Inhibition of tubular cell proliferation by neutralizing endogenous HGF leads to renal hypoxia and bone marrow-derived cell engraftment in acute renal failure

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Ohnishi H, Mizuno S, Nakamura T. Inhibition of tubular cell proliferation by neutralizing endogenous HGF leads to renal hypoxia and bone marrow-derived cell engraftment in acute renal failure. Am J Physiol Renal Physiol 294: F326–F335, 2008. First published November 21, 2007; doi:10.1152/ajprenal.00480.2007.—During the progression of acute renal failure (ARF), the renal tubular S3 segment is sensitive to ischemic stresses. For reversing tubular damage, resident tubular cells proliferate, and bone marrow-derived cells (BMDC) can be engrafted into injured tubules. However, how resident epithelium or BMDC are involved in tubular repair remains unknown. Using a mouse model of ARF, we examined whether hepatocyte growth factor (HGF) regulates a balance of resident cell proliferation and BMDC recruitment. Within 48 h post-renal ischemia, tubular destruction became evident, followed by two-waved regenerative events: 1) tubular cell proliferation between 2 and 4 days, along with an increase in blood HGF; and 2) appearance of BMDC in the tubules from 6 days postischemia. When anti-HGF IgG was injected in the earlier stage, tubular cell proliferation was inhibited, leading to an increase in BMDC in renal tubules. Under the HGF-neutralized state, stromal cell-derived factor-1 (SDF1) levels increased in renal tubules, associated with the enhanced hypoxia. Administration of anti-SDF1 receptor IgG into ARF mice reduced the number of BMDC in interstitium and tubules. Thus possible cascades include 1) inhibition of tubular cell proliferation by neutralizing HGF leads to renal hypoxia and SDF1 upregulation; and 2) BMDC are eventually engrafted in tubules through SDF1-mediated chemotaxis. Inversely, administration of recombinant HGF suppressed the renal hypoxia, SDF1 upregulation, and BMDC engraftment in ARF mice by enhancing resident tubular cell proliferation. Thus we conclude that HGF is a positive regulator for eliciting resident tubular cell proliferation, and SDF1 for BMDC engraftment during the repair process of ARF.

Acute renal failure (ARF) occurs under hypoxic conditions, due to thrombosis, hemorrhagic shock and abdominal compressions (44). The incidence of ARF is now on the increase worldwide, in association with infectious septic diseases (24, 44). During the pathological process, the straight segment (S3) of the proximal tubule is predominantly damaged, especially after renal ischemia and reperfusion (I/R). The loss of tubular continuity leads to a decrease in glomerular filtration rates (GFR) via a tubuloglomerular feedback mechanism (24, 42, 44). Indeed, the proportion of S3 tubules with fragmentation strongly correlates with a decrease in renal blood flow during the progression of ischemic ARF in humans and rodents (44, 47). Consequently, renal hypoxia becomes evident as a result of the loss of tubular continuity, and tubular destruction may be further accelerated by hypoxia, under such a “pathological circuit” (42).

To reverse the S3 tubular injuries, there is an intrarenal repair system by remnant nephrons: surviving tubular epithelial cells are divided to propagate daughter cells, and the defective areas of S3 tubules can be sheeted with newly generated epithelial cells (50). In addition to the local repair system, recent studies have shown that bone marrow-derived cells (BMDC) contribute, more or less, to reconstruction of S3 tubules for reversing ARF (i.e., extrarenal system) (6, 26, 38). Since rapid recovery from morphological damages inhibits further hypoxia and dysfunction (24, 42, 44), it is important to elucidate the mechanism whereby damaged tubular S3 segments are repaired at the cellular and ligand levels. Nevertheless, molecular regulation of the balance between resident epithelial cell proliferation and BMDC recruitment has yet to be addressed.

