Hepatocyte Growth Factor Modulates Matrix Metalloproteinases and Plasminogen Activator/Plasmin Proteolytic Pathways in Progressive Renal Interstitial Fibrosis

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Abstract. Evidence suggests that hepatocyte growth factor (HGF) ameliorates renal fibrosis in animal models of chronic renal disease by promoting extracellular matrix catabolism. This study examined the molecular mechanisms of HGF-induced alterations in matrix degradation both in vitro and in vivo. In vitro, HGF increased the collagen catabolizing activity of human proximal tubular epithelial cells (HKC) that were treated with TGF-β1. Increased collagen catabolism was associated with enhanced activity of both matrix metalloproteinases (MMP) and plasminogen activators (PA)/plasmin proteolytic pathways. HGF abrogated TGF-β1–induced production of the profibrotic tissue inhibitor of metalloproteinase-2 (TIMP-2) and plasminogen activator inhibitor-1 (PAI-1). In addition, HGF induced the production of MMP-9. In vivo, continuous infusion of HGF in the rat remnant kidney model ameliorated renal fibrosis and tubulointerstitial collagen deposition. This was associated with increased tubular expression of MMP-9, enhanced in situ gelatinolytic activity, partially restored plasmin activity and decreased expression of TIMP-2 and PAI-1 in tubular cells, and upregulation of renal TIMP-3 expression. Conversely, blocking of endogenous HGF by an anti-HGF neutralizing antibody increased renal fibrosis and interstitial collagen. This was accompanied by decreased tubular expression of MMP-9, less in situ proteolytic activity, and elevated expression of TIMP-2 and PAI-1 in tubular cells. Collectively, these findings demonstrate that HGF ameliorates renal fibrosis by enhancing extracellular matrix catabolism via both MMP and the PA/plasmin proteolytic pathways.

Renal fibrosis, including glomerulosclerosis and tubulointerstitial fibrosis, is the final common pathway for diverse chronic renal diseases (1). Clinical observations and experimental data suggest that tubulointerstitial injury is the histopathologic parameter that correlates best with renal functional impairment (1–3). As renal disease progresses, there is inexorable accumulation of extracellular matrix (ECM) (3,4), which must result from an imbalance between the rates of synthesis and degradation of matrix components (4,5). Among the many cytokines that are implicated in the regulation of these processes (5), TGF-β1 has been widely accepted as a key fibrogenic factor for the kidney (5). Evidence suggests that TGF-β1 activates renal fibroblasts (6), induces tubular atrophy (7), promotes the transdifferentiation of tubular epithelial cells to myofibroblasts (EMT) (8), and inhibits matrix degradation (9), leading ultimately to ECM accumulation and renal scarring. As yet, no intervention has been completely successfully in counteracting the harmful actions of TGF-β1 (10).

Hepatocyte growth factor (HGF) ameliorates renal fibrosis (11) in several models of chronic renal disease, including murine hereditary nephropathy (12), aristolochic acid nephropathy (13), diabetic nephropathy (14,15), cyclosporin A nephropathy (16), unilateral ureteral obstruction (UUO) (17–19), and remnant kidney (20,21). A complex interaction between HGF and TGF-β1 in which HGF antagonizes the fibrogenic effects of TGF-β1 has been proposed (21–23); however, the molecular mechanism(s) for HGF’s antifibrotic effect are still incompletely understood. One possibility is that TGF-β1 decreases matrix catabolism (24) and that HGF might modulate this effect.

ECM is mainly degraded via two distinct pathways (24): the matrix metalloproteinases (MMP) degrading pathway (25) and the plasminogen activators (PA)/plasmin proteolytic axis (26). Both pathways have specific inhibitors: tissue inhibitors of metalloproteinases (TIMP) and plasminogen activator inhibitors (PAI), respectively. In the present study, we investigated the effect of HGF on the matrix collagen catabolism in human tubular epithelial cells exposed to TGF-β1. Tubulointerstitial depositions of collagen as well as alterations in matrix regulating molecules were also examined in remnant kidneys from rats that were given HGF or an anti-HGF neutralizing antibody.

