Enhanced mobilization of bone marrow cells does not ameliorate renal fibrosis

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Abstract

Background. The plasticity of bone marrow-derived stem cells, also comprising haematopoietic stem cells, has been shown to extend to renal epithelial lineages. Yet, the low rate of their contribution to the injured kidney has led to questions regarding their significance in tissue repair after acute injury. We describe here the effect of stem cell mobilization therapy on the progression of renal fibrosis in a mouse model of chronic obstructive nephropathy.

Methods. Mice were subjected to unilateral ureter obstruction (UUO) and treated with stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) or saline. Circulating cells were analysed by flow cytometry; labelled bone marrow c-KIT HIGH cells were injected into animals subjected to UUO. Granulocytes, macrophages, cellular proliferation or apoptosis and myofibroblasts were detected by immunostaining. Collagen deposition was determined by measuring renal hydroxyproline contents. Cytokine levels were measured by ELISA.

Results. SCF/G-CSF treatment of mice induced significant haematopoietic stem and progenitor cell mobilization from the bone marrow. Although these cells are able to migrate to the obstructed kidney, they did not influence renal damage, fibrosis and inflammatory cell influx.

Conclusions. Although SCF/G-CSF treatment significantly enhanced the availability of haematopoietic stem cells to the obstructed kidney, the progression of renal fibrosis could not be delayed or halted. Our results indicate that effective stem cell mobilization does not alter renal fibrosis.

Keywords: cytokine; fibrosis; kidney; obstructive nephropathy; stem cell; UUO

Introduction

Bone marrow-derived stem cells (BMSC) such as haematopoietic stem cells (HSC) possess a higher degree of plasticity than previously recognized, indicated by the fact that they contribute to the restoration of injured peripheral tissue [1,2]. The supposed mechanisms underlying this regenerative response are that BMSC transdifferentiate into or fuse with the principal cells of the injured tissue [3,4], although there are also indications that a more paracrine fashion of support may arise by providing relevant growth factors [5,6]. Among the non-haematopoietic tissues targeted by BMSC also the kidney can be found [7,8].

Renal injury induced in animals led to cells of bone marrow (BM) origin to emerge as different glomerular cell types [9] and tubular epithelial cells (TEC) [7,8]. Likewise, evidence for a similar response in humans was reported in studies where cells from recipient origin were detected in tubuli of renal allografts [10], suggesting the existence of an extrarenal source for tissue maintenance.

In line with these studies, we have found that BMSC were traceable in the kidney after ischaemia–reperfusion (I/R) injury in mice, but their number was too low to provide any substantial contribution to the observed improved kidney function [11]. Recent studies support this finding in that they report the prime tissue regenerative response to be of renal and not bone marrow (BM) origin [12] or that virtually no TEC of BM origin were detected [13].

An important notion not addressed in the above cited studies is the intrinsic capacity of the kidney to regenerate itself which takes place in most experimental models, regardless of therapy, thereby possibly omitting the necessity of any extrarenal stem cell involvement whatsoever. All studies with respect to the process of kidney regeneration by the BM use acute forms of renal injury such as I/R [7,8], folic acid [14] or cisplatin-induced [15] injury. An irreversible, chronic model of renal damage, such as the unilateral ureter obstruction model (UUO), may provide a more
appropriate way to study whether any significant contribution of the BM to restoration of the kidney can occur, thus bypassing any interference of naturally occurring processes of tissue regeneration.

Mobilization of HSC can effectively be induced by administration of stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) (reviewed by Duarte and Frank [16]). HSC mobilization after treatment of mice with SCF and G-CSF has been shown to improve heart function after experimental myocardial infarction and to reduce tissue fibrosis [17]. Ohtsuka et al. [18] report enhanced neovascularization and reduced apoptosis of ischaemic myocardial tissue following SCF and G-CSF treatment. Although we reported that HSC mobilization with SCF and G-CSF also led to earlier recovery of renal function after I/R injury, this was not dependent on increased incorporation of BM-derived TEC but rather on altered infiltration of granulocytes after injury [11]. As mentioned, HSC incorporation could, in this instance, be overruled by the normally occurring healing process of the kidney which is initiated after ischaemia. Here, we studied whether mobilization of HSC using SCF and G-CSF contributes to renal tissue remodelling or fibrosis during chronic obstructive nephropathy induced by UUO in mice. Hallmark characteristics of UUO injury are macrophage and myofibroblast accumulation, matrix deposition, capillary regression and tubular atrophy [19]. We have investigated whether any of these parameters were altered in UUO injury following SCF/G-CSF treatment to determine if this approach was capable of slowing down, or blocking, progressive renal injury as it is reported to do in the heart [17,18].

