Prevention of Neutrophil Extravasation by Hepatocyte Growth Factor Leads to Attenuations of Tubular Apoptosis and Renal Dysfunction in Mouse Ischemic Kidneys

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Ischemia and reperfusion (I/R) injuries occur in numerous organs under pathophysiological conditions. In this process, neutrophils play important roles in eliciting parenchymal injuries. Using a murine model of renal I/R, we show that hepatocyte growth factor (HGF) is a natural ligand that inhibits endothelial injuries and neutrophil extravasation. In mice after renal I/R, plasma HGF levels increased, along with c-Met/HGF receptor phosphorylation in the vascular endothelium. However, this c-Met activation was transient, associated with a decrease in endogenous HGF level, and followed by neutrophil infiltration and renal dysfunction. Suppression of endothelial c-Met phosphorylation by anti-HGF IgG led to rapid progressions of neutrophil extravasation, tubular apoptosis, and renal dysfunction. Inversely, enhancement of the c-Met activation by exogenous HGF blocked endothelial/tubular apoptotic injuries and acute renal failure. In this process, HGF prevented endothelial nuclear factor κB activation and inhibited induction of an adhesion molecule (ICAM-1), resulting in attenuated vascular edema and neutrophil infiltration. Thus, we conclude that 1) the HGF/c-Met system of endothelial cells confers an initial barrier to block neutrophil infiltration, and 2) transient and insufficient HGF production allows manifestation of postischemic renal failure. Our study provides a rationale for why HGF supplementation elicits therapeutic effects in ischemic kidneys. (Am J Pathol 2005, 166:1895–1905)

Neutrophil-endothelial interactions are the initial event involved in the onset of inflammatory diseases. Especially in ischemia/reperfusion (I/R) injuries, circulating neutrophils selectively infiltrate ischemic tissues and contribute to tissue destruction. During the progression of I/R injuries, endothelial barrier is impaired within a few hours by hypoxia or neutrophil-released toxic products. Along with vascular damage, circulating neutrophils attach and transmigrate between injured endothelial cells, the result being peri-vascular edema (so called as extravasation). In neutrophil-infiltrated organs, tissue damages are accelerated in part via neutrophil-released radicals or proteinases such as elastases. Thus, current opinion defines neutrophils as a key mediator for inflammation under acute stress (including ischemic damage).

In kidneys, ischemia is one of the most important causes of acute renal diseases. Especially in renal transplantation, initial I/R injury affects long-term outcome of renal graft survival. In I/R-undergone kidneys, tubular epithelial apoptosis participates in nephron destruction and renal dysfunction. In this process, infiltrated neutrophils (associated with endothelial injuries) play an important role in nephron destructions. In other words, inhibition of neutrophil extravasation can be a target for abrogating tubular injuries and dysfunction. There may be an intrinsic defense system to suppress or retard initial neutrophil-endothelial interactions. If so, enhancement of this system may lead to avoidance of I/R-related renal injuries. However, it is still poorly understood how renal tissues are physiologically protected from postischemic challenge.

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Hepatocyte growth factor (HGF), originally identified and cloned as a mitogen for mature hepatocytes,\textsuperscript{13,14} has mitogenic, motogenic, and morphogenic activities in various cell types via c-Met/HGF receptor phosphorylation.\textsuperscript{15–17} In renal tissues, HGF is protective and regenerative toward tubular epithelial cells (as well as glomerular cells).\textsuperscript{15,16} HGF prevents onsets of tubular injuries and acute renal failure in various animal models, while more importantly, nephron regeneration is accelerated by HGF even after the onset of acute renal failure.\textsuperscript{18–20} HGF is also cytoprotective for vascular endothelial cells.\textsuperscript{15} Nevertheless, whether and how HGF modulates neutrophil-endothelial interaction (before or during inflammation) remained to be determined.

