Mechanisms of renal injury and repair: role of stem cells, chemokines and the nodosome

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Hematopoietic stem cell migration to the ischemic damaged kidney is not altered by manipulating the SDF-1/CXCR4-axis

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Abstract

Background
Hematopoietic stem cells (HSC) have been shown to migrate to the ischemic kidney. The factors that regulate the trafficking of HSC to ischemic damaged kidney are not fully understood. The stromal cell-derived factor-1 (SDF-1)/CXCR4-axis has been identified as the central signaling axis regulating trafficking of HSC to the bone marrow. Therefore, we hypothesized that SDF-1/CXCR4 interactions are implicated in the migration of HSC to the injured kidney.

Methods
HSC were isolated from mouse bone marrow and labeled with a cell-tracker. Acceptor mice were subjected to unilateral ischemia and received directly after reperfusion intravenously HSC. In addition, in separate groups of acceptor mice endogenous SDF-1 or HSC-associated CXCR4 was blocked or kidneys were injected with SDF-1.

Results
Exogenous HSC could be detected in the tubules and interstitium of the kidney 24 hours after ischemic injury. Importantly, the amount of HSC in the ischemic kidney was markedly higher compared to the contralateral kidney. Neutralizing endogenous SDF-1 or HSC-associated CXCR4 did not prevent the migration of HSC. No increase in the number of labeled HSC could be observed after local administration of SDF-1, as was also determined in bilateral kidney ischemia.

Conclusion
In conclusion, systemically administered HSC preferentially migrate to ischemic injured kidney. This migration could not be prevented by blocking the SDF-1/CXCR4-axis or increased after local administration of SDF-1.
Introduction

Acute renal failure (ARF) is a major clinical problem that affects up to 5% of hospitalized patients and has a mortality rate of 50-80% in these hospitalized patients\(^1\). Ischemia/reperfusion (I/R) injury is the major initiator of ARF and is caused by a sudden transient drop in blood flow to the kidney frequently occurring in shock, sepsis and during renal transplantation. I/R injury leads to a cascade of events affecting mainly tubular epithelial cells (TEC) in the corticomedullary region of the kidney, resulting in structural alterations including loss of brush border and cell polarity, and necrosis and apoptosis of damaged TEC\(^2\). Depending on the severity of injury, renal tubules have the capacity to regenerate and eventually restore the tubule with normal epithelium resulting in functional recovery of the kidney. Hematopoietic stem cells (HSC) are suggested to be involved in this regenerative process. Several studies have found Y-chromosome-positive TEC in renal female grafts transplanted into male recipients, and in kidneys of female mice transplanted with male bone marrow\(^3-5\). However, this was a rare event, occurring only in a small percentage of tubules. Although initial reports showed a high number of bone marrow-derived cells (BMC) with an epithelial phenotype after renal I/R injury\(^6,7\), careful designed experiments by the same and other groups could only observe a few epithelial-like BMC in renal tubules after ischemic injury\(^8-10\). Since the levels of BMC that engraft the injured tubules and develop into functional TEC are very low, their contribution to renal repair is thought to be minor. Enhancing the migration of HSC to the injured kidney may result in a significant contribution of these HSC to renal repair, and hence has therapeutic potential.

The mechanisms that regulate the trafficking of HSC to ischemic damaged kidney remain however unclear. A better understanding of the factors that regulate the migration of HSC into the injured kidney is essential for the development of strategies to improve HSC kidney engraftment and repair. The chemokine stromal cell-derived factor-1 (SDF-1, also known as CXCL12), and its receptor CXCR4 have been identified as the central signaling axis regulating the trafficking of HSC. In immune-deficient mice the SDF-1/CXCR4-axis is essential for efficient homing of human stem cells as indicated by the impaired repopulation of the bone marrow after blocking stem cell-associated CXCR4\(^11-13\). In addition, local administration of recombinant SDF-1 results in an increased homing of stem cells to the liver, spleen and bone marrow\(^11,12\).

