Mesenchymal Stem Cells Are Renotropic, Helping to Repair the Kidney and Improve Function in Acute Renal Failure

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Abstract. Injury to a target organ can be sensed by bone marrow stem cells that migrate to the site of damage, undergo differentiation, and promote structural and functional repair. This remarkable stem cell capacity prompted an investigation of the potential of mesenchymal and hematopoietic stem cells to cure acute renal failure. The model of renal injury induced in mice by the anticancer agent cisplatin was chosen. Injection of mesenchymal stem cells of male bone marrow origin remarkably protected cisplatin-treated syngeneic female mice from renal function impairment and severe tubular injury. Y chromosome–containing cells localized in the context of the tubular epithelial lining and displayed binding sites for Lens culinaris lectin, indicating that mesenchymal stem cells engraft the damaged kidney and differentiate into tubular epithelial cells, thereby restoring renal structure and function. Mesenchymal stem cells markedly accelerated tubular proliferation in response to cisplatin-induced damage, as revealed by higher numbers of Ki-67–positive cells within the tubuli with respect to cisplatin-treated mice that were given saline. Hematopoietic stem cells failed to exert beneficial effects. These results offer a strong case for exploring the possibility that mesenchymal stem cells by virtue of their renotropic property and tubular regenerative potential may have a role in the treatment of acute renal failure in humans.

Acute renal failure (ARF) is a common condition that affects up to 7% of hospitalized patients, especially those in medical and surgical intensive care units (1–4). ARF most frequently ensues upon an ischemic or toxic insult to the kidney and is potentially reversible, however, being often just one element of multiple organ damage. Actually, the mortality rate in hospital-acquired ARF still ranges from 30 to 80%. Modern dialysis techniques, such as continuous renal replacement therapy, had no significant impact on overall mortality. The quest for a pharmacologic therapy that could improve survival after an ARF episode has been largely unsuccessful. Dopamine, furosemide, mannitol, calcium channel blockers, atrial natriuretic peptide, and several other hormonal or pharmacologic substances proved effective in experimental models but almost invariably failed in clinical protocols (5,6).

Dysfunction and loss of tubular epithelial cells play central roles in the process underlying the failure of the kidney after ischemic or toxic challenge (2,7,8). After detachment from the tubular basement membrane, both sublethally injured cells and dead cells (9) can obstruct the tubular lumen, leading to increased intratubular pressure that along with “backleak” of filtrate may contribute to dysfunction (2,7,8). Conversely, the epithelial lining of the tubule has remarkable capacity to recover. In animal models, the rate of recovery depends strictly on the replacement of damaged and/or dead epithelium with a new functioning one. Growth factors, such as IGF-1, hepatocyte growth factor, and epidermal growth factor, have been used consistently to potentiate tubular regeneration in experimental ARF (10,11). Protection may relate both to stimulatory actions on the regenerative potential of surviving tubular cells and to cell “rescue.” One major limit to such healing is the requirement for a critical number of surviving cells to restore structural integrity. An alternative or additional strategy should consider the local supply of new cells to direct the replacement of damaged cells.

Tissue-based stem cells have traditionally been viewed as multipotential precursor cells that are capable of generating tissue-specific differentiated cells (12). In recent years, it has become clear that adult stem cells have remarkable plasticity to the extent that they can differentiate into lineages other than the tissue of origin. After mesenchymal or hematopoietic stem cell transplantation, donor cells have been shown to differentiate into cardiac myoblasts, hepatic epithelium, and neuroectodermal cells (13–19). Adult bone marrow–derived cells can also contribute to renal remodeling (20–22). Chimeric rats carrying enhanced green fluorescence protein bone marrow cells re-
sponded to anti-Thy1 antibody-induced mesangiolysis with a dramatic repopulation of the mesangium by enhanced green fluorescence protein cells, mainly of nonhematopoietic lineages (20). Furthermore, female mice recipients of male bone marrow grafts carried the Y chromosome in up to 7.9% of cortical tubular epithelial cells, indicating that bone marrow cells participated in the normal tubular turnover (21). The possibility that extrarenal cells of bone marrow origin may take part in tubular regeneration in humans rests on findings of Y chromosome–positive epithelial cells in sex-mismatched kidney transplants that sustain damage as a consequence of acute tubular necrosis (21,22). Moreover, recent studies have reported on the role of bone marrow–derived hematopoietic stem cells in the regeneration of the renal tubular epithelium after ischemia/reperfusion in mice (23,24). This newly described pathway of tubular regeneration provides opportunities for therapeutic intervention.

