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 κ B 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)

cially in ischemia/reperfusion (I/R) injuries, circulating neutrophils selectively infiltrate ischemic tissues and contribute to tissue destruction.^{2–4} During the progression of I/R injuries, endothelial barrier is impaired within a few hours by hypoxia or neutrophil-released toxic products.^{1,4,5} 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.^{1,4} 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.⁶ Especially in renal transplantation, initial I/R injury affects long-term outcome of renal graft survival.⁷ In I/R-undergone kidneys, tubular epithelial apoptosis participates in nephron destruction and renal dysfunction.^{8,9} In this process, infiltrated neutrophils (associated with endothelial injuries) play an important role in nephron destructions.^{4,10} In other words, inhibition of neutrophil extravasation can be a target for abrogating tubular injuries and dysfunction.^{11,12} 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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Neutrophil-endothelial interactions are the initial event involved in the onset of inflammatory diseases.¹ Espe-

Hepatocyte growth factor (HGF), originally identified and cloned as a mitogen for mature hepatocytes,^{13,14} has mitogenic, motogenic, and morphogenic activities in various cell types via c-Met/HGF receptor phosphorylation.^{15–17} In renal tissues, HGF is protective and regenerative toward tubular epithelial cells (as well as glomerular cells).^{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.^{18–20} HGF is also cytoprotective for vascular endothelial cells.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,^{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 preventions 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; Slc, Hamamatsu, Japan) were anesthetized with ketamine chloride (80 mg/kg, subcutaneous (s.c.)) and xylazine 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.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.^{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 μ g/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.^{18,22,23} Immediately after the release of warm ischemia, mice were given saline or rh-HGF (500 μ g/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.^{22,23} Tissue sections were cut at a thickness of 4 μ m, 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 (Apo-Tag; Intergen, Purchase, NY).²³ Furthermore, c-Met activation was evaluated on the kidneys, using rabbit IgG against phosphospecific c-Met (1:400) (pMet-1234/1235; Biosource, Camarillo, CA). To evaluate the endothelial inflammation, nuclear factor κ B (NF- κ B) and ICAM-1 were detected using anti-NF- κ B (p65 subunit) rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, 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- κ B-stained or TUNEL-stained sections, endothelial cells were identified using an anti-mouse-CD31 rat IgG (BD Biosciences) and an alkaline phosphatase-conjugated anti-rat IgG rabbit IgG (Vector). 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- κ B, 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 $\times 200$ 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 $\times 200$ fields. Immunoreactive signals for endothelial ICAM-1 were observed in >20 randomly chosen $\times 200$ fields and scored on the scale of 0, no staining; 0.5, trace staining; 1, light staining; 2, moderate staining; and 3, intense staining.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 $\times 200$ fields: 0, absent; 1, mild; 2, moderate; and 3, severe. Furthermore, vascular apoptosis was checked in >20 chosen $\times 200$ 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 Jeffe's method, respectively.^{22,23} The renal extract was obtained as reported^{22,23}, and c-Met expression and phosphorylation were detected as follows:²⁵ 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.^{22,23} Furthermore, the human HGF level was monitored during rh-HGF administrations, using a human HGF-specific ELISA.²⁵ To quantify the degree of infiltrated neutrophils, myeloperoxidase (MPO) activity before and after renal I/R was determined, as described previously.²⁶

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,²⁷ with slight modifications. Umbilical vein endothelial cells (HUVECs) (Japan Health Sciences, Tokyo, Japan) were adjusted to a density of 1×10^4 cells/well and incubated at 37°C for 48 hours in 0.1% 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),²⁷ with a >93% purity on Giemsa's staining. This neutrophil suspension was adjusted at 2×10^5 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.²⁷

ICAM-1 and Active NF- κ B Detections

For the immunoblot of ICAM-1, HUVEC lysates were obtained from whole cells (3×10^5 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 ly-

sates were subjected to SDS-PAGE, electrotransfer, 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,²² using an ECL kit (Amersham Pharmacia, Little Chalfont, UK). Endothelial ICAM-1 and NF- κ B 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- κ B 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- κ B levels on ECL immunoblots. Finally, NF- κ B activation was quantified using the TransAM NF- κ B kit (Active Motif, Rixensart, Belgium), as reported previously.²⁸ Briefly, whole-cell extracts were prepared from 5×10^6 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- κ B DNA-binding site (5'-GGGACTTTCC-3'). The primary antibody used to detect NF- κ B recognized an epitope on p65 subunit that is accessible only when NF- κ B 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 $\pm$ 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.²¹ 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-medullary 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-

