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Renal SDF-1 signals mobilization and homing of CXCR4-positive cells to the kidney after ischemic injury

FLORIAN TÖGEL, JORGE ISAAC, ZHUMA HU, KATHY WEISS, and CHRISTOF WESTENFELDER

Department of Medicine, Division of Nephrology, University of Utah and VA Medical Centers, Salt Lake City, Utah; Department of Pathology, University of Utah, Salt Lake City, Utah; and Department of Physiology, University of Utah, Salt Lake City, Utah

Renal SDF-1 signals mobilization and homing of CXCR4-positive cells to the kidney after ischemic injury.

Background. Stem cell and leukocyte migration during homeostasis and inflammation is regulated by a number of chemokines. Stromal cell–derived factor-1 (SDF-1) and its receptor CXCR4 are important mediators of leukocyte homeostasis. The postischemic kidney has been shown to recruit different leukocyte populations, including bone marrow–derived stem cells. Therefore, we investigated the SDF-1/CXCR4 system in the kidney after ischemic acute renal failure (ARF).

Methods. We used immunohistochemistry, in situ hybridization, enzyme-linked immunosorbent assay (ELISA) and real-time reverse transcription-polymerase chain reaction (RT-PCR) to detect SDF-1 and CXCR4 in the normal kidney and the kidney after ischemia/reperfusion (I/R) ARF. Mobilization was assessed by flow cytometry for CD34 and colony assays.

Results. We show that SDF-1 is expressed in the normal mouse kidney and tubular cells express CXCR4. SDF-1 expression in the kidney increases after I/R induced ARF and decreases in the bone marrow, thereby reversing the normal gradient between bone marrow and the periphery. This causes mobilization of CD34-positive cells into the circulation and their subsequent homing to the kidney with ARF. In vitro and in vivo chemotaxis of bone marrow cells toward damaged kidney epithelium is reversibly inhibited by anti-CXCR4 antibodies.

Conclusion. Our data show that renal SDF-1 is a currently unrecognized mediator of homing to and migration of CXCR4 expressing cells in the injured kidney. Because certain cells that express CXCR4 may have renoprotective effects, our results suggest that SDF-1 may be a major signal involved in kidney repair.

Stem cell and leukocyte migration during homeostasis, inflammation, and tissue damage is a complicated and highly regulated process coordinated by a large number of growth factors, cytokines, and chemokines, all of which are derived from normal or damaged tissue or from inflammatory cells infiltrating such tissues [1]. Chemokines are important in leukocyte homeostasis and inflammation and exert a number of critical functions such as providing directional signals for leukocyte migration, activation of leukocytes, regulation of hematopoiesis and also angiogenesis [1]. Receptors for chemokines are expressed on leukocytes in a specific manner; however, there is some redundancy in function and a number of chemokines are known to be promiscuous in terms of their receptor binding capacity. Unlike other chemokines, stromal cell–derived factor-1 (SDF-1) is unique in that it binds only to its receptor, CXCR4, and its importance in hematopoiesis, cardiac and neuronal development is furthermore underscored by the fetal lethality of either CXCR4 or SDF-1 knockout mice [2]. Contrary to the selective induction of most chemokines by certain stimuli, SDF-1 is constitutively expressed by most organs, although it is known that an up-regulation of its expression takes place after injury or DNA damage [3, 4].

Expression of SDF-1 in the kidney has so far only been shown in the developing kidney [5], and there is not much knowledge about its role in the adult or injured kidney. There is evidence that SDF-1 is a major factor both in hematopoietic stem cell (HSC) mobilization and HSC homing to the bone marrow [6]. However, SDF-1 is not only a major factor for HSC migration, it has also been shown to be involved in metastasizing of rhabdomyosarcoma, breast cancer, prostate cancer, and neuroblastoma [7–9], as well as in attracting bone marrow–derived fibroblasts to bleomycin-damaged lung tissue in an animal model of pulmonary fibrosis [10].

SDF-1 is expressed as two different splice variants, SDF-1α and SDF-1β, the latter having four additional amino acid residues at the carboxy terminus [11]. Although it has been stated that there is no functional difference between those two isoforms [12], Stumm et al [13] showed that endothelial SDF-1β controls cerebral infiltration by leukocytes after ischemia, while the up-regulation of the neuronal SDF-1α/CXCR4 system contributes to neuronal plasticity and repair.

Key words: SDF-1, CXCR4, acute renal failure, mobilization, homing, stem cells.

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There is currently a significant lack of knowledge about the involvement of the SDF-1/CXCR4 system in kidney homeostasis and disease. It has been shown recently that following ischemic acute renal failure (ARF), the number of circulating Sca-1+/lineage-negative cells, also a property of HSCs, is increased [14, 15]. The precise significance of this finding remains unknown. However, with the recently discovered plasticity of HSCs [16], it is tempting to speculate that mobilized HSCs contribute to the repair of the injured kidney. Korbling and Estrov [17] proposed various models of organ repair by bone marrow and circulating adult stem cells. One of those models hypothesizes that HSCs have a default pathway for hematopoietic lineage differentiation, but circulating HSCs are able, under the influence of certain stimuli derived from damaged tissues, to overcome lineage barriers and to contribute, through differentiation, to resident organ cells. Although unproven, this hypothesis would give a plausible explanation for recent reports of transdifferentiation of HSCs in mice with organ damage, a phenomenon not observed in normal mice [18].

HSCs are currently best defined using flow cytometry, as being negative for lineage-specific surface markers (lineage-negative) and expressing c-kit and Sca-1 [19]. CD34, on the other hand, is a surface marker of mobilized HSCs [20] as well as different progenitor cell populations, including endothelial and endothelial progenitor cells (EPCs) [21]. Since cell populations expressing CD34 have been shown to be protective in ARF [14, 22], we chose to use CD34 as a marker in our studies.

EPCs are an important contributor to vascular repair after ischemic damage [23]. EPCs are bone marrow–derived and can circulate in the blood. They express CXCR4, the receptor of SDF-1, and share characteristics with HSCs (e.g., CD34 expression and the capacity to form colonies in vitro). Different conditions such as vascular trauma, cytokines and drugs are known to mobilize EPCs from bone marrow into the circulation [24, 25], and a reduction in the number of peripheral EPCs has been shown to be a risk factor for vascular events [26].

We hypothesized in the present study that kidney injury causes up-regulation of SDF-1 and subsequent mobilization of bone marrow–derived cells, as well as their homing to the kidney. The aim of this study was, therefore, to investigate the expression patterns of the SDF-1/CXCR4 system in the normal kidney and after ischemia/reperfusion (I/R), and its possible role in homing of CXCR4-expressing cells to the kidney with ARF, a response that may contribute to cellular renal repair.