Hepatocyte growth factor (HGF) was originally identified and cloned as a potent mitogen for mature hepatocytes (35, 36). HGF acts on various types of cells through its receptor, c-Met, and exhibits pleiotropic activities during embryogenesis and tissue repair (3, 12). In the kidney, HGF has biological actions for regeneration of renal tubules, for example, by stimulating epithelial cell proliferation and morphogenesis (2, 4, 17, 30), or by reducing tubular cell death (27, 29). Importantly, supplement therapy with HGF accelerates rapid recovery from ARF in rodents, and this is associated with the enhancement of tubular cell proliferation (18, 21, 31). HGF can prevent the onset of tubular apoptosis after renal I/R via inhibiting vascular inflammation in an initial stage of ARF (33). On the other hand, HGF is capable of stimulating incorporation of BMDC into parenchymal areas in the case of severe and extensive injury (19, 49). These backgrounds prompted us to hypothesize that HGF modulates repair fashions either by epithelial cell proliferation or by BMDC recruitment.

To test our hypothesis, we used a model of renal I/R, since it is reported that both tubular cell proliferation and BMDC engraftment contribute to tubular repair (26, 38, 50). Using anti-HGF IgG or recombinant HGF in the ARF model, we provide evidence that endogenous HGF is required for tubular repair, and such an HGF-mediated effect is achieved mainly via tubular cell proliferation. We wish to emphasize

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that induction of tubular cell proliferation by HGF primarily accounts for functional recovery from ARF, while recruitment of BMDC through hypoxic events may be an adaptive response, perhaps to compensate for the loss of epithelial cell proliferation.

METHODS

Bone marrow transplantation and renal injury in mice. We used a strain (i.e., C57BL/6) of green fluorescent protein (GFP) transgenic male mice, which systemically express GFP under the control of a β-actin promoter (37), as donors 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 injection (ip) of 1 × 10^6 bone marrow cells from male GFP transgenic mice. After reconstitution of bone marrow for 5 wk, the recipient mice were subjected to renal I/R: under anesthesia, both renal arteries were clamped for 40 min at 38°C to induce ARF. The mice were killed at 0, 2, 4, 6, or 8 days posts ischemia to characterize the natural course of ARF. Some mice were treated with 5-bromo-2'-deoxuryridine (BrdU; 50 mg/kg ip, Nacalai, Kyoto, Japan) or Hypoxyprobe-1 (60 mg/kg ip, Chemicon, Temecula, CA) 1 h before the autopsy. All animal experiments were done in accordance with National Institutes of Health guidelines, as dictated by the Animal Care Facility at Osaka University Graduate School of Medicine.

Neutralization or administration of HGF in vivo. To neutralize HGF in mice, an anti-HGF rabbit IgG specific for rat HGF was prepared in our laboratory. This antibody cross-reacted with mouse HGF and worsened renal apoptosis and fibrosis in mice (32, 33). The mice that had undergone renal I/R were randomly divided into two groups and injected with anti-rat HGF rabbit IgG (n = 9) or normal rabbit IgG (300 μg ip, n = 9) between 36 and 96 h after the renal I/R treatment. A recombinant form of human HGF (i.e., rh-HGF) was purified from a medium of Chinese hamster ovary cells transfected with a vector containing human HGF cDNA of the 5-amino acid deleted type (>98% of purity on SDS-PAGE) (18, 21, 33). To test the therapeutic effects of HGF on ischemic ARF, 36 mice that had undergone I/R injury were injected with saline or rh-HGF (500 μg/kg−1·h−1·sc).

Neutralization of CXCR4/stromal cell-derived factor 1 receptor. To inhibit stromal cell-derived factor 1 (SDF1) signaling during the progression of ARF, anti-CXCR4 IgG (eBioscience, San Diego, CA) was administered, as in a previous study (45). Briefly, eight mice were subjected to the renal I/R and randomly divided into two groups: four mice were injected with the anti-CXCR4 IgG (100 μg/48 h ip), and the remaining four mice were treated with normal IgG (100 μg/48 h ip). All of these mice were killed at 4 days after I/R, and the renal tissues were collected for analysis, as described below.

Blood chemistry. To evaluate the renal dysfunction, blood urea nitrogen (BUN) levels were measured by a urease indophenol method with a kit (urea nitrogen-B test, Wako, Osaka, Japan).

