Hematopoietic Stem Cell Mobilization–Associated Granulocytosis Severely Worsens Acute Renal Failure

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Abstract. Acute renal failure (ARF), resulting from ischemic or toxic insults, remains a major health care problem because of its grave prognosis and the limited effectiveness of available treatment modalities. On the basis of the recent demonstration that hematopoietic stem cells can differentiate into renal cells and the authors’ observation here that ARF results in a rise in peripheral CD34+ cells, the authors tested whether a further increase in circulating stem cell numbers, induced by their mobilization from the bone marrow, would improve renal function and outcome in mice with ischemic ARF. Unexpected, it was found that the boosting of peripheral stem cell numbers failed to exert any renoprotective effects but rather was associated both with greatly increased severity of renal failure and mortality. Because identical ischemic injury in neutropenic mice resulted in milder renal insufficiency and significantly reduced mortality, it was deduced that the adverse effects of pharmacologic stem cell mobilization are primarily mediated by the concomitant induction of marked granulocytosis. In this manner, high numbers of activated granulocytes seem to obscure the potential renoprotective and positive survival effects of pluripotent hematopoietic stem cells, mediated by both their injurious renal and systemic actions. The data strongly argue against the clinical use of granulocytosis-inducing hematopoietic stem cell mobilization protocols for the prevention or treatment of ischemic ARF. Additional caution with this regimen may be warranted in patients with underlying renal insufficiency and those who develop renal insufficiency while undergoing stem cell mobilization in preparation for an autologous bone marrow transplant.

Clinical acute renal failure (ARF) is characterized by an abrupt decline in renal function caused by ischemia (50%), nephrotoxic injury (35%), interstitial nephritis, or acute glomerulonephritis (15%) (1). Although 50% of the cases appear in multimorbid patients, ARF is an independent determinant of morbidity and mortality (2). Treatment options are essentially limited to supportive measures; as a consequence, recovery of adequate function relies almost exclusively on the kidney’s exceptional autoregenerative capability. The latter, however, seems to be inadequate in severe forms of ARF, which explains why clinical outcomes remain dismal and why the urgent need for the development of new treatment modalities exists.

Recent stem cell research shows that hematopoietic stem cells (HSC) and other tissue-specific stem cells are capable of crossing tissue and even germ-line barriers and thus can give rise to a remarkable range of cell types (3). This plasticity of stem cells is thought to be useful in therapeutic strategies designed to enhance tissue regeneration after severe organ injury.

Anderson et al. (4) proposed a model of endogenous tissue regeneration according to which resident stem cells and, to a lesser extent, blood-derived stem cells physiologically repair destroyed tissue, and as the need for tissue repair rises with increasingly severe organ injury, circulating stem cells would progressively assume the task of tissue repair that endogenous stem cells may not be able to meet. Applied to the kidney, in severe ARF the kidney’s autoregenerative capacity thus may be inadequate for prompt and adequate reconstitution of functional tissue, thereby resulting in permanent loss of renal function.

It was shown recently that the number of circulating Sca-1–positive HSC is increased after ischemic ARF (5). However, their precise impact on the process of regeneration remains unknown. An HSC mobilization procedure used to achieve “sufficient” stem cell numbers in the circulation uses cytokines and is commonly used in autologous bone marrow transplantation in cancer patients. Stem cell populations that can be mobilized include HSC (6), mesenchymal stem cells (7), angioblasts (8), and smooth muscle progenitor cells (9). It is of note that routinely used clinical HSC mobilization regimens not only mobilize stem cells into the circulation but also greatly increase circulating leukocyte numbers; the functional impact of high leukocyte numbers on ischemic tissue, however, remains controversial (10).

