Hematopoietic Stem Cell Mobilization Therapy Accelerates Recovery of Renal Function Independent of Stem Cell Contribution

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Acute renal failure and tubular cell loss as a result of ischemia constitute major challenges in renal pathophysiology. Increasing evidence suggests important roles for bone marrow stem cells in the regeneration of renal tissue after injury. This study investigated whether the enhanced availability of hematopoietic stem cells, induced by stem cell factor and granulocyte colony-stimulating factor, to the injured kidney provides an adequate strategy for stem cell–based therapy to counteract renal ischemia/reperfusion injury. It is interesting that cytokine treatment before injury resulted in significant enhancement of function recovery of the kidney. This, however, was not due to increased incorporation of tubular epithelial cells from bone marrow origin. Importantly, cytokine treatment resulted in impaired influx of granulocytes into the injured kidney. Although cytokine treatment improved renal function rapidly after ischemic injury, the results show that the underlying mechanism likely is not based on stem cell transdifferentiation but rather on altered inflammatory kinetics.


Materials and Methods

Mice
Six- to 8-wk-old male and female C57BL/6 mice and transgenic enhanced green fluorescence protein (eGFP) expressing male mice (C57BL/6-Tg[ACTbEGFP]1Osb) were respectively purchased from Charles River (Maastricht, The Netherlands) and Jackson Laboratories (Bar Harbor, ME).

Experimental Procedures
Mice were anesthetized by an intraperitoneal injection of 0.08 mg/ml fentanyl-citrate, 2.5 mg/ml fluanison (Janssen Pharmaceuticals, Beerse, Belgium), and 1.25 mg/ml midazolam (Roche, Mijdrecht, The Netherlands). Both renal arteries were clamped for 45 (wild type [WT]) or 75 min (chimeras) followed by reperfusion, as predetermined by using a range of occlusion times. Sham-operated mice received identical treatment except for clamping of the renal arteries. One hour before mice were killed, 5-bromo-2′-deoxyuridine (BrdU; Sigma Chemicals, Zwijndrecht, The Netherlands) was injected intraperitoneally (50 mg/kg body wt). WT mice (n = 10 per group) were killed at 1, 3, 7, and 14 d after ischemia (shams at day 3), and chimeric mice (n = 8 per group) were killed at 1, 7, 14, or 28 d after ischemia (shams at day 7). Blood samples were obtained via heart puncture and transferred to heparin
BM Transplantation

Total BM was collected from male eGFP mice by flushing femurs and tibiae with sterile PBS that contained 10% FCS (Invitrogen, Breda, The Netherlands) and penicillin (500 units)/streptomycin (500 μg; Invitrogen). Female C57BL/6 mice were lethally irradiated with two doses of 4.5 Gy, divided by 3 h minimally, using a 137Cs irradiator (CIS Bio International, Gif, France). After the last irradiation dose, mice received an intravenous injection of 5 × 10^6 eGFP-BM cells and 2 × 10^7 female WT spleen cells to induce radioprotection in a total volume of 300 μl/mouse.

Six weeks after transplantation, mice received sterile, acidified water (12 × 10^−3 M HCl) that contained 0.16% neomycin sulfate (Sigma Chemicals). Chimeras with donor BM engraftment of 50% or higher received an injection of SCF and G-CSF or saline and were subjected to ischemia.

Cytokine-Induced HSC Mobilization

For inducing mobilization, mice received a subcutaneous injection of 50 μg/kg per d recombinant rat SCF and 200 μg/kg per d recombinant human G-CSF in saline (a gift from Amgen, Breda, The Netherlands). Cytokines were administered daily starting 5 d before induction of ischemia up to 3 d afterward (8). Control mice received an injection only of saline. For analyzing expression of adhesion molecules, cytokines or saline was administered daily for 5 d per group, after which the mice were killed the next day; blood samples were handled as described below.