Materials and Methods
Cell Culture
Human proximal tubular epithelial cells (HKC-8), provided by Dr. L.C. Racusen (John Hopkins University, Baltimore, MD), were cul-
tured in Dulbecco’s modified Eagle’s medium (DMEM/F12 supplemented with 5% FBS (Life Technologies, Grand Island, NY). HKC were plated at approximately 60% confluence for 24 h and then subjected to serum starvation for another 24 h. Human recombinant HGF (Genentech, South San Francisco, CA) and recombinant TGF-β1 (Sigma, St. Louis, MO) were added with fresh serum-free medium at concentrations of 20 and 2 ng/ml, respectively. Cells and conditioned media were harvested at the indicated time points.

Animal Experimental Design
Male Sprague-Dawley rats that weighed 180 to 210 g were purchased from Harlan Sprague-Dawley (Indianapolis, IN), housed in the Central Research Facilities of Rhode Island Hospital, and fed standard diet. Remnant kidneys were created as described (27). Two weeks later, rats were randomly assigned to one of the three groups (n = 6): (1) HGF rats were given recombinant human HGF (Genentech) as a continuous infusion into the jugular vein by micropump at a dose of 100 μg/kg per d for 7 d; (2) α-HGF rats received daily injections of a high-titer, polyclonal anti-HGF neutralizing antibody intravenously through tail vein for 7 d; or (3) remnant kidney rats received a daily intravenous injection of rabbit preimmune IgG dissolved in 0.9% saline solution. Sham-operated rats were used as controls. Three weeks after surgery, kidneys were perfused with iced saline and parts were fixed in 10% phosphate-buffered formalin, frozen for cryostat sectioning, or snap-frozen in liquid nitrogen and stored at −80°C.

Isotopic Labeling and Pulse Chase of Matrix Collagen in HKC Cells
For determining the degradation rates of matrix collagen, cells labeled with [3H]-proline were pulse chased. Briefly, HKC cells (4 × 10^5/well) grew on 12-well plates. Media were removed and replaced with DMEM/F12 supplemented with 50 μg/ml l-ascorbic acid phosphate (Sigma) and 20 μCi/ml [3H]-proline (Amersham Biosciences, Piscataway, NJ). After 24 h, cells were rinsed with prewarmed PBS and chased in DMEM/F12 media in the presence of HGF, TGF-β1, or plasminogen (5 μg/ml; Sigma) for 48 h. Then cells were washed with cold PBS and lysed in RIPA buffer (1% Nonidet P-40, 0.1% SDS, 100 μg/ml PMSF, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 2 μg/ml aprotin, 2 μg/ml leupeptin, 5 mM EDTA in PBS). Proteins were precipitated with cold 10% TCA, washed in cold acetone, and then solubilized and analyzed in a Beckman LS6500 scintillation counter.

Western Immunoblot Analysis and Immunoprecipitation
Tissue homogenates, cell lysates, and conditioned media were processed for SDS-PAGE and immunoblot as described previously (27). Antibodies against c-Met, u-PA receptor (u-PAR), and PAI-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and those for MMP-9, TIMP-1, and TIMP-2 were from Sigma.

Semiquantitative Reverse Transcriptase–PCR
Total RNA was extracted from approximately 2 × 10^6 HKC cells or 0.2 g frozen tissue. The first strand cDNA was prepared using Superscript RT reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA was amplified using primers (Table 1) for a number of cycles in the linear range as determined in preliminary experiments. Glyceraldehyde-3-phosphate dehydrogenase and ribosomal protein S26 (RPS26) were used as normalizing genes for HKC cells and kidneys. PCR products resolved in 1.5 to 2% agarose gels were photographed under ultraviolet light.