Although there is no definite stem cell population identified in the kidney to date, HSC have been shown to contribute to the population of tubular epithelial and glomerular mesangial
cells (11,12). Normally, circulating HSC numbers are exceptionally low, but they can be increased, as in current clinical practice, by mobilization from the bone marrow with granulocyte colony-stimulating factor (G-CSF), stem cell factor, or cyclophosphamide (13). A recent study reported in a myocardial infarction model in mice that increasing circulating HSC by mobilization with stem cell factor and G-CSF significantly improved subsequent myocardial repair, function, and survival (14). The authors suggested that this beneficial effect was due to differentiation of circulating HSC into cardiomyocytes at the site of injury, a view supported by their earlier experiments in which purified HSC were injected directly into the injured myocardium (15). More recently, Kale et al. (5) showed that bone marrow stem cells, in principle, are able to support directly the repair of damaged tubular epithelial cells in ARF and suggest that therapeutic strategies aimed at enhancing the circulating stem cell pool as proposed by Anderson et al. (4) might be of therapeutic utility. However, mobilization of HSC by currently used clinical protocols results not only in an up to 300-fold increase in circulating stem and progenitor cells but also in a massive increase of leukocytes, most of them granulocytes.

It is well established that ARF has a prominent inflammatory component, yet some controversy exists regarding the exact role that different leukocyte populations have in the pathophysiology of ischemic ARF (16). Nevertheless, it is believed that increased circulating leukocyte numbers aggravate renal damage.

Our present study was designed to investigate the influence of a commonly used HSC mobilization regimen on the course of ischemic ARF, with a primary focus on the elucidation of the roles that circulating HSC and peripheral leukocyte numbers play, respectively. We found that increasing the circulating HSC pool with cyclophosphamide and G-CSF greatly increases severity of and mortality in ARF. We conclude that this adverse outcome is principally mediated by the associated granulocytosis, because neutropenic mice developed milder renal insufficiency and had much lower mortality rates. Finally, we believe it advisable that HSC mobilization protocols that lead to marked leukocytosis be avoided in patients with ARF and possibly those with underlying renal insufficiency.

Materials and Methods

Animals

For all studies adult male FVB mice (FVB/NJ; Jackson Laboratory, Bar Harbor, ME), weighing 20 to 25 g, were used in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Animals were maintained at a 12-h day and 12-h night cycle. They ate a regular mouse diet and had free access to water.

Ischemic ARF

Animals were anesthetized with isoflurane on an EZ Anesthesia system animal inhalation machine (Euthanex, Palmer, PA) and kept on a water-perfused temperature-regulated pad to maintain their core temperature at 37°C throughout surgery. ARF in mice was induced, using a transabdominal approach, by clamping of both renal pedicles for 60 min with nontraumatic microvascular clamps (Roboz, Gaithersburg, MD). Reperfusion was confirmed visually before the abdominal incision was closed. All mice received 0.5 ml of saline subcutaneously at the end of surgery to replace fluid losses. Pilot experiments in control mice showed that 60 min of bilateral ischemia induces severe ARF with a serum creatinine rise to 1.5 to 2.2 mg/dl and a mortality of ~60% on day 3. Shorter clamping times resulted in mild renal injury, and longer clamping times resulted in death of all animals. Therefore, 60 min of bilateral ischemia was chosen to induce severe renal failure. Blood samples for serum creatinine measurement (Beckman Creatinine Analyzer; Beckman Instruments, Palo Alto, CA) were obtained from the tail vein before ischemia and once daily thereafter. For determination of leukocyte numbers, retro-orbital sinus blood from anesthetized animals was analyzed by an automated Coulter Counter (Beckman Coulter, Fullerton, CA).

Mobilization of HSC by ARF

For assessing whether ischemic ARF per se results in a rise in peripheral HSC numbers, ARF was induced as above and CD34+ cell numbers were determined by FACS analysis in control and in ARF animals at 24 h after ARF.

Pharmacologic HSC Mobilization

HSC were mobilized with cyclophosphamide (200 mg/kg intraperitoneally) given on day 1 and G-CSF (125 μg/kg twice daily subcutaneously) given on days 4 to 6. For measuring stem and progenitor cell mobilization kinetics, assays of colony-forming units of cells were performed on days 5, 6, and 8 after the start of cyclophosphamide. Peripheral blood was collected from the retro-orbital sinus of anesthetized mice, and 2 × 10^6 leukocytes were seeded into 3 ml of MethoCult murine methylcellulose medium (StemCell Technologies, Vancouver, British Columbia, Canada), according to the manufacturer’s guidelines. Colony numbers were counted on day 12.