Antibodies

Phycocerythrin-conjugated antibodies to CD11b, Gr-1, and Sca-1 and allophycocyanin-conjugated antibodies to c-Kit and CD62L were purchased from BD Biosciences (Alphen a/d Rijn, The Netherlands), to very late antigen-4 (VLA-4) were purchased from Cymbus, and to CXCR4 were purchased from Chemicon (both in Chandlers Ford, UK). Rabbit anti-GFP antibody was purchased from Molecular Probes (Leiden, The Netherlands), antibody to F4/80 was purchased from Serotec (Oxford, UK), active caspase-3 was purchased from Cell Signaling Technologies (Beverly, MA), α-smooth muscle actin (α-SMA) was purchased from DAKO (Glostrup, Denmark), pan-cytokeratin was purchased from Sigma Chemicals, CD10 was purchased from Neomarkers (Fremont, CA), and osteopontin was purchased from R&D (Abingdon, UK). Secondary antibodies conjugated to horseradish peroxidase or alkaline phosphatase were purchased from DAKO.

Flow Cytometric Analyses

White blood cells were counted on a Coulter ACT diff2 (Beckman Coulter, Mijdrecht, The Netherlands). Erythrocytes were lysed in 160 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA (pH 7.4). eGFP-BM engraftment efficiency in recipients was determined by analyzing circulating leukocytes for GFP expression. Analysis of adhesion molecules was performed by incubating leukocytes with labeled antibodies for 1 h. Before analysis, cells were fixed in PBS that contained 2% paraformaldehyde. Analyses were performed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

Histology, Renal Function, and Immunohistochemistry

Kidneys were fixed in 10% buffered formalin for 20 h and embedded in paraffin using standard procedures. For examining renal histology, sections (4 μm) were stained with periodic acid-Schiff reagent and hematoxylin. Injury to tubuli was assessed by determining the percentage of affected tubules per 10 fields (magnification, ×400) in the corticomedullary region according to the following criteria: Tubular dilation, epithelial necrosis, cast deposition, and loss of brush border. Injury was graded on a scale from 0 to 5: 0, 0%; 1, <10%; 2, 10 to 25%; 3, 25 to 50%; 4, 50 to 75%; and 5, >75%. For assessing renal function, serum creatinine and urea concentrations were measured by standard diagnostic procedure. For detection of F4/80, active caspase-3, and GFP, sections were boiled in 0.3% citrate buffer (pH 6); for BrdU, sections were treated with 2 M HCl and 0.4% pepsin (Sigma Chemicals). Sections were stained using 3 to 3′ diaminobenzidine dihydrochloride, 3-amino-9-ethylcarbazole, or the Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Peterborough, UK). Counterstaining was performed in a 2% methyl-green solution. For quantifying granulocytes, macrophages, and cellular proliferation (BrdU) or apoptosis of tubular epithelial cells (caspase-3), positive cells were counted per 10 high-power fields (×400) in the corticomedullary region. Osteopontin- and α-SMA–stained sections were analyzed using Image-Pro Plus version 4.5.1 software package from MediaCybernetics (Gleichen, Germany) by determining the percentage of staining in six nonoverlapping fields in the corticomedullary region (×200).

Y Chromosome Fluorescence In Situ Hybridization

Detection of y chromosomes was performed on 6-μm cryostat sections, using a mouse Y chromosome–specific FITC-labeled probe (Starfish; Cambio, Cambridge, UK) following the procedure described by Kanazawa et al. (10). Sections then were washed according to the manufacturer’s instructions. Sections were counterstained with Texas Red–X phallolidin (Molecular Probes) and mounted with Vectashield that contained 4,6-diamidino-2-phenylindole (Vector Laboratories).

ELISA

Frozen kidneys were blended in PBS that contained 1% Triton X-100, 1 mM EDTA, and 1% protease inhibitor cocktail II (Sigma Chemicals). Keratinocyte-derived chemokine (KC), IL-1β, and monocyte chemotactic protein-1 (MCP-1) DuoSet ELISA-kits (R&D Systems) were performed according to the supplied protocols. Cytokine levels were corrected for total protein content per sample using Bio-Rad Protein Assay (Biorad, Veenendaal, The Netherlands).