Here we tested the hypothesis that the treatment with mesenchymal stem cells or hematopoietic stem cells of adult bone marrow origin could improve renal function and attenuate tubular injury in ARF induced in mice by injection of cisplatin. As the regenerative process takes place, events that occur during renal development are reactivated as shown by reapparance of mesenchymal molecules (25–28), but the role of extrarenal stem cells has not been fully appreciated. We found that mesenchymal stem cells but not hematopoietic stem cells contributed to restore renal tubule structure and ameliorated renal function.

Materials and Methods

Murine Model of ARF

C57BL6/J female or male mice (Charles River Italia s.p.a., Calco, Italy), 2 mo of age at the start of the experiments, 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 (ECC Council Directive 86/609, OJL 358, Dec 1987; NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Animals were housed in a constant temperature room with a 12-h dark 12-h light cycle and fed a standard diet. ARF was induced in female mice by i.v. injection of cisplatin. As the regenerative process takes place, events that occur during renal development are reactivated as shown by reappearance of mesenchymal molecules (25–28), but the role of extrarenal stem cells has not been fully appreciated. We found that mesenchymal stem cells but not hematopoietic stem cells contributed to restore renal tubule structure and ameliorated renal function.

Isolation and Purification of MSC and HSC

Bone marrow (BM) was obtained from 2-mo-old male C57BL6/J mice. Briefly, mice were killed, and femurs and tibias were aseptically removed. BM was flushed from the shaft of the bone with DMEM medium (Sigma) containing 5% FCS (Invitrogen, Paisley, Scotland) plus penicillin/streptomycin (100 U/ml to 0.1 mg/ml; Invitrogen) and then filtered through a 100-μm sterile filter (Falcon) to produce a single-cell suspension. MSC were recovered from BM by their tendency to adhere tightly to plastic culture dishes, as described previously (29). Filtered BM cells were plated in DMEM plus 10% FCS and penicillin-streptomycin (100 U/ml to 0.1 mg/ml) and allowed to adhere for 6 h. Nonadherent cells were then removed. Medium was changed regularly every 3 d; after 2 to 3 wk, adherent cells were detached by trypsin-EDTA (0.5 to 0.2 g/L; Invitrogen), washed with PBS, and used for the in vivo experiments.

In addition, experiments were performed using cells isolated from male and female mice and injected into C57BL6/J mice. Briefly, blocking with PBS that contained 0.5% BSA (Sigma), cells were incubated for 20 min with rat anti-mouse CD45 antibody, 0.2 μg/10^6 cells (Caltag Laboratories, Burlingame, CA). After washing, cells were incubated with magnetic microbeads coated with goat anti-rat IgG (Miltenyi Biotec, Caldarla di Reno, Bologna, Italy), and CD45^- MSC were isolated by magnetic cell-sorting separation system.

For isolating HSC (30,31), total BM cells were suspended in PBS that contained 1% FCS and incubated on ice with rat anti-mouse mAb specific for the following lineage markers: CD4 (0.125 μg/10^6 cells) and CD8 (0.5 μg/10^6 cells; T lymphocytes), CD45R (0.05 μg/10^6 cells; B lymphocytes), CD11b (0.5 μg/10^6 cells; macrophages), Gr-1 (0.125 μg/10^6 cells; granulocytes), and Ter-119 (0.5 μg/10^6 cells; erythrocytes; Caltag Laboratories). After washings, cells were incubated with magnetic microbeads and magnetically cell sorted as described above. The obtained lineage negative cells (Lin^-), which represented 10% of the total BM cells, were incubated with rat mAb against mouse CD117 (c-kit) PE conjugate (Caltag Laboratories). A wavelength of 488 nm was used to excite c-kit–PE–labeled cells. The c-kit^POS cells were sorted on a FACS Vantage cytofluorimeter (Becton Dickinson, BD Bioscience, Milan, Italy) to obtain purified HSC preparations.