Figure 1. Changes in HGF levels during progression of neutrophil infiltration and tubular apoptosis in mice after renal I/R. **A:** Changes in the number of neutrophils (identified as Gr1-positive interstitial cells per $\times 200$ field), noted in interstitial spaces of I/R-undergone kidneys (original magnification, $\times 330$). The means of infiltrated neutrophils in each time point were calculated based on individual values ($n = 6$), which were determined on at least 20 fields per mouse. **B:** Alterations of tubular epithelial cell apoptosis (as determined by TUNEL staining) in ischemic kidneys ($\times 330$). **C:** Changes of plasma and renal HGF levels (as determined by a rodent HGF-specific ELISA system) after the release from warm ischemia. Statistical differences: * $P < 0.05$, ** $P < 0.01$ versus pre-ischemia group (ie, 0-hour). For abbreviations, see text.

lial desquamation (Figure 1B). In the murine model, plasma and renal HGF levels were biphasic with peaks 1 and 24 hours after renal I/R treatment (Figure 1C). The later peak correlated well with G₁/S progression in a cell cycle of the tubular cells (data not shown), indicating a renotropic role of HGF, as reported previously.²⁹ In contrast, it is still unknown whether initial HGF elevation may alter fate of postischemic renal diseases. Thus, we hypothesized that the initial increases in HGF levels may be involved in protecting nephrons against I/R-induced challenges.

Acceleration of Tubular Apoptosis and Neutrophil Infiltrations by anti-HGF IgG

To test our hypothesis, we injected an antibody specific to rodent HGF into mice immediately after renal I/R. In normal IgG-treated control mice, both the expression and phosphorylation of c-Met were evident at 1 hour after release from ischemia, whereas c-Met expression and activation were inhibited to below about 20% of basal levels by anti-HGF IgG treatment (Figure 2A). The loss of total c-Met expression levels with HGF neutralization may suggest that renal c-Met

expression is in part dependent on ligand HGF, as reported previously.³⁰ 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-medullary 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.²⁷ As a result, TNF- α alone increased the neutrophil adhesion to a 2.8-fold of the BSA level, whereas

Figure 2. Acceleration of renal injuries and dysfunction in ischemic mouse kidneys by an anti-HGF IgG. **A:** Suppression of c-Met protein expression and phosphorylation by an antibody specific for rodent HGF. **Left:** Renal tissues were collected 1 hour after the I/R treatment, and then renal extract was immunoprecipitated with an anti-c-Met IgG and probed on PVDF membranes with an anti-phosphospecific c-Met IgG, as reported previously.²⁵ **Right:** The band density was quantified using a densitometric analysis, with aid of computer software (NIH image). Data are expressed as mean $\pm$ SD ($n = 4$). ** $P < 0.01$ versus normal IgG. **B:** Effects of the HGF-neutralizing treatment on BUN and plasma creatinine (Cr) levels. Mice were treated with anti-HGF IgG immediately after the surgical treatment of renal I/R and killed 8-hour postischemia. Data are shown as mean $\pm$ SD ($n = 6$). * $P < 0.05$, ** $P < 0.01$ versus normal IgG group. **C:** Aggravating effects of anti-HGF IgG on tubular apoptosis in the renal I/R-undergone mice (8 hours postischemia). Representative findings of the apoptotic tubules in TUNEL-stained sections ($\times 160$). **D:** Acceleration of interstitial neutrophil infiltrations in the kidneys at 8 hours after HGF neutralization, as evidenced by counting Gr1-positive interstitial cells per $\times 200$ -field.

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.^{1,4} 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- κ B, a key transcription factor for ICAM expression. In immunoblot analysis of the nuclear extraction of HUVECs, the NF- κ B 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- κ B expression levels were suppressed to 31% of the level, as noted in stimulation with TNF- α alone. Likewise, nuclear NF- κ B 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- κ B (Figure 4C, middle): NF- κ B-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 endo-

thelial NF- κ B activation: HGF (30 ng/ml) reduced the NF- κ B-to-DNA binding by 30% of the level when HUVECs were stimulated with TNF- α alone (Figure 4C, bottom).

HGF-MediatedSuppressions of Endothelial Injuries 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- κ B was detected in the nucleus of the CD31-positive endothelium, using anti-NF- κ B 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

Figure 3. c-Met localization in mouse ischemic kidneys. **A:** Western blotting analysis for c-Met expression levels in kidneys after I/R. β -actin was detected as an internal control. Representative data were shown, and the density was quantified using a densitometric analysis, with aid of computer software (NIH image). **B:** Immunohistochemical findings of phosphorylated c-Met/HGF receptor in vascular endothelial cells in mice before and after renal I/R (original magnification: $\times 360$). Renal tissues were fixed in 70% ethanol and then subjected to a process for paraffin embedding. Anti-phosphorylated c-Met IgG (pMet-1234/1235) was applied on the dewaxed sections, followed by an ABC immuno-peroxidase method. **Insets:** Normal rabbit IgG was applied on renal tissues (1 and 18 hours postischemia) as a negative control. **Arrowheads:** Positive signals in tubules.

mouse IgG and normal rabbit IgG (Figure 6A, top inset). Of note, such NF- κ B-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- κ B 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.¹⁻⁴ On the other hand, it is well-known that HGF protects numerous organs (including kidneys) from I/R.^{19,29,31-33} 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.⁴ We discuss sequential events involved in HGF-mediated beneficial effects to antagonize postischemic renal damage.