METHODS

Cells and animals

FVB mice, female or male, 20 to 30 g, were used for all experiments. All studies were approved by the institutional animal care and use committee (IACUC). Animals were housed at constant temperature with a 12-hour dark-light cycle and had unrestricted access to a standard diet and tap water. Bone marrow cells were harvested from anesthetized mice by flushing femurs and tibias with phosphate-buffered saline (PBS). Cells were passed through a 40 μm nylon mesh (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged. Granulocyte depletion was accomplished by density centrifugation with Histopaque-1077 (Sigma Chemical Co., St. Louis, MO, USA), and the mononuclear cell fraction was collected, washed with PBS and centrifuged. For in vivo and in vitro tracking, cells were incubated with 25 μmol/L carboxyfluorescein diacetate (CFDA) (Molecular Probes, Eugene, OR, USA), a green fluorescent cell tracking dye, for 30 minutes in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co.) and thoroughly washed with PBS thereafter. Adequacy of fluorescence staining was confirmed by microscopy before performing experiments. Primary mouse kidney tubular cells were generated by mincing kidney cortices from 2-week-old animals and digestion with collagenase I (Sigma Chemical Co.) for 1 hour at 37°C. Cells were passed through a 40 μm nylon mesh and seeded on collagen I–coated 6-well plates. Once confluent and prior to use, the epithelial nature of the cells was confirmed by cytokeratin (pan-cytokeratin antibody) (Sigma Chemical Co.) and aquaporin-1 (AQP1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) staining.

Kidney injury in the in vivo homing study was induced by clamping both renal pedicles for 60 minutes under general anesthesia with isofluorane as described earlier [27]. Adequate reflow was assured by visual inspection post clamp removal. This ischemia time was needed in FVB mice in order to induce severe renal insufficiency with a mortality of about 60% on day 3. For homing studies, kidneys were examined on day 1 after injury.

HSC mobilization in ARF

FVB mice were anaesthetized with isofluorane and subjected to severe ARF by clamping both renal pedicles for 60 minutes [27, 28]. In order to determine the amount of circulating CD34-positive cells, anesthetized mice were sacrificed at 24 hours after ARF and blood was sampled by cardiac puncture. Mononuclear cells were isolated with Histopaque-1077 (Sigma Chemical Co.) and stained with biotin-conjugated rat antimouse CD34 monoclonal antibody and Streptavidin-PE (Pharmingen, San Diego, CA, USA) for subsequent analysis with a FACScan Analyzer (BD Biosciences, San Jose, CA, USA). Clonogenic and blood forming potential of mobilized HSCs was assessed by culture of mononuclear cells (MNCs) in methylcellulose medium known to promote growth of colony-forming units (CFU) cells (CFU-C). MNCs were isolated with Histopaque and 1.5×10^4 cells were

suspended in 2 mL of Methocult (StemCell Technologies, Vancouver, Canada). All formed colonies (CFU-GM, CFU-G, and CFU-M) were counted collectively after 12 days of incubation, thereby including both stem as well as progenitor cell–derived colonies.

In situ hybridization

In situ hybridization for SDF-1 and CXCR4 was carried out with RNA sense (negative control) and antisense probes derived by in vitro transcription from linearized plasmids containing mouse SDF-1 cDNA (generously provided by Dr. Ralf Stumm, Magdeburg, Germany) and CXCR4 cDNA (generously provided by Dr. Ohl, Hannover, Germany), using digoxigenin-uridine triphosphate (UTP) (DIG) (Roche Diagnostics, Mannheim, Germany) as label.

Kidney sections of mice with ARF with or without prior HSC mobilization were fixed in 10% formalin for less than 24 hours and paraffin embedded; 4 μm tissue sections were deparaffinized with clear solvent solution (Richard-Allan Scientific, Kalamazoo, MI, USA) and rehydrated with decreasing concentrations of ethanol followed by incubation in diethyl pyrocarbonate (DEPC)-water and PBS. After fixation in 4% paraformaldehyde, slides were washed in PBS and endogenous peroxidase was quenched with 1.5% H_2O_2 for 25 minutes. Sections were acetylated for 10 minutes with triethanolamine and acetic anhydride and postfixed in 4% paraformaldehyde (PAF), followed by prehybridization (30 minutes at 42°C). Hybridization with the riboprobe was performed overnight at 42°C with an empirically tested concentration of 100 to 400 ng as quantified by DIG quantification strips (Roche Diagnostics). After hybridization, the probe was washed off under high stringency conditions at 60°C in buffer containing 10 mmol/L dithiothreitol (DTT) and 2 × standard sodium citrate (SSC). The hybridized probe was detected by overlaying sections with anti-DIG-POD antibody for 2 hours in a humidified chamber and developing the color reaction with diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA, USA) and a DAB enhancement reagent. Finally, sections were covered with mounting medium. To control for false positive and unspecific staining, hybridization with sense probes was included in all sections. Experiments with RNAse incubation of tissue sections showed that after RNAse treatment no signal was detectable, thereby confirming the specificity and RNA nature of the obtained signal.

Immunohistochemistry

Four micron sections of formalin-fixed, paraffin-embedded tissue were deparaffinized with xylene and hydrated using decreasing concentrations of ethanol. The primary antibody was a rabbit antimouse polyclonal antibody to SDF-1α (eBioscience, San Diego, CA, USA). Kidneys from control FVB mice, mice subjected to 60 minutes ARF and HSC mobilization, as well as control ARF mice were stained for SDF-1. Control experiments included omission of either the primary or secondary antibody. All sections for SDF staining were subjected to antigen retrieval using immersion in a 0.1 mol/L citrate buffer (pH 6.0) for 27 minutes, followed by heating in an electrical pressure cooker for 3 minutes. The primary SDF antibody was applied at a dilution of 1:100. This was sequentially followed by incubation for 30 minutes with a streptavidin peroxidase–conjugated secondary antirabbit IgG antibody (Sigma Chemical Co.) at a 1:300 dilution and with color development using DAB (Dako, Carpenteria, CA, USA) for 5 minutes.

Real-time polymerase chain reaction (PCR)

RNA for real-time PCR was extracted with the RNeasy kit (Qiagen, Valencia, CA, USA), including a DNase digestion step to exclude contaminating DNA. Reverse transcription (RT) was performed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for 60 minutes at 42°C.

Real-time PCR with relative quantification of SDF-1 copy numbers in relation to β-actin transcripts was carried out using the following primers: SDF-1 forward TGA GGC CAG GGA AGA GTG AG; SDF-1 reverse GAC ACA TGG CGA TGA ATG GA; β-actin forward AGA GGG AAA TCG TGC GTG ACA; β-actin reverse CAC TGT GTT GGC ATA GAG GTC; CXCR4 forward TCA GTG GCT GAC CTC CTC TT; and CXCR4 reverse TTT CAG CCA GCA GTT TCC TT.

The Smart-Cycler system (Cepheid, Sunnyvale, CA, USA) was used to monitor real-time PCR amplification using SYBR Green I (Molecular Probes), a nonspecific double-stranded DNA intercalating fluorescent dye. All reactions were carried out in a total volume of 25 μL with TaKaRa Ex Taq^{TM} R-PCR Version (TaKaRa Bio Inc., Shiga, Japan). Reaction conditions were hot start for 120 seconds at 95°C, melting at 95°C for 10 seconds, annealing at 63°C for 12 seconds, and amplification at 72°C for 15 seconds. Reading of the fluorescent product was set to be 2°C below the specific melting peak of the product in order to eliminate reading of nonspecific products and primer dimers and was performed at 85°C for 6 seconds after each cycle for SDF-1. Optimal annealing and melting temperatures were determined for the primers prior to running the samples. Melting temperature analysis for the reaction mix revealed a characteristic melting profile with a single sharp peak at the typical melting temperature for the product. Specificity of the product was determined by a melting curve and gels were run to control for the formation of unspecific bands. Samples were run in duplicate and the average threshold cycle (Ct) value was used for calculations. The Ct, which is the cycle at

which the amount of amplified gene of interest reached a threshold above background fluorescence, was determined in order to quantitate initial starting copy amount. Relative quantitation of SDF-1 mRNA expression was calculated with the comparative Ct method using the following formula:

$$\text{eff}\Delta C(\text{target})/\text{eff}\Delta C(\beta-\text{actin}) = 2^{(\Delta C(\text{target})-\Delta C(\beta-\text{actin}))}$$

The relative quantitation value of target, normalized to an endogenous control β-actin gene, is expressed as a number, which indicates the relative expression compared to that gene. To avoid the possibility of amplifying contaminating DNA and unspecific amplification, the following precautions were taken: (*1*) a DNase-digestion step was included in the RNA-extraction protocol, (*2*) some primers were designed to include an intron sequence inside the cDNA to be amplified, (*3*) reactions were performed with appropriate negative controls (template-free controls), (*4*) a uniform amplification of the products was rechecked by analyzing the melting curves of the amplified products (dissociation graphs), and (*5*) gel electrophoresis was performed to confirm both the correct size of the amplification products and the absence of unspecific bands, respectively.