Immunohistochemistry. The renal tissues were embedded in paraffin in a routine fashion. The tissues were cut at 4 μm, dewaxed, and subjected to the following procedures. To detect the proliferative events, BrdU and tyrosine-phosphorylated c-Met (phospho-Met) were identified in 70% ethanol-fixed sections, using an anti-BrdU IgG (Oxford Biotechnology, Oxfordshire, UK) or anti-phospho-Met-1234/1235 IgG (Biosource, Camarillo, CA), followed by the second reaction with biotin-labeled anti-IgG (Vector, Burlingame, CA). An avidin-biotin coupling reaction was performed on the renal sections, using a kit (Elite, Vector). To detect hypoxic changes, anti-Hypoxyprobe-1 IgG (Novus Biologicals, Littleton, CO) and anti-mouse SDF1 IgG (R&D, Minneapolis, MN) were used as the primary antibodies. All antigens were visualized as brown, with 3,3′-diaminobenzidine (Nacalai).

Immunofluorescence staining. The GFP-positive signals were observed under a confocal microscope LSM-PASCAL (Carl Zeiss). The cryosection was washed with PBS and then incubated with the primary antibodies such as anti-Na^+-K^+-ATPase IgG (LSL, Tokyo, Japan) and anti-c-kit IgG (eBiosciences) at 4°C overnight, followed by fluorescent staining with Alexa 546- or Alexa 647-conjugated secondary antibodies (Invitrogen, Carlsbad, CA). The nuclei were detected with TOPRO-3 (Invitrogen). For Y-FISH, the cryosection was stained with anti-Na^+-K^+-ATPase IgG and incubated with 1 M sodium thiocyanate at 80°C for 10 min, followed by digestion with 0.4% pepsin. After fixation with 4% paraformaldehyde, the sections were dehydrated and air-dried. Slides were overlaid with a probe (STAR-FISH Y chromosome paint, Cambio, Cambridge, UK) and then incubated at 60°C for 10 min followed by overnight incubation at 37°C.

Morphometric scores. The remaining renal tissues were stained with a solution of periodic acid-Schiff (PAS). Tubular injury was graded as follows: 0, 0%; 1, <10%; 2, 10–25%; 3, 25–50%; 4, 50–75%; 5, >75% of the tubules were affected, as reported (33). The BrdU-positive cells were counted in >1,000 tubular cells in the kidneys (21). The appearance of HIF-1α, SDF1-, phospho-Met-, and GFP-positive cells at the outer medulla was semiquantified by counting positive cells in >15 randomly chosen nonoverlapping high-power fields (×400 magnification). Furthermore, c-kit-positive- and GFP-positive cells and Y chromosome-positive and Na^+-K^+-ATPase-positive cells were counted in >15 fields (×400). The overall means of these parameters in each group were calculated based on individual values. For quantification of hypoxia, photographed images were analyzed using image-analysis software (WinRoof, Mitani, Fukui, Japan) to determine the extent of hypoxic areas in the outer medulla.

ELISA. Plasma HGF levels of ARF mice were measured using an ELISA kit for rodent HGF (Institute of Immunology, Tokyo, Japan) (32, 33). To prepare the renal extract, kidney tissues were homogenized in 10 volumes of buffer composed of 20 mM Tris·HCl (pH 7.5), 2 M NaCl, 0.01% Tween 80, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA (Nacalai). The homogenate was centrifuged at 15,000 rpm for 30 min, and the supernatant was used as a tissue extract. SDF1 levels in the renal extract were determined using an ELISA kit (R&D).

Real-time PCR. Total RNA was prepared from kidneys, using ISOGEN (NipponGene, Tokyo, Japan). One microgram of total RNA was reverse-transcribed into first-strand cDNA with a random hexamer primer using Superscript II reverse transcriptase (Life Technologies, Rockville, MD). Quantitative PCR was performed to detect SDF1 mRNA, using an ABI PRISM 7700 system (PerkinElmer Biosystems, Foster City, CA), according to the manufacturer’s instructions. (For primer designs, see catalog no. Mm00445552-m1.)