Surgical treatment of I/R induces neutrophil infiltration and tubular apoptosis in rodent kidneys,\textsuperscript{8,11,12} which mimics ischemic renal diseases in humans. Using the well-documented model, we provide herein evidence that HGF is an intrinsic ligand to inhibit neutrophil infiltration and endothelial/tubular apoptosis, contributing to prevention of nephron destruction and dysfunction. Based on all available data, we discuss physiological and therapeutic functions of HGF to block vascular inflammatory response, a common event in ischemic organs.

Materials and Methods

Animal Treatments

Eight-week-old female ICR mice (26–28 g; SIC, Hamamatsu, Japan) were anesthetized with ketamine chloride (80 mg/kg, subcutaneous (s.c.)) and xyladine sulfate (8 mg/kg, s.c.). Under general anesthesia, they underwent ischemia of the left renal artery with a vascular clamp (38°C for 30 minutes). After release of this ischemia, contra-lateral (ie, right) nephrectomy was done to elicit acute renal dysfunction.\textsuperscript{21} For analysis of histopathological and biochemical changes during progression of renal I/R, 36 mice were killed at 3, 6, 12, and 18 hours after the surgical treatment of renal I/R. At the time of necropsy on six mice, renal tissues were fixed in 70% ethanol (or in part, 10% formalin) at 4°C overnight for immunohistochemistry, as described below. Remaining tissues were rapidly frozen in liquid nitrogen and stocked at −80°C for biochemical analysis.

For neutralization of endogenous HGF, an anti-HGF antibody specific for rat HGF was used. This antibody shows cross-reactivity with mouse HGF (but not human HGF) and accelerates tissue injuries in kidneys.\textsuperscript{22,23} Thus, the renal I/R mice were randomly divided into two groups and injected with rabbit anti-rat HGF IgG (n = 6) or normal rabbit IgG (n = 6) (250 μg/mouse, intraperitoneal) immediately after the renal I/R treatment. To determine the role of endogenous HGF, mice were killed at 1 and 8 hours after injecting the anti-HGF IgG.

To evaluate the therapeutic effects of exogenous HGF on the progression of renal injuries, 24 renal I/R mice were generated and injected with saline or recombinant human HGF (rh-HGF), which was purified from medium of chinese hamster ovary (CHO) cells transfected with a vector containing HGF cDNA of the 5-amino acid-deleted type, with >98% of purity on SDS-PAGE.\textsuperscript{18,22,23} Immediately after the release of warm ischemia, mice were given saline or rh-HGF (500 μg/kg, every 6 hour, s.c.) and then killed at 3 and 18 hours after the renal I/R (n = 6 per group).

Immunohistochemistry

Renal tissues were fixed in 70% ethanol and embedded in paraffin, as reported.\textsuperscript{22,23} Tissue sections were cut at a thickness of 4 μm, dewaxed, and stained with hematoxylin and eosin (H&E). To identify neutrophil infiltration, anti-mouse neutrophil Gr1 rat monoclonal IgG (BD Biosciences, San Jose, CA) was applied to the dewaxed sections as the primary reaction, followed by a second reaction with biotin-labeled anti-rat IgG goat IgG (Vector, Burlingame, CA). Finally, an avidin biotin coupling (ABC) reaction was done on sections, using a kit (Vectastain Elite; Vector). To detect apoptosis, an in situ end-labeling method was applied on renal sections, using a kit (ApopTag; Intergen, Purchase, NY).\textsuperscript{23} Furthermore, c-Met activation was evaluated on the kidneys, using rabbit IgG against phosphospecific c-Met (1:400) (pMet-1234/1235; Intergen, Purchase, NY).\textsuperscript{23} Biosciences, San Jose, CA) and anti-mouse ICAM-1 hamster IgG (BD Biosciences), respectively. These antigens were visualized using the ABC method, with 3–3’-aminobenzidine (ie, as brown signals). In the NF-κB-stained or TUNEL-stained sections, endothelial cells were identified using an anti-mouse-CD31 rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-mouse ICAM-1 hamster IgG (BD Biosciences), respectively. The CD31-positive signals were visualized as red signals, with a new Fuchsin kit (DAKO, Glostrup, Denmark). In the immunohistochemistry of phosphorylated c-Met and NF-κB, normal rabbit IgG (DAKO) was used as negative control.