Enzyme-linked immunosorbent assay (ELISA)

SDF-1 protein quantification in kidneys before ($N = 3$) and day 1 and day 3 after ARF ($N = 3$), bone marrow supernatant, and peripheral blood were carried out by ELISA (RnD Systems, Minneapolis, MN, USA). Decapsulated kidney tissues were minced, sonicated, lysed with RIPA buffer [1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxylate, 0.1% sodium dodecyl sulfate (SDS) and protease inhibitor] for cell and tissue lysis and protein quantified by BCA protein assay reagent assay (Pierce, Rockford, IL, USA). Bone marrow from femurs of mice ($N = 3$) was flushed with 500 μL PBS and cells were spun down. Cell fractions and supernatants were analyzed for SDF-1. Equal protein amounts were assayed for SDF-1; 96-well plates were coated with 100 μL of SDF-1 antibody (final concentration 1 μg/mL) and incubated overnight. Wells were washed with 400 μL buffer (0.05% Tween 20 in PBS) and incubated for 1 hour at room temperature with 300 μL blocking buffer [1% bovine serum albumin (BSA) and 5% sucrose in PBS with 0.05% NaN_3]. One hundred microliters of sample were loaded and incubated for 2 hours at room temperature. After three washing steps, anti-SDF-1 antibody was applied and samples were incubated for 2 hours at room temperature. After washing, horseradish peroxidase conjugate was added to the reaction mixture and incubated for 20 minutes at room temperature. The reaction was stopped and optical density was determined with a microplate reader set at 450 nm. Wavelength correction was set to 570 nm. Sample results were calculated from a standard curve generated by dilutions of a known amount of recombinant SDF-1 protein. The SDF-1 concentration in each sample is the average of duplicate assays.

Migration assays

In the transfilter assay, primary kidney tubular cells from 1- to 2-week-old FVB-mice were grown to confluence in 6-well plates and were adenosine triphosphate (ATP) depleted by incubation in glucose free medium for 30 minutes with Antimycin A 1.7 μmol/L (Sigma Chemical Co.) in which glucose was substituted by 10 mmol/L 2-deoxy-glucose (90% ATP depletion was achieved in this setting as determined by luminescence assays) (Sigma Chemical Co.) (data not shown). After ATP depletion the cells were washed twice and allowed to recover for 60 minutes in normal medium before the bone marrow cells were put onto the transwell filter insert. Bone marrow MNCs (10^5 cells), immunomagnetically (Dynal Biotech, Brown Deer, WI, USA) enriched CD34-positive cells (10^3 cells) or bone marrow (10^5 cells) incubated with CXCR4 antibody (10 μg/mL for 30 minutes) (eBioscience) to block SDF-1 signaling were put on top of a polycarbonate filter insert (transwell) coated with gelatin (8 μm pore size) (Nunc, Naperville, IL, USA). After incubating at 37°C for 2 hours, cells on the top surface of the filter were removed and cells that had migrated through the filter to the undersurface were fixed in methanol and stained with hematoxylin. Cell numbers of at least ten high-power fields (40×) were counted for experimental and control groups and the experimental groups were expressed as fold increase or decrease compared to control groups, respectively. Results are given as the average of three experiments.

In vivo homing studies

To determine the number of homing cells in bilaterally or unilaterally clamped kidneys, 10^6 cells of whole bone marrow from normal mice were stained with CFDA and injected into the tail vein immediately after 60 minutes of renal pedicle clamping. Animals were sacrificed at 24 hours and kidneys were obtained for further analysis. One quarter of each kidney was digested with collagenase for 60 minutes at 37°C. After centrifugation and washing with PBS, kidney cells were resuspended and put into a Neubauer chamber for counting. Injected and homed cells were clearly recognizable by their bright green CFDA staining. CFDA-positive cells as well as total cell numbers were counted in at least two samples of the same kidney and averaged. Bone marrow was obtained by flushing both femurs with PBS and quantification of homed cells was carried out by counting CFDA-positive

cells in relation to bone marrow cells in a Neubauer chamber. To determine the role of CXCR4 in the homing of CXCR4-expressing cells to the injured kidney, bone marrow cells were preincubated with 10 μg anti-CXCR4 blocking antibody (eBioscience) for 30 minutes and cells were injected after washing and centrifugation. Kidney and bone marrow were examined for CFDA-positive cells as described above. Results are given as average of three experiments.

Data analysis

Data are expressed as means ± SD. Differences between data means were analyzed by analysis of variance (ANOVA) or Student *t* test using Prism software (GraphPad, San Diego, CA, USA). A *P* value of less than 0.05 was considered significant.

RESULTS

SDF-1 and CXCR4 are up-regulated in postischemic kidneys

In order to determine expression of SDF-1 and CXCR4 on a protein level in the healthy and postischemic kidney, we subjected tissue sections from normal and ARF kidneys to immunohistochemical analysis. The staining for SDF-1 in the normal kidney cortex showed a patchy pattern under low magnification. Cytosol of distal tubular cells stained with SDF-1, while most proximal tubular cells were negative or stained only weakly (Fig. 1, upper panel). Except for glomerular endothelial cells, glomeruli were negative. After I/R injury, virtually all surviving cells in cortex and outer medulla stained positive for SDF-1 (Fig. 1, lower panel) (i.e., demonstrating injury-induced up-regulation of SDF-1). Control incubation with secondary antibody alone did not result in any staining (not shown).

CXCR4 (fusin), the receptor of SDF-1, is expressed on tubular cells, with more intense staining of distal tubular compared to proximal tubular cells (Fig. 2, upper panel).

Glomeruli did not stain for CXCR4. Twenty four hours after ARF, surviving proximal tubular cells showed increased expression of CXCR4, indicating that there is both induction of SDF-1 as well as CXCR4 after ischemic injury (Fig. 2, lower panel).

RNA in situ hybridization shows up-regulation of SDF-1 after ischemia

To confirm the immunohistochemical results and to identify SDF-1–producing cells, we performed RNA in situ hybridization. All negative controls showed only minimal background staining, whereas sections subjected to treatment with anti-sense probe showed clear staining consistent with a positive hybridization signal. Staining patterns of SDF-1 and CXCR4 by in situ hybridization were essentially the same as those detected by immunohistochemistry with expression of SDF-1 mRNA mainly in distal tubular cells and in the papillae, while glomeruli were negative and normal proximal tubules stained rarely and with low intensity, thus corroborating our immunohistochemical results (Fig. 3). In situ hybridization in kidneys postischemic injury showed SDF-1 expression in almost all tubule segments that were not necrotic or apoptotic, as well as in detached tubular cells in the lumen (Fig. 4, upper left panel). Some cells in glomeruli stained weakly (Fig. 4, lower left panel), while necrotic tubule segments showed no staining, thus excluding nonspecific background staining.