Gelatin and Casein Zymographic Analysis
Frozen tissues were lysed in RIPA buffer including a mixture of protease inhibitors except EDTA and other MMP inhibitors. Gelatinease activity of MMP in conditioned media from cultured HKC cells or in tissue lysates was detected by SDS-PAGE gelatin zymography as described (27).

For casein zymographic analysis of PA, remnant kidney lysates were obtained after tissue homogenization in RIPA buffer without PMSF. PA activity in tissue lysates, conditioned media, or cell lysate was detected as described (28).

### Table 1. Nucleotide sequences of the primers used for RT-PCR

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PA Plaque Assay

The PA plaque method of Vassalli et al. (29) was used to detect single-cell PA activity. HKC cells were seeded at low density. After different treatments for 24 h, cells were washed with PBS; an agarose overlay mixture (3 ml of DMEM/F12 containing 0.8% agarose, 2.0% Carnation nonfat dry milk, and 5 μg/ml plasminogen) was pipetted onto the cells and incubated at 37°C for 20 h. Cell-associated PA activity was estimated indirectly by plasmin-dependent caseinolytic activity, revealed by the area of clearing of the agarose overlay under darkfield illumination.

Plasmin Activity Assay

The activity of plasmin in the conditioned media or in tissue homogenates prepared for casein zymography was measured spectrophotometrically by incubation with a specific chromogenic substrate, H-D-Val-Leu-Lys-paranitroanilide (S-2251; Chromogenix, Milano, Italy), at 37°C for 30 min (30). The increase in absorbance per minute at 405 nm is proportional to the plasmin enzymatic activity as confirmed with standards containing plasmin (Chromogenix).

Fluorescence In Situ Gelatin Zymography

The net functional activity of MMP in the remnant kidney was assessed by in situ zymography based on the method of Galis et al. (31). Briefly, Oregon-green conjugated gelatin (1 mg/ml) was mixed (1:1) with 1% agarose in incubation buffer (50 mmol/L Tris-HCl [pH 7.5], 10 mmol/L CaCl₂, 5 mM PMSF, 0.05% Brij 35). A thin film of the mixture was spread on glass slides and allowed to gel at 4°C. Frozen sections were mounted onto the gelatin-coated slides; incubated at 37°C in a light-protected, humidified chamber for 20 h; and then detected by fluorescence microscopy. Gelatinase activates the quenched fluorescence substrate, producing areas of fluorescence against a dark background. The specificity of the reaction was determined by incorporating 100 mM EDTA in the incubation buffer. Alternatively, PMSF was depleted from the system to test the contribution of serine proteases to gelatinolytic activity in vivo.

Morphology and Immunohistochemistry Studies

Formalin-fixed kidneys were embedded in paraffin and prepared in 3-μm-thick sections. Sections were stained with Masson-Trichrome to examine matrix collagen deposition. One observer performed morphologic scoring in a blinded manner. Immunoperoxidase staining was performed with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). As a negative control, the primary antibody was replaced by nonimmune serum from the same species. The extent of fibrosis was performed with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). As a negative control, the primary antibody was replaced by nonimmune serum from the same species. The extent of fibrosis was determined using a densitometer and an NIH image analysis program. All data are expressed as mean ± SD. Statistical analysis of the data from multiple groups was performed by one-way ANOVA followed by Student-Newman-Kuels test. Data from two groups were compared by t test. P < 0.05 was considered significant.

Results

HGF Reverses TGF-β1 Inhibition of Matrix Degradation through Non-Plasmin and Plasmin-Mediated Pathways

Shown in Figure 1, in the absence of growth factors, the accumulation of the labeled collagen was not significantly altered by the addition of plasminogen. Accumulation of radioactive collagen tended to decline in the presence of HGF alone, and significant degradation occurred in the presence of HGF and plasminogen (P < 0.05). TGF-β1 significantly retarded the degradation of the prelabeled collagen with and without plasminogen in the media (P < 0.05). TGF-β1-induced inhibition of degradation was overcome by HGF in the absence of plasminogen and was significantly (P < 0.05) abrogated by HGF in the presence of plasminogen.