Fibroblast-Like Colony-Forming Unit Assay

Fibroblast colony growth was evaluated on primary cells grown on tissue culture six-well dishes (32). Total BM-derived cells were plated at the density of 25 × 10^5 cells/well. After 7 d, the capability of MSC

Diagnetics, Indianapolis, IN). BUN levels that exceeded 30 mg/dl were considered abnormal (normal range in our laboratory: 14 to 29 mg/dl). Kidneys were harvested and processed for histologic ultrastructural analysis after 1, 2, and 3 d after cisplatin injection. In situ hybridization revealed the expression of Ki-67, a proliferation marker, was also evaluated. Normal mice served as controls. A group of mice that received cisplatin were killed after 1 d for light and electron microscopy studies. At this time, BUN levels were within the normal range. An additional group of mice (n = 12) 1 d after cisplatin received an i.v. injection of MSC immunodepleted of hematopoietic precursor CD45-pos cells (2 × 10^5 cells). Renal function was evaluated before (day 0) and at day 4 after cisplatin and compared with BUN of mice that received saline (n = 10) or MSC (n = 8). Renal histology was assessed at day 4.

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to form fibroblast-like colonies was assessed. Images that showed MSC morphology were acquired by contrast-phase microscope.

**In Vitro Differentiation Assays**

BM-derived cells obtained by plastic adhesion, as described above, were studied to verify their mesenchymal potential to differentiate toward osteoblasts, adipocytes, and chondroblasts. MSC were grown until confluence, and the growth medium was replaced with the inductive medium consisting of Iscove’s modified Dulbecco’s medium (Invitrogen), 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.05 mM β-mercaptoethanol supplemented with specific differentiation reagents as follows.

**Osteogenesis.** Cultures were fed twice a week for 3 wk with 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid 2-phosphate, and 10⁻⁹ M dexamethasone (33). Then cells were fixed with 10% formalin for 20 min at room temperature and mineralization—presence of calcium-rich hydroxyapatite—of the extracellular matrix was assessed by staining for 20 min with 2% wt/vol Alizarin Red S, adjusted to pH 4.1 with ammonium hydroxide (all reagents were from Sigma) (34).

**Adipogenesis.** Cells were incubated for 3 wk with 5 μg/ml insulin (Sigma) and 10⁻⁹ M dexamethasone. Adipogenic differentiation was visualized in phase-contrast microscopy by the presence of highly refractive intracellular lipid vacuoles (33). Oil Red O (Sigma) staining was used to assay the accumulation of lipid droplets in these vacuoles (34).

**Chondrogenesis.** MSC were harvested and 6 × 10⁵ cells were centrifuged to form a pellet on the bottom of a 15-ml polypropylene tube (Falcon). The micromass was cultured in 500 μl of chondrogenic medium that consisted of 50 μg/ml ascorbic acid 2-phosphate and 1 ng/ml TGF-β1 (Sigma) (33). After 3 wk of culture, cell clumps were harvested, embedded in paraffin, cut into 3-μm sections, and stained for glycosaminoglycans using 0.1% safranin O (Sigma).

**Renal morphology. Light microscopy.** Fragments of renal cortex were fixed overnight in Dubosq-Brazil, dehydrated in alcohol, and embedded in paraffin. Sections of 3-μm thickness were stained with hematoxylin and eosin, Masson’s trichrome, or periodic acid-Schiff (PAS) reagent. Slides were scored for the following changes: luminal hyaline casts, tubular cell degenerative changes (cytoplasmic vacuolization, swelling, cell flattening, PAS-positive droplets, nuclear fragmentation, cell debris), and cell loss (denudation of the tubular basement membrane). Nonoverlapping fields of the entire section (up to 28 fields for each mouse) were analyzed at high magnification using a ×40 objective (high-power field [HPF]). Lesions were focal in distribution, and the scores ranged from 0 to 3+ as follows: 0, no changes; 1+, very occasional tubular profiles (usually <3/section and <2/HPF) affected by lesions; 2+, more evident lesions affecting a minor percentage of tubuli in each affected area; 3+, lesions in most tubuli within affected areas. Sections were analyzed by the same pathologist, in a single-blind manner.

**Electron microscopy.** Fragments of kidney tissue were fixed for 4 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and washed repeatedly in the same buffer. After postfixation in 1% OsO₄, specimens were dehydrated through ascending grades of alcohol and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a Philips Morgagni electron microscope.