Study Groups

Six groups (G1 to G6) of animals were examined: G1, control ARF with 60 min of renal pedicle clamping and no further treatment (n = 9); G2, HSC mobilization followed by ARF on day 6 after mobilization start (n = 6); G3, animals with neutropenia induced by cyclophosphamide (150 mg/kg twice daily intraperitoneally on 2 subsequent days) and ARF (n = 7); G4, sham-operated animals without ARF and without mobilization (n = 6); G5, HSC mobilization without ARF (n = 4); and G6, ARF after a short course of cyclophosphamide (200 mg/kg given on 2 consecutive days) and G-CSF (250 μg/kg subcutaneously) for the next 3 d (n = 6). In this group, ARF was induced at day 3 after cyclophosphamide, a time at which neither significant leukocytosis nor HSC mobilization had yet occurred. All animals were monitored for 3 d.

Histology and Injury Scores

We stained coronal sections of fixed kidneys with hematoxylin and eosin and scored the degree of tubular injury in randomly selected inner cortical fields, using a previously reported method (17). Accordingly, one of us used a graticule grid with 25 squares with a ×20 objective, and 100 intersections between tubular profiles and the grid for each kidney were examined. A score for each tubular cross-section per intersection was assigned: 0, normal histology; 1, tubular cell swelling, loss of brush border, nuclear condensation (apoptosis), up to one third of tubular cross-section showing nuclear loss (necrosis); 2, same as for score 1, except for greater than one third and less than two thirds of nuclear loss per tubular cross-section (necrosis); 3, greater than two thirds of tubular cross-section shows nuclear loss (necrosis).
The total score per kidney was calculated by addition of all 100 scores with a maximum possible injury score of 300.

Immunocytochemistry on kidney tissues was performed on paraffin sections after antigen retrieval, followed by staining with a primary antibody against ED-1, a macrophage marker (BD Pharmingen, San Diego, CA). Mouse spleen sections were used as positive control.

**Statistical Analyses**

Data are expressed as means ± SEM. Differences between data means were analyzed by paired or unpaired \( t \) test, where appropriate. \( P < 0.05 \) was used to indicate significant differences between data means.

**Results**

Induction of ARF increased peripheral circulating CD34+ numbers at 24 h after reperfusion to 6% compared with 1.7% observed in control animals (\( n = 2 \)) as determined by FACS analysis, an observation also made by another group (5).

HSC mobilization with cyclophosphamide and G-CSF resulted in a significant increase in circulating peripheral blood stem and progenitor cells measured by colony-forming units of cells, with highest numbers on day 6 after the first dose of cyclophosphamide (Figure 1). There was also a concomitant increase in peripheral leukocyte numbers up to \( 7 \times 10^4/\mu l \) (Figure 2) and a shift from lymphocyte- to a granulocyte-predominant pattern. Leukocyte numbers in all experimental groups were measured on day 1 after ARF or sham surgery, and mobilized ARF animals (G2) had significantly higher numbers of peripheral white blood cells than all other groups (Figure 2).

We next determined whether the increase in circulating peripheral stem cell numbers has an impact on ischemic ARF. Control ARF animals (G1) had severe renal failure with a significant increase in serum creatinine from control levels of 0.4 to 1.5 ± 0.1 mg/dl (± SEM) on day 1, 1.6 ± 0.3 mg/dl on day 2, and 0.7 ± 0.1 mg/dl on day 3 (Figure 3). Renal function

![Figure 1](image1.png)

**Figure 1.** Colony-forming units of cells (CFU-C) numbers (No.) in peripheral blood of mice mobilized with cyclophosphamide and granulocyte colony-stimulating factor (G-CSF). The peak number of circulating stem and progenitor cells, derived from \( 2 \times 10^4 \) seeded leukocytes, occurred on day 6 after mobilization start, the time point subsequently chosen to induce ischemic acute renal failure (ARF). Normal mice had 0 to 3 CFU-C (not shown).

![Figure 2](image2.png)

**Figure 2.** Peripheral blood leukocyte numbers on day 1 after induction of renal ischemic injury or sham surgery. Mobilized animals (group 2 [G2]) had a significantly higher number \((*P < 0.05)\) of circulating white blood cells compared with all other groups (G1, G3, G4, and G6).