Real-time quantitative PCR corroborates the observed up-regulation of SDF-1

SDF-1 RNA levels increased significantly 2.5-fold as early as $1^1/_2$ hours after I/R in the kidney and were increased twofold at 24 hours after ARF (Fig. 5). This response corroborates the results seen with in situ hybridization. Our in vitro model of tubular injury also showed an increase in SDF-1 expression after injury. Specifically, a primary tubular cell monolayer subjected to graded ATP depletion showed a 1.5-fold increase in SDF-1 RNA expression immediately after and up to 3 hours after ATP depletion.

SDF-1 protein levels in kidney and plasma are elevated after ARF while bone marrow levels are reduced

While normal renal cortical levels of SDF-1 were 5.5 ± 1 ng/mg protein, there was a significant increase to 10.8 ± 3.3 ng/mg protein in the cortex at 24 hours after ARF (Fig. 6A). SDF-1 levels in whole kidney increased without reaching statistical significance. At 3 days after ARF, there was still an increase in protein levels of SDF-1 in the whole kidney. SDF-1 levels in plasma were significantly increased at 24 hours after ARF and remained elevated up to day 3 (Fig. 6B). To determine if the kidney is the main source of the SDF-1 elevation, mice were nephrectomized and plasma SDF-1 levels were measured at day 1. SDF-1 plasma levels were significantly increased to the same level as in ARF animals, caused both by accumulation of SDF-1 produced by nonrenal sources as well as absent elimination by the kidney. Clamping of only one kidney resulted in an increase of SDF-1 in the plasma as well. SDF-1 levels in the bone marrow supernatant were significantly decreased after ARF (Fig. 6), thereby reversing the gradient from bone marrow to blood, which is a potent stimulus for stem cell mobilization [29]. We found that the reduced bone marrow levels are due to reduced production of SDF-1 by bone marrow cells (data not shown).

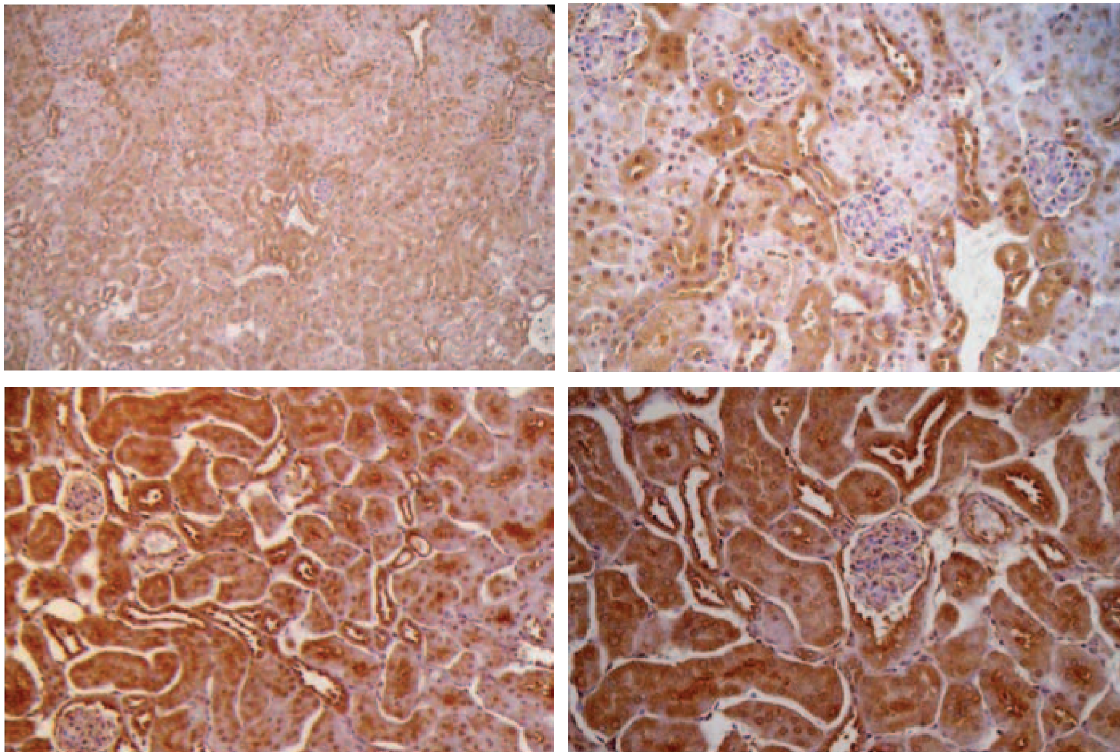

Fig. 1. Stromal cell–derived factor-1 (SDF-1) is up-regulated in acute rental failure (ARF). Immunohistochemisty of normal kidney. SDF-1 is expressed, by immunohistochemistry, mostly by distal tubular cells in the cortex (upper panels). Twenty-four hours after induction of ARF, most regions of the kidney cortex strongly express SDF-1 (bottom panels) (upper left panel original magnification ×10; lower left panel ×20; upper right panel ×40; lower right panel ×40).