**Immunohistochemistry and In Situ Hybridization**

To verify the presence of male mesenchymal or hematopoietic stem cells and to characterize their phenotype in the kidney of female recipients, we stained sections for lectin *Lens culinaris* binding sites in the brush border of proximal tubuli and further processed for Y-chromosome detection using in situ hybridization. Renal biopsies were fixed in Dubosq-Brazil, routinely processed, and paraffin wax embedded. Slides were dewaxed by xylene, taken through graded alcohols (100, 90, 70, and 50%) to PBS, and incubated with trypsin (0.1% in CaCl₂ 0.1%) at 37°C for 15 min. After washing with PBS, slides were incubated with biotinylated lectin (Vector Laboratories, Burlingame, CA) for 30 min (1:200), followed by treatment with streptavidin-alkaline phosphatase (1:50). Vector Red substrate (Vector Laboratories) was then applied for 15 min at room temperature. After washing in PBS, the slides were processed using the in situ hybridization protocol as described previously (21). Sections were washed again in PBS and digested with 0.4% wt/vol pepsin (Sigma) in 0.1 M HCl for 10 min at 37°C. For quenching the reaction, 0.2% glycine was used and then sections were postfixed with 4% paraformaldehyde and dehydrated through graded alcohols. Samples were air dried and incubated with FITC-labeled mouse Y-chromosome paint (Star-FISH; Cambio, Cambridge, UK). The probe was added to the sections, and a 10-min incubation at 60°C was performed followed by overnight incubation at 37°C. Sections were washed with 50% formamide/2× SSC and then 0.1× SSC followed by PBS. The sections were incubated with 1/50 peroxidase-conjugated antifluorescein antibody (Boehringer Mannheim), developed in 3,3′-diaminobenzidine, counterstained with hematoxylin, and mounted with either an aqueous body (Boehringer Mannheim), developed in 3,3′-diaminobenzidine, or 60% glycerol. Slides were analyzed under a light microscope. In additional experiments, sections were further stained with PAS reagent after initial examination and removal of the coverslip to stain tubular basement membrane structures for more precise localization of Y chromosome–positive cells.

Proliferating tubular cells were immunohistochemically identified by labeling with mAb against the proliferating cell nuclear antigen Ki-67 (Novoceastra Laboratories, Newcastle, UK) as follows (35). Sections fixed in Dubosq-Brazil were deparaffinized and dehydrated. For obtaining an adequate signal with Ki-67 antibody, the sections were microwaved twice for 5 min in citrate buffer, and for reducing background, they were blocked for 30 min in 1% BSA (Sigma). The sections were incubated overnight with anti–Ki-67 Ab (1:100) at 4°C. After rinsing with PBS, sections were incubated with biotinylated sheep anti-mouse IgG (Chemicon International, Temecula, CA), 1:100 for 30 min at room temperature. Then streptavidin-alkaline phosphatase conjugate (Roche Diagnostic) was applied at a dilution of 1:100 for 30 min. The reaction product was visualized by incubation with Fast Red substrate (Vector Laboratories). Counterstaining was performed by using Harris hematoxylin (Bio-Optica, Milan, Italy). For establishing whether MSC may be proliferating, serial sections of kidneys of selected female mice at 4 d were stained as above for Y chromosome and Ki-67, respectively. In control experiments, the first antibody was omitted or replaced by normal serum. Signals in negative control sections were negative. Ki-67–positive cells within tubuli were counted in 10 randomly selected HPF (×400) for each animal (n = 5 mice for each group).

**Statistical Analyses**

The results are expressed as mean ± SEM. ANOVA with Tukey-Cicchetti test for multiple comparisons was used to analyze BUN data. Histology and immunohistochemistry data were analyzed using the nonparametric Kruskal-Wallis test or Mann-Whitney test, as appropriate. Statistical significance level was defined as *P* < 0.05.
Results

MSC Characterization

MSC after isolation from BM by their adherence to plastic consisted of an heterogeneous cell population with a predominant spindle-shaped morphology and were able to form fibroblast-like colonies (Figure 1a). Under appropriate culture conditions, primary MSC were able to produce extracellular mineral deposition, indicating that MSC possess osteogenic potential (Figure 1b). In addition, MSC differentiated to adipocytes, as indicated by the accumulation of neutral lipid vacuoles, visualized by phase-contrast microscopy or stained with Oil Red O (Figure 1c). Finally, the accumulation of glycosaminoglycans within the extracellular matrix suggested that chondrogenesis had occurred (Figure 1d).

MSC Can Cure ARF

To determine whether MSC and HSC obtained from BM of male C57BL6/J mice ameliorated acute renal dysfunction, we injured the kidney of female C57BL6/J mice with cisplatin, an antitumor drug whose clinical use is accompanied by a high incidence of nephrotoxicity, mainly in the form of renal tubular damage. In the murine model of cisplatin nephrotoxicity, renal function, as measured by serum BUN and creatinine, was impaired between days 4 and 7 after drug administration (10,36). In our setting, subcutaneous injection of 12.7 mg/kg cisplatin induced significant increases in serum BUN, which peaked at days 4 and 5, declined at day 7, and stabilized at days 11 and 29 to values slightly higher than baseline (Figure 2). Intravenous injection of $2 \times 10^5$ MSC obtained from the BM of male mice into syngeneic female mice on day 1 after receiving cisplatin strongly protected renal function at days 4 and 5 as reflected by significantly lower BUN values with respect to cisplatin-treated mice that were given saline (Figure 2).

