Mechanisms of renal injury and repair: role of stem cells, chemokines and the nodosome
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SDF-1 provides morphological and functional protection against renal ischemia/reperfusion injury

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Abstract

Background
The chemokine stromal cell-derived factor-1 (SDF-1) is thought to be involved in mediating tissue repair by promoting migration of bone marrow stem or progenitor cells to the site of injury. Increased levels of renal SDF-1 are found after kidney injury. However, recently we showed that SDF-1 does not play an important role in the migration of hematopoietic stem cells to the postischemic kidney. The function of increased postischemic renal SDF-1 expression in modulating renal ischemia/reperfusion injury remains therefore unknown.

Methods
We studied the role of SDF-1 in renal ischemia/reperfusion injury by locally decreasing SDF-1 expression and subsequent SDF-1 signaling in the corticomedullary region of the kidney using antisense oligonucleotide treatment in mice.

Results
Renal SDF-1 protein increased significantly in the early phase of ischemia/reperfusion injury. Antisense treatment resulted in a reduction of corticomedullary SDF-1 expression which was accompanied by severely increased tubular injury and decreased renal function. We did not observe any difference in mobilization or retention of CXCR4 positive hematopoietic stem or progenitor cells after induction of renal ischemia. Rather, antisense treated animals showed markedly increased apoptosis of the tubular epithelium accompanied by an increased renal inflammatory response.

Conclusion
These data indicate a new role for SDF-1 in renal pathogenesis by mediating tubular epithelial protection against ischemic injury and suggest that SDF-1 by itself is not crucial for the influx of hematopoietic stem or progenitor cells towards the ischemic injured kidney.
SDF-1 and renal I/R injury

Introduction

The chemokine SDF-1 (CXCL12) and its receptor CXCR4 have been shown to be involved in tissue repair by mediating migration of circulating stem or progenitor cells to the site of peripheral injury in various tissues where they promote angiogenesis or are engaged in other mechanisms of repair. A similar mechanism has been postulated for renal repair, whereby expression of SDF-1 by tubular epithelial cells (TEC) following renal ischemia/reperfusion (I/R) injury is increased and in this way could mediate migration of bone marrow derived cells to the kidney. We however recently showed that SDF-1 does not play a significant role in the migration of purified hematopoietic stem cells (HSC) during renal I/R injury. Although HSC preferentially migrate to the injured kidney following renal ischemia, local administration of SDF-1 recombinant protein did not increase HSC migration to the kidney during I/R injury. Using the opposite approach, neutralization of SDF-1 or blocking of HSC-associated CXCR4 with neutralizing antibodies also did not inhibit HSC migration. Hence, the functional significance of the increased postischemic SDF-1 expression by TEC in modulating renal I/R injury remains unknown. Recent studies implicate that SDF-1 besides regulating the migration of cells, also has other functional activities. SDF-1 can enhance cell survival by inhibiting apoptosis in CD34+ cells, CD4+ cells, myeloid precursor cells, embryonic retinal ganglionic cells, and mesenchymal stem cells. However, nothing has been reported about a possible role for SDF-1 in the function of TEC during injury.

In the present study we aim to understand the role of SDF-1 expression of TEC during I/R damage by studying disruption of SDF-1 expression in mice using specific antisense oligonucleotides (ASON). These ASON are mainly targeted to the kidney, with the highest accumulation in renal epithelial cells and are therefore highly suitable to study the role of renal-specific SDF-1 in the ischemic damaged kidney. We found that a local decrease of SDF-1 expression in the corticomedullary area severely increased renal dysfunction and injury whereas this had no effect on stem cell mobilization to the circulation.
Subjects and methods