Histological Scores

The degree of neutrophil infiltration was expressed as a Gr1-staining score, determined by counting numbers of polynuclear cells positive for Gr1, in >20 randomly chosen ×200 fields per mouse (n = 6). To evaluate the degree of tubular apoptosis, TUNEL score was determined by counting numbers of positive tubular cells in >20 ×200 fields. Immunoreactive signals for endothelial ICAM-1 were observed in >20 randomly chosen ×200 fields and scored on the scale of 0, no staining; 0.5, trace staining; 1, light staining; 2, moderate staining; and 3, intense staining.\textsuperscript{24} To quantify edematous lesions, renal sections were examined under a light microscope, and the degree of renal expression was expressed as a mean value of the following histochemical scales evaluated in at least 20 randomly chosen ×200 fields: 0, absent; 1, mild; 2, moderate; and 3, severe. Furthermore, vascular apoptosis was checked in >20 chosen ×200 fields, and its degrees were expressed as TUNEL-positive cells per
CD31-positive vascular lumen. These quantitative analyses were all made in a blinded fashion.

Biochemical Analysis

To evaluate renal functions after renal I/R, blood urea nitrogen (BUN) and plasma creatinine levels were determined using a urease indophenol method and by Jaffe’s method, respectively. The renal extract was obtained as reported, and c-Met expression and phosphorylation were detected as follows: total and phosphorylated c-Met were visualized on polyvinylidene difluoride (PVDF) membranes, with Western blotting. In the immunoblot analysis, β-actin was detected as an internal control. The c-Met expression and phosphorylation levels were quantified by a densitometric analysis, using computer software (NIH image). Endogenous HGF (ie, mouse HGF) levels in plasma and kidneys were determined, using an enzyme-linked immunosorbent assay (ELISA) for specifically detecting mouse (but not human) HGF. Furthermore, the human HGF level was monitored during rh-HGF administrations, using a human HGF-specific ELISA. To quantify the degree of infiltrated neutrophils, myeloperoxidase (MPO) activity before and after renal I/R was determined, as described previously.

Neutrophil Adhesion Assay

To determine the effects of HGF on neutrophil-to-endothelial adhesion, an in vitro model was prepared, according to a reported method, with slight modifications. Umbilical vein endothelial cells (HUVECs) (Japan Health Sciences, Tokyo, Japan) were adjusted to a density of 1 × 10⁶ cells/well and incubated at 37°C for 48 hours in 1% fetal bovine serum (FBS)-containing MCDB-131 (Gibco, Grand Island, NY). After washing twice with the medium, HUVECs were incubated in the presence of recombinant human tumor necrosis factor-α (TNF-α; 5 ng/ml) (R&D, Minneapolis, MN). Furthermore, HGF (3–30 ng/ml) or vehicle (0.1% bovine serum albumin (BSA)) was added into the HUVEC model 30-minute before adding TNF-α. The HUVECs were washed with medium 3 hour after adding TNF-α and used as an endothelial monolayer. Neutrophils were isolated from human peripheral blood with Ficoll-Conray gradients (P = 1.077 g/ml), with a >93% purity on Giemsa’s staining. This neutrophil suspension was adjusted at 2 × 10⁷ cells/well, placed onto the HUVEC monolayer, and kept for 30 minutes under static conditions to induce cell attachment. Each culture well was gently washed twice with medium to remove nonadherent cells, and then attached neutrophils were quantified using a MPO assay.