![Figure 3](image3.png)

**Figure 3.** Renal function after stem cell mobilization. Serum creatinine (mg/dl) was significantly increased \((*P < 0.05)\) in the group of hematopoietic stem cells (HSC) mobilized animals (black; G2) at all time points versus all other groups. Neutropenic ARF mice (red; G3) had an insignificantly better renal function compared with the control ARF group (white; G1), whereas sham-operated animals (green; G4) had no increase in serum creatinine. G-CSF/cyclophosphamide short course ARF animals (blue; G6) had the same severity of renal failure as the control ARF group (G1).

in animals with HSC mobilization and ARF (G2) was significantly worse than in control ARF animals(G1) throughout the study, with a serum creatinine of 2.2 ± 0.2, 3.4 ± 0.2, and 3.1 mg/dl on days 1, 2, and 3, respectively.

To assess the influence of high circulating white blood cell numbers on the course of ARF, we subjected neutropenic ARF
animals (G3) to partial bone marrow ablation, using a higher dose of cyclophosphamide (150 mg/kg intraperitoneally on 2 consecutive days), which results in peripheral leukocytopenia. ARF was induced at the nadir of the leukocyte count at day 4, and animals were followed for 3 d. These neutropenic ARF animals (G3) had insignificantly better renal function on day 1 versus control ARF animals (G1); however, recovery of renal function, as assessed by serum creatinine, was delayed in parallel with the return of peripheral leukocyte numbers (Figure 3). Sham-operated animals (G4) had no change in renal function and no mortality. HSC mobilization without ARF (G5) in mice that were given G-CSF and cyclophosphamide also had no impact on kidney function as determined by serum creatinine (data not shown).

For determining the impact of cyclophosphamide and G-CSF alone, i.e., without HSC mobilization, on the severity of ARF, animals in G6 were subjected to a short course of cyclophosphamide and G-CSF, and ARF was induced on day 3 after start of treatment. In this manner, animals were exposed to both drugs, but HSC mobilization and granulocytosis had not yet occurred when ARF was induced. This treatment had no effect on the severity of ARF and animal survival, i.e., both were not different from those in G1 (data not shown).

Mortality, a hard end point in ARF, was significantly higher in animals that were subjected to HSC mobilization and ARF on day 3 compared with control ARF (G1) and neutropenic ARF (G3) animals (P < 0.05). In fact, neutropenic ARF animals were significantly protected from the consequences of severe ARF, reflected by their low mortality of only 15% compared with 63% in control ARF animals (G1) and 84% in mobilized ARF animals (G2; Figure 4A). Renal injury scores were highest in HSC mobilized animals with ARF (300 in G2 versus 244 ± 37 in G1; P < 0.05; Figure 4B). The increase in histologic kidney injury score in mobilized animals with ARF (G2) was furthermore associated with massive infiltration of polymorphonuclear granulocytes in the renal cortex and outer medulla and was most extensive in capillaries surrounding necrotic tubules (Figures 4C and 5).

Because macrophages are thought to play a role in ischemia/reperfusion ARF (18), we assessed their presence in the renal infiltrates by ED-1 immunocytochemistry and detected, on day 3, only very low numbers among the extensive inflammatory cell infiltrates (Figure 6).

Discussion

Our results have significant clinical implications. Most important, they demonstrate that an increase in circulating leukocyte numbers worsens renal tubular injury, renal function, and animal survival. ARF has an important inflammatory component that contributes to renal damage (18). This is illustrated by the fact that HSC mobilization-induced leukocytosis greatly aggravated ARF, whereas neutropenic ARF animals had a significantly lower mortality and slightly better renal function. The main cell type involved in increasing tissue injury in this model is the granulocyte, whereas infiltrating macrophages seem to play only a subordinate role.

A number of studies have shown that by reducing intrarenal neutrophil accumulation, by generation of either adhesion molecule–deficient mice or other depletion methods, ischemic renal injury is alleviated (19,20). Those results are corroborated by our data, showing that neutropenic mice had a better survival. However, the influence of markedly elevated circulating granulocyte numbers on the severity of ischemic ARF has not been systematically investigated to date. Although it is clear from the literature and our data that ischemic renal injury can occur in the absence of neutrophils, our results clearly show that high neutrophil numbers at the time of reperfusion
cause greater renal damage and higher mortality rates, the latter likely as a result of adverse systemic effects.