Western blot analysis. Renal tissues were homogenized in a lysis buffer (50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 25 mM glycerophosphate, 50 mM NaF, 1 mM Na3VO4, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml antipain, peptatin A, leupeptin, and 1% aprotinin, pH 7.4), incubated with anti-HIF-1α IgG (as same as in Immunohistochemistry) at 4°C overnight and then precipitated with protein-G/A (Amersham Pharmacia, Little Chalfont, UK), as reported (33). These samples were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The anti-HIF-1α IgG was applied to PVDF membranes, followed by a second reaction with peroxidase-labeled antibodies (Dako, Glostrup, Denmark). The HIF-1-positive signals were visualized on PVDF membranes, using a kit (ECL system, Amersham Pharmacia). Similarly, phospho-Met and total Met signals in the renal tissue extraction were detected as immunoblots, as reported (33).

Statistical analysis. All data are expressed as means ± SD. Student’s t-test, ANOVA, a Mann-Whitney U-test, or Kaplan-Meier analysis.
analysis for the survival rate was used to compare the group means, and a value of \( P < 0.05 \) was considered to be significant.

RESULTS

Changes in plasma HGF levels during tubular cell proliferation and BMDC recruitment in ARF mice. In this study, we used chimeric mice in which the ration of the GFP-positive cells in total bone marrow cells was >85%. All mice manifested tubular injuries (such as losses in brush border, cast accumulation, and leukocyte infiltration) at the outer medulla of S3 segments within 2 days postischemia, and this was associated with increases in injury scores and BUN levels (day 2: \(-120 \text{ mg/dl}\) (Fig. 1A). Such histological damage and renal dysfunction became mild after 4 days of ischemia, thus indicating the reversibility of ischemic ARF in mice. In this process, BrdU-positive signals were identified mainly in injured tubules at the corticomedullary areas, with a peak at 2 days (Fig. 1B). Furthermore, GFP-positive donor cells (i.e., BMDC) were localized around or in \( \text{Na}^+\text{-K}^+\text{-ATPase} \)-positive tubular areas, especially at 6 and 8 days after renal I/R (Fig. 1C). The three-dimensional analysis of fluorescence sections strengthened the involvement of BMDC in renal tubules (supplementary Fig. S1; all supplementary material is available in the online version of this article at the journal website). In these regenerative processes, plasma HGF levels increased at 2 days after I/R, to a fivefold level of basal control (1.5–2 ng/ml) and remained higher during tubular repair (Fig. 1D). In the sham operated group, there was no significant increase in plasma HGF levels, except for a slight increase at 3 h after renal I/R, as reported (33). Thus we hypothesized that endogenous HGF may be involved in reversing S3 tubular injury after the onset of ARF in mice.

Inhibition of tubular cell proliferation and functional recovery from ARF by anti-HGF IgG in mice. To test our hypothesis, anti-HGF IgG was injected into the mice between 36 and 96 h postischemia (Fig. 2A). Anti-HGF IgG treatment accelerated mortality during the progression of ischemic ARF (\( P = 0.081 \)) (Fig. 2B). To identify the cells targeted by HGF, we examined the localization of c-Met tyrosine phosphorylation (e.g., phospho-Met). In normal IgG-treated ARF mice, a phospho-Met-
positive signal was detected in the S3 tubular areas, but not in vascular endothelial areas (Fig. 2C). This result was distinct from previous observations (33), probably due to the difference between inflammatory (3-h) and regenerative (96-h) phases. Thus we focused on the roles of HGF in tubular events and found that anti-HGF IgG suppressed c-Met expression and tyrosine phosphorylation, as evidenced by Western blotting (Fig. 2Ca), being in agreement with our previous report (33). Immunohistochemically, phospho-Met-positive signals were identified specifically in the S3 tubular area of the outer medulla, while the number of phospho-Met-positive tubular cells was significantly reduced in ARF mice, treated with anti-HGF IgG (Fig. 2Cb). Consistent with the lower expression of phospho-Met, the number of BrdU-positive tubular epithelial cells was decreased in the anti-HGF IgG-treated mice compared with the control mice (3.8 ± 1.6 vs. 7.8 ± 2.4%, P < 0.05) (Fig. 2D). Of note, some BrdU-labeled tubular cells were positive for phospho-Met staining (supplementary Fig. S1Ac), indicating a direct mitogenic role of HGF toward tubular cells. Tubular atrophy, loss of brush border, and cast accumulation became moderate at 4 days in the normal IgG group, whereas such lesions were still severe at the same time-point in the HGF-neutralized group (Fig. 2E), with a significant difference in the injury score between normal IgG and anti-HGF IgG groups. Overall, BUN levels were higher in the HGF-neutralized mice than in the control mice (Fig. 2F).