ICAM-1 and Active NF-κB Detections

For the immunoblot of ICAM-1, HUVEC lysates were obtained from whole cells (3 × 10⁶ cells) and collected into lysis buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 10 mmol/L EDTA, and 0.5%-Triton X-100, pH 7.4). The lysates were subjected to SDS-PAGE, electrophoresis, and blotting procedures. Anti-human ICAM-1 mouse IgG (1:500, Sigma, St. Louis, MO) was applied on PVDF membranes as mentioned. After reaction with peroxidase-conjugated anti-mouse IgG (DAKO), immunoreactive signals were visualized on the PVDF membranes, using an ECL kit (Amersham Pharmacia, Little Chalfont, UK). Endothelial ICAM-1 and NF-κB were immunocytochemically detected after incubation for 3 hours in the presence of TNF-α or HGF plus TNF-α. These cells were fixed on a glass disk in cold 70% ethanol for 30 minutes, then subjected to the ABC technique, using a primary antibody against NF-κB or against ICAM-1, as mentioned. On the other hand, we obtained the nuclear extraction from remaining HUVEC cell suspension with a slow centrifugation and checked the nuclear NF-κB levels on ECL immunoblots. Finally, NF-κB activation was quantified using the TransAM NF-κB kit (Active Motif, Rixensart, Belgium), as reported previously. Briefly, whole-cell extracts were prepared from 5 × 10⁶ HUVECs, and protein concentrations of cell extracts were determined using a kit (DC protein assay; Bio-Rad, Hercules, CA). Ten micrograms per well of cell extracts was incubated in a 96-well plate on which have been immobilized double-strand oligonucleotides containing the sequence NF-κB DNA-binding site (5’-GGGACCTTCC-3’). The primary antibody used to detect NF-κB recognized an epitope on p65 subunit that is accessible only when NF-κB is activated and bound to its target DNA. After incubation with a peroxidase-conjugated secondary antibody and the developing solution, absorbance was read at 450 nm, according to a manufacturer’s instruction.

Statistical Analyses

All data were expressed as mean ± SD. A Student’s t-test or analysis of variance test for parametric values and Mann-Whitney U-test for non-parametric values were used to compare group means, with P < 0.05 accepted as significant.

Results

Changes in HGF Levels during Progressions of Neutrophil Infiltration and Tubular Apoptosis after Renal I/R in Mice

To determine changes in endogenous HGF levels during a process of postischemic renal injuries, we prepared a murine model of renal I/R, according to a previous report. In our model, BUN levels rapidly increased within 24 hours (0 hour, 21.2 ± 2.28 mg/dl; 6 hours, 56.3 ± 7.13 mg/dl; 24 hours, 108.1 ± 6.23 mg/dl). In the renal tissues, infiltration of neutrophils (ie, Gr1-positive round cells) was noted around cortico-medullar areas especially from 3 hours after the I/R treatment (Figure 1A). Being in parallel with the enhanced neutrophil infiltration, tubular epithelial apoptosis (as evidenced by TUNEL staining) became severer, concomitantly with tubular dilatation and epithe-
expression is in part dependent on ligand HGF, as reported previously. Under such HGF-neutralized conditions, there were significant increases in BUN and plasma creatinine levels at 8 hours postischemia in the HGF-neutralized mice (Figure 2B). Furthermore, anti-HGF IgG significantly enhanced tubular apoptosis at 8 hours after the renal I/R treatment (Figure 2C). The tubular TUNEL score of the anti-HGF IgG group increased to a twofold level of the placebo control group (P < 0.05). Consistently, tubular atrophic changes (such as tubular dilatation, lumen casts, and epithelial desquamation) were severer in the HGF-neutralized mice than in the control mice (data not shown). Of importance, anti-HGF IgG treatment led to a significant increase in the number of infiltrated neutrophils (ie, Gr1-positive interstitial cells) (Figure 2D), especially around renal cortico-medullar regions.

