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Stromal cell-derived factor-1 (SDF1)-dependent recruitment of bone marrow-derived renal endothelium-like cells in a mouse model of acute kidney injury

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ABSTRACT. Ischemic acute kidney injury (AKI) is the most key pathological event for accelerating progression to chronic kidney disease through vascular endothelial injury or dysfunction. Thus, it is critical to elucidate the molecular mechanism of endothelial protection and regeneration. Emerging evidence indicates that bone marrow-derived cells (BMCs) contribute to tissue reconstitution in several types of organs post-injury, but little is known whether and how BMCs contribute to renal endothelial reconstitution, especially in an early-stage of AKI. Using a mouse model of ischemic AKI, we provide evidence that incorporation of BMCs in vascular components (such as endothelial and smooth muscle cells) becomes evident within four days after renal ischemia and reperfusion, associated with an increase in stromal cell-derived factor-1 (SDF1) in endothelium and that in CXCR4/SDF1-receptor in BMCs. Notably, anti-CXCR4 antibody decreased the numbers of infiltrated BMCs and BMC-derived endothelium-like cells, but not of BMC-derived smooth muscle cell-like cells. These results suggest that reconstitution of renal endothelium post-ischemia partially depends on a paracrine loop of SDF1-CXCR4 between resident endothelium and BMCs. Such a chemokine ligand-receptor system may be attributable for selecting a cellular lineage (s), required for renal vascular protection, repair and homeostasis, even in an earlier phase of AKI.

KEY WORDS: acute kidney injury, bone marrow-derived cell, chemokine (C-X-C motif) receptor-4, endothelial cell, stromal cell-derived factor-1

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The kidney plays a central role in blood clearance via glomerular filtration and urination for sustaining systemic homeostasis. Glomerular vascular component is essential for primitive urination and peri-tubular capillary network for supporting tubular metabolisms (such as electrolyte exchanges). There is now growing evidence that a decrease in peri-tubular vessels (*i.e.*, vascular rarefaction) is a common cause for chronic kidney disease (CKD), regardless of an initial etiology [2]. Especially, under renal ischemia, damage or dysfunction of endothelial cells (ECs) often triggers a rapid progression of acute kidney injury (AKI) to CKD, associated with advanced fibrosis [2, 3]. Thus, it is important to elucidate the molecular mechanism of renal vascular protection or repair against ischemic challenges.

During recovery from AKI, proliferation of tubular epithelial cells is a major event for restoring structural continuity with function [17, 19]. In the early 2000s, several lines of studies demonstrated the involvement of bone marrow-derived cells (BMCs) in injured kidneys [13, 22]. Y-chromosome-positive BMCs were detected in renal tubules (and in part, in vessels) of a female recipient who underwent bone marrow transplantation from a male donor [22]. Similar results are also obtained in adriamycin-treated nephrotic mice: BMC-derived ECs were seen near the tubules at an advanced stage of CKD (*i.e.*, 4 weeks post-challenge) [15]. However, such a chronic model is not advantageous for identifying an initial mechanism of vascular remodeling, due to the secondary complicated events, such as inflammation, hence raising a demand of an "acute" model.

Warm ischemia and reperfusion (I/R) elicits typical patterns of AKI, such as tubular and endothelial damages [2, 3]. Indeed, apoptotic changes in peri-tubular ECs become evident in mice 24 hr post-renal I/R, followed by neutrophil extravasation and tubular injuries [18]. However, it is still unclear whether BMCs participate in renal vascular remodeling (including EC-like phenotype) in the earlier phase of AKI. Stromal cell-derived factors-1 (SDF1) is induced by hypoxic stresses and contributes to BMC-based tissue remodeling [5]. For example, tumor angiogenesis is mediated via SDF1-dependent recruitment of BMCs [1]. SDF1 is also critical for regenerative events of injured organs, especially for BMC-mediated angiogenesis [23, 31].