Enhancements of BMDC engraftment and hypoxia in HGF-neutralized kidneys in mice. We examined whether HGF-neutralizing treatment alters the BMDC engraftment in this ARF model. There were few GFP-positive cells (i.e., BMDC) in the renal tubules (<1% in the outer medulla) within 4 days postischemia (Fig. 3Aa). Of note, anti-HGF IgG injections increased the number of “GFP-positive and Na+K+-ATPase-positive” tubular cells to a twofold level of the normal IgG group (P < 0.05). Similarly, Y-FISH analysis strengthened the enhancement of tubular BMDC engraftment by HGF neutralization: the percentage of Y chromosome-positive and tubular Na+K+-ATPase-positive (i.e., tubular BMDC) increased to a significant level by anti-HGF IgG (Fig. 3Ab). Since local hypoxia may facilitate BMDC engraftment in injured tissues (7), we next examined the hypoxic events in the kidney. Renal immunohistochemical examination revealed that hypoxic tubules (identified as Hypoxyprobe-1 deposition) were extensively noted at 2 days, especially around corticomedullary
regions, but these changes were attenuated within 4 days in the normal IgG group (Fig. 3B). In contrast, anti-HGF IgG inhibited the recovery from renal hypoxia, as evaluated by measuring the Hypoxyprobe-1-positive tubular areas.

**Upregulation of SDF1 levels and recruitment of BMDC in the kidney by anti-HGF IgG.** Consistent with the change in renal hypoxia, HIF-1α expression increased between 6 and 48 h, then returned to the basal level at 4 days postischemia (Fig. 4A). In contrast, the expression of HIF-1α by renal tubules was sustained at a higher level in HGF-neutralized ARF mice, as evidenced by immunoblotting and immunohistochemistry. We next focused on SDF1, a ligand for homing BMDC into injured tissues (7). Along with the enhanced expression of HIF-1α, renal SDF1 mRNA and protein levels markedly increased at 2 days, but gradually decreased in the normal IgG-treated mice by 4 days after the renal I/R surgery (Fig. 4A). In contrast, anti-HGF IgG increased the number of SDF1-positive tubules, as noted at 4 days postischemia. Renal SDF1 levels correlated with the number of c-kit-positive and GFP-positive cells (including bone marrow-derived progenitor cells): both parameters reached a peak between 2 and 4 days postischemia, but decreased at 6 days after the I/R surgery. Of note, anti-HGF IgG treatment sustained the “interstitial infiltration” of c-kit-positive BMDC (Fig. 4C), and this was associated with the increase in renal SDF1 levels. Thus we predict that SDF1 may be responsible for infiltration of BMDC into renal “interstitium,” especially under persistent renal hypoxia.

**Importance of SDF1/CXCR4 for BMDC infiltration into injured kidneys.** To test our prediction, we injected an anti-CXCR4/SDF1 receptor IgG into the ARF mice, using an experimental protocol (Fig. 5A). About 35% of GFP-positive renal cells expressed CXCR4 (data not shown). In our ARF model, administration of anti-CXCR4 IgG suppressed the interstitial infiltration of c-kit-positive and GFP-positive cells (including bone marrow-derived progenitor cells) (Fig. 5B). As a result, the number of GFP-positive and Na⁺-K⁺-ATPase-positive cells (i.e., tubular BMDC) was decreased.