**c-Met Levels and Its Localization in the Ischemic Kidneys**

Because endogenous HGF is now critical to protect kidneys from ischemia, it is important to determine HGF-targeted cells in vivo. Therefore, we checked expression and localization of c-Met during the course of ischemic acute renal failure in this murine model. Western blot analysis revealed gradual increases in c-Met levels during the present experimental periods, whereas β-actin expression remained unchanged (Figure 3A). c-Met expression levels increased especially between 3 and 18 hours after renal I/R to more than twofold levels of a pretreatment basal level. Using an antibody specific to phosphorylated c-Met, we next explored the site of c-Met activation. In normal kidneys (without ischemia), phosphorylated c-Met was not detected, but the HGF receptor was activated especially on interstitial vascular endothelial cells (and in part, tubular epithelial cells) at 1 hour after renal I/R treatment (Figure 3B). In contrast, significant signals were not seen at any time points in normal IgG-applied sections (Figure 3B, insets). In renal tissues at 3 hour after the renal I/R, c-Met phosphorylation became faint, but tubular c-Met phosphorylation was in part evident, especially at 18 hours after renal ischemia (Figure 3B, arrowheads). Together, our results mean that renal c-Met levels increase under local ischemia, but activation of c-Met appears predominant on renal endothelial cells, probably due to the increased plasma HGF level (<1 hour).

**Inhibitory Effects of HGF on Neutrophil Recruitments by Endothelium in Vitro**

We now have evidence that endogenous HGF targets vascular endothelium, whereas anti-HGF IgG treatment increased the neutrophil extravasation. Thus, we determined the effect of HGF on attachment of peripheral blood neutrophils to endothelial cells, using an in vitro model. As a result, TNF-α alone increased the neutrophil adhesion to a 2.8-fold of the BSA level, whereas
HGF dose-dependently suppressed the TNF-α-mediated increase in neutrophil attachment levels (Figure 4A). We next examined the effect of HGF on ICAM expression, a critical molecule for developing neutrophil-to-endothelium adhesion. Of interest, HGF (30 ng/ml) counteracted the TNF-α-mediated endothelial ICAM-1 induction, as evidenced by Western blotting analysis or immunocytochemistry (Figure 4B). In this culture system, HGF alone had little effect on the ICAM-1 induction.

We finally checked effects of HGF on the nuclear localization of NF-κB, a key transcription factor for ICAM expression. In immunoblot analysis of the nuclear extraction of HUVECs, the NF-κB expression levels markedly increased at 3 hours after adding TNF-α (Figure 4C, top). When HGF was added into the HUVEC model 30 minute before adding TNF-α, nuclear NF-κB expression levels were suppressed to 31% of the level, as noted in stimulation with TNF-α alone. Likewise, nuclear NF-κB was immunocytochemically detected in HUVEC at 3 hour after adding TNF-α, whereas pretreatment of HUVEC with HGF (30 ng/ml) repressed the TNF-α-induced nuclear localization of NF-κB (Figure 4C, middle): NF-κB-positive nuclei were much fewer in the HGF+TNF-α group than in the TNF-α group (P < 0.05). Furthermore, DNA-binding ELISA supported the inhibitory effects of HGF on endothelial NF-κB activation: HGF (30 ng/ml) reduced the NF-κB-to-DNA binding by 30% of the level when HUVECs were stimulated with TNF-α alone (Figure 4C, bottom).