Statistical Analyses

Results are expressed as means ± SEM. Data were analyzed with the nonparametric Mann-Whitney U Test, using SPSS software (SPSS, Inc., Chicago, IL). Values of P ≤ 0.05 were considered statistically significant.

Results

Cytokine Treatment Improves Renal Function after I/R Injury

To determine whether cytokine-induced HSC mobilization would affect renal function, we subjected mice to bilateral renal ischemia after treatment with either SCF, G-CSF, or saline. Analysis of peripheral blood revealed a 15-fold increase in the total number of c-KIT+Sca-1+ cells in cytokine-treated animals compared with controls (0.34 ± 0.09 versus 0.02 ± 0.01 × 10^6 cells/ml, respectively). Cytokine treatment led to increased numbers of circulating granulocytes compared with controls (2.31 ± 0.62 versus 0.23 ± 0.08 × 10^6 cells/ml, respectively) as has previously been reported (11,12).

Renal function was assessed by the measurement of urea and
creatinine in the serum of the animals. One day after ischemia, serum urea (Figure 1a) and creatinine (Figure 1b) concentrations increased in both groups, indicating loss of renal function. Three days after the induction of ischemia, the renal function of cytokine-injected mice showed a better improvement than that of control animal. Additional animals (n = 10 per group) were operated on and killed at day 3, resulting in similar outcome (data not shown).

To correlate these findings to histology (Figure 2a), we determined the degree of tubular injury by assessing the percentage of damaged tubuli (Figure 2b). Although cytokine-treated animals showed a lower degree of tubular damage, resembling the course of renal function, no statistically significant differences were found in the percentage and degree of injured tubuli in kidney sections from both the cytokine-treated and control animals. The rate of proliferation of TEC (Figure 3a), as determined by BrdU incorporation, and apoptosis (Figure 3b), as determined by staining for active caspase-3, were found not to differ significantly between cytokine-injected and control mice.

Figure 1. Cytokine treatment resulted in earlier recovery of renal function. (a) Serum urea values in cytokine-treated animals (■) were lower at day 3 after ischemia as compared with controls (●; NS, P = 0.08). (b) Cytokine-treated mice (■) showed lower serum creatinine levels 3 d (NS, P = 0.06) and 7 d after ischemia (*P = 0.01) compared with controls (●). Data are expressed as mean and SEM.

No Evidence for Cytokine-Induced Increase in BM-Derived Tubular Epithelium after I/R Injury

To investigate whether the increase of renal function after cytokine treatment is attributable to the recruitment of BM-derived cells into the kidney after I/R injury, we generated chimeras by syngenic transplantation of male eGFP-expressing BM cells into lethally irradiated female WT recipients. In a pilot experiment, the clamping time needed to induce a degree of injury and renal dysfunction comparable to that induced in the nonirradiated animals was appointed at 75 min (as opposed to 45 min for nonirradiated animals). No significant increase in serum creatinine was detected when clamping times of 45 or 60 min were used, confirmed by the lack of significant histologic indications of injury (data not shown).

One week after ischemia, a large amount of GFP+ cells were observed in kidneys from both groups of mice (Figure 4a). Because of their peritubular localization, the vast majority of these cells were not recognized as TEC (Figure 4c) but were mainly leukocytes and myofibroblasts (data not shown). Double immunostainings for GFP and the epithelial marker metalloproteinase CD10 (Figure 4e) or cytokeratins (Figure 4f) identified only a few BM-derived TEC in both groups. Sporadically, glomerular epithelial cells of BM origin were detected regardless of the received treatment (Figure 4d). Two and 4 wk after ischemia, the number of GFP+ cells decreased to levels comparable to sham-operated chimeras, whereas no BM-derived TEC were detected.