SDF-1 is upregulated after injury in diverse experimental models including skin ischemia\(^14\), toxic liver injury\(^11\), myocardial infarct\(^15,16\) and DNA damage\(^17\). In the kidney we (unpublished data) and others\(^18\) have shown that after I/R injury SDF-1 protein levels are upregulated. Tögel et al. also observed a selective homing of exogenous administered BMC to the injured kidney and could inhibit this by blocking bone marrow-associated CXCR4\(^18\). Taken together, current literature suggests a role for the SDF-1/CXCR4-axis in HSC migration to injured organs.
In the present study we thoroughly characterized the involvement of the SDF-1/CXCR4-axis in the migration of HSC to the injured kidney.

**Subjects and methods**

**Mice**

Eight-week old male C57Bl/6 (B6) mice were purchased from Charles River (Maastricht, the Netherlands) and housed under specific pathogen-free conditions receiving food and water *ad libitum*. All experimental procedures were approved by the local Animal Care and Use Committee of the University of Amsterdam, the Netherlands.

**Isolation of HSC**

Total bone marrow was collected from male B6 mice by flushing femurs and tibiae. Erythrocytes were lysed in 160mM NH$_4$Cl, 10mM KHCO$_3$, and 0.1mM EDTA (pH 7.4, 0.22µm filtered). Cells were labeled with an APC-conjugated monoclonal antibody to c-Kit (BD Biosciences, Alphen a/d Rijn, the Netherlands) for 20 minutes, washed with FACS buffer (0.5% BSA, 0.35 mM EDTA in PBS) and the population of cells expressing high levels of c-Kit (c-Kit$^{\text{high}}$) was collected using a FACSaria cell sorter (Becton Dickinson, Breda, the Netherlands). The sorted c-Kit$^{\text{high}}$ population was labeled with the fluorescent cell-tracker CM-DiI (Invitrogen) according to the manufacturer’s protocol. Unlike other membrane stains, this dye binds covalently to cellular thiols and therefore is well retained in cells throughout fixation steps. After washing, cells were resuspended in sterile saline. For CXCR4 neutralization, 10$^6$ c-Kit$^{\text{high}}$ BMC were preincubated with 20 µg anti-murine CXCR4 neutralizing mAb (R&D, Abingdon, UK) for 30 minutes at 37°C.

Analysis of the expression of lineage markers, Sca-1 and CXCR4 on the sorted c-Kit$^{\text{high}}$ BMC was performed by incubating the cells with a cocktail of rat-anti-mouse lineage markers (Ter119, CD45R, Ly6, CD8a, CD4, CD11b, all from BD Biosciences) for 30 minutes followed by a FITC-conjugated polyclonal antibody to rat-IgGs (Dako, Heverlee, Belgium) and a PE-conjugated monoclonal antibody to Sca-1 (BD Bioscience) for 30 minutes, or by incubating with a FITC-conjugated monoclonal antibody to CXCR4 (BD Bioscience) for 30 minutes. Analyses were performed on a FACS Calibur (Becton Dickinson) and showed that after cell-sorting the c-Kit$^{\text{high}}$ BMC expressed no lineage markers, as previously reported$^{19}$, and that at least 85% of the c-Kit$^{\text{high}}$ population expressed Sca-1. Therefore the population of cells used in this study are highly enriched in c-Kit$^+$, Sca-1$^+$ and Lin$^-$ cells, which are considered to represent HSC$^{20}$ and will hereafter be referred to as HSC. After cell-sorting, 90% of our HSC population expressed CXCR4, the receptor for SDF-1.
In vitro migration assay

*In vitro* migration assays were performed in triplicate with 5 µm pore size transwells (Corning Life Sciences, Schipol-Rijk, Netherlands) coated with 0.5 µg/mL VCAM-1 (R&D) in PBS. To the lower compartment medium (DMEM; Gibco, Invitrogen cell culture, Breda, Netherlands) alone or supplemented with 200 ng/mL recombinant murine SDF-1α (recSDF-1; PeproTech EC, London, UK) was added. To the upper compartment 0.1 x 10⁶ CM-Dil-labeled HSC were applied and allowed to migrate for 2 hours at 37°C. The neutralizing capacity of anti-SDF-1 was determined by adding 17 µg/mL neutralizing anti-human/mouse SDF-1α (anti-SDF-1, R&D) to medium with recSDF-1 in the lower compartment. The neutralizing capacity of anti-CXCR4 was determined by adding HSC pre-incubated with CXCR4 (20µg/10⁶ HSC) to the upper compartment. The amount of migrated CM-Dil-labeled HSC was determined on a FACSCalibur (Becton Dickinson) and expressed as a percentage of the input.