The identity of the inflammatory cell types that mediate the aggravation of ischemic renal injury in ARF remains controversial (16). Whereas some investigators have presented data demonstrating the involvement of T cells, others have shown that Rag-deficient mice are not protected against ARF (21,22). Macrophages have been shown to be involved later in the course of ARF (18); however, our data do not show an increased number of infiltrating macrophages at 72 h after induction of ARF. The number of circulating neutrophils in our “mobilized” animals was greatly increased, and it has been previously demonstrated that the mobilization protocol used in this study results in activation and enhanced adhesion of mobilized granulocytes (23), both responses that likely contribute to the noted aggravation in kidney injury and escalation in mortality in the “mobilized” group, respectively.

Our data show the highest mortality rate of 84% at day 3 in mobilized ARF animals, whereas neutropenic ARF animals had a mortality rate of only 15%, and this despite slower recovery of renal function and an almost comparable degree of histologic injury. We suggest that this marked difference in outcome is significantly mediated by adverse systemic effects elicited by the large number of activated neutrophils. Specific sequelae of this response, as has been proposed, include increased leukocyte infiltration and adherence in vital organs such as the lungs and heart, higher cell loss as a result of apoptosis, and raised local and systemic levels of proinflammatory cytokines (24).

A potential limitation of our study in showing renoprotective effects of increased numbers of circulating stem cells in the injured kidney could be the relatively short time span of 3 d between induction of ARF and assessment of outcome (recovery or death). This may be a valid concern, because we have shown that transdifferentiation of HSC into renal epithelial cells, one of the cell types to be “replaced” by mobilized stem cells, requires >3 d (25). As a consequence, we would not expect that incompletely differentiated HSC would be able to significantly replace damaged tubular cells, assume their function, and thus improve renal function. In addition, the very high degree of tubular cell necrosis and microvascular compromise seen in our experimental animals may have limited or blocked the delivery of pluripotent hematopoietic stem cells to the site of injury, thereby preventing the postulated expression of the repair-potentiating effects of HSC. Nevertheless, the time frame of our study is directly relevant to the ongoing search for novel interventions that afford protection in the early phase of severe clinical ARF. In this regard, our data show clearly that stem cell mobilization protocols that are routinely used in clinical autologous stem cell transplantation are unsuited for the prevention of ARF in patients.

A recent study described the spontaneous mobilization of Sca-1-positive HSC into the circulation after induction of renal ischemia and suggested a lower regenerative capacity of the ischemic kidney after previous bone marrow ablation by radiation, a negative response that was mitigated by previous bone

Figure 5. Hematoxylin and eosin–stained histologic sections of kidney inner cortex and outer medulla. Kidney section from control ARF (G1; A), from HSC mobilized ARF (G2; B), and from neutropenic ARF animals (G3; C). Note the massive capillary accumulation of granulocytes (black arrow) surrounding necrotic tubules (arrowhead) in HSC mobilized animals with ARF (B), whereas in neutropenic ARF animals (C), granulocyte infiltrates are essentially absent. Magnification, ×400.
marrow transplantation (9). These data are in support of the concept that HSC play a significant role in renal repair.

The contribution of HSC per se to the renal tubular cell population has been shown (3,9), and their organ protective effects, even when mobilized with associated leukocytosis, have been suggested in a myocardial infarction model in the mouse induced by permanent coronary artery ligation (5). It seems from these and other data that the experimental model investigated plays an important role in determining outcome. Specifically, when posts ischemic reperfusion was allowed in a myocardial infarction model in nonhuman primates, preconditioning with HSC mobilization significantly worsened outcome (26), i.e., a negative response similar to that seen in our present ischemia/reperfusion model of ARF.

We conclude, therefore, that our data strongly argue against the use of unmodified HSC mobilization protocols in ARF. Furthermore, caution is advisable, we believe, when patients have underlying renal disease or develop renal insufficiency while undergoing HSC mobilization that is accompanied by granulocytosis. Under these circumstances, HSC mobilization should not be undertaken or interrupted until renal function has normalized, respectively. We believe, however, on the basis of our own recent observations (25) that treatment strategies that augment circulating stem cell numbers without concomitant granulocytosis may prove both safe and promising as novel therapy in clinical ARF. The latter strategies are currently under investigation.

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