To confirm these findings, we determined the presence of Y chromosomes. In accordance with immunostainings for GFP, few Y chromosome–containing TEC were observed 1 wk after injury (Figure 4b), and none were detected 4 wk after induction of ischemia, although some clustered peritubular donor-derived cells were found.

Cytokine Treatment Leads to Impaired Granulocyte Migration to the Injured Kidney

I/R injury is characterized by the influx of granulocytes that are deleterious for renal function (13–15). Although cytokine treatment results in increased levels of circulating granulocytes, infiltration into the injured kidneys of cytokine-treated mice by granulocytes was significantly less than in control mice at 1 d after ischemia (Figure 5a), as assessed by immunostaining (Figures 5, b and c). At later time points, the number of granulocytes in both groups was found to converge and returned to low, normal levels in both groups of animals (Figure 5a).

Cytokine Treatment Results in Impaired Adhesion of Granulocytes but Not of HSC

Because G-CSF has been reported to have anti-inflammatory properties, consisting of lowering secretion of proinflammatory cytokines (16), we measured the concentrations of IL-1β and the chemokine KC in kidney homogenates. No differences in concentrations of both IL-1β and KC were detected.

To investigate whether differences in expression of adhesion molecules at the day of injury are responsible for the impaired influx of granulocytes after cytokine treatment, we analyzed granulocytes for expression of L-selectin and CD11b. Circulat-
ing granulocytes from cytokine-treated animals showed significant lower expression of L-selectin, whereas expression of CD11b was unaffected compared with controls (Figure 6a). Likewise, c-KIT Sca-1+ cells were examined for expression of CXCR4, CD11b, and VLA-4. In contrast, cytokine treatment did not result in altered expression of the chemokine receptor CXCR4 and the integrins CD11b on VLA-4 by c-KIT Sca-1+ cells (Figure 6b).

Figure 2. The progression and resolution of ischemic renal injury and organ reperfusion. Left and right columns display, respectively, representative examples of periodic acid-Schiff–stained kidney tissue sections from saline- and cytokine-treated mice. Ischemia was induced for 45 min by clamping of both renal arteries. (a) Animals were killed, and renal histology was examined. After 24 h, tubular dilation in the corticomedullary area was observed as well as migration of inflammatory cells into the interstitium. At the third day after ischemia, injury had progressed and resulted in necrosis of tubular epithelial cells (TEC), leading to denudation of tubuli. One week after induction of ischemia, the normal tissue histology had been replaced by areas of dedifferentiated cell types. Two weeks after the initial ischemic insult, kidney morphology had returned to normal; only few tubules showed signs of minor damage. (b) Semiquantitative scoring of the percentage of damaged tubuli revealed no significant difference in tissue damage between controls (▫) and cytokine-treated animals (●). Data are expressed as mean and SEM.

Figure 3. Cytokine treatment in mice (●) did not affect overall TEC proliferation or apoptosis compared with saline-injected animals (▫). (a) No statistically significant differences in the number of 5-bromo-2-doxuryridine–positive TEC were observed between both groups at all time points. (b) Similarly, no significant differences were detected in the number of apoptotic TEC present after ischemia between both groups. Data are expressed as mean and SEM.
Cytokine Treatment Leads to Increased Macrophage Influx into the Injured Kidney

Because in this study cytokine treatment seemed to affect the migrational potential of leukocytes, we also determined the presence of macrophages. Infiltration of macrophages peaked at 7 d after ischemia and was significantly higher in kidneys from cytokine-injected mice compared with controls (Figure 7a).

MCP-1 is an important chemokine associated with increased macrophage numbers in kidney diseases (17). Therefore, MCP-1 levels were measured in renal homogenates. No significant difference was detected in MCP-1 concentrations between both groups (data not shown).