In vivo migration assay

B6 mice received 0.6 x 10⁶ CM-Dil-labeled HSC, or CM-Dil-labeled HSC pre-incubated with a CXCR4 blocking antibody (anti-CXCR4 group; 20µg/10⁶ HSC) or CM-Dil-labeled HSC mixed with free anti-SDF-1 (anti-SDF-1 group; 2 mg/kg) intravenously. Mice were killed 24 hours after injection and bone marrow aspirates were analyzed by flow cytometry for the presence of injected HSC.

Renal I/R injury model

Unilateral renal I/R injury was induced by clamping the left renal artery for 55 minutes under general anesthesia (0.1 mg/10 g mouse of fentanyl citrate fluanisone midazolam mixture, containing 1.25 mg/ml midazolam [Roche, Mijdrecht, the Netherlands], 0.08 mg/ml fentanyl-citrate, and 2.5 mg/ml fluanisone [Vetapharma, Leeds, UK]). Contralateral (right) kidney was used as internal control. In the recSDF-1-treated group, mice received an intrarenal injection of 2 µg (25 µg/mL) recSDF-1 in a 0.2% peptide hydrogel (PuraMatrix; BD Biosciences) in both the ischemic and contralateral kidney. Bilateral renal I/R injury was induced by clamping both renal arteries for 45 minutes under general anesthesia, immediately followed by intrarenal injection of 2x1 µg recSDF-1/0.2% hydrogel in the left kidney. The right kidney (control) was injected with the same volume of vehicle. After surgery, mice received intravenously 0.6 x 10⁶ CM-Dil-labeled HSC, or CM-Dil-labeled HSC pre-incubated with a CXCR4 blocking antibody (anti-CXCR4 group, 20µg/10⁶ HSC) or CM-Dil-labeled HSC mixed with free anti-SDF-1 antibody (anti-SDF-1 group; 2 mg/kg, R&D) in a total volume of 200 µL. For analgesic purposes, mice received subcutaneously 50 µg/kg buprenorphin (Temgesic; Schering-Plough, Brussels, Belgium) after closing the abdomen and mice were killed 24 hours after surgery. Kidneys were harvested and blood samples were obtained via heart puncture and transferred to heparin tubes.
Detection of fluorescent-labeled HSC in the kidney

Formalin-fixed kidneys were embedded in paraffin. Four µm thick serial sections were cut and every tenth section was used for examination. Sections were deparaffinized in fresh ethanol gradient series and subsequently embedded in Vectashield HardSet mounting medium with DAPI (Vector Laboratories, Amsterdam, the Netherlands). The presence of injected HSC was examined by fluorescence microscopy using a Leica CTR5000 (Leica Microsystems, Rijswijk, the Netherlands) equipped with the Nuance Multispectral Imaging System (Cambridge Research, Woburn, MA, USA). Kidney sections stored in cold medium were used for flow cytometric analysis. Kidneys were minced through a 70 µm gauze and a single cell suspension was made. Erythrocytes were lysed in 160 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA (pH 7.4). After washing, the kidney cells were analyzed on a FACSCalibur (Becton Dickinson). For background correction, ischemic and contralateral kidneys of mice not injected with CM-Dil-labeled HSC were used.