Using an AKI mouse model, we provide herein evidence that engraftment of BMCs by peri-tubular vessels is detectable at latest from 2 days post-renal I/R in an SDF1-dependent manner. We will discuss the roles of hypoxia in BMC-involved endothelial reconstruction at the initial phase

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of ischemic AKI, a key cause of acute-on-CKD progression.

MATERIALS AND METHODS

Bone marrow transplantation and AKI induction in mice: The C57Bl/6 strain of green fluorescent protein (GFP) transgenic male mice was used as the donor for bone marrow implantation. Wild-type female mice were used as the recipients. The wild-type mice were irradiated at a dose of 10 Gy using an X-ray generator. Soon after the X-irradiation, the wild-type female mice received an intravenous injection of 1×10^6 bone marrow cells from male GFP-transgenic mice. After reconstitution of bone marrow for 5 weeks, the recipient mice were subjected to renal I/R: both renal arteries were clamped for 40 min at 38°C to induce AKI under general anesthesia. The mice were sacrificed at 0, 2, 4, 6 and 8 days post-challenge to characterize the natural course of AKI. To evaluate the renal hypoxia, some mice were treated with pimonidazole (Chemicon, Temecula, CA, U.S.A.) (60 mg/kg, i.p.) at 1 hr before the autopsy, as reported [19].

Blood chemistry: To confirm the successful induction of AKI in mice, blood urea nitrogen (BUN) levels were measured with a commercial kit (Urea nitrogen-B test®, Wako, Osaka, Japan), as reported [18].

Neutralization of SDF1 in AKI-manifested mice: To neutralize SDF1 signaling during the progression of AKI, an anti-CXCR4 rabbit IgG (eBioscience, San Diego, CA, U.S.A.) was administered, as reported [25]. Briefly, 8 mice were subjected to the renal I/R and randomly divided into 2 groups: 4 mice were injected with the anti-CXCR4 IgG (100 μg/48 hr, i.p.), and the remaining 4 mice were treated with normal IgG (100 μg/48 hr, i.p.). All mice were sacrificed at 4 days post-challenge, and the renal tissues were analyzed, as described below.

Immunohistochemistry: The renal tissues of mice were fixed with 10% formaldehyde in phosphate buffered saline (PBS), dehydrated through a graded series of ethanol and then embedded in paraffin. The tissues were cut at 4 μm, dewaxed and subjected to the following procedures. The hypoxic changes were detected on tissue sections, using an anti-pimonidazole mouse IgG (Chemicon) as the primary antibody, followed by the second reaction with biotin-labeled anti-mouse IgG (Vector, Burlingame, CA, U.S.A.). An avidin-biotin coupling reaction was performed on the sections, using a kit (ABC-HRP Elite®, Vector). The hypoxic antigen was visualized as brown, with 3,3′-diaminobenzidine (Nacalai, Kyoto, Japan).

Immunofluorescence staining: The renal tissues were fixed in 4% paraformaldehyde at 4°C for overnight, subsequently cryoprotected in 10% and 20% sucrose in PBS and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Tokyo, Japan) for the following antigen immunostaining. The cryosection was cut at 5 μm in a cryostat, washed with PBS and then incubated with the primary antibodies, such as anti-c-Kit rat IgG (eBiosciences), anti-CD31 rat IgG (*i.e.*, EC marker) (BD Biosciences, San Jose, CA, U.S.A.), anti-Na^+-K^+-ATPase rabbit IgG (*i.e.*, renal tubular epithelium marker [19]) (LSL, Tokyo, Japan) and anti-α-smooth muscle actin (α-SMA) mouse IgG (*i.e.*, SMC marker) (Dako Japan, Kyoto, Japan), followed by fluorescence staining with Alexa546-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, U.S.A.). The nuclei were also detected with TOPRO-3 (Invitrogen). The GFP fluorescence signals were observed under a confocal microscope LSM-PASCAL (Carl Zeiss, Thornwood, NY, U.S.A.).