Mice and experimental model
Eight to 10 week old wild type male C57BL6 mice were purchased from Charles River (Maastricht, The Netherlands). I/R injury was induced by bilateral clamping of the renal arteries for 45 minutes as described previously\textsuperscript{11,13}. Mice (n=8 per group) were treated twice (i.p.) with 4nmol ASON or nonsense oligonucleotides (NSON) per injection dissolved in saline. To compensate for possible adverse effects, control animals were treated with NSON. Both nonsense and antisense phosphorothioate-labeled oligonucleotides targeted to SDF-1 were purchased from Biognostik (Göttingen, Germany). Each first dose was given 24 hours prior to I/R injury, the second dose just after release of the clamps. Animals were sacrificed by cervical dislocation 24 hours following reperfusion. Blood samples were obtained via heart puncture and transferred to heparin tubes. Kidneys were snap frozen in liquid nitrogen and stored at -80°C or fixed in 10% formalin for 10 hours prior to further processing. Bone marrow was flushed from tibia and femurs with phosphate buffered saline (PBS) supplemented with 10% foetal calf serum (FCS; Hyclone, Etten-Leur, Netherlands) and stored on ice until further analysis. Sham operated mice (n=8) were handled similarly with the exception of clamping of the renal arteries. To examine the \textit{in vivo} distribution of ASON, mice were treated twice i.p. with 4nmol FITC-labelled ASON (Biognostik) and subjected to sham or renal I/R surgery as described above.

To determine expression of SDF-1 in time, mice were subjected to bilateral ischemia and sacrificed after 1, 5 or 10 days (n=8 per group). Sham operated animals received identical treatment without clamping of the renal arteries and were sacrificed at day 1 after ischemia (n=6 per group). All experimental procedures were approved by the Animal Care and Use Committee of the Academic Medical Center.

Antibodies
Antibodies used for immunostainings and FACS analyses were the following. Fluorescein isothiocyanate labelled anti-mouse Gr-1/Ly6G, phycoerythrin labelled anti-mouse Sca-1 and allophycocyanin labelled anti-mouse c-KIT were purchased from BD Biosciences (Alphen a/d Rijn, Netherlands). The rabbit monoclonal antibody to Ki.67 (Sp7) was purchased from Neomarkers (Fremont, CA), the rabbit monoclonal to cleaved caspase 3 from Cell Signalling Technologies (Beverly, MA). A rabbit-anti-mouse SDF-1a antibody was from eBioscience (San Diego, CA). The rabbit antibody to FITC was from DAKO. PowerVision poly-HRP anti rabbit IgG was from Immunologics (Duiven, Netherlands), all other secondary antibodies were purchased from DAKO (Glostrup, Denmark).
Histology, Renal Function, and Immunohistochemistry

Formalin fixed tissue was embedded in paraffin using standard procedures. Four μm thick sections were cut and used for all stainings. For examining renal histology, sections were stained with periodic acid-Schiff reagents after diastase digestion (PAS/D). Injury to tubules was assessed by determining the percentage of affected tubules per 10 fields (magnification, x400) in the corticomedullary region according to the following criteria: Tubular dilation, epithelial necrosis, cast deposition, and loss of brush border. Injury was graded on a scale from 0 to 5: 0, 0%; 1, <10%; 2, 10 to 25%; 3, 25 to 50%; 4, 50 to 75%; and 5, >75%. For assessing renal function, plasma urea concentrations were measured by standard diagnostic procedure, suitable for detection of samples of murine origin. To stain for Gr-1, tissue sections were treated for 30 minutes with 0.25% pepsin in 0.1M HCl at 37°C, for detection of active caspase 3, Ki.67, and FITC sections were boiled for 10 minutes in a 0.3% citrate buffer (pH 6). For all stainings, sections were incubated with primary antibodies for 2 hours and relevant peroxidase conjugated secondary antibodies for 30 minutes in PBS and stained using 3 to 3’ diaminobenzidine dihydrochloride (DAB). To quantify neutrophils, proliferation (Ki.67) or apoptosis of tubular epithelial cells (caspase-3), positive cells were counted per 10 high-power fields (HPF, magnification: 400x) in the corticomedullary region.