Osteopontin is a glycoprotein that is expressed on TEC during I/R injury and also implicated attraction and binding of macrophages (18,19). Immunostaining revealed a significant increase in expression of osteopontin by TEC in the corticomedullary area in the kidneys of cytokine-treated mice compared with expression in controls at day 7 after ischemia, coinciding with the increased level of macrophages (Figure 7b).

Macrophages are correlated with the progression of fibrosis
Discussion

Tissue regeneration after injury, based on the contribution by BM-derived stem cells, is thought to offer an attractive and practical solution to organ failure in patients but may be limited by the often low and biologically insignificant involvement of these newly formed stem cell–derived tissue cells to the function of the target organ. SCF- and G-CSF-mediated mobilization has been proposed to present a potential answer by increasing the availability of HSC to the target organ. In a previous study, amelioration of heart function was observed in an experimental model of myocardial ischemia after treatment with SCF and G-CSF (8). In accordance, our results show that cytokine treatment based on SCF and G-CSF accelerates renal function recovery after ischemic injury. However, the underlying mechanism for the observed effect is not based on increased HSC or other BM stem cell involvement but rather on altered characteristics of the inflammatory processes after the initial ischemic insult.

Despite the 9.5-fold increase in circulating granulocytes after cytokine treatment, the number of granulocytes present in the injured kidney was reduced by a factor of 3.2 compared with saline-treated mice. This finding is likely to be responsible for the rapid improvement of renal function in mice that were treated with SCF and G-CSF, because postischemic granulocyte recruitment into the kidney has been recognized as a key factor in the early development of acute ischemic renal failure (13, 21). Adhesion of leukocytes is initiated by the interaction between L-selectin and its ligands on the activated endothelium; subsequent interactions between integrins expressed on the leukocyte and cellular adhesion molecules on the endothelium result in tight adhesion (22). Furthermore, L-selectin is implicated in so-called secondary tethering of granulocytes to already adherent cells (23). Although L-selectin has been shown to be important in I/R injury in different tissues, such as the liver (24), skeletal muscle (25), and heart muscle (26), its role in renal I/R injury is controversial (15). Integrins implicated in the inflammatory response to renal ischemia are the β2 integrins CD11a and CD11b (27, 28), which are known to bind to intercellular adhesion molecule-1 on the endothelium. This binding may ultimately result in transmigration of the leukocyte across the endothelial monolayer and the underlying extracellular matrix into the renal interstitium. Previous reports demonstrate G-CSF–mediated downregulation of L-selectin on neutrophils in humans (29, 30) and release of CD11b from granules in vitro (31). In our model, cytokine treatment resulted in downregulation of L-selectin on granulocytes at the day of injury, whereas expression of CD11b was unaltered. Importantly, IL-1β and KC were not affected by the cytokine treatment. Therefore, the lower degree of migration of granulocytes to the injured kidney, despite their increased numbers in the circulation, is likely the result of decreased selectin-mediated rolling of these cells on the endothelium, which leads to an incapability to adhere and is not the result of suppression of inflammatory processes as a whole.

A recent report (32) described mobilization of HSC by means of cyclophosphamide and G-CSF on I/R injury. It was concluded that this mobilization regimen increased renal damage as a result of neutrophilia. The discrepancy between both studies is probably attributable to the type of mobilization therapy. The short G-CSF course is most likely the reason for the increased migration of granulocytes in the circulation observed by Tögel et al. (32), contrasting with our study in which a longer G-CSF mobilization course combined with SCF was used, which resulted in impaired migration capacity of granulocytes without increasing tissue damage or affecting HSC adhesion. Furthermore, they did not investigate whether HSC migrated to the kidney and whether successful transdifferentiation had occurred. The conclusions of both studies are opposite, but both illustrate the important role of granulocytes in early tissue damage during the onset of I/R injury.

