SDF-1 provides morphological and functional protection against renal ischaemia/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 haematopoietic stem cells to the post-ischaemic kidney. The function of increased post-ischaemic renal SDF-1 expression in modulating renal ischaemia/reperfusion injury remains, therefore, unknown.

Methods. We studied the role of SDF-1 in renal ischaemia/reperfusion injury by locally decreasing SDF-1 expression and subsequent SDF-1 signalling 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 ischaemia/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 haematopoietic stem or progenitor cells after induction of renal ischaemia. Rather, antisense-treated animals showed markedly increased apoptosis of the tubular epithelium accompanied by an increased renal inflammatory response.

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

Keywords: apoptosis; ischaemia/reperfusion; SDF-1; tubular epithelial cells

Introduction

The chemokine stromal cell-derived factor-1 (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 [1,2]. A similar mechanism has been postulated for renal repair, whereby expression of SDF-1 by tubular epithelial cells (TEC) following renal ischaemia/reperfusion (I/R) injury is increased and in this way could mediate migration of bone marrow-derived cells to the kidney [3]. We, however, recently showed that SDF-1 does not play a significant role in the migration of purified haematopoietic stem cells (HSC) during renal I/R injury [4]. Although HSC preferentially migrate to the injured kidney following renal ischaemia, local administration of SDF-1 recombinant protein did not increase HSC migration to the kidney during I/R injury [4]. Using the opposite approach, neutralization of SDF-1 or blocking of HSC-associated CXCR4 with neutralizing antibodies also did not inhibit HSC migration [4]. Hence, the functional significance of the increased post-ischaemic 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 [5], CD4⁺ cells [6], myeloid precursor cells [7], embryonic retinal ganglionic cells [8] and mesenchymal stem cells [9]. 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 [10–12], and are therefore highly suitable to study the role of renal-specific SDF-1 in the ischaemic damaged kidney. We found that a local decrease in 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.

Materials 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 min as described previously [11,13]. Mice (n = 8 per group) were treated twice (i.p.) with 4 nmol non-sense oligonucleotides (NSON) or ASON per injection dissolved in saline. To compensate for possible adverse effects, control animals were treated with NSON. Both nonsense and antitumor phosphorothioate-labelled oligonucleotides targeted to SDF-1 were purchased from Biognostik (Göttingen, Germany). Each first dose was given 24 h prior to I/R injury, and the second dose just after the release of the clamps. Animals were sacrificed by cervical dislocation 24 h 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 h prior to further processing. Bone marrow was flushed from tibia and femurs with phosphate-buffered saline (PBS) supplemented with 10% fetal calf serum (FCS; Hyclone, Etten-Leur, the 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 in vivo distribution of ASO, mice were treated twice i.p. with 4 nmol fluorescein isothiocyanate (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 ischaemia 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 ischaemia (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: FITC-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, the Netherlands). The rabbit monoclonal antibody to Ki.67 (Sp7) was purchased from Neomarkers (Fremont, CA, USA), the rabbit monoclonal to cleaved caspase 3 from Cell Signaling Technology (Beverly, MA, USA). A rabbit anti-mouse SDF-1 antibody was from ebioscience (San Diego, CA, USA). The rabbit antibody to FITC was from DAKO. PowerVision poly-HRP anti-rabbit IgG was from Immunologics (Duiven, the Netherlands), and 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-micrometre-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, ×400) in the corticomedullary region according to the following criteria: tubular dilation, epithelial necrosis, cast deposition and loss of brush border. Injury was graded on a scale from 0 to 5: 0, 0%; 1, <10%; 2, 10–25%; 3, 25–50%; 4, 50–75%; 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 min with 0.25% pepsin in 0.1 M HCl at 37°C; for detection of active caspase 3, Ki.67 and FITC, sections were boiled for 10 min in a 0.3% citrate buffer (pH 6). For all stainings, sections were incubated with primary antibodies for 2 h, and relevant peroxidase-conjugated secondary antibodies for 30 min in PBS and stained using 3,3′-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, ×400) in the corticomedullary region.

Flow cytometric analyses

White blood cells and bone marrow cells were counted on a Coulter ACT diff® (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 min. Before analysis, cells were fixed in PBS that contained 2% paraformaldehyde. FACS analyses were performed on a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA).

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, the 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 (Bio-Rad, Veenendaal, the Netherlands).

Quantitative real-time RT–PCR

Total RNA was extracted from kidneys using the TRRoeq reagent (Invi- trogen, Breda, the Netherlands) and converted to cDNA. Quantitative real-time RT–PCR was performed on a LightCycler® 480 System (Roche, Mijdrecht, the 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 ggttcttcgagagccacatc and reverse cagctgctaagaacgccgcta (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 ASON which blocks transcription of SDF-1.