**HGF-MediatedSuppressions of EndothelialInjuries in the Mice after Renal I/R**

We predict that insufficient increases in plasma HGF levels may fail to completely block I/R-related renal injury. To test our hypothesis, rh-HGF was administrated into the murine model of renal I/R, based on an experimental protocol (Figure 5A). During the injection periods, plasma human HGF was detected in rh-HGF-treated mice at ranges of 3 to 20 ng/ml (Figure 5B), which were shown to be effective in vitro. In contrast, no human HGF was detectable in plasma at any time points in the saline group. Under such rh-HGF-supplemented states, c-Met phosphorylation in vascular endothelium (and partially in tubular cells) was still evident at least 3 hours after the renal I/R treatment (Figure 6A). In the kidneys, NF-κB was detected in the nucleus of the CD31-positive endothelium, using anti-NF-κB p65 rabbit IgG (Figure 6A, bottom inset). In contrast, no signals were detected in the negative control, where the tissue was stained with anti-CD31.
mouse IgG and normal rabbit IgG (Figure 6A, top inset). Of note, such NF-κB-positive signals were much fewer in the endothelium of rh-HGF-treated mice (Figure 6A). In the saline group, ICAM-1 expression of endothelial cells became evident at 3 hours of postischemia, along with the increase in endothelial NF-κB signals (Figure 6B). In contrast, there was a significant suppression of endothelial ICAM-1 expression in the rh-HGF-treated group (vascular ICAM-1 score: saline 0.99 ± 0.37 versus HGF 0.16 ± 0.07, P < 0.05).

We next asked whether supplemented HGF alters endothelial cell survival under I/R-related stresses: TUNEL-positive signals were occasionally evident in CD31-positive endothelial cells at 3 hours after surgical I/R treatment, whereas rh-HGF significantly reduced the numbers of apoptotic endothelium (Figure 6C). There were peri-vascular edematous changes in renal tissues, characterized by swelling lesions, with the inflammatory cell accumulation (Figure 6D, arrowhead). In contrast, the peri-vascular edema became milder in kidneys after rh-HGF supplement therapy (Figure 6D). Actually, vascular edema score was decreased by rh-HGF administrations to below a half-level of the saline-injected I/R group (P < 0.05).

Inhibitory Effect of HGF on Neutrophil Infiltrations, Tubular Apoptosis, and Renal Dysfunction in Ischemic Mouse Kidneys

We finally determined whether HGF-induced endothelial protection would alter the pathological events after renal I/R treatment. In the saline group, interstitial neutrophil infiltration was moderate at 3 hours after the renal I/R, whereas rh-HGF treatment reduced the number of infiltrated neutrophils (Figure 7A). Likewise, MPO activities in the ischemic kidneys were lowered in the HGF group to 50 and 36% levels in the control group at 3 and 18 hours after the I/R, respectively. Concomitantly with the suppressed neutrophil infiltration, there was a significant decrease in apoptotic tubules in the rh-HGF group (Figure 7B). Overall, rh-HGF supplementation inhibited increases in BUN levels, as noted in the saline-injected I/R mice. Furthermore, plasma creatinine levels were significantly lower in the rh-HGF-treated mice than in the saline-injected mice (18 hours, 0.62 ± 0.18 mg/dl versus 1.28 ± 0.13 mg/dl, P < 0.01), hence the inhibitory effect of HGF on onset of acute renal failure was demonstrated.

Discussion

Neutrophils mobilized from peripheral blood provide a common mechanism whereby not only endothelial but also parenchymal injuries progress under ischemic states. On the other hand, it is well-known that HGF protects numerous organs (including kidneys) from I/R. Based on these backgrounds, we predicted that HGF may suppress the common mechanism underlying I/R-related injuries. Using cultures and animal models of renal I/R, we showed that HGF targets vascular endothelium and maintains endothelial homeostasis, leading to avoidance of postischemic acute renal failure. This is the first report revealing a compensatory system to inhibit extravasation of neutrophils, a mediator for tissue inflammation. We discuss sequential events involved in HGF-mediated beneficial effects to antagonize postischemic renal damage.
Several reports described that renal endothelial injuries and dysfunction cause postischemic renal failure via recruitment of neutrophils. Thus, we focused on the effects of HGF in vascular pathogenesis in our mouse model of renal I/R: when endothelial c-Met activation was abolished by anti-HGF IgG, there were rapid progressions of neutrophil infiltration, tubular injuries, and acute renal failure; whereas vice versa in cases of rh-HGF administration. In this process, vascular lesions (such as apoptosis and edema) became evident in the natural course of renal I/R, whereas HGF suppressed these endothelial injuries, accompanied with an attenuated neutrophil infiltration. Because neutrophils per se are cytotoxic toward endothelial cells, HGF-mediated inhibition of neutrophil recruitment can be of central importance in suppressed endothelial injuries. In addition, HGF is capable of blocking hypoxia-induced apoptosis in the endothelium. Thus, such direct and indirect effects by HGF may provide protective mechanisms for the endothelium.