Next, we investigated whether the renal function improvement by MSC treatment was associated with preservation of tubular structure. By light microscopy, no significant changes were detectable in the kidneys taken 1 d after cisplatin injection. Focal and severe tubular changes were observed at day 4 (Table 1, Figure 3b). Proximal tubuli revealed loss of brush border, cytoplasmic vacuolization, flattening and loss of the epithelial cells, PAS-positive droplets, nuclear fragmentation, and luminal cell debris. Hyaline casts were prominent. Distal nephron segments revealed cytoplasmic swelling, fragmented nuclei, and luminal debris. The most damaged tubuli displayed areas of apparent denudation of the tubular basement membranes. Scores of tubular damage reached the highest values at day 4 (Table 1). At day 7, tubular changes were still present. They recovered within 29 d, except for sparse casts (Figure 3c). Like the effect on renal dysfunction, MSC had an impressive effect on renal pathologic changes. Injection of MSC almost

Figure 1. Photomicrographs of mesenchymal stem cells (MSC) showing differentiative potential. (a) MSC after 1 wk of culture grew in colonies that contained heterogeneous small spindle-shaped fibroblastoid cells and more rounded cells. (b through d) In vitro differentiation of MSC maintained in culture with specific inductive media. The differentiation toward osteoblasts is indicated by the formation of calcium-rich hydroxyapatite detected with Alizarin red (b). MSC-adipocyte differentiation is visualized by highly refractive intracellular lipid vacuoles (c, left) and by Oil Red O staining (c, right). Chondrogenesis was induced by incubating MSC as a micromass pellet in chondrogenic medium. Glycosaminoglycans stained by safranin O indicate chondroblast differentiation (d). Magnifications: ×100 in a; ×40 in b; ×400 in c and d.
completely abrogated tubular damage. In particular, the kidneys of MSC-treated mice that were killed on day 4 after cisplatin exhibited low degrees of proximal tubular cell damage, cast formation, and focal cell loss (Table 1, Figure 3d).

Electron microscopy analysis of the kidneys of all cisplatin-treated mice revealed changes on day 1 at the time of MSC injection, both in proximal tubuli and in segments of the distal nephron (Figure 4, c and d). Tubular cells showed loss of brush border, cytoplasmic vacuolization, apical blebbing, phagolysosomes, and enlarged mitochondria with irregular cristae. Cell lysis and detachment from the tubular basement membrane were prominent in kidneys taken at day 4 after cisplatin injection (Figure 4, e and f). In contrast, the kidneys of mice that received MSC and were killed on day 4 showed much less severe ultrastructural changes. Remarkably, areas of cell detachment were either absent or detectable in just a few scattered tubuli (Figure 4, g and h).

MSC Engraft and Differentiate to Tubular Epithelial Cells

To assess whether the protective effects of MSC in cisplatin-induced ARF could be associated with recruitment of the cells into the renal parenchyma, we analyzed kidneys of cisplatin-injected mice for the presence of Y chromosome by in situ hybridization. The Y chromosome was detected in the majority of cell nuclei in sections of male control mouse kidneys (Figure 5a). No signal was present in kidneys of female controls (not shown) or of the female mice that received an injection of cisplatin and were left untreated (Figure 5b). Sections of kidney cortex from mice that received an injection of cisplatin and were treated with MSC revealed Y chromosome-positive cells (Figure 5c).

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**Table 1. Effect of mesenchymal and hematopoietic stem cells on renal histology**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (days)</th>
<th>Casts</th>
<th>Tubular Cell Degeneration</th>
<th>Cell Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin + saline</td>
<td>4</td>
<td>1.77 ± 0.20f</td>
<td>1.54 ± 0.14f</td>
<td>1.38 ± 0.18f</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.60 ± 0.30g</td>
<td>1.40 ± 0.22e</td>
<td>0.60 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0.33 ± 0.16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>0.43 ± 0.20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cisplatin + MSC</td>
<td>4</td>
<td>0.50 ± 0.19d,g</td>
<td>0.62 ± 0.18c,h</td>
<td>0.12 ± 0.12d,h</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.60 ± 0.24</td>
<td>0.40 ± 0.24c</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0.25 ± 0.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>0.17 ± 0.17</td>
<td>0.17 ± 0.17</td>
<td>0</td>
</tr>
<tr>
<td>Cisplatin + HSC</td>
<td>4</td>
<td>1.67 ± 0.32</td>
<td>1.67 ± 0.21</td>
<td>1.33 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0.33 ± 0.33</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>0</td>
<td>0</td>
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</tr>
</tbody>
</table>