Influence of HGF and TGF-β1 on MMP Proteolysis In Vitro

The MMP system accounts for the bulk of the non-plasmin-mediated proteolytic activity. Using gelatin zymography, we found that HKC constitutively secreted MMP-2, as evidenced by the 72-kD gelatinolytic band. TGF-β1 significantly increased the activity of MMP-2, whereas HGF had little effect (Figure 2A). Another gelatinolytic band (95 kD), MMP-9, was produced by HKC after HGF treatment for 48 h but was barely detectable in control cells. Although TGF-β1 seemed to have little effect on the activity of MMP-9, simultaneous stimulation with HGF considerably enhanced the gelatinolytic activity was determined using a densitometer and an NIH image analysis program. All data are expressed as mean ± SD. Statistical analysis of the data from multiple groups was performed by one-way ANOVA followed by Student-Newman-Kuels test. Data from two groups were compared by t test. P < 0.05 was considered significant.

Statistical Analyses

For immunoblot analysis, reverse transcriptase–PCR (RT-PCR), and zymography, bands were scanned and the integrated pixel density was determined using a densitometer and an NIH image analysis program. All data are expressed as mean ± SD. Statistical analysis of the data from multiple groups was performed by one-way ANOVA followed by Student-Newman-Kuels test. Data from two groups were compared by t test. P < 0.05 was considered significant.

Figure 1. Hepatocyte growth factor (HGF) attenuates TGF-β1 inhibition of collagen-degrading activity in HKC cells; enhanced effect in the presence of plasminogen. Values represent the mean ± SD of six individual experiments. *P < 0.05 versus other groups without plasminogen; †P < 0.05 versus other groups with plasminogen; ‡P < 0.05 versus cells that were treated with the same growth factors but without plasminogen.
The absolute activity of MMP in vivo depends on the balance between MMP and their physiologic inhibitors, TIMP (34,35). HKC cells constitutively expressed TIMP-2, and this was slightly downregulated by HGF. TGF-β1 strongly upregulated the production of TIMP-2. This effect was prominently blocked by co-incubation with HGF (Figure 2, B and C). Semiquantitative RT-PCR revealed that the mRNA level of TIMP-2 was modulated in a similar pattern (Figure 2D). At 24 h, HGF significantly deceased TIMP-3 expression in HKC cells, while TGF-β1 also moderately downregulated it. HGF and TGF-β1 in combination had an additive effect (Figure 2D). In contrast, neither HGF nor TGF-β1 significantly altered TIMP-1 expression (data not shown).

**HGF Promotes the PA/Plasmin Proteolytic Axis In Vitro**

The finding that collagen accumulation decreased after addition of plasminogen prompted us to investigate further the PA/plasmin proteolytic axis in HKC cells. Casein zymographic analysis revealed that HKC secreted an abundant amount of u-PA into the medium (Figure 3A). Cell-associated u-PA activity was considerably less than that in the medium. Neither HGF nor TGF-β1 decreased the activity of u-PA in the media; however, cell-associated u-PA activity was decreased by TGF-β1. Addition of HGF enhanced the activity of cell-associated u-PA, partially reversing the effects of TGF-β1 (Figure 3A).

Of note, in addition to the major u-PA band, a minor band was observed in the conditioned media that probably corresponds to a t-PA/PAI-1 complex (28). Both TGF-β1 and HGF upregulated t-PA activity, and simultaneous incubation with both growth factors produced an additive effect. The t-PA/PA1 band was not seen in the cell-associated samples (Figure 3A).

We used the PA plaque method to examine further the PA/plasmin pathway in single cells (Figure 3B, a). Addition of HGF markedly increased caseinolytic clearing (Figure 3B, b), and this was blocked by an anti-HGF neutralizing antibody (Figure 3B, c). Simultaneous addition of TGF-β1 with HGF failed to attenuate the HGF action (Figure 3B, d). In contrast,
TGF-β1 alone inhibited constitutive caseinolytic activity in HKC cells, suggesting that the in situ activity of PA was lowered (Figure 3B, e). Addition of an anti–TGF-β1 antibody restored activity to normal (Figure 3B, f).