Induction of adhesion molecules on endothelial surfaces is the initial step for neutrophil recruitment under ischemia. In particular, ICAM-1 is essential for onsets of postischemic renal injuries. Thus, we focused on the effects of HGF in vascular pathogenesis in our mouse model of renal I/R: when endothelial c-Met activation was abolished by anti-HGF IgG, there were rapid progressions of neutrophil infiltration, tubular injuries, and acute renal failure; whereas vice versa in cases of rh-HGF administration. In this process, vascular lesions (such as apoptosis and edema) became evident in the natural course of renal I/R, whereas HGF suppressed these endothelial injuries, accompanied with an attenuated neutrophil infiltration. Because neutrophils per se are cytotoxic toward endothelial cells, HGF-mediated inhibition of neutrophil recruitment can be of central importance in suppressed endothelial injuries. In addition, HGF is capable of blocking hypoxia-induced apoptosis in the endothelium. Thus, such direct and indirect effects by HGF may provide protective mechanisms for the endothelium.

**Figure 4.** Inhibitory effect of HGF on neutrophil adhesion and ICAM-1/nuclear NF-κB location *in vitro*. A: HGF-mediated suppressions of adhesions between HUVEC and peripheral blood-derived neutrophils. HUVECs were incubated for 3 hours with TNF-α (5 ng/ml). HGF (0–30 ng/ml) or vehicle (BSA) was pulsed into the HUVEC culture 30 minutes before adding TNF-α. After washing the cells with medium, neutrophils were transferred onto the HUVEC cell layer. The co-culture was kept for an additional 30 minutes, and then neutrophil adhesion was quantified using a MPO assay. Immunoblot analysis of ICAM-1 in whole-HUVEC cell lysates (top), which were harvested 3 hours after adding TNF-α (5 ng/ml), HGF (30 ng/ml) plus TNF-α, or HGF. The remaining HUVECs were fixed on a glass disk with 70% ethanol and then stained using an anti-ICAM-1 IgG (bottom). C: Inhibitory effects of HGF on TNF-α-induced NF-κB activation. Top: Immunoblot analysis of NF-κB (p65) in the nuclear extraction of HUVEC, incubated for 3 hours. Middle: Changes in nuclear location of NF-κB, as revealed by immunocytochemistry. The positive percentage of NF-κB-stained nucleoli were counted in >1000 cells per well (n = 6). Bottom: DNA-binding ELISA for detecting activated NF-κB in the whole-cell lysates of HUVECs. The culture condition was the same as in Figure 4A. Data were shown as means ± SD (n = 6). **P < 0.01 versus BSA; #P < 0.05 versus TNF-α alone.

**Figure 5.** Changes in plasma human HGF levels during administrations of rh-HGF to mice after renal ischemia. A: An experimental protocol of rh-HGF supplement therapy in the mouse model of posts ischemic renal failure. Rh-HGF (or saline) was subcutaneously injected into mice immediately after renal I/R, and s.c. injections were repeated at an interval of 6 hours. The mice were killed at 3 and 18 hours to determine the effect of HGF on posts ischemic renal damages. B: Time course changes in plasma human HGF levels at 1, 3, and 18 hours after start of rh-HGF supplement therapy. The plasma was obtained from postorbital veins, and human HGF levels were determined using a human HGF-specific ELISA kit. S, saline; H, rh-HGF; n.d., not detectable.
inhibition of NF-κB activation by HGF underlies the major mechanisms of suppressed neutrophil extravasation in vivo. Given that inhibition of NF-κB activation or of adhesion and rolling molecules can be therapeutic toward postischemic renal injuries, HGF can serve as a physiological drug to enhance endothelial defense lines against neutrophil attack.