Results

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

To analyse the renal SDF-1 expression during different phases of renal ischaemic injury, mice were subjected to bilateral I/R injury and sacrificed after several time points. This revealed that the level of total (active and inactive) renal SDF-1 increased significantly at Day 1 following ischaemia compared with 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 ASON which blocks transcription of SDF-1.

First, we analysed the renal distribution of ASON uptake using FITC-labelled 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
with NSON-treated control approaching statistical significance (Figure 1b, P=0.056). As demonstrated previously [3], 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 ischaemia. 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 [14]. Concurring, SDF-1 ASON-treated animals showed a 5-fold (P < 0.01) reduction of SDF-1 mRNA expression compared with NSON-treated controls (Figure 1c).
**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 [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 with controls (Figure 3a–c). In accordance with the increase in tubular damage, urea levels were significantly higher in ASON-treated animals compared with NSON-treated animals demonstrating a drastic 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 nonspecific adverse effects. Moreover, no difference in renal function was observed between ASON- or NSON-treated animals subjected to sham surgery (Figure 3d).

**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.

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**Fig. 2.** Distribution of ASON in kidney. The distribution of ASON in kidneys was determined by using FITC-labelled ASON. One day after (a) sham or I/R injury (b), ASON was detected in renal tubular epithelial cells of the corticomedullary region.

**Fig. 3.** Tubular damage and renal function following ASON treatment. Representative pictures of PAS/D-stained kidney sections of NSON- (a) and ASON-treated (b) animals 24 h after renal ischaemia. (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 h after ischaemia compared with 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 with that found in NSON-treated controls (white bar) at 24 h following ischaemia (\( *P < 0.0001 \)). Data are expressed as mean±SEM.
Haematopoietic stem cells (HSC) have been shown to migrate towards the injured kidney following ischaemia [3,4] and may contribute to renal repair [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 haematopoietic progenitors identified by their expression of the markers c-KIT and Sca-1 [17] by FACS analysis. At 24 h following ischaemia, 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 bone marrow stem cell 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 [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 with NSON-treated controls 24 h after ischaemia (Figure 4b–d). Consistent-
ly, 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 ischaemia 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 sections from ASON-treated animals compared with NSON controls after 24 h (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.

Discussion

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 ischaemic kidney [15]. We, however, recently demonstrate in three different and independent manners that migration of purified HSC to the ischaemic damaged kidney occurs independently of the SDF-1/CXCR4-signalling axis [4]. 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 signalling is able to mobilize heterogeneous bone marrow cells during renal ischaemic 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 ischaemic damaged kidney. Indeed, HMGB-1 [19], HGF [1], hyaluronic acid [20] and GROβ [21] have already been shown to play a role in HSC trafficking.

We and others [3] 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 abundant in tubules of kidneys subjected for 1 day to renal I/R injury [3]. When studying additional time points, we now found that SDF-1 was specifically upregulated during the early phase of renal I/R injury. The high SDF-1 expression in post-ischaemic tubules together with the observation that SDF-1 inhibition increases renal ischaemic damage and dysfunction suggests 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 [5–9] and that SDF-1 is not expressed in post-ischaemic tubules that were apoptotic or necrotic [3]. Others suggest that SDF-1 may serve a tissue-protective and tissue-regenerative role in the ischaemic myocardium by MAPK and Akt activation, decreased apoptosis and upregulation of vascular endothelial growth factor (VEGF) protein [22,23]. Our results suggest that TEC, which express both receptors for SDF-1 {i.e. CXCR4 [3,24] and CXCR7 (data not shown)} in the post-ischaemic kidney, are responsive to increased SDF-1 levels which may favour 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 [25]. In addition, several studies have shown a role for CXCR7 in survival [26–28].

Local SDF-1 blockade significantly attenuated neutrophil influx into the lung following LPS exposure [29]. On the contrary, we found a significant increase in neutrophil influx into the ischaemic 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 cells [30].

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 study [11] 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 kidneys is similar to sham-operated kidneys. These findings are in accordance with previous studies by Carome et al. [10] and Rifai et al. [12], where a similar distribution pattern in the 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 ischaemic damage, the proximal TEC. Indeed, we detected SDF-1 in TEC of the corticomedullary area of ASON-treated animals after ischaemia, 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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Conflict of interest statement. None declared.

References
Diazoxide on preserved kidney


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Diazoxide attenuates hypothermic preservation-induced renal injury via down-regulation of CHOP and caspase-12

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Abstract

Background. Successful clinical organ preservations are a prerequisite for organ transplantation. Diazoxide (DE), which shows a concentration-dependent selectivity for mitoK+-ATP over plasma membrane K+-ATP, displays protective effects during organ preservation. The current study investigated possible protective effects of DE on rat kidneys injured by hypothermic preservation.

Methods. Forty-eight Sprague–Dawley rats were randomly divided into six groups (n = 8): Celsior groups with kidneys preserved in Celsior solution for 0, 24 and 48 h and DE groups with kidneys preserved in DE (30 μM) plus Celsior

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