Morphometric scores: The renal tubular injury index was determined, according to the previous method [19]. Photographed images were analyzed using image analysis software (WinRoof®, Mitani, Fukui, Japan) to determine the extent of hypoxic areas in the outer medulla (OM). The appearance of c-Kit-positive and GFP-positive cells at the OM area was semi-quantified by counting positive cells in more than 15 randomly chosen non-overlapping high-power fields (×400 magnification). The ratio of CD31-positive cells, or that of α-SMA-positive cells, to total GFP-positive cells were determined in >15 fields (×400). The overall means of these parameters in each group were calculated based on individual values.

Enzyme-linked immunosorbent assay (ELISA): The renal homogenate was centrifuged at 15,000 rpm for 30 min, and the supernatant was used as tissue extract [18]. SDF1 levels in the renal extract were determined using an ELISA kit (R&D, Minneapolis, MN, U.S.A.).

Real-time polymerase chain reaction (PCR): Total RNA was prepared from kidneys, using ISOGEN (Nippon Gene, Tokyo, Japan). One microgram of total RNA was reverse-transcribed into first strand cDNA with a random hexaprimer using Superscript II reverse transcriptase (Life technologies Inc., Rockville, MD, U.S.A.). Quantitative PCR was performed to detect SDF1 mRNA, using an ABI PRISM 7,700 system (Perkin-Elmer Biosystems, Foster City, CA, U.S.A.), according to the manufacturer's instruction (catalogue no. Mm00445552-m1).

Statistical analysis: All data were expressed as mean ± S.D. A Student's *t*-test, ANOVA analysis or Mann Whitney U-test was used to compare the group means, and a value of $P<0.05$ was considered to be significant.

RESULTS

Renal hypoxia-associated engraftment of BMCs in the ischemic kidneys: We first examined the change in BUN levels post-renal I/R to estimate the degree of renal dysfunction. The BUN level reached a peak at 2 days post-renal I/R challenge (Fig. 1A). The increase of tubular injury index correlated with that of the BUN levels (Fig. 1A), thus indicating the successful onset of ischemic AKI. Local hypoxia is one of the most key events to trigger engraftment of BMCs in injured tissues [5, 31]. Thus, we next examined the degree of hypoxia, based on the accumulation of pimonidazole. Although there is no hypoxic area in the intact kidney, hypoxic areas became evident, especially in the renal OM from 2 days post-release of warm ischemia (40 min) (Fig. 1B), suggesting renal vascular dysfunction, as reported [2, 18]. Indeed, the ratio of pimonidazole-positive area to total area varied from approx. 50% to 20% between 2 and 6 days post-