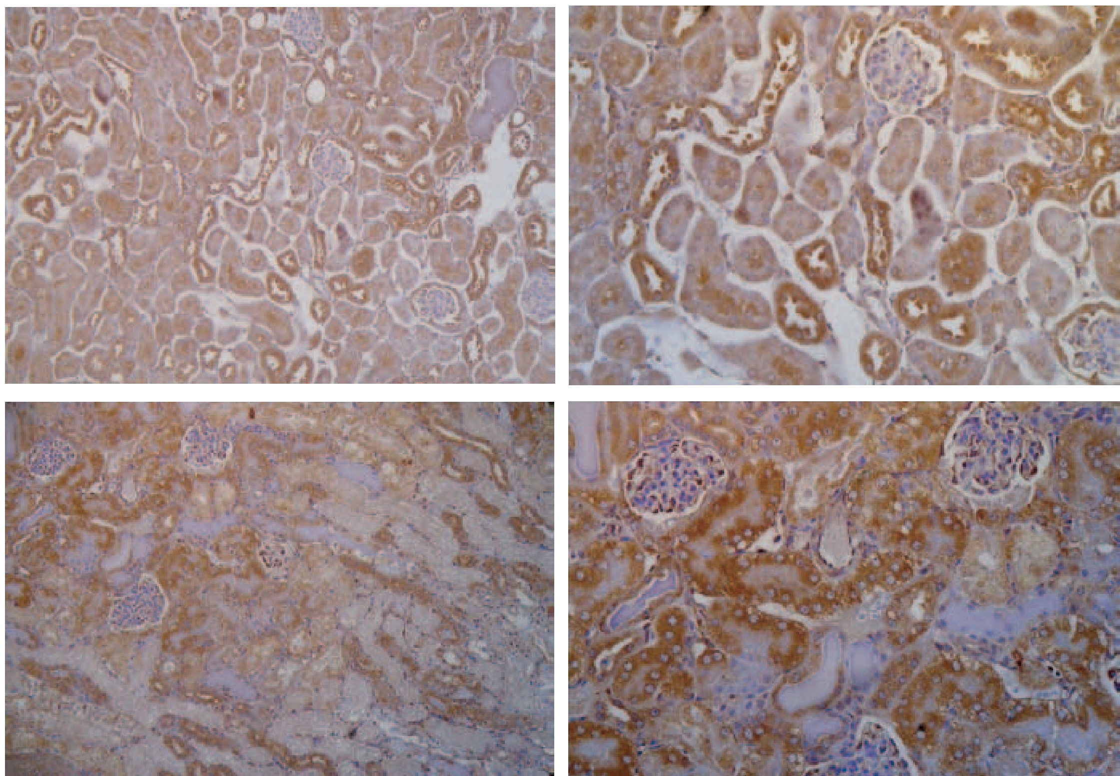

Fig. 2. CXCR4 is up-regulated after acute renal failure (ARF). Immunohistochemistry of normal kidney. CXCR4 is detected in a similar pattern to that of SDF-1 seen in Fig.1 (upper panels). After induction of ARF, CXCR4 is up-regulated in the areas of kidney injury (lower panels) (left panels, original magnification ×10; right panels ×40).

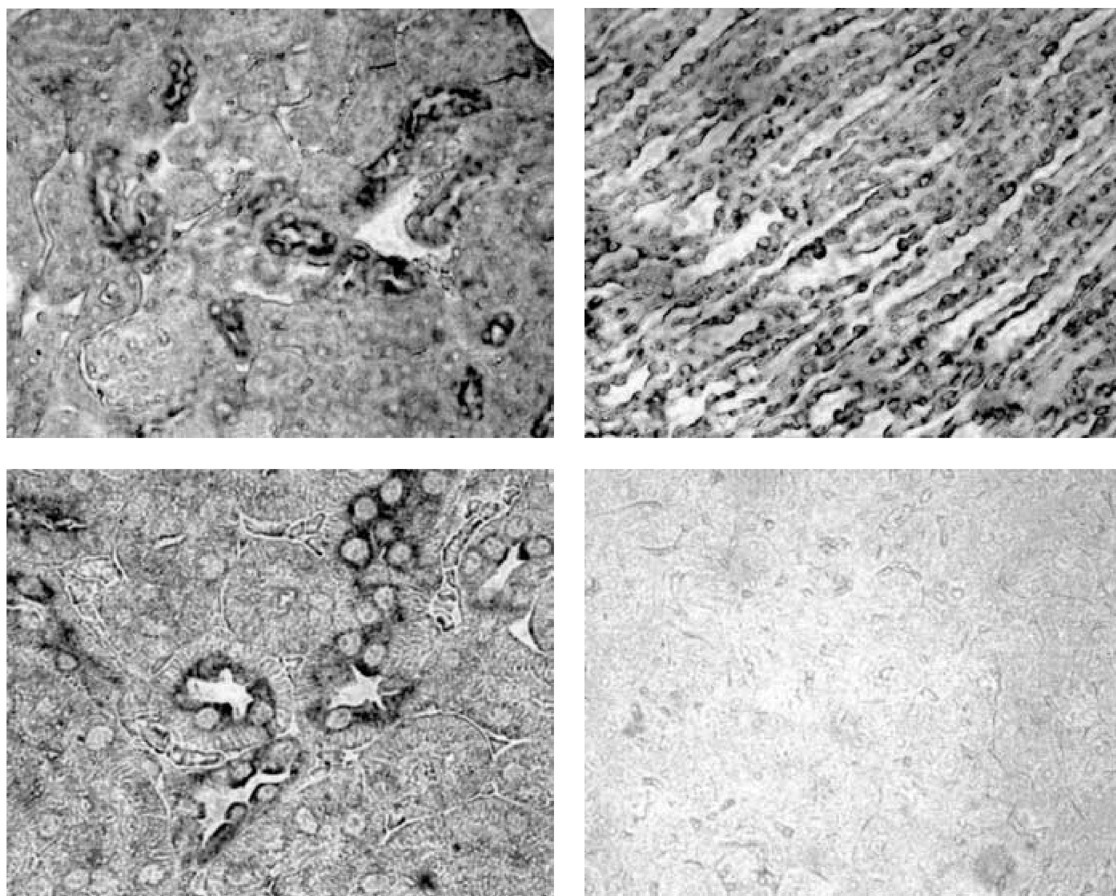

Fig. 3. Stromal cell–derived factor-1 (SDF-1) mRNA in situ hybridization in normal kidney. Normal kidney showing strong staining mainly of distal tubules and weak staining of remaining tubular segments (upper left panel). Glomeruli are negative for SDF-1 expression (original magnification ×20). Expression in the medulla (upper right panel). Higher magnification (lower left panel) of the same kidney (upper left panel) (original magnification ×40). Control hybridization with sense probe (lower right panel). No nuclear counterstain was used.

Bone marrow and CD34-positive cells migrate toward damaged kidney cells in vitro

Primary kidney tubular cells grown from 1- to 2-week-old mice were injured by ATP depletion for 30 minutes in serum- and glucose-free medium containing Antimycin A and 2-deoxy glucose. This procedure led to a significant decrease of ATP levels to 10% of baseline as determined by a luminescence assay (data not shown) and increased expression of SDF-1 (see above). Bone marrow cells expressing CXCR4 seeded onto the top of a transwell insert showed a significant migratory response toward the ATP-depleted monolayer of tubular cells in the bottom well when compared to that observed with undamaged cells (Fig. 7). To determine the role of SDF-1 in this migratory response, the SDF-1 receptor, CXCR4, was blocked with an anti-CXCR4 antibody, which significantly decreased cell migration toward damaged tubular cells. However, since there was still a significant difference between CXCR4-blocked and control cell migration, it is likely that other factors besides SDF-1 are involved in mediating this migratory response. The same migratory response was seen when immunomagnetically isolated CD34-positive cells were tested with this system (data not shown).

Bone marrow cells home to the kidney in vivo

To determine the capacity of injected bone marrow cells to home into the kidney in vivo, we developed a homing assay as described in the **Methods** section. The kidney contained an average of eight injected bone marrow cells per million cells at day 1 after ARF, whereas the bone marrow contained three cells per million cells (Fig. 8). After blocking the SDF-1 pathway with antibodies against CXCR4, the numbers of homing cells in kidney and bone marrow were significantly reduced to 0.6/million cells and 0.13/million cells, respectively. This pattern corroborates the results of the previous in vitro studies showing the importance of the SDF-1/CXCR4 system in chemotaxis.