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**Fig. 3.** Acceleration of BMDC engraftment in renal tubules of ARF mice by anti-HGF IgG under persistent hypoxia. **A:** enhancing effect of anti-HGF IgG on engraftment of BMDC into renal tubules. **a:** typical fluorescence images of the kidney (left) and quantification of GFP-positive tubular cells in OM (right). Green, GFP; red, Na⁺-K⁺-ATPase; blue, TOPRO-3. Arrows indicate tubular BMDC. Inset: expanded image of GFP and Na⁺-K⁺-ATPase. **b:** Y chromosome-positive (red) cells in Na⁺-K⁺-ATPase-positive (green) cells, as checked at 4 days postischemia. **B:** immunohistochemistry for Hypoxyprobe-1 accumulation. The hypoxic areas were expressed as a ratio of Hypoxyprobe-1-positive areas to total areas (×200). Values are means ± SD (n = 4 for anti-HGF IgG group, n = 6 for normal IgG group). *P < 0.05 vs. normal IgG group.
at 4 days postischemia by anti-CXCR4 IgG treatment (Fig. 5C). Thus our data demonstrated a critical role for SDF1 in the infiltration of BMDC into interstitial spaces under renal hypoxia. Although it is still unclear whether tubular SDF1 is directly involved in mobilization of interstitial BMDC into S3 tubules, we wish to at least emphasize that local SDF1 is required for accumulation of BMDC in injured kidneys.

Acceleration of tubular cell proliferation by adding HGF leads to suppression of BMDC engraftment during recovery from renal damage. Inversely, we examined the effect of rh-HGF on the balance of tubular cell proliferation and BMDC engraftment during the course of ARF, using an experimental protocol (Fig. 6A). As expected, delayed treatment of ARF mice with rh-HGF led to rapid decreases in the BUN level and histological injury score up to 6 days postischemia (Fig. 6B). There was a significant difference in BUN levels between saline and rh-HGF groups (day 4: 43.1 ± 12.2 vs. 18.4 ± 3.4 mg/dl, P < 0.01). Similarly, rh-HGF therapy improved tubular injuries, such as tubular dilatation with the loss of tubular brush border, hence reflecting the decrease in the histological injury score. In this process, rh-HGF injections increased the number of BrdU-positive tubular cells (Fig. 6C). On the other hand, rh-HGF treatment reduced the hypoxic area at 3 days postischemia (Fig. 6D). Along with the improved hypoxia, SDF1 levels decreased in rh-HGF-treated kidney at 3 days after I/R (Fig. 6E), associated with the decrease in GFP-positive interstitial cells (data not shown). Overall, the appearance of BMDC in the Na\(^+\)-K\(^+\)-ATPase-positive cells (i.e., tubular BMDC) was suppressed under the HGF-added condition (Fig. 6F).

It is noteworthy that renal tubular BMDC engraftment became evident between 6 and 8 days postischemia, while BUN levels returned to the normal range by 6 days (Fig. 1A), suggesting a minor contribution of BMDC to the functional recovery from ARF. However, histological injury score was still higher at 6 days postischemia. Thus we predict that the recruitment of BMDC by tubules may be involved in the morphological improvement, as noted between 6 and 8 days (Fig. 1A). In other words, a rapid induction of tubular cell proliferation by administration of renotrophic growth factors may be considered as an option for a recovery from morphological and functional abnormality, as reported elsewhere (9, 15, 21, 29, 31).
DISCUSSION

Growing evidence indicated that epithelial cell proliferation after tissue injury becomes faint, associated with the hypoxic conditions (8, 10, 22, 25). Thus local hypoxia can be a risk factor for suppression of organ repair. On the other hand, a recent study showed that the appearance of BMDC preferentially occurs in hypoxic areas (7). Thus we hypothesized that epithelial cell proliferation is primarily responsible for restoring injured tissues, while suppression of epithelial growth by hypoxia may enhance nonepithelial repair, compensated by BMDC. To test this hypothesis, we used an animal model of ARF, in which both resident epithelial cells and BMDC were involved in renal tubular reconstruction (26, 38, 50). We obtained evidence that a differential expression of HGF and SDF1 in kidneys may be critical to regulate which repair mechanism, epithelial cells or BMDC, is involved in a recovery from renal tubular damage.