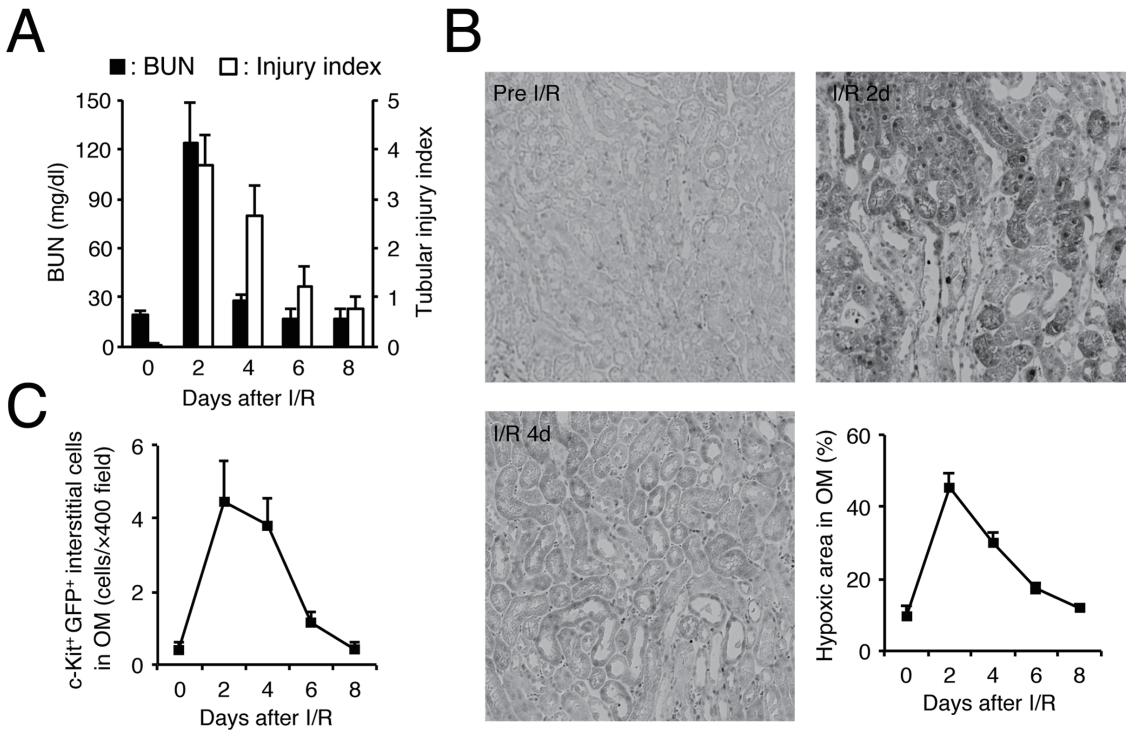

Fig. 1. Expansion of tissue hypoxia and recruitment of BMCs in the course of AKI. (A) Time course of BUN levels (filled bars, left y-axis) and tubular injury index (as assessed in renal sections [19], open bars, right y-axis) in mice post-renal I/R. (B) Tissue hypoxia was detected by immunostaining of pimonidazole, a marker of tissue hypoxia. Graph shows ratios of pimonidazole-positive areas to total areas in renal OM region (×200). (C) Bone marrow-derived stem/progenitor cells were detected by double-staining of c-Kit and GFP in renal sections, and the numbers of double positive cells in OM region (×400) were counted. Data are shown as mean ± SD.

renal I/R challenge. As a result, BMCs (*i.e.*, c-Kit-positive and GFP-positive cells) were detected, especially in the OM area post-I/R challenge (2 days: 4.47 ± 1.10; 4 days: 3.81 ± 0.75) (Fig. 1C), supporting previous reports to show the involvement of BMC engraftment during experimental AKI in rodents [10, 25].

Differentiation of BMCs to vascular ECs and SMCs in the hypoxic kidneys: Growing evidence indicated the potential of BMCs to differentiate to tubular epithelial cells during AKI [10, 19, 22]. However, little information is available whether infiltrated BMCs are incorporated into renal vessels, under AKI-associated pathological conditions. In our model, CD31-positive signals were detected in the GFP-positive cells around peri-tubular (but not glomerular) areas, especially from 2 days post-ischemia, hence suggesting the acquisition of EC-like phenotype by BMCs (*i.e.*, *in situ* trans-differentiation) (Fig. 2A), as seen in a mouse model of CKD [15]. Likewise, some of BMCs acquired the SMC-like phenotypes, as evidenced by α-SMA between 2 and 8 days post-renal I/R challenge (Fig. 2B). The number of α-SMA-positive cells is 17-fold higher than that of CD31-positive cells, while myofibroblasts are also known to be positive for α-SMA [4, 30]. Importantly, BMC-derived myofibroblasts participate in renal remodeling, even during ischemic AKI [4, 30], possibly for collagen deposition. Taken together, we cannot exclude a possibility that the α-SMA-positive BMCs include interstitial myofibroblasts, as discussed later.