It is important to discuss a cause-and-result relationship between neutrophils and tubular destruction. The neutrophils can enhance tubular injuries via releasing oxidant products or elastase-like proteinases. In mice with renal I/R injuries, HGF inhibited the neutrophil infiltration per se, hence this is the first step by which HGF protects tubular cells from I/R-related challenges. On the other hand, HGF has a direct protective effect on tubular epithelium even under hypoxic states. Given that there is a hypoxia-related mechanism in the pathogenesis of renal tubular injuries, HGF appears effective for inhibiting not only neutrophil-mediated but also hypoxia-mediated tubular injuries. Actually, renal hypoxia increased tubular nuclear NF-κB and inflammation, whereas c-Met phosphorylation of tubular cells by HGF led to attenuation of tubular NF-κB expression in vivo (not shown). Of note, tubular apoptosis also elicits or aggravates interstitial neutrophil accumulation. Therefore, we cannot exclude a possibility that anti-apoptotic effect of HGF on tubular epithelium may be in part involved in attenuating tubular death-promoted inflammation. Taken together, we would like to emphasize that HGF is a critical ligand to inhibit renal inflammation (including neutrophil outflux and endothelial injuries), caused by hypoxic and oxidant stresses.

There is now ample evidence that HGF production level rapidly increases during organ injuries and then HGF is delivered to injured sites via endocrine and/or paracrine pathways. In the rodent model of renal I/R, HGF mRNA levels rapidly increased in intact organs (such as liver and lung), whereas renal HGF production (noted in macrophages and endothelial cells) is delayed and insufficient, probably due to local hypoxia. Thus, such an endocrine mechanism seems adaptable to overcome impaired HGF production in hypoxic kidneys. In contrast, local c-Met levels increased after renal I/R, being in parallel with changes of ischemic injuries. With regard to this, possible explanations include: 1) ligand HGF is capable of up-
regulating c-Met gene transcription; and 2) hypoxia itself enhances c-Met expression and activation, possibly both of which lead to phosphorylation of c-Met, specific in hypoxic kidneys. Such effects of hypoxia on HGF production (ie, insufficient supply) versus c-Met expression (ie, up-regulation) provide a rationale as to why HGF supplementation is effective to inhibit pathological changes after renal I/R. Circulating HGF, released from distant organs, could initially target endothelial cells, a front face of blood flow. Thus, injections of rh-HGF (s.c., intramuscular, and intraperitoneal injections) capable of elevating blood HGF levels seem physiological to mimic and enhance the endocrine mechanisms for delivering HGF.

So far, growth factor therapy has been attempted to inhibit postischemic renal disorders. For example, insulin-like growth factor-1 is considered to prevent I/R-related renal diseases, but insulin-like growth factor-1 worsened renal injuries probably via enhancement of neutrophil infiltrations. Vascular endothelial growth factor is an angiogenic ligand that is up-regulated under ischemic states, but vascular endothelial growth factor may enhance vascular permeability and edema formation. In contrast, HGF has numerous merits such as protective, regenerative, and anti-fibrotic effects in nephron tissues. HGF has anti-hypoxic and anti-oxidant functions. Although combined therapy targeting more than one pathophysiological pathway may prove to be the most beneficial approach, HGF monotherapy may mimic the combined strategy. Inhibition of initial tubular injuries by HGF results in a long-term maintenance of renal allografts, hence suggesting a potential use of HGF for example in cases of renal transplantation.

Overall, we conclude that HGF/c-Met axis on renal endothelium can function as a front-line defense switch to block vascular inflammatory cascades. Actually, prevention of neutrophil extravasation by HGF is noted not only in kidneys but also in hearts and lungs after ischemia (unpublished data). Together, investigation into the HGF-mediated protective mechanisms after organ ischemia may provide more insight into the physiological adaptation system of ischemic diseases and reveal additional as well as novel therapeutic interventions.

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References