Subjects and methods

Animals

Six- to 8-week-old male C57BL/6 (B6) mice were purchased from Charles Rivers (Maastricht, Netherlands) and housed under SPF (Specific Pathogen Free) conditions receiving food and water *ad libitum*.

Experimental procedures

To induce mobilization of HSC, mice were injected subcutaneously with 5 μg/kg/day recombinant rat SCF and 20 μg/kg/day recombinant human G-CSF in saline (a gift from Amgen, Breda, Netherlands). Cytokines were administered daily, starting 5 days before induction of UUO and up to 3 days afterwards, in accordance with the study by Orlic et al. [17]. Control mice were injected with saline. To measure cytokine-induced mobilization of HSC and granulocytes, mice were treated for 5 days with SCF/G-CSF or saline (*n* = 5 mice per group). All mice received a pre-operative dose of analgesia (0.15 mg/kg buprenorphine, subcutaneously; Shering-Plough, Brussels, Belgium) and were anaesthetized by inhalation of 3% isoflurane, 0.2% N2O and 2% O2 during the whole surgical procedure. The right ureter was permanently ligated using 6-0 silk (Tyco, Gosport, UK) and mice (*n* = 6 per group) were sacrificed 3, 7 and 14 days after surgery. Blood was obtained via heart puncture and transferred to heparin collecting tubes. All experimental procedures were approved by the Animal Care and Use Committee of the University of Amsterdam, the Netherlands.

(Immune)Histochemistry and antibodies

Renal tissues were fixed in 10% formalin for 12 h and embedded in paraffin in a routine fashion. Four micrometre sections were stained with periodic acid Schiff after diastase digestion (PAS-D). A rat anti-mouse F4/80 antibody to detect mouse macrophages was purchased from Serotec (Oxford, UK), human anti-mouse IgG2a to α-smooth muscle actin (SMA) from DAKO (Glostrup, Denmark), rabbit anti-mouse active caspase 3 (apoptosis) from Cell Signaling (Beverly, MA, USA), rabbit-anti Ki.67 (proliferation) from NeoMarkers (Fremont, CA, USA). Fluorochrome labelled antibodies to Gr-1, CD31, CD34, c-KIT and Sca-1 (all rat anti-mouse) were obtained from BD Biosciences (Alphen a/d Rijn, Netherlands) and rat anti-mouse CD133 from ebioscience (San Diego, CA, USA). All peroxidase (HRP) conjugated secondary antibodies were from DAKO.

Histopathological scoring and renal function

All histopathological scorings were made in the cortex and performed in a blinded fashion. Tubular injury was assessed by grading tubular dilatation, epithelial simplification and brush border loss in 10 randomly chosen, non-overlapping fields (200 × magnification). Lesions were graded on a scale from 0–4: 0 = normal; 1 = mild, involvement of <25% of the cortex; 2 = moderate, involvement of 25–50% of the cortex; 3 = severe, involvement of 50–75% of the cortex; 4 = extensive damage involving >75% of the cortex. Renal function was determined by measurement of plasma creatinine and urea, using standard methods for clinical diagnostics.

Apoptotic or proliferating TEC, neutrophils and macrophages were counted per high power field (hpf, original magnification of 400-fold). Myofibroblast accumulation was determined by measuring the positively stained area per field (expressed as percentage staining of one field) in six non-overlapping fields per kidney (200 × magnification) for α-SMA by image analysis using Image-Pro Plus version 4.5.1 software package (Media Cybernetics, Giechen, Germany).