Hypoxia-associated up-regulation of SDF1 in ECs and of CXCR4 in BMCs: Growing evidence suggests that SDF1 is a key chemokine to elicit engraftment of BMCs into hypoxic organs or tissues [5, 31]. In our model, mRNA and protein levels of SDF1 dramatically raised 2 days post-challenge and then returned near the basal levels at 6 days post-renal I/R (Fig. 3A). Consistent with these biochemical data, SDF1 was not seen in intact kidney, but detected in the OM areas 2 days after renal I/R (Fig. 3B). SDF-1 signal was detected in CD31-positive cells or in Na^+-K^+-ATPase-positive cells, indicating that renal ECs and tubular epithelial cells can be a source of SDF1 production (Fig. 3C). On the other hand, some GFP-positive cells (*i.e.*, bone marrow origin) displayed CXCR4 (Fig. 3D), a functional receptor of SDF1. Thus, we hypothesized that hypoxic kidney-derived SDF1 is responsible for mobilization of BMCs from bone marrow to hypoxic kidneys (*i.e.*, endocrine pathway through targeting of CXCR4 on BMCs).

Critical roles of SDF1-CXCR4 axis for BMCs to convert ECs during renal hypoxia: To test this hypothesis, mice were treated twice with an antibody to disrupt SDF1-CXCR4 signaling (Fig. 4A). The anti-CXCR4 IgG injection decreased the number of c-Kit-positive BMCs (4.49 ± 1.15 vs. 2.27 ± 0.90,

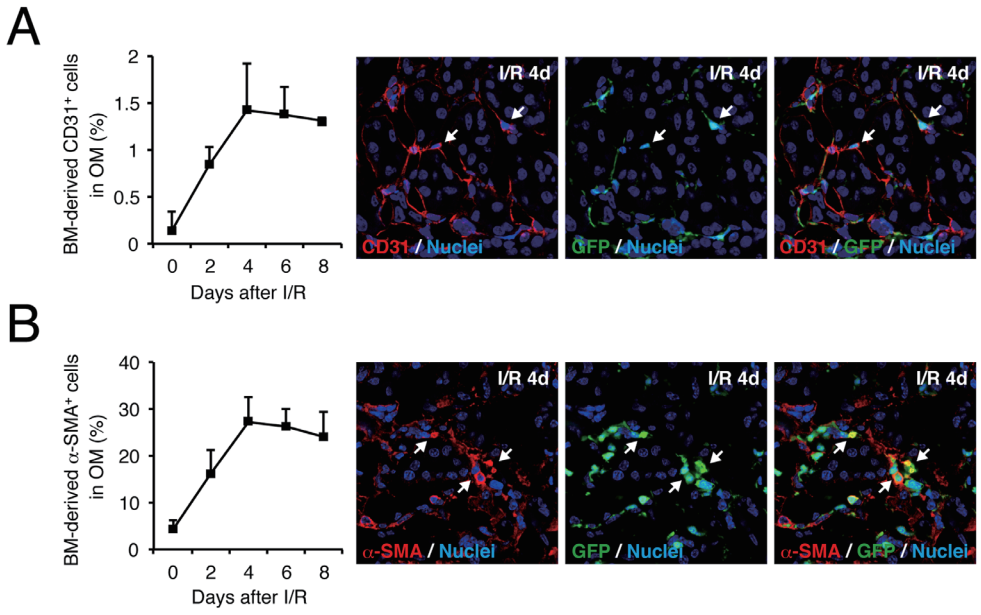

Fig. 2. Engraftment of BMCs with EC or SMC marker in the hypoxic kidneys. Tissues were stained with CD31 (red, in A) or α-SMA (red, in B) and GFP (green), and the percentages of marker-positive cells in BMCs (arrows) were shown. Data are shown as mean ± SD.