In the previous work, we examined the role of HGF “before the onsets” of tubular destruction in a mouse model of ARF: c-Met tyrosine phosphorylation was noted in the renal endothelium at 3 h after renal I/R, with an increase in plasma HGF levels (33). Inhibition of the endothelial c-Met activation by anti-HGF IgG led to an acceleration of neutrophil infiltration and of tubular apoptosis, indicating a key role of circulating HGF for inhibiting endothelial inflammation in the initial stage of ARF (33). In the present study, we investigated the role of endogenous HGF “after the onset” of tubular destruction, focusing on tubular regenerative events, as c-Met activation was noted in renal S3 tubules, but not in endothelium in the advanced stage (i.e., between 48 and 96 h postischemia). Of note, anti-HGF IgG reduced the number of BrdU-labeled tubular cells, indicating that endogenous HGF is a key mitogen of renal tubules after renal damage. In vitro, HGF has been reported to be a mitogen of renal tubular cells (2, 4, 17, 30). Although clinical and experimental studies revealed that HGF and c-Met levels increased during progression of ARF (18, 28, 34, 43), there was no direct evidence to identify a role of endogenous HGF after onset of ARF. Our results strongly suggest that HGF is a physiological regulator for restoration of epithelial integrity in the advanced stage of ARF.

In view of the fact that HGF is a key ligand for tubular cell growth, let us examine whether hypoxia-related events (including BMDC engraftment) may be modified under the HGF-neutralized condition. Indeed, anti-HGF IgG treatment enhanced renal hypoxia in our model, and this was associated with prolonged tubular injuries. Since tubular damage (i.e., a loss of tubular continuity) negatively regulates GFR via tubulo-glomerular feedback (24, 42, 44), acceleration of tubular “discontinuity” by anti-HGF IgG possibly leads to a decrease in renal blood flow and subsequent hypoxia, as reviewed elsewhere (44). As a result, the appearance of BMDC in interstitial (and S3 tubular) areas became evident under the HGF-neutralized (i.e., hypoxic) conditions, where renal tubular cell proliferation was prohibited by anti-HGF IgG. Thus we predict that BMDC-participated tissue repair may be, at least in part, a secondary reaction compensating for the loss of epithelial cells under hypoxia.

Fig. 5. Inhibitory effects of anti-CXCR4 IgG on engraftment of BMDC into the kidney of ARF mice. A: in vivo neutralization of SDF1 signaling using an anti-CXCR4 IgG after renal ischemia. The antibody was administered to mice at 1 and 3 days after renal I/R. B: decrease in the number of c-kit-positive and GFP-positive interstitial cells by anti-CXCR4 IgG, as noted at 4 days postischemia. Arrows, donor-derived c-kit-positive interstitial cells. C: inhibitory effect of anti-CXCR4 IgG on BMDC engraftment into tubules, as evidenced by fluorescence images. Red, Na⁺-K⁺-ATPase; green, GFP; blue, TOPRO-3; arrows, donor-derived tubular cells. Inset: colocalization of Na⁺-K⁺-ATPase and GFP. Values are means ± SD (n = 4). *P < 0.05 vs. normal IgG group.
It is important to explain how renal hypoxia accelerates infiltration and engraftment of BMDC under HGF-neutralized states. We focused on SDF1, since SDF1 is upregulated by hypoxia and responsible for recruiting BMDC into organs (7, 39). In our ARF model, SDF1 was noted in renal tubules, as reported (45), while 35% of BMDC in the kidney were positive for CXCR4. Anti-HGF IgG enhanced tubular SDF1 expression, due to an increase in HIF-1 levels (7), leading to an increase in GFP-positive interstitial and tubular cells. More importantly, anti-CXCR4 IgG reduced the number of BMDC, suggesting a paracrine loop between SDF1-expressing tubules and circulating BMDC for renal accumulation of bone marrow-derived progenitor cells. With regard to this, a recent study delineated possible fusion of BMDC in a rat model of ARF (11). Although it is still unclear whether SDF1 may enhance fusion of BMDC in our ARF model, we wish to emphasize that renal hypoxia, accelerated by anti-HGF IgG, causes SDF1 upregulation, and then infiltration of BMDC into interstitial tissue is facilitated. Interstitial BMDC can acquire a phenotype of collagen-producing myofibroblasts (5, 26), while endogenous HGF is required to suppress renal fibrosis (29, 32). Indeed, anti-HGF IgG treatment enhanced the renal accumulation of bone marrow-derived myofibroblasts at 4 days post-ischemia (data not shown). Thus the increases in BMDC following neutralization of HGF may be due, at least in part, to the loss of the antifibrotic effect of HGF. Another possibility is that interstitial BMDC may protect renal tubules from ischemic stresses via a paracrine production of cytokines or growth factors, as reported (46).