Hydroxyproline measurement assay

Hydroxyproline concentration measurement as approximation of the total collagen content was performed in lysates of kidney tissue in a slightly modified fashion from Kivirikko et al. [20], as described previously [21]. In short, tissue was lysed in 1% Triton x–100, 4 mM EDTA, 1% protease inhibitor cocktail II (Sigma Chemicals) and blended using a PT1300D tissue homogenizer (Kinematica, Lucerne, Switzerland) set at full speed. After centrifugation (10,621 rpm at 4 °C) both the supernatant and pellet fractions were hydrolysed in 6 M HCl (110°C, 6 h). Results were expressed as microgram of collagen per milligram total lysate.

ELISA

Tissue lysates prepared as described earlier were assayed for transforming growth factor-β1 (TGF-β1), tumour necrosis factor-α (TNF-α) and hepatic growth factor (HGF) levels by using mouse-specific Duoset ELISA kits (R&D), in accordance with the supplied protocol. Cytokine levels were corrected for total protein content per sample.

Flow cytometry

White blood 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 surface antigens was performed by incubating leukocytes with monoclonal antibodies for 0.5 h. After washing, cells were fixed in PBS with 2% paraformaldehyde. Gr-1⁺ cells were in addition gated from the neutrophil population. Analyses were performed on a FacsCalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

C-KIT cell transplantation and tracking

Four recipient male B6 were subjected to UUO as described 6 days before transplantation. C-KITHIGH cells were isolated from four B6 male donors by flushing whole BM from tibia and femur with PBS supplemented with 10% FCS and penicillin plus streptomycin; cells were labelled with a monoclonal antibody to c-KIT (BD Biosciences) for 15 min. Cells were washed with PBS and the c-KITHIGH cell population was collected using a FacsAria cell sorter (BD). The sorted cells were labelled with Cell Tracker CM-DiI (Invitrogen, Paisley, UK) according to the accompanying protocol. After washing, cells were suspended in sterile saline and 0.5 × 10⁶ cells were injected in the tail vein of the recipient mice. Both kidneys were removed the following day (i.e. 7 days after UUO), fixed in formalin with 2% paraformaldehyde. Gr-1⁺ cells were in addition gated from the neutrophil population. Analyses were performed on a FacsCalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical analyses

Results are expressed as mean ± standard error of the mean (SEM). Data were first tested for normality using the Kolmogorov-Smirnov test and then analysed using an unpaired t-test. Tubular injury scores were analysed using the non-parametric Mann–Whitney U-test. Values of P ≤ 0.05 were considered statistically significant. All statistical analyses were performed using Graphpad Prism4 (GraphPad Software, San Diego, California, USA).

Results

Stem cell mobilization does not affect tubular damage due to ureter obstruction

To determine the effect of SCF/G-CSF treatment on the composition of the blood, we performed flow cytometric analysis of whole blood samples obtained from mice injected with cytokines for 5 days. This reflects the situation at the time when obstruction was initiated. As reported previously [11,23], we detected a significant increase in c-KIT/Sca-1 double-positive cells after cytokine-treatment (Figure 1A), indicative of HSC mobilization. In addition, we found a 5-fold increase in the level of circulating granulocytes after treatment (Figure 1B). Circulating cells expressing CD34, CD133 but which lack expression of CD31 are broadly enriched for endothelial progenitor cells (reviewed by Hunting et al. [24]). Cytokine treatment did not significantly affect mobilization of CD34/CD133 cells, although a trend towards a lower number of circulating cells was detected (Figure 1C).

Already at day 3, obstructed kidneys were heavily affected as witnessed by tubular dilatation (Figure 2B). At day 14, the renal tissue was severely damaged, whereas contralateral kidneys were unaffected (data not shown). We found a small, but significantly increased rate of damage in obstructed kidneys from cytokine-treated animals compared to controls at day 3. At later time points, no differences were detected in the severity of injury between both groups (Figure 2A). We observed no indications of pyelonephritis resulting from the surgical procedure.