*Histology data are mean scores ± SE. MSC, mesenchymal stem cells; HSC, hematopoietic stem cells.

a Tubular cell degeneration consisted of brush border loss, nuclear changes, vacuolization, periodic acid-Schiff–positive droplets, and cell debris in tubular lumens.

b P < 0.05, d P < 0.01 versus cisplatin + saline at the corresponding time.

c P < 0.05, f P < 0.01 versus cisplatin + saline at 1, 11, and 29 d.

d P < 0.05, h P < 0.01 versus HSC at the corresponding time. At 1 d after cisplatin injection, tubular changes were not observed (casts: 0; tubular cell degeneration: 0; cell loss: 0).
both in proximal tubuli and in distal tubuli in focal areas at day 4. The distribution of Y chromosome–positive cells closely reproduced the focal distribution of injury otherwise found in mice that were given cisplatin. The morphology of the tubuli that contained Y-positive cells was well preserved or even comparable to normal (Figure 5, c through e). Y chromosome–positive cells were aligned within epithelial areas showing well-recognizable brush borders positive for the tubular differentiation marker binding sites for \textit{Lens culinaris} lectin. In addition, Y chromosome–positive cells were focally found in peritubular areas in the proximity of tubular profiles. The localization of the cells in close contact to the outer aspect of the tubular basement membrane (Figure 5e) was confirmed on PAS-stained sections (Figure 5f), possibly consistent with migration into the tubule from the surrounding peritubular structures. Y chromosome–positive cells were also detected within the context of the tubular epithelium on day 29 in cisplatin-treated mice that were given MSC (Figure 5, g and h). These findings suggest local recruitment of MSC through renotropism at sites of injury and provide evidence that mesenchymal cells actively participate to the reconstitution of the differentiated epithelial lining.
MSC Accelerate Tubular Epithelial Cell Regeneration

The effect of engrafted MSC on tubular cell regeneration was explored by analyzing the proliferation marker Ki-67 in cisplatin-treated mice at different time intervals after injection with saline or MSC. In normal control kidneys and in cisplatin-treated mice at day 4, low numbers of Ki-67–positive cells were detected, reflecting low frequency of tubular cell turnover (control, 2.5 ± 0.5; cisplatin day 4, 4.6 ± 1.9 cells/HPF). The numbers of Ki-67–positive cells in kidneys of cisplatin-treated mice were significantly higher since day 11 as compared with normal controls (day 11, 6.2 ± 1; day 29, 24.5 ± 5.1 cells/HPF).

Figure 5. Detection of Y chromosome-positive cells by in situ hybridization in mouse kidney sections simultaneously stained with the tubular cell marker *Lens culinaris* lectin, and comparison with periodic acid-Schiff (PAS) staining in sequential sections to identify relationships with the tubular basement membrane. (a) Male control shows staining for the Y chromosome (brown color). (b) Cisplatin-treated female mouse that received an injection of saline and was killed at 4 d. Tubular damage is severe in one area with extensive disruption of *Lens culinaris* lectin sites (red color). (c through f) Cisplatin-treated mice that received injections of MSC and were killed at 4 d. Signal for Y chromosome is seen in tubular epithelial linings (arrows). Y chromosome–positive nuclei are recognizable in the context of the epithelium of proximal tubuli showing differentiated brush borders (arrows in d and e). One Y chromosome–positive cell is located at the periphery of a proximal tubular profile (arrowhead in e). (f) PAS staining of the same section confirms the localization of the Y–chromosome–positive cells at either the tubular (double arrows) or the interstitial aspect (double arrowheads) of the basement membranes. (g and h) Cisplatin-treated mouse that received an injection of MSC and was killed at 29 d. Magnifications: ×400 in a, b, c, and g; ×1000 in d, e, f, and h.
cells/HPF; \( P < 0.05 \) and \( P < 0.01 \) versus control, respectively). The effects of MSC on cell proliferation over time in cisplatin-treated mice are shown in Figure 6. Injection of MSC into cisplatin-treated mice resulted in a fourfold increase in tubular cell proliferation at day 4 over cisplatin-treated mice that were given saline (\( P < 0.05 \); Figure 6a). With respect to cisplatin-treated mice showing sparse Ki-67–positive cells (Figure 6b), high numbers of Ki-67–positive cells were detected at this time both in proximal tubuli and, to a lesser extent, in distal nephron profiles after MSC injection (Figure 6c). Higher numbers of Ki-67–positive cells within tubuli in MSC transplant recipients were also detected on day 11 (\( P < 0.05 \) versus cisplatin + saline).