We next examined the localization of HGF and SDF1 in our ARF model. HGF was detected in the interstitium of “nonhypoxic” areas near the BrdU-positive tubules (supplementary Fig. S2), thus indicating a paracrine interaction between renal mesenchyme and parenchyma. On the other hand, HGF expression became faint at the hypoxic area, as reported (8). It is known that hypoxia per se suppresses HGF mRNA expression in vitro and in vivo (16, 41). Thus a decrease in HGF production by local hypoxia may lead to acceleration of renal damage in ARF, as reported in liver diseases (8). In contrast, SDF1 was identified in the hypoxic tubules, along with the BMDC engraftment (supplementary Fig. S2). Thus possible cascades are 1) under nonhypoxic conditions, HGF is produced by peritubular interstitial cells, and then the defective area is covered by newly generated epithelium; and 2) HGF production is sup-
pressed by hypoxia, and in return, SDF1 levels increase in tubules to facilitate homing of BMDC into renal interstitium and tubules. Such a local balance of HGF and SDF1 may regulate a contribution of resident epithelial cells or of BMDC to morphological recovery from S3 tubular injuries.

In addition to the local HGF delivery system, it is critical to discuss the role of endocrine delivery of HGF. In our ARF model, HGF mRNA levels significantly increased in the lung and liver in the later stage (not shown), as reported (20), and this may reflect the increase in blood HGF levels (day 6, P < 0.05 vs. day 0). As a result, GFP-positive cells appeared in renal tubules after 6 days of I/R, despite the lower SDF1 expression. In this process, we noted that the tubular BMDC entered G1/S progression in the late stage (data not shown), as reported (11, 26). Since HGF is a key mitogen for renal epithelium, proliferation of tubular BMDC by blood HGF may explain the increase in GFP-positive tubules (>6 days). Recently, it was reported that systemic HGF is capable of enhancing migration of BMDC if tissue hypoxia is severe (19, 49). Together, circulating HGF may also contribute to increasing the GFP-positive tubular areas in the later stage (>6 days), probably as a migration factor and/or an in situ mitogen.

Renal hypoxia causes tubular damage, and vice versa during the progression of ARF (42). Thus the effect of hypoxia on organ regeneration should be discussed. As HGF production is suppressed by hypoxia (16, 41), the loss of local HGF production may underlie the mechanism whereby “organ repair failure” occurs under hypoxia. Inversely, rh-HGF supplementation restores hypoxia-mediated failure to regenerate. In this point, recent studies revealed that HIF-1α is a key nuclear switch for cell growth arrest under hypoxia (13, 25), while HGF reduced renal HIF-1α levels. Such an inhibitory effect of HGF may contribute to a release of the growth arrest of tubular epithelium. It is known that renal tubular cells undergo dedifferentiation during G1/S progression (44, 50). Of interest, HGF can stimulate dedifferentiation (i.e., epithelialization) of renal tubular cells (2). Therefore, these biological effects by HGF may produce regenerative outcomes during the morphological repair postischemia. Although hypoxia elicits apoptotic and inflammatory changes (40, 42), HGF is capable of inhibiting these hypoxia-related pathological events, such as apoptosis (27, 48) and inflammation (14, 33). Overall, multiple actions of HGF would lead to a rapid recovery from ARF.

A dual repair mechanism via epithelial cell proliferation and BMDC engraftment has been observed in several organs (23, 39). HGF is now a potent mitogen for resident epithelial cells in numerous tissues or organs (3, 12, 30). Together, we predict that HGF may be an innate regulator to stimulate epithelium-based regeneration at an early stage of tissue injury. HGF may also enhance BMDC-related tissue repair at a later stage, if induction of resident cell proliferation is difficult under severe injuries (19, 49). Our study would shed more light on an understanding of the self-defense system and directing a therapeutic orientation for overcoming hypoxic organ diseases.

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REFERENCES

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