Although it is generally accepted that during UUO injury the contralateral kidney is capable of taking over the function of the obstructed kidney, we have determined whether cytokine treatment may affect proper functioning of the contralateral kidney due to a possible inflammatory response caused by increased numbers of circulating neutrophils. Creatinine and urea plasma levels did not differ significantly between any time points between both groups (data not shown) indicating that cytokine treatment did not affect renal function of the contralateral kidney.

![Fig. 1. SCF and G-CSF induce HSC mobilization. Flow cytometric analyses of whole blood samples of mice subjected to cytokine (filled square) or saline (square) treatment of (A) c-KIT/Sca-1 cells (*) to determine the number of circulating HSC or early hematopoietic progenitors, (B) Gr-1⁺ cells gated from neutrophil population (**P = 0.01) and (C) CD34⁺/CD133⁺/CD31NEG (NS, P = 0.25). Data are expressed as mean and SEM.](https://academic.oup.com/ndt/article-abstract/23/2/483/1851944/1851944)
No differences were noted in the levels of apoptosis of TEC between either treatment groups as determined by staining for the active form of caspase 3 (data not shown). In contrast, proliferation of TEC, as determined by the presence of the nuclear antigen Ki.67, was significantly decreased 7 days after UUO in the cytokine treated group but not affected at other time points (Figure 3).

To determine whether HSC or their more differentiated progenitors are capable of entering the obstructed kidney, we used BM cells expressing high levels of c-KIT (c-KIT^{HIGH}) for in vivo tracking. This small subset of BM cells is enriched for cells, which do not express lineage-specific markers and which have haematopoietic reconstitution activity [25], which we confirmed by FACS analysis (data not shown). We sorted c-KIT^{HIGH} cells from BM, labelled this population of cells and injected these cells into the tail vein of animals that were subjected to UUO 6 days before. The kidneys were removed the next day (seven days after UUO) and examined for infiltration by labelled cells (Figure 4). Single labelled c-KIT^{HIGH} cells (data not shown) or clusters of c-KIT^{HIGH} cells (Figure 4A–C) were detected in the lumen of dilated tubules. Labelled cells were readily detected intraluminal, whereas cells were only rarely detected in the interstitial compartment (Figure 4I). In contrast, only sporadically could we detect a labelled cell in the interstitium of contralateral kidneys (Figure 4F). Whereas cells found in the obstructed kidneys appeared more lymphoid; labelled cells in the contralateral kidneys had a rather granular appearance (compare Figure 4C and F, respectively). Spectral analysis of tissue sections was used to discriminate between proper fluorescence and non-specific auto-fluorescence. Freshly labelled cells were fixed and embedded in paraffin. Digital image analysis was applied to sections of obstructed and contralateral kidneys. The wavelength associated with the fluorescent tag of freshly labelled cells was set as standard; next, fluorescent signals with a higher or lower wavelength were removed from the original image (Figure 4D), whereby only proper fluorescent labelling was visualized (Figure 4E).

Immunostainings of serial sections confirmed that labelled cells did not express the myeloid markers Ly6C and Ly6G, excluding the possibility that these cells represent intraluminal host inflammatory cells displaying auto-fluorescence (Figure 4G and H). These data strongly indicate that HSC or progenitors are able to migrate into the fibrotic kidney and are not entangled in the glomerular vasculature but rather transmigrate into the luminal space of downstream tubules.

**Inflammatory cell influx**

Neutrophil-mediated tissue damage has been reported to occur during post-ischaemic reperfusion injury [26] and pyelonephritis [27]. Although excessive neutrophil
influx is not reported to occur after UUO, we determined whether granulocytosis after cytokine-treatment affected obstruction-induced kidney injury. Tissue sections were stained for the granulocyte marker Gr-1/Ly6G (Figure 5A). No significant differences in the amount of infiltrated granulocytes could be detected between groups at any time points. We have previously demonstrated that granulocytes from cytokine-treated animals have a lower level of L selectin expression [11], which might explain the discrepancy between increased numbers of circulating granulocytes and the disproportional low number of granulocytes present in the kidney.