Figure 6. Effects of MSC on tubular cell regeneration after cisplatin. (a) Counts of Ki-67–positive nuclei within tubuli of cisplatin-treated mice that were treated with saline or MSC at different time intervals (4, 11, and 29 d). (b through e) Ki-67 staining in kidneys of cisplatin-treated mice at 4 (b) and 29 d (d) and effects of MSC at 4 (c) and 29 d (e). Data are mean \( \pm \) SEM. HPF, high-power field. \( ^{o}P < 0.05 \) versus cisplatin + saline 4 and 11 d; \( ^{*}P < 0.05 \) versus cisplatin + saline at the corresponding time.

Similar degrees of proliferation were recorded thereafter in both groups (Figure 6, a, d, and e). Staining for Y chromosome and Ki-67 on serial sections revealed co-localization of both markers to the same nuclei (Figure 7), indicating actual proliferation of MSC within tubule.

MSC Immunodepleted of CD45\(^{+} \) Cells Protect Cisplatin-Treated Mice against Renal Damage

On the basis of the evidence (32) that murine MSC cultures established from various inbred mice contain heterogeneous populations, including hematopoietic precursors, we performed addi-
tional experiments by injecting an enriched population of MSC immunodepleted of CD45<sup>+</sup> cells (CD45<sup>-</sup> MSC), 1 d after cisplatin. Injection of CD45<sup>-</sup> MSC significantly reduced serum BUN in cisplatin-treated mice at day 4 to levels that were comparable to MSC (CD45<sup>-</sup> MSC, 44.2 ± 5.8 and MSC, 32 ± 4.4 versus saline, 103.6 ± 8.9 mg/dl; P < 0.01). The light microscopy analysis confirmed the protective effect on renal structure (score values: cisplatin+CD45<sup>-</sup> MSC, casts 0.75 ± 0.16; tubular degeneration 0.25 ± 0.16; cell loss 0 versus cisplatin + saline, casts 1.8 ± 0.24; tubular degeneration 1.8 ± 0.24; cell loss 2 ± 0.21; P < 0.01 for all lesions).

**HSC Fail to Protect Cisplatin-Treated Mice against ARF**

To examine whether HSC may have the same protective potential as MSC against renal injury, we assessed the effect of Lin<sup>-</sup> c-kit<sup>POS</sup> HSC purified from BM of male mice. Injection of HSC into female cisplatin-treated mice (2 × 10<sup>5</sup> cells given intravenously on day 1) did not ameliorate renal function, as indicated by serum BUN levels that were similar to those of cisplatin-treated mice that were given saline, at any time points considered (Figure 2). By histology, at day 4 after cisplatin, mice that received HSC had severe tubular changes comparable to those of mice that received saline (Table 1, Figures 3f and 8a). As shown in Figure 8b, occasional Y chromosome–positive cells were localized, mostly in the least severely damaged tubular structures, at day 4 in kidneys of cisplatin-treated mice that received HSC.

**Discussion**

BM stroma-derived MSC are progenitors of skeletal tissue components such as bone, cartilage, hematopoiesis-supporting stroma, and adipocytes (29,37–39). Recent experimental findings have revealed the potential of MSC to differentiate along multiple cell lineages, such as neuronal, myogenic, and hepatocyte-like cells (13,15,17,40). As such, MSC are both an important paradigm of postnatal nonhematopoietic stem cells and an easy source for regenerative therapy.