Figure 1. HSC preferentially migrate to the ischemic injured kidney and this migration is not altered by manipulating the SDF-1/CXCR4-axis. One day after unilateral ischemia for 55 min and subsequent intravenous administration of CM-Dil-labeled HSC, kidneys were harvested and analyzed by flow cytometry for the presence of exogenous HSC (i.e. CM-Dil and c-Kit positive). Flow cytometry plots of (a) a contralateral and (b) an ischemic kidney demonstrate a higher percentage of exogenous HSC in the ischemic kidney. (c) Exogenous HSC in contralateral (white bars) and ischemic (black bars) kidneys of mice with I/R injury and intravenously HSC (control) and of mice with additional neutralization of endogenous SDF-1 (anti-SDF-1), neutralization of HSC-associated CXCR4 (anti-CXCR4), and intrarenal recSDF-1 (recSDF-1), showed an increase in exogenous HSC in the ischemic kidney compared to the contralateral. However, this was only significant in the control and recSDF-1 mice (both *P=0.03). Anti-SDF-1 or anti-CXCR4 treatment did not result in a decreased migration of HSC towards the ischemic kidney. Intrarenal recSDF-1 did not increase the migration of HSC towards the ischemic or contralateral kidney. Background fluorescence of ischemic and contralateral kidneys without HSC administration was subtracted to correct for autofluorescence of (dead)cells. Data are expressed as mean ± SEM, n=4.
Statistical Analyses
Results are expressed as mean ± standard error of the mean (SEM), unless mentioned otherwise. Data were analyzed using a non-parametric Mann-Whitney U Test. Values of $P \leq 0.05$ were considered statistically significant.

Results

HSC preferentially migrate to ischemic damaged kidney
To study the migration of HSC towards the injured kidney mice were subjected to unilateral I/R injury, after which they received $0.6 \times 10^6$ CM-DiI-labeled HSC into the tail vein. Twenty-four hours later, there was a significant ($P=0.03$) increase in HSC migrating to the ischemic injured kidney compared to the contralateral kidney as assessed by flow cytometric analysis (Figure 1). The background fluorescence of ischemic and contralateral kidneys without HSC administration was subtracted to correct for autofluorescence of (dead) cells.

Figure 2. Exogenous HSC migrate to tubules and the interstitium. Fluorescent detection with TX2 filter of intravenously administered CM-Dil-labeled HSC in the injured kidney one day after I/R. (a and b) High magnification (40x) of the renal corticomedullary region. Two bright fluorescent cells are present in/close to injured tubules. (b) Spectral Imaging was used to reduce the autofluorescent signal of the kidney, showing that the bright fluorescent cells are not endogenous kidney or inflammatory cells. (c) Overlay of the (c) TX2-filter image with (d) DAPI staining image corresponding to the boxed area in a. Arrows point the two bright exogenous cells, a clear overlay is seen with the DAPI staining.
To localize the exogenous HSC in the injured kidney, we visualized the presence of these cells in renal tissue. Spectral Imaging was used to make a clear distinction between the exogenous HSC and endogenous kidney and inflammatory cells by reducing the autofluorescent background, thereby reducing possible false positive hits (Figure 2a and b). Of interest, exogenous HSC were mainly detected in the corticomedullary area, the part of the kidney most affected by I/R injury. More specifically, we found that these cells were localized in dilated tubules, in close proximity to dilated tubules and in the interstitium (Figure 2).

**SDF-1 and CXCR4 neutralizing antibodies inhibit HSC migration in vitro and in vivo**

To prove the efficacy of recSDF-1 as well as the neutralizing antibodies against SDF-1 and CXCR4, *in vitro* migration assays were performed. As depicted in Figure 3a, medium supplemented with recSDF-1 resulted in an 8-fold increase of HSC migration compared to medium alone. Adding anti-SDF-1 to the assay or pre-incubating the HSC with anti-CXCR4 resulted in reduced migration of HSC cells by 65% and 40% respectively.

To validate whether anti-SDF-1 and anti-CXCR4 were also able to inhibit the migration of HSC *in vivo*, HSC were injected into the tail vein of naive mice with or without blocking either endogenous SDF-1 or HSC-associated CXCR4 with a monoclonal antibody as previously described. Under physiological conditions the bone marrow is the niche for HSC; the basal level of hypoxia in the bone marrow microenvironment may explain the constitutive and regional expression of SDF-1 in the bone marrow and the CXCR4-dependent stem and progenitor cell tropism to the bone marrow. Twenty-four hours after administration we could detect CM-DiI-labeled HSC in the bone marrow (Figure 3c). Subtraction of background signal from non-injected animals (Figure 3b) revealed that 0.21% ± 0.01% of total bone marrow consisted of CM-DiI-labeled HSC that had migrated towards the bone marrow. However, after neutralizing SDF-1 (Figure 3d) or CXCR4 (Figure 3e) no CM-DiI-labeled HSC could be detected in the bone marrow indicating that we have established a reliable *in vivo* system to disrupt the SDF-1/CXCR4-mediated migration of HSC.