Flow Cytometric Analyses

White blood cells and bone marrow cells were counted on a Coulter ACT diff2 (Beckman Coulter, Mijdrecht, The Netherlands). Erythrocytes were lysed in 160 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA (pH 7.4). Analysis of c-Kit, Sca-1 and CXCR4 expression was performed by incubating cells with fluorochrome labelled monoclonal antibodies for 30 minutes before analysis, cells were fixed in PBS that contained 2% paraformaldehyde. FACS analyses were performed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

ELISA

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

Quantitative real-time RT-PCR

Total RNA was extracted from kidney using the TRIzol® reagent (Invitrogen, Breda, Netherlands) and converted to cDNA. Quantitative real-time RT-PCR was performed on a LightCycler® 480 System (Roche, Mijdrecht, Netherlands) using LightCycler® 480 SYBR Green I Master mix (Roche). Specific gene expression was normalized towards the housekeeping gene peptidylprolyl isomerase A (PPIA). Primer sequences are
as follows: SDF-1 forward ggtcttcagagccacatc and reverse ttcttcagcgtcaaca PPIA forward tgccagggtgtgactttac and reverse gatgccaggacctgtatgct.

**Statistical Analyses**

All statistical analyses were performed using Graphpad 4 software (San Diego, CA). Data were analyzed using the Student T test; tubular injury scores were analyzed using the nonparametric Mann-Whitney U Test. Results are expressed as means ± SEM. A value of $P < 0.05$ was considered statistically significant.

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**Figure 1.** SDF-1 expression following ASON treatment

(a) Levels of SDF-1 were determined by ELISA using whole kidney homogenates obtained from sham operated animals or animals sacrificed at day 1, 5 or 10 following ischemia. The amount of SDF-1 at day 1 was significantly higher compared to that found in sham operated animals (*P=0.004). (b) Kidney homogenates from mice subjected to I/R injury and treated with NSON (white bar) or ASON (black bar) were analyzed for SDF-1 using ELISA. ASON treatment resulted in a decrease of SDF-1 levels compared to the concentration found in homogenates from NSON treated animals that reached statistical significance (*P=0.056). Animals were sacrificed 24 hours following ischemia. (c) Total mRNA isolated from kidneys of mice subjected to I/R injury and treated with NSON (white bar) or ASON (black bar) were analyzed for SDF-1 using quantitative real-time RT-PCR. ASON treatment resulted in a 5-fold decrease of SDF-1 mRNA compared to NSON treated animals (*P<0.01). Animals were sacrificed 24 hours following ischemia. Kidney sections from NSON (d) or ASON (e) treated animals 24 hours after ischemia were stained for SDF-1 protein expression. Staining revealed a basolateral expression pattern for SDF-1 in TEC from the corticomedullary area in NSON treated animals (as indicated by the arrow), which was reduced in ASON treated animals. Magnification: 200x. Data are expressed as mean ± SEM.
Results

SDF-1 expression is affected during renal I/R injury and after antisense treatment

To analyze the renal SDF-1 expression during different phases of renal ischemic injury mice were subjected to bilateral I/R injury and sacrificed after several timepoints. This revealed that the level of total (active and inactive) renal SDF-1 increased significantly at day 1 following ischemia compared to that in kidneys from sham operated animals and decreased steadily to control levels in the following days (figure 1a). Since the highest total SDF-1 level was found on day 1, we next studied the effect of SDF-1 on renal pathogenesis at this time point by treating animals with antisense oligonucleotides (ASON) which blocks transcription of SDF-1.

First, we analyzed the renal distribution of ASON uptake using FITC-labeled ASON. ASON were targeted to renal tubular cells (Figure 2a). Even after renal I/R injury ASON accumulated in renal tubular cells including the damaged tubules (Figure 2b). ELISA analysis of whole kidney homogenates demonstrated that total SDF-1 concentrations were decreased in SDF-1 ASON treated animals compared to NSON treated control approaching statistical significance (figure 1b, P=0.056).