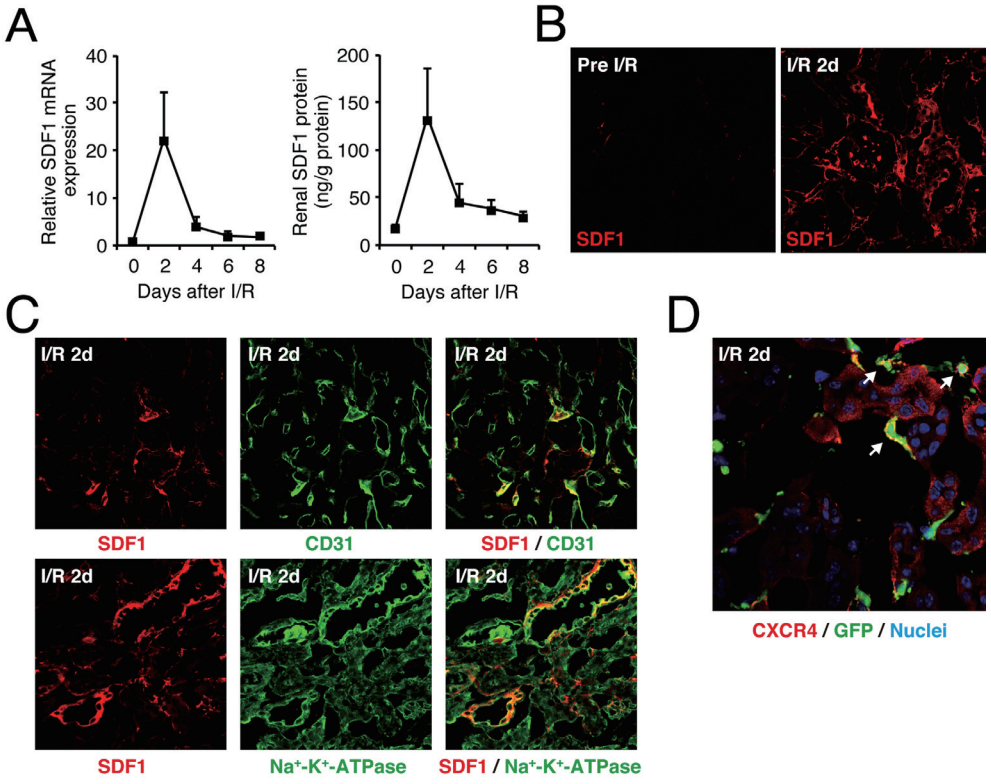

Fig. 3. Up-regulation of SDF1 and appearance of BMCs expressing CXCR4 during AKI. (A) Expressions of SDF1 mRNA (left) and protein (right) in the ischemic kidney were measured by real-time PCR and ELISA, respectively. (B) Localization of SDF1 expression (red) was detected by immunohistochemistry. (C) Double immunostaining of SDF1 (red) and CD31 (upper panel, green) or Na^+-K^+-ATPase (lower panel, green) in the kidney after renal I/R. (D) BMCs (green) positive for CXCR4 immunostaining (red) were detected in the kidney after renal I/R (arrows). Data are shown as mean ± SD.