Interstitial macrophages are known to produce fibrosis-inducing cytokines and extracellular matrix products; therefore, we evaluated macrophage influx after UUO. We observed a gradual increase in interstitial F4/80-positive macrophages in time after initiation of obstruction; however, no difference in cell numbers was detected between groups (Figure 5B).

**Myofibroblast accumulation and collagen deposition**

To determine if cytokine treatment affected myofibroblast influx, tissue sections were stained for α-SMA...
The percentage of the positive stained area was measured using digital image analysis. No effect of cytokine treatment on myofibroblast accumulation in obstructed kidneys was detected at any time points compared to controls (Figure 6A). Staining for α-SMA in contralateral kidneys was confined to vessel walls only (Figure 6B and C).

To determine whether cytokine treatment leads to altered deposition of collagen, we measured the hydroxyproline contents of the kidney. We detected a gradual increase in hydroxyproline in obstructed kidneys, whereby no significant difference was detected between groups at all time points (Figure 7).

Seven days after obstruction, the concentration of active TGF-β1 was significantly increased in renal homogenates compared to day 3 after obstruction, but no statistical difference was detected between the groups (Figure 8A).

In contrast, HGF levels steadily decreased after UUO, where at day 7, a significantly lower concentration was measured in cytokine-treated animals (Figure 8B).

Increases in TNFα levels have been reported to correlate with fibrosis [28]. In accordance, we detected increased levels of TNFα after ligation peaking at day 7. Similarly, to TGF-β1 levels, cytokine treatment did not significantly affect TNFα concentrations (Figure 8C), indicating that the treatment did not target the kidney besides inducing mobilization of HSC.

Regression of the peritubular capillary network

Loss of capillary density during UUO injury is an important determinant for the induction of renal tissue atrophy [29]. To measure regression of the capillary network, we stained sections with an antibody to the sialomucin CD34 (Figure 9). Digital image analysis demonstrated that no difference was detected in the cumulative area of capillary CD34 expression per hpf.
between groups at all time points. Nevertheless, a strong decrease of staining for CD34 was observed in time, although the relative increase in staining at day 3 after obstruction compared to the contralateral situation may be due to proliferation of the endothelium as has been reported previously [30].

**Discussion**

BMSC have been connected to non-haematopoietic tissue regeneration after injury, but recent studies suggest that the kidney is less prone to accepting support from the BM [11–13]. Here, we show that in a model of chronic kidney injury with increased availability of HSC, no significant alteration in injury progression was achieved, despite their capacity to successfully penetrate into the fibrotic kidney. As has been shown recently by Sugimoto et al. [31], BM-derived cells have the capability to migrate through the glomerular basement membrane and can thus end up in the luminary space of Bowman’s capsule, thereby crossing the obstacle set up by the basement membrane.

We obtained similar results as those described earlier, when cytokine treatment was started after establishment of UUO injury, in order to provide HSC mobilization as a therapeutic intervention (data not shown). This suggests that neither high circulating HSC levels prior to UUO injury or during established UUO injury significantly affect renal fibrosis.

Our findings are in sharp contrast to previous results obtained from a model of myocardial ischaemia [17]. After experimental myocardial infarction in mice, tissue remodelling of the affected tissue starts, resulting in the formation of scar tissue. Treatment of animals with G-CSF, as well as G-CSF combined with SCF, was found to decrease formation of fibrous scar tissue and increase capillary density after myocardial infarction [18]. Another study showed that G-CSF induces over-expression of metalloproteinases, which reduce collagen accumulation [32]. More recently, G-CSF was shown to induce cell survival of cardiomyocytes [33]. Although similar mechanisms were also considered at a forehand, our results do not indicate that either of the beneficial effects of cytokine treatment occurs during chronic obstructive nephropathy.