As demonstrated previously, expression of SDF-1 in the healthy kidney is predominantly localized at the cortex, whereas it is expanded to the corticomedullary area of the kidney during I/R injury (figure 1d). In line, we detected SDF-1 in the corticomedullary area of NSON treated animals after ischemia. However, this localized expression was considerably reduced in renal tissue sections of ASON treated animals (figure 1e).

ASON-induced protein knockdown is achieved by inhibiting translation of the protein and by reduction of mRNA via a RNase H-dependent mechanism. Concurring, SDF-1 ASON treated animals showed a 5-fold (P<0.01) reduction of SDF-1 mRNA expression compared to NSON treated controls (Figure 1c).

Figure 2. Distribution of ASON in kidney
The distribution of ASON in kidneys was determined by using FITC-labeled ASON. One day after (a) sham or (b) I/R injury ASON was detected in renal tubular epithelial cells of the corticomedullary region.
SDF-1 antisense treatment increases renal damage and dysfunction during I/R injury

Next we determined the impact of ASON treatment on tubular injury, by using a semi-quantitative scoring system\textsuperscript{11,13}. Despite the mild but specific inhibition of SDF-1 expression, ASON treated animals demonstrated a significant increase in tubular damage of the corticomedullary area compared to controls (figure 3a-c). In accordance with the increase in tubular damage, urea levels were significantly higher in ASON treated animals compared to NSON treated animals demonstrating a drastically decrease in renal function (figure 3d). Animals that were subjected to I/R injury and treated with NSON or vehicle only, showed a similar degree of renal dysfunction (data not shown), demonstrating that the treatment with phosphorothioate capped oligonucleotides itself does not induce any non-specific adverse effects. Moreover, no difference in renal function was observed between ASON or NSON treated animals subjected to sham surgery (figure 3d).

![Figure 3. Tubular damage and renal function following ASON treatment](image_url)

Representative pictures of PasD stained kidney sections of NSON (a) and ASON (b) treated animals 24 hours after renal ischemia. (c) Tubular damage was scored semi-quantitatively using PAS/D stained kidney tissue sections in a blinded fashion. ASON treatment (black bar) significantly increased tubular damage 24 hours after ischemia compared to NSON treated animals (white bar) (*$P=0.01$). (d) Plasma urea levels were measured to determine renal function. ASON treatment (black bar) significantly increased plasma urea compared to that found in NSON treated controls (white bar) at 24 hours following ischemia (*$P<0.0001$). Data are expressed as mean ± SEM.
Figure 4. ASON treatment and inflammation
(a) The number of circulating and bone marrow c-KIT/Sca-1/CXCR4 triple positive cells was determined by FACS analysis. No differences were found in the number of positive cells in bone marrow samples (left) or blood samples (right) obtained from NSON treated (white bars) or ASON treated (black bars) animals at 24 hours following ischemia. Neutrophil influx into the kidney was determined by staining tissue sections with an antibody to the marker Gr-1 expressed by neutrophils. Representative pictures of Gr-1 stained kidney sections of NSON (b) and ASON (c) treated animals 24 hours after renal ischemia. Positive cells were counted in 400-fold magnified fields (high power field, HPF) in the corticomedullary area. (d) Significantly more Gr-1 positive cells were counted in tissue sections from ASON treated animals compared to the number from NSON treated controls at 24 hours following ischemia (*P=0.0415). (e) The concentration of KC in whole kidney homogenates was measured by ELISA. Samples obtained from ASON treated animals (black bars) contained a significantly higher concentration of KC at 24 hours after ischemia compared to ASON treated controls (white bars) (*P=0.006). Data are expressed as mean ± SEM.
Migration of HSC is not prevented by blocking SDF-1 with antisense oligonucleotides