To determine whether PAI-1 was involved in the modulation of caseinolytic activity, we analyzed the conditioned media by Western immunoblot. Shown in Figure 3, C and D, PAI-1 was nearly undetectable in conditioned media from control cells and from cells that were treated with HGF alone. TGF-β1 profoundly induced PAI-1 expression, and this effect was partially blocked by HGF (P < 0.05).

Because the activity of PA/plasmin pathway can be affected...
by regulation of u-PAR, we examined the influence of TGF-β1 and HGF on the u-PAR by Western immunoblot (Figure 3C). Although there was a slight decrease in u-PAR with TGF-β1 treatment (Figure 3D), the transcriptional data (Figure 3E) clearly demonstrate that the dominant effect of TGF-β1 is on PAI-1.

To examine further the PA/plasmin axis in HKC, we performed a direct activity assay for plasmin (Figure 3F), the final component of this pathway. In the culture system without plasminogen, plasmin activity in the conditioned media was low. Nevertheless, the suppressive effect of TGF-β1 was still markedly attenuated by HGF (P < 0.05). Addition of exogenous plasminogen increased plasmin activity by >10-fold. HGF significantly enhanced the plasmin activity (P < 0.01) and markedly blunted the inhibitory action of TGF-β1 (P < 0.01).

Antifibrotic Action of HGF in Remnant Kidney

To examine the effect of HGF on the matrix collagen accumulation in vivo, we infused HGF or an anti-HGF neutralizing antibody into rats with remnant kidneys. As previously reported (20,27), renal injury was ameliorated by administration of HGF (Figure 4D) and severely aggravated in anti-HGF antibody–treated rats (Figure 4C) as compared with controls (Figure 4B). Compared with kidneys from sham-operated rats (Figure 4A) and control remnant kidneys (Figure 4B), interstitial collagen deposition was increased in antibody-treated rats (Figure 4C) and dramatically lessened in HGF-treated animals (Figure 4D). A semiquantitative score for collagen deposition is presented in Figure 4E. The exact collagen content in the remnant kidney was significantly decreased in response to HGF (P < 0.05) and increased after HGF neutralization (P < 0.05; Figure 4F).

Alterations in the HGF/c-met Axis and TGF-β1/TβR Axis In Vivo

HGF might attenuate fibrosis by modulating the expression of TGF-β1 or its receptors (22,36). We examined this hypothesis by detecting the mRNA levels for both HGF/c-met and TGF-β1/TβR1 receptor (TβR1) axes in remnant kidneys by RT-PCR (Figure 5). Three weeks after renal ablation, TGF-β1 expression was significantly greater in remnant than in normal kidneys. After HGF infusion for 1 wk, HGF expression was modestly lowered, whereas the expression of c-met, TGF-β1, and TβR1 and TβR1 remained unchanged. Anti-HGF treatment downregulated the mRNA levels of c-met, HGF, and TβR1 but only slightly decreased TGF-β1 expression, which was still much greater than in normal kidney. Collectively, the antifibrotic effect of HGF seemed not to be primarily mediated via the downregulation of TGF-β1 or its receptors.