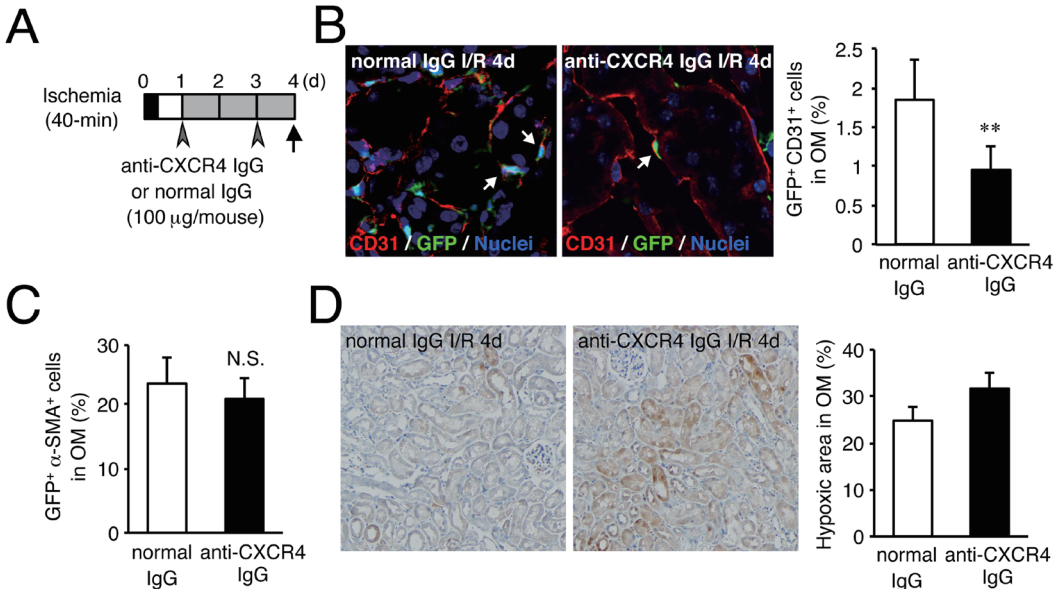

Fig. 4. Blocking CXCR4 during AKI decreased engraftment of BM-derived ECs. (A) An experimental protocol of CXCR4 neutralization after renal I/R. (B) Inhibitory effect of CXCR4 blocking on CD31-positive BMC engraftment. Tissues were stained with CD31 (red) and GFP (green), and the percentages of double positive cells (arrows) were measured. (C) Effect of CXCR4 neutralization on the differentiation of BMCs into SMC-like cells. The percentages of cells double positive for α-SMA and GFP were shown. (D) Effect of CXCR4 neutralization on the improvement in renal tissue hypoxia. Hypoxic areas in OM region were measured by pimonidazole staining, and ratios of pimonidazole-positive areas to total areas (×200) are shown. Data are shown as mean ± SD. ***P*<0.05 vs. normal IgG group. N.S., not significant.

P<0.05). Consistent with the suppressed BMC engraftment, anti-CXCR4 IgG decreased the number of BMC-derived ECs (*i.e.*, CD31-positive and GFP-positive cells) to 48% of normal IgG-treated mice (Fig. 4B). In contrast, this antibody did not modify the number of BMC-derived SMC-like cells (*i.e.*, α-SMA-positive and GFP-positive cells) (Fig. 4C). Concomitant with the decreased number of CD31-positive and GFP-positive cells, renal hypoxia was slightly accelerated by the anti-CXCR4 IgG treatment (Fig. 4D). However, there was no significant difference in BUN levels (not shown), suggesting that the aggravation of renal hypoxia by this antibody was mild, failing to the acceleration of renal dysfunction. Overall, kidney-produced SDF1 was shown critical for the BMC recruitment (and possible differentiation) to ECs, rather than SMCs, as discussed later.

DISCUSSION

During AKI, vascular EC injury is an important event to trigger peri-tubular fibrosis, followed by CKD progression [2, 3]. Actually, preservation of vascular structure by growth factors inhibits the secondary events post-AKI [14, 18]. Although administration of BMCs improves the pathological status of AKI in rodents [6, 20], little is known whether endogenous BMCs are engrafted into renal vessels in an early phase of AKI. We previously reported the early onset of EC apoptosis within 24 hr post-renal I/R [18]. In the present study, we found that engraftment of CD31-positive BMCs in the renal vessels became evident within a few days post-challenge. This is, to our knowledge, the first report to show an early contribution (*i.e.*, 2 days post-I/R) of BMCs to endothelial remodeling during AKI.

In our AKI model, SDF1 up-regulation was noted in vascular ECs (and tubules), while its receptor, CXCR4, was seen specifically in BMCs. Anti-CXCR4 IgG repressed the BMC homing. Thus, it is likely that surviving ECs release a chemo-attractant SDF1 to move BMCs from blood to the renal vessels (*i.e.*, paracrine loop). In general, BMCs include endothelial progenitor cell (EPC) lineages, and SDF1 is required for homing of this lineage [21]. As a result, CD31-positive EC-like cells were also reduced by anti-CXCR4 IgG, due to the possible reflection of the decreased EPCs, as reported in skin healing [31]. Thus, we speculate that SDF1 secreted from EC may confer an initial gate system for selecting an appropriate lineage (s) (such as EPCs) to sustain endothelial barrier integrity.