**Migration of HSC is not prevented by blocking the SDF-1/CXCR4-axis**

In order to investigate the involvement of the SDF-1/CXCR4-axis in the migration of HSC to the injured kidney, we utilized two independent approaches to inhibit SDF-1/CXCR4 function. Unexpectedly, neutralization of endogenous SDF-1 or HSC-associated CXCR4 did not result in a significant decreased migration of exogenous HSC to the ischemic injured or contralateral kidney (Figure 1c). On the contrary, in both groups there was an increase in exogenous HSC in the ischemic kidney compared to the contralateral kidney, although this was not significant.
HSC migration is not influenced by exogenous SDF-1 in vivo

In contrast to other studies\textsuperscript{11,13,18,21}, we were unable to significantly influence the migration of HSC to the ischemic injured kidney by blocking the SDF-1/CXCR4-axis. Therefore, we hypothesized that endogenous SDF-1 levels in ischemic kidneys may be insufficient to properly attract HSC. To test this hypothesis, both contralateral and I/R injured kidneys received recSDF-1 (at a double dose as described before\textsuperscript{11,12}). Twenty-four hours after intrarenal injection of recSDF-1, there was still a 4.3x ($P<0.01$) increase in renal SDF-1 as compared with vehicle injected kidney. In accordance to the previous experiments, significantly ($P=0.03$) more HSC migrated towards the ischemic injured kidney compared to the contralateral kidney (Figure 1c). However, we could not observe an increased migration of the HSC towards the ischemic or contralateral kidneys locally injected with recSDF-1 compared to the control group.
Subsequently, we hypothesized that a more severe and systemic type of danger might be necessary. Therefore, bilateral I/R injury was induced and directly after reperfusion local injections of recSDF-1 in the left and saline in the right kidney were performed. Immediately after surgery, mice received HSC into the tail vein and 24 hours later the kidneys were analyzed. Again, we could not observe any effect of the locally injected recSDF-1 on the migration of HSC since both kidneys were engrafted with the same amount of exogenous administered HSC (Figure 4.)

Figure 4. HSC migration is not influenced by locally administered SDF-1. One day after bilateral ischemia and local administration of saline or recSDF-1 followed by intravenous injection of CM-Dilabeled HSC, no difference was observed in migration of the exogenous HSC. Flow cytometry plots of (a) a saline and (b) a recSDF-1 treated ischemic kidney. (c) Graphical representation of the mean (± SEM) percentage of exogenous HSC in the saline (control) and recSDF-1 treated ischemic kidneys shows no difference between the groups. Data are expressed as mean ± SEM, n=4.
Discussion

The aim of our study was to establish the importance of the SDF-1/CXCR4-axis in the migration of HSC to the ischemic injured kidney, in order to eventually manipulate this axis for therapeutic purposes. The contribution of HSC in post-ischemic renal tissue repair is controversial. First of all, the capacity of HSC to engraft tubules after ischemic injury is much lower than originally reported. Initial studies in chimeric mice stated that 21% or even 80% of the tubules contained BMC. However, some of these numbers are thought to be overestimated due to false-positive results as a consequence of β-gal leaking by the BMC. Sex-mismatched and GFP chimeric mice studies published in 2005 showed only a few BMC in the tubules after I/R injury, whereas Duffield and colleagues could not observe any BMC in post-ischemic tubules. Secondly, the biological significance of HSC in repair after I/R injury is challenged, since low numbers of BMC in the tubules do not make a significant contribution to renal functional recovery. Therefore, mechanisms to increase the migration of HSC may result in a significant contribution of these cells to tubular repair and hence has therapeutic potential.