SDF-1 is thought to be responsible for migration of circulating stem or progenitor cells which mediate tissue repair. HSC have been shown to migrate towards the injured kidney following ischemia\textsuperscript{3,4} and may contribute to renal repair\textsuperscript{15,16}. Since decreased SDF-1 expression following ASON treatment correlated with increased renal damage, we determined whether we could observe a difference in the number of mobilized, circulating HSC or hematopoietic progenitors identified by their expression of the markers c-KIT and Sca-1\textsuperscript{17} by FACS analysis. At 24 hours following ischemia we could not observe a difference in the number of CXCR4 positive c-KIT/Sca-1 cells that were present in the circulation or the bone marrow (figure 4a). This suggests that ASON treatment did not result in HSC retention but also did not affect their mobilization. Therefore disrupted BM stem or progenitor cell mobilization following ASON treatment is unlikely to be the underlying cause for the increase in renal damage we observed.

SDF-1 antisense treatment increases an intrarenal inflammatory response during I/R injury

Neutrophils are important early mediators of I/R injury\textsuperscript{18}. To determine whether ASON treatment affected neutrophil influx we stained tissue sections using an antibody to the Gr-1 epitope expressed by neutrophils. We found that significantly more neutrophils were present in kidneys from ASON treated animals compared to NSON treated mice 24 hours following ischemia (figure 4b-d). Consistently, the level of neutrophil chemoattractant KC was increased in kidney homogenates from ASON treated animals (figure 4e). Both neutrophil influx and KC levels were similar between ASON and NSON treated sham animals (figure 4d-e).

SDF-1 antisense treated animals have an increased amount of apoptotic TEC during I/R injury

We next determined whether ASON treatment following ischemia also affected TEC. Apoptosis of TEC was determined by performing immunostainings to the active form of caspase 3. Interestingly, we found significantly more apoptotic TEC in kidney section from ASON treated animals compared to NSON controls after 24 hours (figure 5a). This suggests that SDF-1 may act on TEC and induce protection against I/R injury. To determine the rate of proliferation, we stained tissue sections of kidneys from both groups for Ki.67 expression which is present in cycling cells and counted the positive TEC (figure 5b). No difference was however observed in the number of cycling TEC between both groups. Together these data suggest that SDF-1 may act on TEC during renal I/R injury by inducing protection against I/R-mediated apoptosis.
Figure 5. TEC apoptosis and proliferation
(a) Tissue sections obtained from NSON or ASON treated animals (white and black bars, respectively) were stained with an antibody to the active form of caspase 3 as a marker for cell apoptosis. We counted significantly more caspase 3 positive TEC per HPF in ASON treated animals, compared to NSON treated animals 24 h following ischemia (*P=0.0008). (b) Tissue sections were analyzed for proliferation of TEC via staining for Ki.67 expression. We counted a similar number of Ki.67 positive TEC per HPF on sections from ASON treated animals (black bars) compared to NSON treated controls (white bars) at 24 hours following ischemia or sham operation. Data are expressed as mean ± SEM.
SDF-1 has been shown to be important for tissue repair by mediating the trafficking of circulating stem or progenitor cells to the site of peripheral injury in various tissues. The current study provides evidence for a new function for SDF-1 in mediating renal repair following I/R injury which is independent of the migration of HSC. SDF-1 in the kidney was originally proposed to be involved in mediating migration of bone marrow cells to the ischemic kidney. We however recently demonstrated in three different and independent manners that migration of purified HSC to the ischemic damaged kidney occurs independently of the SDF-1/CXCR4 signaling axis. In accordance with this study, we found here that reduced expression of SDF-1 during I/R injury by a fourth manner, namely ASON treatment, did not result in a consequent reduction in HSC mobilization. Apparently SDF-1/CXCR4 signaling is able to mobilize heterogeneous bone marrow cells during renal ischemic injury while this axis is not exclusively responsible for the migration of HSC. Conceivably, other mechanisms might also be involved in HSC migration to the ischemic damaged kidney. Indeed, HMGB-1, HGF, hyaluronic acid and GROβ have already been shown to play a role in HSC trafficking.