We further discuss the molecular basis of BMC-involved EC reconstitution, focusing on a possible cellular event. Emerging evidence delineates a pivotal role of VEGF for BMC-to-EC differentiation [9]. Actually, VEGF and its receptor, VEGFR2, are up-regulated in the rodent kidneys post-ischemic AKI [11, 27]. Of note, SDF1 is a potent inducer of VEGF [32]. Thus, we predict that SDF1 is critical not only for "vascular BMC accumulation" but also for "EC differentiation" through up-regulating VEGF in ischemic kidney. The number of GFP-positive ECs increased within 4 days post-ischemia, while VEGF is, as its name indicates, a potent mitogen of ECs. Thus, VEGF likely contributes to

the increase in bone marrow-derived ECs via inducing differentiation (*i.e.*, primary effect) and promoting subsequent proliferation (*i.e.*, secondary effect).

It is important to note that renal accumulation of α-SMA-positive cells is not modified by anti-CXCR4 IgG, suggesting an alternative pathway (s) other than SDF1. BMCs also include a small population of SMC progenitor cells [26]. In a mouse model of atherosclerosis, CX3CR1-positive SMC progenitors contribute to the neointimal hyperplasia of SMCs, in response to its ligand, fractalkine [12]. In contrast, SDF1-CXCR4 axis is critical in another model [33], raising a controversial issue. Although further studies are required to determine the role of fractalkine-CX3CR1 in our model, we at least emphasize that distinct mechanisms (*i.e.*, SDF1-dependent for ECs and SDF1-independent for SMCs) might participate in BMCs-mediated vascular reconstitution. BMCs are a new source of myofibroblasts during renal fibrosis [4, 30]. The possible accumulation of BMC-derived myofibroblasts may be independent on SDF1, as reported [30], and future studies will shed light on this notion.

We next discuss the impact of local hypoxia on BMC-based renal reconstitution. Hypoxia induces SDF1, VEGF and HGF-receptor/c-Met through HIF1-dependent transcriptional pathways [5]. Hypoxic apoptosis of ECs is responsible for endothelial dysfunction [18], while SDF1 can protect ECs from apoptosis [32]. Thus, it is likely that EC-derived SDF1 attenuates hypoxia-induced death of ECs through an autocrine loop. Furthermore, BMCs produce nitric oxide, a vasodilator, in VEGF-dependent manners [8]. Indeed, BMC administration increased the renal blood flow in a pig model of AKI [7], possibly via local vasodilation. These data may explain the reason why the decrease in renal BMCs by anti-CXCR4 IgG leads to tendency of accelerated hypoxia. In other words, hypoxia-induced SDF1 may confer an adaptive system to attenuate local hypoxia, partly via recruiting nitric oxide-producing BMCs.

We finally discuss the possible dual role of SDF1 in epithelial repair, because SDF1 signal is required for BMCs to acquire a phenotype of tubular epithelium [19]. Tubular epithelium produces SDF1 in response to hypoxia [16, 28], while HGF is produced in renal interstitial cells in a model of AKI [19]. Notably, SDF1 and HGF cooperatively stimulate the migration of BMCs across matrix-based Matri-gel [24], implying critical roles of SDF1 and HGF for migration of BMCs to injured tubules across basement membrane. HGF signal is also required for differentiation of stem cells to renal epithelial cells in kidneys [29]. Thus, we hypothesize that a molecular switch of SDF1→VEGF and that of SDF1→HGF may be involved in BMC-EC and BMC-epithelial conversion, respectively. We are now investigating the contribution of growth factors to BMC differentiations.

In summary, we found the engraftment of BMCs in peritubular vessels at the latest from 2 days post-AKI challenge. In this process, there was a CXCR4-dependent pathway for BMC-based engagement of ECs, and possibly, CXCR4-independent cascade for that of SMCs. Such a chemokine ligand-receptor axis seems to serve as a biological filter to select a cellular lineage (s), in cooperation with various effects of BMC-secreted cytokines (including paracrine effects) [16]. The AKI mouse model will be a tool to elucidate cytokine networks of BMC-mediated tissue remodeling.

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