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.²⁷ **B:** 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.

Several reports described that renal endothelial injuries and dysfunction cause postischemic renal failure via recruitment of neutrophils.^{34,35} 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 progres-

sions 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,³⁶ 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.³⁷ 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.^{1,4} In particular, ICAM-1 is essential for onsets of postischemic renal injuries.³⁸ In our culture model, HGF suppressed TNF-α-mediated endothelial ICAM-1 induction. HGF is also capable of suppressing *de novo* induction of E-selectin,³⁹ another key molecule for leukocyte-to-endothelial adhesion. In our HUVEC model, HGF suppressed activation of NF-κB, a transcription factor for up-regulating ICAM-1 and E-selectin,¹ suggesting that

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 postischemic acute 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 postischemic 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.²⁵ S, saline; H, rh-HGF; n.d., not detectable.

Figure 6. HGF-induced suppressions of endothelial nuclear NF-κB and ICAM-1 expressions, related to attenuated vascular injuries in mice. **A:** Immunohistochemical detections of phosphorylated c-Met and nuclear NF-κB in renal vessels of the ischemic mice. The kidneys were collected at 3 hours after renal I/R and then subjected to immunohistochemistry for phosphorylated c-Met as well as for NF-κB (brown signals) and CD-31 (red signals), respectively. **Arrowheads,** vascular endothelium. #, tubular epithelial areas. **Top inset:** The time-matched renal tissue was immunostained with normal rabbit IgG and anti-CD31 mouse IgG (ie, negative control). **Bottom inset:** Expanded finding of the NF-κB-positive nuclear staining. **B:** Changes in endothelial ICAM-1 scores in kidneys, as determined by histological scores.²⁴ Renal tissues were sampled before or 3 hours after renal I/R and then subjected to immunohistochemical procedures. Typical findings of endothelial ICAM-1 expression were shown. **Arrowheads,** endothelial surface. **C:** Suppression of endothelial apoptosis by rh-HGF in renal tissues, obtained at 3 hours after renal I/R. The kidneys were fixed in cold 70% ethanol, sectioned, and immunostained using an anti-mouse CD31 IgG. The CD31-stained tissue sections were refixed in 10% formalin and then subjected to TUNEL staining, as reported.²³ **D:** Changes in peri-vascular edematous lesions (H&E staining). **Arrowheads,** edematous lesions. #, inflammatory cell accumulation. All data are shown as means ± SD (*n* = 6). In these experiments, statistical differences are expressed as **P* < 0.05; ***P* < 0.01.

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,^{11,40,41} 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.⁴ 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.^{42,43} Given that there is a hypoxia-related mechanism in the pathogenesis of renal tubular injuries,⁴ 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.²⁶ 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.^{17,31,44} 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 cells²⁹) is delayed and insufficient, probably due to local hypoxia.⁴⁵ 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-

Figure 7. Preventive effects of rh-HGF supplement therapy toward neutrophil infiltration, tubular apoptosis, and renal dysfunction in mice that have undergone renal I/R. **A:** Changes in neutrophil accumulations in interstitial spaces of ischemic kidneys, treated with or without rh-HGF therapy (Gr1 staining, $\times 300$). Renal tissues were collected at 3 and 18 hour after the renal I/R challenge and then subjected to immunohistochemical procedures. Furthermore, granulocyte-specific MPO activities were measured in a part of samples used in immunohistochemistry. In this assay, the saline control level (3 hours postischemia) was defined as 100%. **B:** Decrease in tubular apoptosis levels and of BUN levels in the mouse model of renal I/R injury by rh-HGF treatment. Data are shown as means $\pm$ SD ($n = 6$). * $P < 0.05$; ** $P < 0.01$ compared with the saline-injected control group.

regulating c-Met gene transcription;³⁰ and 2) hypoxia itself enhances c-Met expression and activation,^{46,47} 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.⁴⁸ Vascular endothelial growth factor is an angiogenetic ligand that is up-regulated under ischemic states, but vascular endothelial growth factor may enhance vascular permeability and edema formation.⁴⁹ In contrast, HGF has numerous merits such as protective, regenerative, and anti-fibrotic effects in nephron tissues.^{18–20,23,29} HGF has anti-hypoxic and anti-oxidant functions.^{31,37,43} 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,⁵⁰ 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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