Mobilization of HSC by G-CSF has been shown to be accompanied by downregulation of the integrin VLA-4 and the cytokine receptor CXCR4. We hypothesized that c-KIT+Sca-1+ cells use an adhesion mechanism comparable to that used in homing of HSC to the BM, which involves VLA-4 (33) and CXCR4 (34), the latter of which has also been shown to be involved in the successful migration of BM-derived cells toward the injured kidney and is not the result of suppression of inflammatory processes as a whole.
which indicates that normal adhesion of c-KIT expression of VLA-4, CD11b, and CXCR4, explain the low degree of HSC transdifferentiation. No differ-

bility could lead to impaired migration of HSC as well as Therefore, we investigated whether the cytokine-induced mo-

liver (35). In addition, expression of CD11b was examined. Therefore, we investigated whether the cytokine-induced mo-

bilization could lead to impaired migration of HSC as well as explain the low degree of HSC transdifferentiation. No differ-

ence was observed in expression of VLA-4, CD11b, and CXCR4, which indicates that normal adhesion of c-KIT+ Sca-1+ cells can take place.

In contrast to impairment of granulocyte migration, we ob-

served an increased macrophage influx in cytokine-treated an-

imals. Macrophage influx during renal injury has been linked to progressive fibrosis by the accumulation of myofibroblasts (20). Attraction of macrophages seemed not to rely on MCP-1 production but rather on increased expression of the glycoprotein osteopontin, which is a well-described macrophage attractant (19) and is likely responsible for the increased influx and re-

tention of macrophages in the kidneys of cytokine-treated mice. Osteopontin is expressed by TEC after ischemia and is associ-

ated with cell survival (19) and thus may also contribute to recovery of renal function. Previously, different cytokines have been shown to induce upregulation of osteopontin in vitro (19), which also indicates that the influx of macrophages is induced by the upregulation of osteopontin in response to cytokine treatment and is not the result of HSC differentiation as a consequence of enhanced mobilization from the BM.

In our study, a small fraction of all TEC was of BM origin in both cytokine-treated and control animals. This is in accordance with previous studies (3,4), although these studies demonstrated a more substantial contribution of BM cells to tubular regeneration. One week after injury, we did detect GFP+ cells coexpressing the marker endopeptidase CD10 or forms of cy-

tokemulators and were part of a tubular structure. At later time points after injury, however, no GFP+ TEC were detected. To rule out the possibility that donor-derived cells were over-

looked as a result of loss of expression of GFP, we performed fluorescence in situ hybridization for Y chromosomes, which gave similar results. Thus, we conclude that in our model, BM contribution to TEC after injury is only modest and most likely transient. More recent studies concerning the same model used by Orlic et al. (8) reported less significant outcomes (36,37), and results are attributed to enhanced angiogenesis or neovascular-

ization. In agreement, no BM-derived cardiomyocytes were detected upon cytokine treatment using Flt-3 ligand and gran-

ulocyte/macrophage colony-stimulating factor (38).

A surprising feature that arose during a pilot experiment for this study was the increased clamping time of the renal arteries of chimeric animals needed to result in decreased renal func-

tion. Full-body ionizing radiation before BM transplantation induces oxidative stress in the exposed kidney (39). The ability of the kidney to respond and adapt in response to previous oxidative injury such as ischemia is termed preconditioning and leads to decreased injury upon a new ischemic insult (40). Therefore, it is likely that full-body irradiation confers some degree of renal protection as a result of preconditioning.

In light of recent studies concerning the plasticity of HSC, we investigated whether therapy that is based on the increased availability of HSC would provide a potential therapeutic inter-

vention in the treatment of renal I/R injury. Here we show that the beneficial effect of cytokine treatment on renal function after I/R injury is not based on mobilization and increased circulation levels of HSC but rather on decreased granulocyte influx in the damaged kidney. Although we did observe some BM-derived TEC, we do not consider the level of their contri-

bution biologically relevant. Nevertheless, here we described one of the first attempts of stem cell–based therapy using SCF and G-CSF in the treatment of renal I/R injury offering new leads for future studies.
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References