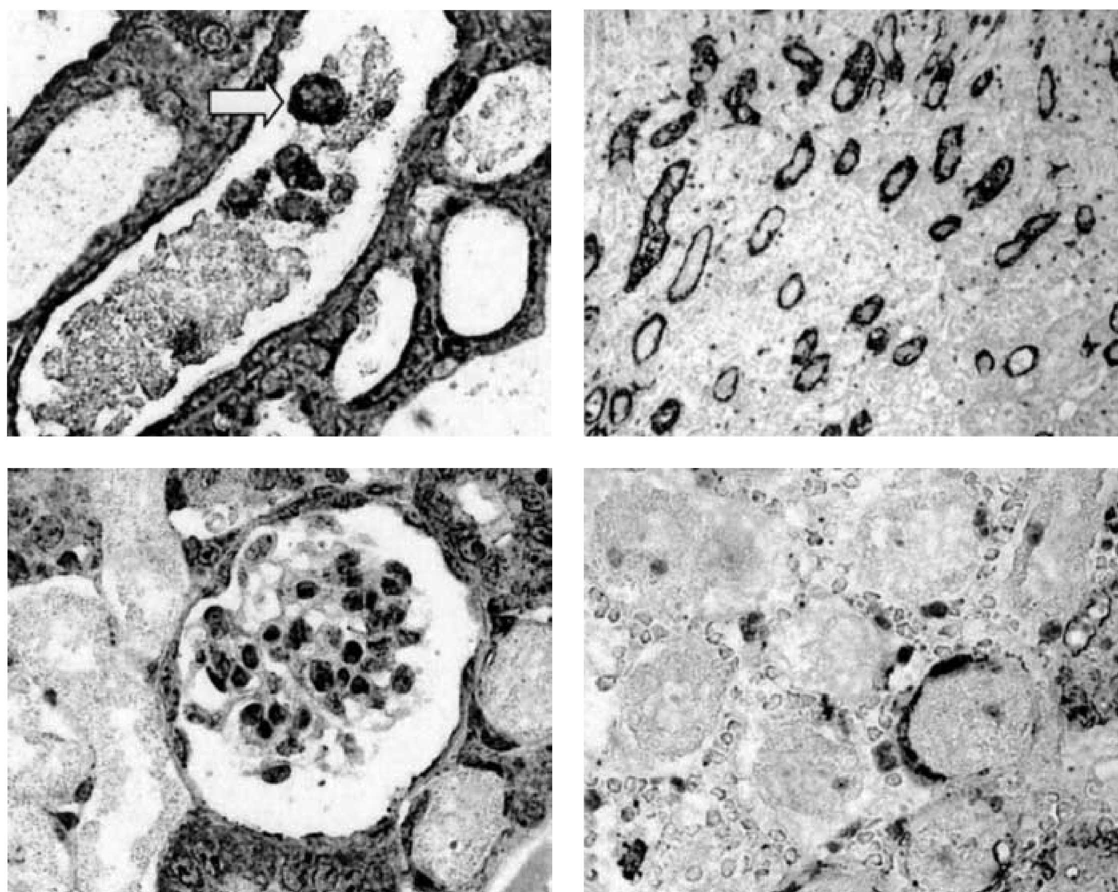

Fig. 4. Stromal cell–derived factor-1 (SDF-1) mRNA in situ hybridization in kidney after acute renal failure (ARF). Injured but surviving cells highly express SDF-1 mRNA (left upper panel), while necrotic areas stain negative (upper right panel) (original magnification ×10; other panels ×100). Note the expression of SDF-1 in detached cells in the tubulus lumen (arrow, upper left panel). Nuclei are counterstained with hematoxylin (original magnification in lower panels show higher magnifications ×40).

Homing is specific to the injured kidney

Unilateral clamping of the left kidney and injection of bone marrow showed that the homing process is selective for the injured organ (data not shown). The damaged kidney contained the same amount of cells as seen in bilateral clamping experiments, whereas the noninjured kidney did not contain any cells 24 hours after the infusion. Involvement of the SDF-1/CXCR4 system was also demonstrated by the almost complete absence of homed cells in the damaged kidney and bone marrow after blocking CXCR4 (Fig. 8).

ARF causes mobilization of CD34-positive cells

SDF-1 is a known stem cell and leukocyte-mobilizing chemokine [28]. Since we showed an increase of SDF-1 plasma levels after ARF, we asked if there is an increase in circulating CD34-positive cells during recovery from ARF. Levels of circulating CD34-positive cells in normal animals are 1% of leukocytes, and after ARF, there is a significant increase ($P < 0.01$) to 7% of total leukocytes (Fig. 9). These data correspond well with the increase in SDF-1 plasma levels, although it is likely that other factors are involved in causing the rise of peripheral circulating CD34-positive cells. Paralleling the increase of circulating peripheral blood CD34-positive cells, we detected an increase of CFU-C in peripheral blood (500 μL) after ARF. Control mice had a mean of 1.1 ± 0.5 ($N = 4$) CFU-C/500 μL peripheral blood and 24 hours after induction of ARF this increased to a mean of 14 ± 1.6/500 μL ($P < 0.05$).

DISCUSSION

This study was performed because we hypothesized that the SDF-1/CXCR4 system is important in the regulation of stem and progenitor cell trafficking after ischemic ARF. Our results demonstrate that SDF-1 is expressed in the normal kidney and up-regulated after ARF. SDF-1 serum levels increase after ARF while bone marrow SDF-1 levels decrease, leading to a reversal of the normal gradient from bone marrow to peripheral blood, in turn resulting in mobilization of CD34-positive cells that have colony forming ability as shown by CFU-C assay. We also

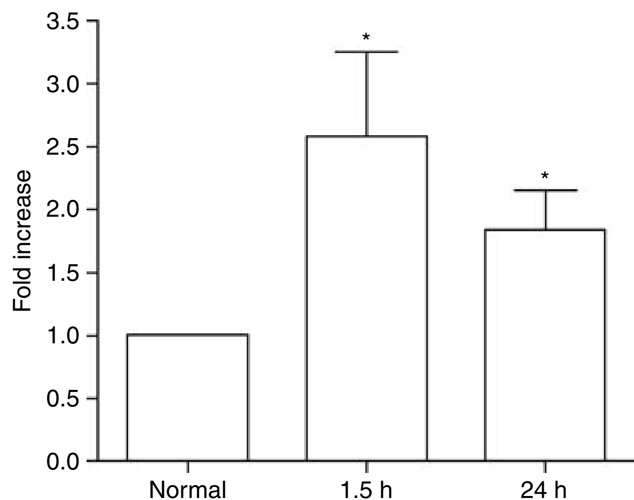

Fig. 5. Stromal cell–derived factor-1 (SDF-1) mRNA levels early after acute renal failure (ARF). Real-time polymerase chain reaction (PCR) quantification of SDF-1 mRNA showed a significant increase in transcription as early as $1\frac{1}{2}$ hours after ischemia, and with still significantly elevated levels at 24 hours. $^{*}P < 0.05$, vs. normal.

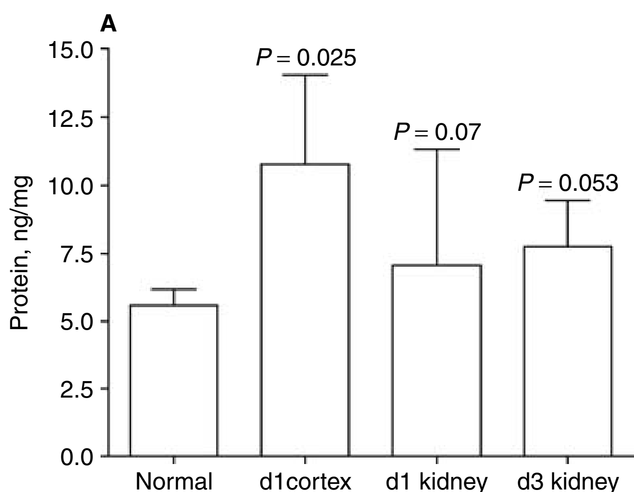

Fig. 6. Stromal cell–derived factor-1 (SDF-1) protein levels in kidney (*A*), plasma and bone marrow (*B*) after acute renal failure (ARF). SDF-1 protein in the kidney cortex (A) was significantly elevated at 24 hours after ARF compared to normal kidney levels, but not in the whole kidney on day 1(d1) and day 3 (d3) after ARF. SDF-1 plasma levels (B) were significantly elevated at day 1(d1) and day 3 (d3) compared to baseline. Total nephrectomy (total nx) also led to elevated SDF-1 levels compared to baseline. Bone marrow (BM) SDF-1 levels were reduced after ARF compared to bone marrow baseline levels, thereby reversing the normal bone marrow blood gradient.

show that CXCR4, the receptor for SDF-1, is expressed on tubular cells and expression is up-regulated after ischemia. Because cells, such as HSCs and EPCs, that express CXCR4 may have renoprotective effects and SDF-1 is not an inflammatory but a homeostatic chemokine, our results suggest that SDF-1 may be a currently unrecognized signal involved in kidney repair.