We and others found a profound increase in the amount of total SDF-1 in the kidney after renal I/R injury. Immunohistochemistry and in situ hybridization showed that SDF-1 expression is abundantly in tubules of kidneys subjected for 1 day to renal I/R injury. When studying additional timepoints, we now found that SDF-1 was specifically upregulated during the early phase of renal I/R injury. The high SDF-1 expression in postischemic tubules together with the observation that SDF-1 inhibition increases renal ischemic damage and dysfunction suggest that this chemokine may have a particular and protective role during renal I/R injury. Indeed, we found that decreased SDF-1 levels following TEC-directed ASON treatment resulted in an imbalance between tubular epithelial cell apoptosis and proliferation which was accompanied with impaired renal function. This is in agreement with findings demonstrating that SDF-1 can suppress apoptosis in several different cell types and that SDF-1 is not expressed in postischemic tubules that were apoptotic or necrotic. Others suggest that SDF-1 may serve a tissue-protective and regenerative role in the ischemic myocardium by MAPK and AKT activation, decreased apoptosis and upregulation of vascular endothelial growth factor (VEGF) protein. Our results suggest that TEC, which express both receptors for SDF-1 (i.e. CXCR4 and CXCR7), in the post-ischemic kidney are responsive to increased SDF-1 levels which may favor TEC survival by preventing apoptosis. This survival is most likely regulated via CXCR7. Recently Hattermann et al. described that SDF-1/CXCR7 interactions inhibit apoptosis of glioma cells. In addition, several studies have shown a role for CXCR7 in survival. Local SDF-1 blockade significantly attenuated neutrophil influx into the lung following LPS exposure. On the contrary, we found a significant increase in
neutrophil influx into the ischemic kidney after SDF-1 ASON treatment. It may well be that the increased amount of apoptotic TEC increased the need and signals (such as KC) for neutrophils to infiltrate the kidney in order to clear damaged cells. These neutrophils can in their turn lead to collateral tubular damage with a decrease in renal function. It seems unlikely that the negative effect of SDF-1 antisense treatment on renal damage and dysfunction is due to impaired tubular repair by intrinsic renal stem/progenitor cells. Humphreys et al. show that injured tubules are predominantly repaired by intrinsic, surviving tubular epithelial cells after renal I/R injury and not by intrinsic renal stem/progenitor cells30.

To block SDF-1 expression specifically in the corticomedullary area we used ASON. We have previously examined the distribution of phosphorothioate-capped oligonucleotides in a separate study11 by treatment of animals with oligonucleotides that were conjugated to FITC. Accumulation of oligonucleotides was predominantly in the liver and kidney, with the highest accumulation in TEC as well as epithelial cells from the Bowman’s capsule. In addition, in the present study we show that ASON distribution in I/R injured kidney is similar to sham operated kidney. These findings are in accordance with previous studies by Carome et al.10 and Rifai et al.12 where a similar distribution pattern to kidney and liver was reported. Although we found only moderate total SDF-1 protein inhibition when animals were treated with ASON, SDF-1 mRNA expression was significantly reduced. In addition, we found a dramatic effect on renal dysfunction and damage emphasizing the strong potential of this compound. This strong effect could be explained by the fact that ASON very locally target those cells which are most vulnerable for ischemic damage, the proximal TEC. Indeed, we detected SDF-1 in TEC of the corticomedullary area of NSON treated animals after ischemia, whereas expression was reduced in sections of ASON treated animals. Apparently, the local targeting of ASON can influence greatly the effect on renal function and injury.

Our data demonstrate that SDF-1 provides morphological and functional protection against I/R injury and suggest a new function for SDF-1 in the damaged kidney. Although postulated to induce migration of renoprotective cells from the bone marrow, renal SDF-1 seems more important for the induction of resistance of TEC to apoptosis.

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References