HGF Regulates MMP-9 Expression in Tubular Epithelial Cells In Vivo

Consistent with the in vitro studies, the antifibrotic action of HGF in remnant kidney was concordant with an increase in tubular expression of MMP-9 (Figure 6, A to E) primarily in a cytoplasmic distribution. In normal rat kidney, few epithelial cells expressed MMP-9 (Figure 6B). Surprising is that the expression of MMP-9 was upregulated in the remnant kidney (Figure 6C). Treatment with anti-HGF antibody reduced the expression of MMP-9 (Figure 6D) to normal. In contrast, HGF markedly upregulated MMP-9 expression (Figure 6E). Objective scoring of these immunohistochemistry patterns (Figure 6F), along with Western immunoblot analysis of tissue homogenates (Figure 6G) with the respective densitometric measurements (Figure 6H), confirmed that HGF enhanced MMP-9 expression in vivo. Gelatin zymography on kidney homogenates (Figure 6, I and J) demonstrated the predominant activity of MMP-9 in all groups. HGF infusion strongly increased MMP-9 activity (P < 0.05) and MMP-2 to a lesser extent. In contrast, HGF neutralization significantly decreased MMP-9 activity (P < 0.05) as compared with controls.

HGF Modulates TIMP in Remnant Kidney

Compared with normal kidneys, remnant control kidneys express significantly higher levels of TIMP-2 mainly in tubular cells (Figure 7, B and C), which might mitigate MMP activity. Immunohistochemistry (Figure 7, A to F) revealed that renal expression of TIMP-2, the most potent TIMP for gelatinases (34), was blunted by HGF and enhanced after HGF blocking. Furthermore, Western immunoblot analysis on kidney tissue homogenates (Figure 7G) and the corresponding densitometric measurements (Figure 7H) corroborated the immunohistochemistry findings. The expression of two other less potent TIMP, TIMP-1 and -3, was also examined by RT-PCR (Figure 7, I and J). Renal expression of TIMP-1 was markedly higher than normal in all remnant groups but was not affected by treatment. TIMP-3 was expressed in normal kidneys. Renal ablation modestly decreased TIMP-3 expression, and HGF blockade further enhanced this decline, whereas HGF supplementation restored it to almost normal levels.

HGF Promotes Net Activity of MMP-Mediated Proteolysis in Remnant Kidney

MMP may be activated during SDS-PAGE gelatin zymography (25), and TIMP may mitigate matrix degradation by MMP in vivo even if the zymogens are activated. Therefore, gelatin zymography on kidney homogenates may not accurately reveal the location or function of MMP (31). To examine more accurately MMP proteolysis, we used fluorescence in situ zymography. In normal kidney, MMP activity was moderate in the peritubular interstitium and very weak in tubular cells (Figure 8B). MMP activity declined in remnant kidneys (Figure 8D). HGF neutralization further reduced peritubular gelatinolytic activity (Figure 8E), whereas HGF administration enhanced it (Figure 8F). Of note, in situ gelatinolytic activity was enhanced in the absence of PMSF (Figure 8C), suggesting that serine proteases might contribute to collagen degradation.
Figure 4. HGF ameliorates, whereas blockade of the endogenous HGF worsens, interstitial collagen accumulation in remnant kidneys. (A to D) Representative pictures of Masson trichrome staining in kidney sections from the sham-operated group (NCtrl; A), the remnant kidney group (Rem; B), α-HGF antibody (C), and HGF group (D). (E) Semiquantitative collagen scores for each group (n = 6); *P < 0.01 versus Rem and α-HGF; #P < 0.01 versus all other groups. (F) Total collagen content in rat kidneys (n = 6) for all groups; *P < 0.05 versus Rem and α-HGF; #P < 0.05 versus all other groups. Magnification, ×100 (×200 for inserts).
HGF Modulates the PA/Plasmin Proteolytic Pathway in Remnant Kidney

To determine whether HGF modulates the PA/plasmin axis in remnant kidneys, we first examined the expression of PAI-1 (Figure 9, A to F). Similar to the findings in vitro, profibrotic PAI-1 expression was evident in tubular cells (Figure 9C) and increased further with the anti-HGF antibody (Figure 9D). HGF infusion produced a marked suppression of renal PAI-1 (Figure 9E) that coincided with the decline in interstitial matrix accumulation. Western immunoblot analysis of kidney tissue homogenates (Figure 9, G and H) corroborated the immunohistochemistry findings. Casein zymography of kidney homogenates showed that, regardless of the treatment, remnant kidneys had higher-than-normal activity of u-PA, which was similar in all groups (Figure 9, I and J). Conversely, the plasmin activity decreased in remnant kidney homogenates and after HGF blocking but markedly (P < 0.05) recovered after HGF infusion (Figure 9K). In addition, no significant changes in u-PAR expression was found in vivo by Western immunoblot analysis of whole-tissue homogenates (data not shown). Collectively, these data suggest that HGF stimulated the PA/plasmin pathway mainly by modulating PAI-1.