To determine whether the SDF-1/CXCR4-axis is the driving force for the recruitment of HSC to the injured kidney, we followed the migration of exogenous HSC in a unilateral renal I/R model. This model gives the opportunity to compare the migration of HSC in injured and normal tissue within one mouse, in contrast to experimental models such as cisplatin-induced renal injury and bilateral renal I/R injury. Here we demonstrate that HSC migrate to the injured kidney and are even able to migrate into damaged tubules. In line with our data, Lin et al. and Tögel et al. have shown that the homing process of BMC is selective for the ischemic injured kidney. Our study however extends these findings as we have studied migration of purified HSC instead of whole bone marrow which is a heterogeneous population of cells consisting, amongst others, of inflammatory cells, mesenchymal stem cells and only a small percentage of HSC. Surprisingly, manipulating the SDF-1/CXCR4-axis in three different and independent manners did not result in a significant altered migration of exogenous HSC.

Previous studies have shown the importance of the SDF-1/CXCR4-axis in the migration of BMC. This was done either by blocking endogenous SDF-1, blocking BMC-associated CXCR4 or increasing locally SDF-1. Our results are at variance with the current opinion that SDF-1 is the mediator of HSC trafficking to injured organs. The neutralizing antibodies were chosen based on their proven functionality in previous studies. Neutralization with the same function-blocking anti-SDF-1 monoclonal antibody and dose as used in the present study significantly attenuated HSC accumulation within the growing platelet-rich thrombus in a arterial thrombosis mouse model. In order to neutralize HSC-associated CXCR4, the same protocol was used as reported previously to be effective in blocking the recruitment of BMC or HSC. In addition, our in vitro and in vivo HSC migration
assays demonstrated a decreased migration towards high recSDF-1 levels and bone marrow respectively, after neutralizing SDF-1 or CXCR4. Therefore, we believe that our experimental set-up is highly reliable to study the role of this axis. Despite this, we could not observe a significant effect of neutralizing SDF-1 or its receptor CXCR4 on the migration of HSC to the injured kidney. Since these results do not support the current reports on SDF-1-involvement in HSC-migration, we conducted a third independent experiment to determine the effect of local SDF-1 administration on the migration of exogenous HSC. Despite the previously reported preferred migration of HSC towards high SDF-1 gradient in liver, bone marrow and spleen\cite{11,12}, we could not observe this in both the unilateral and bilateral renal I/R injury model. However, in accordance with our results, it has been reported that BMC migration to the lung could not be influenced significantly by manipulating the SDF-1/CXCR4-axis in both a non-injured\cite{12} and a bleomycine-injured\cite{27} mice model. This indicates that other mechanisms may be involved in HSC migration to the kidney and lung. We also did not find an effect of the local administration of recSDF-1 in the non-injured (contralateral) kidney on renal engraftment of HSC compared to the non-treated contralateral kidney. These results suggest that injury is necessary for HSC migration and that SDF-1 alone is not sufficient for migration. This was also observed in the heart, where overexpression of SDF-1 without injury could not induce the homing of BMC\cite{16}. In addition, the results from the bilateral I/R model, where both the SDF-1 and saline injected kidney had the same percentage of migration, indicate that the exogenous administered HSC cells did not migrate preferentially towards high SDF-1 gradient.

Our results indicate that I/R injury induces the homing of HSC in the injured kidney and that this preferred migration is not exclusively dependent on the SDF-1/CXCR4-axis. Homing of HSC towards the ischemic damaged kidney could be much more complex. Several other factors are described to be involved in stem cell migration and in addition are known to be induced after I/R injury. These factors include the chemokine GROβ\cite{28}, the glycosaminoglycan hyaluronic acid\cite{29}, the nuclear factor HMGB1\cite{30}, and the growth factor HGF\cite{11}. Interestingly, in a recently published report a flexible hierarchy of homing molecules was proposed, providing an explanation for some of the contradictory findings in the literature\cite{31}. Herein the authors show that in a mouse model of HSC transplantation, SDF-1 was not required for stem cell homing due to compensatory action via the VLA-4/VCAM-1 interaction\cite{31}.

In conclusion, our initial hypothesis that SDF-1/CXCR4 interactions are implicated in the migration of HSC to the injured kidney is not supported by the results in this report. In sharp contrast to studies in other injured organs, our results indicate that migration of HSC to the ischemic damaged kidney is not solely dependent on the SDF-1/CXCR4 signaling axis.
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