture, cisplatin-pretreated PTEC were incubated with test medium or MSC at the density of $90 \times 10^3/cm^2$ for 4 d. MSC in test medium served as controls. The nature of proliferating cells was assessed using two different techniques. First, PTEC or MSC were alternatively chased (before co-culture) with the DNA labeling BrdU (100 μM for 4 h; Sigma) that marks cells during mitosis. Second, MSC were labeled with PKH-26 red fluorescence cell linker (Sigma-Aldrich) and then co-cultured with cisplatin-treated PTEC. Four days after cisplatin treatment, cells were detached for FACS analysis. For Transwell experiments, MSC were seeded on top of polycarbonate inserts of wells that were previously plated with PTEC that were pretreated or not for 6 h with cisplatin. After 4 d, PTEC proliferation was estimated.

The role of IGF-1, TGF-β, and IL-10 was studied in the co-culture system at 4 d by use of blocking Ab: Anti–IGF-1 Ab (0.25 μg/ml; R&D Systems, Minneapolis MN), anti–TGF-β Ab (20 μg/ml; Genzyme Corp., Cambridge, MA),[49] anti–IL-10 Ab (10 μg/ml; R&D Systems), or the respective irrelevant IgG (Goat IgG purified [Chemicon Int., Millipore, Millerica, MA], Hamster IgG Isotype Control [Caltag Laboratories, Burlingame, CA], and 13C4 irrelevant murine Ab [Genzyme Corp.]).

The involvement of MSC-produced IGF-1 on PTEC proliferation was studied in MSC that were transfected with si-irrel or si-IGF-1 RNA for 4 h and then maintained in test medium 2 d before their addition to co-culture. IGF-1 mRNA and protein were evaluated before and after 4 d of co-culture by reverse transcription–PCR (RT-PCR) and ELISA immunoassay (R&D Systems).

FACS Analysis

Cell suspensions were fixed in p-formaldehyde, permeabilized, and incubated with FITC-conjugated anti-BrdU Ab (Oxford Biotechnology, Kidlington, UK). BrdU-positive cells were detected by flow cytofluorimetric analysis (FACSort; Becton Dickinson, Franklin Lakes, NJ).

The percentage of PKH-26 labeled-MSC was quantified by FACS in the co-culture after 4 d from cisplatin treatment. Labeling efficacy was >98%; cell viability was >96%.

Apoptosis was evaluated by assessment of caspases 3 and 7 expression (FLICA reagent) and propidium iodide staining (Vybrant FAM caspases 3 and 7 assay kit; Molecular Probes, Eugene, OR) following the manufacturer's instructions. Cells were analyzed by FACS at 488 nm excitation, green emission for the FLICA-stained cells, and red emission for propidium iodide.

RNA Interference

siRNA was selected 364 bases downstream of the start codon in a region common to both the mouse IGF-1 transcript variants (NCBI ref. seq. no. NM-010512 and NM-184052). si-IGF-1 and si-irrel RNA were synthesized by Ambion (Austin, TX). siRNA (100 pmol) was introduced in MSC cells that were grown on six-well plates by 4 h of transfection with siPORTAMINE (Ambion) according to the manufacturer's instructions. Forty-eight hours after transfection, MSC were collected and used for the experiments.

RT-PCR and Quantitative Real-Time RT-PCR

Total RNA was extracted from PTEC and/or MSC using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR and quantitative RT-PCR were performed as described previously.[50,51] Primer sequences are shown in Table 1.

In Vivo Experiments

C57BL6/J female 2-mo-old mice (Charles River Italia S.p.a., Calco, Italy) were used. Animal care and treatment were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n.116, G.U., suppl 40, 18 Febbraio 1992, Circolare No. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, Dec 1987; NIH Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996). Mice were housed in a constant temperature room with a 12-h dark/12-h light cycle and fed a standard diet. AKI was induced by subcutaneous injection of cisplatin (12.7 mg/kg). One day after cisplatin administration, mice were divided in three groups that received an intravenous injection as follows: Group 1, saline ($n = 12$); group 2, si-irrel RNA–transfected MSC (2×10^5 cells; $n = 12$); and group 3, si-IGF-1–transfected MSC (2×10^5 cells; $n = 12$). Mice were killed 4 d after cisplatin administration.[16] Mice in groups 2 ($n = 6$) and 3 ($n = 4$) were administered an intravenous injection of PKH-26–labeled MSC transfected or not with si-irrel or si-IGF-1. Renal function was assessed as BUN in heparinized blood by the Reflotron test (Roche

Table 1. Primers used

Primers used for RT-PCR of mouse IGF-1, TGF-βR1 and -βR2, and IL-10 receptors

Target	Sequence	bp
IGF-1	for 5′-agtgactcggatggcttcgtt-3′	
	rev 5′-tttcacaggaagctcgctctc-3′	600
TGF-βR1	for 5′-tgcccatcgagatctgaagag-3′	
	rev 5′-tcgggtctgagaaccatctgt-3′	400
TGF-βR2	for 5′-tccgacatcagctccacgt-3′	
	rev 5′-tgtatcttgccgttcccacc-3′	600
IL-10	for 5′-ggccctcaaacagtacggaaa-3′	
	rev 5′-aaacactgctcctccgacca-3′	500

Primers used for real-time RT-PCR of mouse IGF-1 and 18S ribosomal RNA

Target	Sequence	nM
IGF-1	for 5′-gaagtccccgtccctatcga-3′	300
	rev 5′-ccttctcctttgcagcttcg-3′	300
18S	for 5′-acggctaccacatccaagga-3′	50
	rev 5′-cgggagtggggtaatttgcg-3′	50

Diagnostics Corp., Indianapolis, IN) at baseline and 4 d. BUN levels >30 mg/dl were considered abnormal. Kidneys were taken for histologic analysis. Normal mice served as controls.

Renal Morphology

Kidney fragments were fixed in Duboscq-Brazil. Paraffin sections (3 μm thickness) were stained with hematoxylin and eosin or periodic acid-Schiff reagent. Luminal hyaline casts and cell loss (denudation of tubular basement membrane) were assessed in nonoverlapping fields (up to 28 for each section) using a ×40 objective (high-power field). Numbers of casts and tubular profiles showing necrosis were recorded in a single-blind manner.

For PKH-26–labeled MSC quantification, samples were fixed in 4% paraformaldehyde for 4 h, infiltrated with 30% sucrose/PBS, embedded in OCT compound, and fresh-frozen in liquid nitrogen. Eight-micrometer-thick sections were stored at −70°C. Sections were fixed in acetone (10 min) and incubated (15 min) with FITC-labeled Wheat Germ Agglutinin (WGA Lectin; Vector Laboratories, Burlingame, CA). Nuclei were stained with SYTOX Green (SelectFX Nuclear Labeling Kit; Invitrogen). Slides were then mounted with Moviol and analyzed for PKH-26–positive cells.

Statistical Analyses

The results are expressed as means ± SEM. Statistical analysis was performed using ANOVA followed by Tukey Cicchetti test for multiple comparisons or non parametric Kruskal-Wallis test, as appropriate. Statistical significance level was defined as $P < 0.05$.

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DISCLOSURES

None.

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