SDF-1 is a unique chemokine being highly conserved in mammals. It is the only chemokine necessary for embryonic survival, it binds to and signals exclusively through CXCR4, and is essential for stem cell homing. Although it has been shown to be a chemoattractant for T cells, movement of T cells is concentration-dependent and there is a selective repulsion of T-cell subpopulations at higher levels of SDF-1, which explains the low frequency of T cells in compartments like the bone marrow [30]. SDF-1 is known to play a key role in CD34-positive cell trafficking [28], and to mediate mobilization of HSCs and progenitor cells from the bone marrow by chemotaxis [28, 31]. This system is also important in the homeostasis of a number of other leukocyte types such as lymphocytes, monocytes, and neutrophils. Although CXCR4 and SDF-1 are induced by hypoxia [32] or DNA damage [4], SDF-1 is primarily a homeostatic rather than an inflammatory chemokine. This has been demonstrated in patients with unstable angina, where SDF-1 in high concentrations has anti-inflammatory and matrix stabilizing effects [33].

We show in the present study that the SDF-1/CXCR4 system appears activated in ARF. It stimulates CD34-positive cell mobilization from the bone marrow as shown by increased numbers of circulating CD34-positive cells by FACS analysis and CFU-C, and it directs recruitment of CXCR4 expressing bone marrow cells to the injured kidney, as shown by in vitro and in vivo migration assays.

ARF, a common complication among hospitalized patients, is still affected with a disturbingly high mortality rate. Treatment options are limited to largely supportive measures. New stem cell–based treatment approaches have been tested, but to date there is only one report showing that administered MSCs are of functional benefit in cis-platinum–induced ARF [34]. HSCs have been shown to contribute to the tubular cell pool [14, 15]; however, their functional impact on the severity of ischemic ARF and their contribution to regeneration in this form of ARF remains to be established. Only recently has the microvascular component of ARF come to broader attention [35], and the targeting of vascular damage seems a promising new treatment approach in ARF. HSCs and EPCs are derived from a common progenitor cell, the hemangioblast [36]. Our results show that circulating CD34-positive cells with the capacity to form CFU-Cs are increased after ARF and that this is mediated, at least in part, by reversal of the SDF-1 gradient from blood to bone marrow. Since EPCs are also characterized by CD34 expression and high numbers of CXCR4 receptors in their cell membrane, mobilization of EPCs and HSCs, induced by ischemic ARF, is possibly an adaptive response, mainly mediated by SDF-1, a response that has the potential to support the repair of the injured kidney.

There are various models for stem cell repair of damaged tissues. Tissue repair is facilitated in many cases

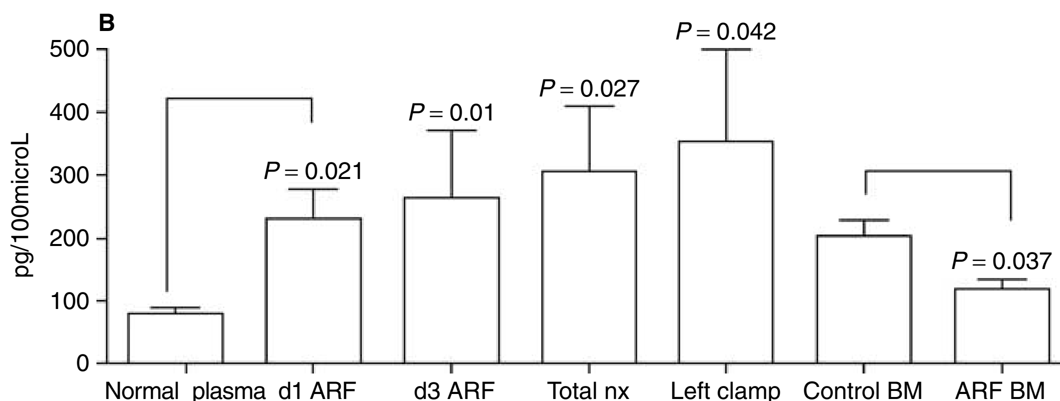

Fig. 6. (continued)

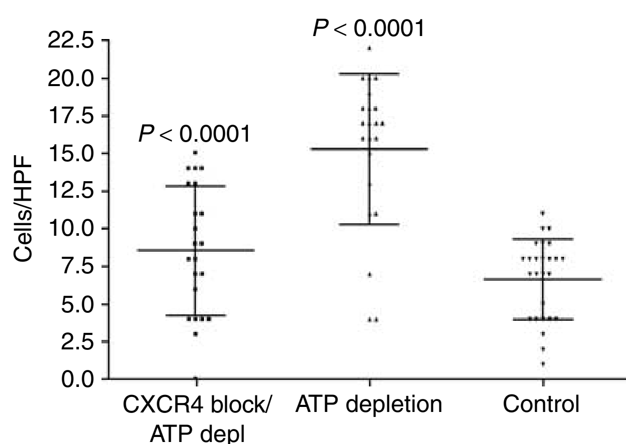

Fig. 7. In vitro migration assay. Bone marrow (BM) cells migrate toward adenosine triphosphate (ATP)-depleted primary kidney tubular cells. By blocking CXCR4 with an antibody, migration is significantly reduced compared to ATP-depleted monolayers, but it remains significantly higher than compared to control experiments, suggesting that other migration-stimulating factors are involved as well.

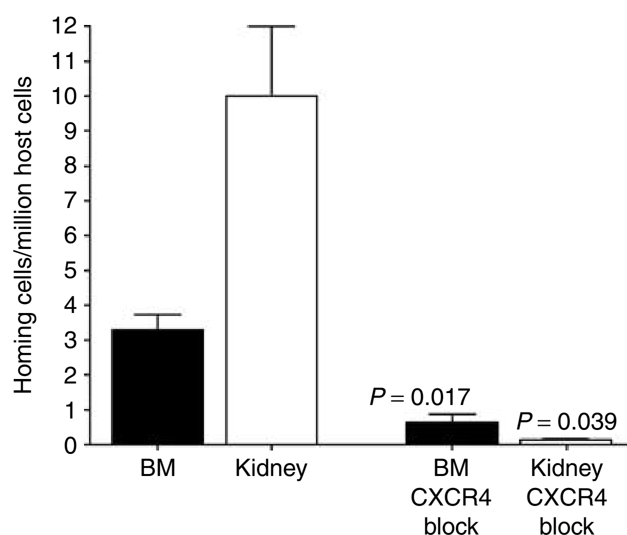

Fig. 8. In vivo homing of whole bone marrow (BM) cells in kidney and bone marrow. Intravenously injected bone marrow cells home to the injured kidney after 24 hours as well as to the bone marrow. This homing process can be significantly inhibited compared to homing without blocking by preincubation with a blocking antibody to CXCR4.