SCF is primarily recognized as an inducer of differentiation in various cell types of the haematopoietic system [34], but has also been involved in neurogenesis [35], development of melanocytes [36] and proliferation of hepatocytes [37]. Here, we detected no beneficial effect on proliferation of TEC by systemically injected SCF but rather a transient decrease in Ki.67 positive TEC at day 7 after UUO (Figure 3). This diminished rate of TEC proliferation coincided with a lower level of renal HGF (Figure 8B). Binding of HGF to its receptor c-met induces proliferation of TEC [38]; therefore, the observed decrease in proliferation may be related to the concomitant decrease of available HGF, yet both these phenomena were not associated with an increased progression of fibrosis. In addition, no significant effect was noted in the number of apoptotic TEC. However, we did detect increased concentrations of endogenous SCF in renal homogenates of saline-treated animals at day 7 after UUO (data not shown), demonstrating that SCF may...
be involved in UUO injury when expressed in the kidney itself but not when administrated systemically. The role of SCF in renal injury is currently being investigated by our group.

Here, we report that cytokine treatment using SCF and G-CSF does not alter progression of fibrosis in obstructive nephropathy in mice. SCF and G-CSF treatment (i) did not alter the progression of tubular damage, (ii) did not affect collagen deposition, (iii) did not affect myofibroblast or macrophage influx, both considered important mediators of fibrotic processes [39], (iv) did not decrease regression of the peritubular capillary network and (v) did not alter TGF-β1, TNF-α or HGF (with the exception of day seven after UUO) levels in the obstructed kidney. When considering growth factor signalling, the UUO model is, like several other nephropathies, characterized by altered expression of a number of growth factors. Of these, the fibrosis-inducing TGF-β1 and anti-fibrotic HGF are recognized as important players during progression of fibrosis [40].

BM-derived stem cells have been implicated in tissue restoration after injury in peripheral tissues, yet there is also the suspicion that these cells give rise to cells with a myofibroblast-like phenotype. Although it is hypothesized that these represent intermediate stages of differentiating stem cells [41], the BM is recognized to be a source of myofibroblasts that appear in the kidney during fibrosis [42]. We have previously shown that cytokine treatment transiently increases myofibroblast levels in the kidney upon I/R injury, and was accompanied by increased macrophage influx [11]. Although we did not detect a progression of fibrosis in this instance, this finding could nonetheless indicate that BM-derived cells do not just contribute to restoration of renal function and tissue repair, but may also give rise to generation of non-functional tissue and fibrosis. Renal myofibroblasts are reported to be derived from different sources: from proliferating interstitial fibroblasts, from the transition of TEC into myofibroblasts and from the BM [42]. Fibrocytes are circulating blood-borne cells displaying leukocyte surface markers and which produce extracellular matrix proteins. Fibrocytes are involved in wound repair, upon TGFβ1 exposure may express α-SMA [43], are thought to be important in mediating pulmonary fibrosis [44] and recently have been implicated in renal fibrosis [45]. The total number of α-SMA expressing cells found after UUO did not differ between both treatment groups (Figure 6), suggesting that if fibrocytes are the potential source of BM-derived myofibroblasts in the kidney, their contribution to fibrosis was not altered by cytokine induced mobilization in our study. In accordance, collagen type I deposition by BM derived (myo)fibroblasts in UUO injury was found to be insignificant compared to that of fibroblasts of renal origin [46].

Our initial hypothesis, being that HSC or their immediate downstream progeny could in any way halt or hinder fibrosis induced by UUO, is not supported by our results. Although we detected increased levels of c-KIT/Sca-1 double-positive cells after cytokine treatment, no biologically important effects were noted. As discussed earlier, no significant effects of cytokine treatment were found when we studied the separate injury pathways that play a role in UUO injury, acting on different cell types, such as tubular atrophy, capillary regression or myofibroblast influx. Cytokine treatment could in theory lead to mobilization of one specific subset of BM progenitor cells that is unrelated to HSC, but capable of affecting one of these processes. However, we have investigated all processes separately and found no differences between cytokine-treated animals or controls, making it in our opinion unlikely that any biological significant effect can come from SCF/G-CSF treatment with respect to mobilization of a distinct subset of any putative progenitor subset. Our results show that findings regarding altered tissue remodelling or fibrosis in other organs, induced by either HSC or the anti-fibrotic properties of the cytokines used for mobilization could not be extrapolated to the kidney.

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Conflict of interest statement. None declared.

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