From adult male mouse BM, we established a cell population with morphologic and functional characteristics of multipotent mesenchymal progenitors. The transplantation of MSC attenuated severe epithelial cell injury in mice with ARF and improved function. This is to the best of our knowledge the first report to show that MSC accelerate the structural recovery of the kidney after ARF and, more importantly, that they confer therapeutic benefit. Functional protection by MSC can possibly be the consequence of the capacity of MSC to engraft the damaged kidney and integrate/differentiate within tubuli. Consistent with this possibility are the present data documenting that Y chromosome-containing MSC were recruited into the kidney. Detection of MSC at day 4 and at day 29 in the context of the well-differentiated tubular epithelial lining indicates that MSC repopulate the tubule, most presumably by recruitment at peritubular sites in which the Y-chromosome MSC were also visualized. MSC exploit their potential to generate tubular epithelial cells, as shown by the simultaneous evidence of specific lectin-binding sites in the proximal tubular brush border. In support of our interpretation, adult BM-derived cells can indeed traffic into the kidney, contributing to tubular renewal in a sex-mismatched mouse model of BM transplantation (21). Moreover, in the setting of acute tubular damage Y chromosome–positive cells are detectable in human biopsy specimens taken from female kidneys upon grafting into male recipients (21,22).

Recently, cell fusion between transplanted donor BM cells and recipient tissue has been claimed as an alternative novel mechanism to transdifferentiation, which can occur in vivo (41) and produce functional cells in liver (42) and brain (43). This is, however, a controversial issue, because in other experimental systems—skeletal muscle and pancreatic islets of Langershans—the cell fusion process has been excluded as a way to explain BM stem cell plasticity (44,45). Whether in our setting MSC-driven regeneration of tubular cells might also occur by steps that suggest cell fusion of MSC with resident cells is an issue that at present cannot be completely ruled out.

Another significant finding of the present work is that MSC engrafting the kidney accelerated to a remarkable extent tubular cell proliferation in response to cisplatin-induced damage as...
shown by high numbers of Ki-67–positive cells within the tubuli at day 4, time at which renal function was ameliorated. The evidence of nuclear colocalization of Y-chromosome and Ki-67 staining in tubuli indicates that at least some MSC may proliferate and directly reconstitute the tubular epithelium. We also suggest that the functional benefit of MSC could be due to their ability to produce growth and trophic factors (46–49). That the local production of factors by stem cells may occur and play a role in tissue repair has been suggested by data in a mouse model of pancreatic regeneration (50). Among those factors, in vitro MSC express hepatocyte growth factor and bone morphogenetic proteins acting to promote mitogenic, antiapoptotic, and morphogenic activities of renal tubular epithelial cells (46,48).

HSC have a clearly defined therapeutic potential in liver, heart, and brain reconstitution (14,16,19). Actually, highly purified HSC can differentiate into mature hepatocytes, restoring biochemical function of the liver in a murine model of tyrosinemia type I (16). In mice with infarcted myocardium, locally delivered Lin− c-kitPOS HSC generated de novo cells exhibiting markers and morphology of myocytes, endothelial cells, and smooth muscle cells, thus ameliorating heart function (14). In keeping with such kinds of differentiation plasticity, we wanted to assess whether purified HSC may have a renoprotective effect in cisplatin-induced ARF. Systemic injection of Lin− c-kitPOS HSC failed to protect cisplatin-treated mice against renal function impairment and tubular damage. Notably, scattered HSC were detected by staining for Y chromosome in tubular structures of cisplatin-treated mice after injection of male HSC, indicating that HSC can localize to the injured renal tissue. However, in the cisplatin model, this occurred to a much lesser extent than with MSC. The rarity of Y chromosome–positive cells in the kidney of mice that received HSC injections could simply reflect that donor HSC cannot survive in the host, possibly because of lack of engraftment in nonirradiated mice. If so, then the clinical application of HSC, albeit considered important, seems to be less relevant in respect to MSC that did not require irradiation to engraft and to exert beneficial effect. One can also speculate that HSC may be more susceptible to the cytotoxic effect of cisplatin or that the kidney exposed to cisplatin may generate chemoattractants with more specific activity toward MSC.

Recent studies have reported that Lin− Sca-1POS c-kitPOS cells engraving the BM of irradiated mice were mobilized by renal ischemia/reperfusion into the circulation and homed specifically to injured regions of the tubule, where they differentiated into tubular epithelial cells (23,24). Consistent with our data, injection of Lin− BM cells had no protective effect on renal function impairment of ischemic mice (24). However, such manipulation limited further worsening of BUN induced by BM ablation in mice with ischemic ARF, suggesting that BM stem cells contributed to spontaneous repair after ischemia.

In summary, our findings indicate that MSC contribute to the recovery of the kidney during ARF. In the context of regenerative therapies, renotropic preparations of autologous adult stem cells can be proposed as a safe strategy in humans. Combined administration with growth factors or molecular engineering of MSC to deliver specific factors to the site of injury would hopefully aid in maximizing their therapeutic potential.

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