(e.g., liver injury or kidney tubule damage, by dedifferentiation, migration, proliferation and redifferentiation of cells). However, repair is also thought to involve selective recruitment of circulating stem and progenitor cell populations. A model proposed by Anderson, Gage, and Weissman [37] suggests that an equilibrium exists between apoptotic cell loss and replacement of cells by tissue intrinsic stem cells. In addition, blood-derived stem cells of heterologous lineages are thought to contribute to tissue repair, although to a much lower extent. In the case of tissue damage (e.g., when apoptosis exceeds the repair capacity of intrinsic stem cells), recruitment of circulating stem cells serves as back-up response. This response may be mediated by the SDF-1/CXCR4 system. However, if damage is very severe, intrinsic repair capacity will be exhausted, resulting, in turn, in disease such as irreversible ARF. Our data provide evidence in support of this model, since we demonstrate both activation of the SDF-1/CXCR4 system in ARF as well as mobilization of CD34-positive cells into the circulation.

Accordingly, we suggest that kidney tissue damaged by ischemia initiates repair by extrinsic cells via up-regulated expression of SDF-1, which, in turn, leads to the mobilization and homing of CXCR4/CD34-positive cells. Furthermore, the concentrations of SDF-1 we found in the kidney reach the concentration detected in the bone marrow, a level shown to induce repulsion of T cells and thereby having an anti-inflammatory effect [30]. However, the mobilization of CD34-positive cells might not be adequately protective in the case of severe injury. A possible way of enhancing this chemokine response could be the administration of SDF-1 or the enhancement of EPC mobilization with sulfated polysaccharides [38].

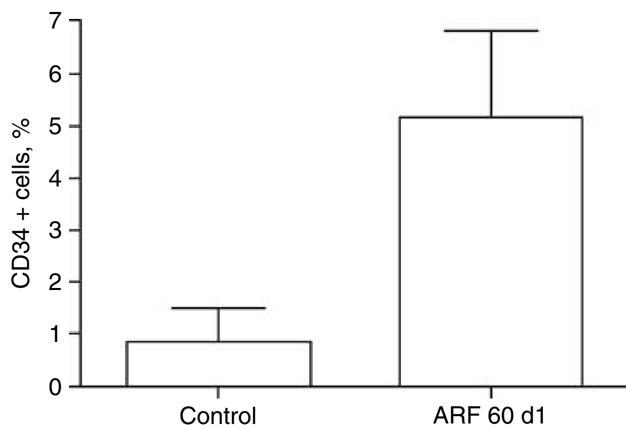

Fig. 9. Circulating CD34-positive cells in peripheral blood (PB) of control animals and animals with 60 minutes ischemic acute renal failure (ARF). The number of circulating CD34-positive cells is significantly ($P < 0.0014$) increased at 24 hours after induction of ARF.

We also show CXCR4 expression on tubular cells and up-regulation after ischemia. CXCR4 has been described in different epithelial cell populations like alveolar epithelial cells as well as colonic epithelial cells [39]. CXCR4 is up-regulated by hypoxia and has also been implicated in cancer metastasis and leukocyte migration [40]. Hypoxic induction of CXCR4 may be a protective mechanism, since it was demonstrated that increased CXCR4 expression enhanced proliferation of epithelial cells by activating the mitogen-activated protein (MAP)/Erk kinase pathway, and acceleration of angiogenesis by increasing vascular endothelial growth factor (VEGF) secretion [41]. Although it is speculative, it is possible that the increase of SDF-1 expression in the kidney directly protects the epithelial cells that have up-regulated CXCR4 by the MAP/Erk kinase pathway as mentioned above.

Bone marrow–derived circulating EPCs participate in the vascular regeneration after various injuries. For example SDF-1 was observed to attract EPCs to ischemic regions in a hind-limb injury model, a response that prevented apoptosis and led to improved vasculogenesis and neovascularization [42]. It was recently reported that fibroblasts, engineered to express SDF-1, and when injected into ischemic cardiac tissue attract mobilized stem cells, which in turn resulted in improved cardiac function after ischemia [43]. In kidney allografts, the extent of replacement of donor endothelial cells lining the peritubular capillaries by those of the recipient is related to the severity of concurrent vascular injury [44]. Since ARF also has an important vascular injury component, this vascular repair process might also play a role in physiologic as well as therapeutic regeneration.

Endothelial injury, an important component of ARF, leads to increased recruitment of inflammatory cells causing further cellular damage. Targeting this component of the injury cascade by enhancing repair of damaged vessel segments through mobilization of EPCs may be a promising new treatment approach. Preliminary data from our laboratory [abstract; Westenfelder C et al, *J Am Soc Nephrol* 14:570A, 2003] and others [22] have shown that infused, differentiated endothelial cells are renoprotective after ARF. Certain other stem cell populations such as mesenchymal stem cells with endotheliogenic potential are also under investigation.

Induction of SDF-1 induction after ischemia is mediated by hypoxia-inducible factor-1 (HIF-1) [45], a transcription factor and the central mediator of cellular responses to hypoxic conditions. It is readily degraded in normoxic cells and HIF-2 has been shown to be an important mediator of adaptive responses after ischemic ARF [46].

Ratajczak et al [47] found that there are circulating progenitor cells for different lineages in the peripheral blood after injury and hypothesized that these are mobilized as a repair response induced by damaged organs. They also showed an increase in SDF-1 mRNA in the kidney after CCl_4 induced liver and secondary kidney injury, corroborating our present results.

Hatch et al [48] showed that SDF-1 contributes to hepatic oval cell activation and bone marrow stem cell recruitment after liver injury. The involvement of SDF-1 as well as hepatocyte growth factor (HGF) and matrix metalloproteinase-9 (MMP-9) in human stem cell recruitment to the injured liver has also been demonstrated by Kollet et al [49]. Taken together, these reports support our finding that the SDF-1/CXCR4 system plays an important role after renal damage.

We have previously shown that mobilization of HSC leads to a more severe form of ARF with higher mortality and greater histologic damage [27]. However, the mobilization regimen used in the previous study is entirely different from the mobilization of CD34-positive cells described here, because granulocyte-colony-stimulating factor (G-CSF) and cyclophosphamide administration leads to massive leukocytosis with a large increase in circulating granulocyte numbers, a cell type that is significantly involved in the pathogenesis of ARF. Furthermore, ARF was induced at the time when circulating neutrophils numbers peaked. The mobilization of CD34-positive cells, as described here, mainly takes place due to a reversal of the SDF-1 gradient from bone marrow to peripheral blood, which is a stimulus for mobilization of progenitor cells [29]. Although it is known that SDF-1 can induce leukocytosis as well [28], the time frame for this response differs from the course of ARF in our model.

CONCLUSION

Our results describe the SDF-1/CXCR4 system in the kidney after ischemic ARF and its role in the recruitment and mobilization of CXCR4-positive cells. This adaptive

response to ARF likely serves to support organ repair by cells extrinsic to the kidney, and, if it can be therapeutically enhanced, it may prove of therapeutic utility.

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Reprint requests to Dr. Christof Westenfelder, Section of Nephrology (111N), VA Medical Center, 500 Foothill Boulevard, Salt Lake City, UT 84148.
E-mail: c.westenfelder@uofu.net

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