Discussion

Accumulation of extracellular matrix is the morphologic hallmark of progressive chronic renal disease leading to end-stage renal failure. Consistent with previous reports (12–21), the current study demonstrates that HGF retards ECM accumulation and ameliorates interstitial fibrosis in chronic renal disease. However, previously, the antifibrotic effect of HGF was attributed either to inhibition of apoptosis (13,17,18) or to blockade of EMT (12,16–20,36). Our data provide direct evidence for another mechanism: HGF specifically antagonizes the profibrotic effects of TGF-β1 and promotes matrix catabolism in cultured renal epithelial cells and in remnant kidneys. Of note, HGF treatment failed to prevent the rise in TGF-β1 expression in remnant kidneys, yet interstitial fibrosis was still attenuated. This suggests that HGF counteracts the profibrotic effects of TGF-β1 by modulating downstream pathways.

We examined the influence of HGF on MMP and PA/plasmin pathways in renal tissue remodeling. As reported previously (37,38), HKC cells constitutively express plasminogen as well as other components of PA/plasmin axis. However, plasmin activity was found to be very low in the absence of exogenous plasminogen (Figure 3F). This suggests that endogenous matrix degradation depends primarily on MMP, although u-PA–dependent effects may also be involved (38). HGF attenuates TGF-β1–retarded collagen catabolism, and this effect was enhanced in the presence of exogenous plasminogen, demonstrating that HGF provokes matrix degradation through dual pathways.

Regulation of the activity of MMP pathway is complex and occurs at several levels (25,27,39), including transcription and extracellular zymogen activation. Ultimately, net proteolytic activity depends on the final balance between active MMP and TIMP (3,25,39). Although TGF-β1 induced the expression and activity of MMP-2 in HKC cells, it also significantly increased production of TIMP-2, which overcame upregulation of MMP-2 and resulted in net inhibition of collagen degradation. When HGF was added to this system, MMP-2 expression was not suppressed, but TGF-β1–induced production of TIMP-2 was prevented. As a result, the net effect of combined treatment with HGF and TGF-β1 was antifibrotic. It is interesting that we did not find any alternation in the expression of the
ubiquitous but less potent TIMP-1 (34). This is consistent with findings in MDCK cells (40) but conflicts with a previous report (27) demonstrating inhibition of TIMP-1 production in HKC cells transfected to produce HGF. It seems likely that the marked, tonic overexpression of HGF in the transfected cells might have altered the cell phenotype and enhanced their invasive ability. Also of note and consistent with a previous report (40), both HGF and TGF-β1 suppressed TIMP-3 expression at 24 h and had an additive effect when combined.

The PA/plasmin proteolytic pathway also has multiple
points of regulation, including the main protease u-PA, the u-PAR, t-PA, and plasmin, as well as their specific inhibitors (26), the most important being PAI-1 (38,41). Consistent with previous reports (42), we found that TGF-β1 significantly upregulated the expression of PAI-1 in HKC cells and that HGF abolished this effect. It has also been reported that HGF increases expression of u-PA in MDCK (43); however, in the present study, u-PA expression in HKC cells was not altered by...
HGF or by TGF-β1. This apparent inconsistency might have resulted from the different cell types studied or from variation in the timing or dose of HGF. However, although u-PA expression was not affected, TGF-β1 still reduced cell-associated u-PA activity, and this was reversed by HGF, as demonstrated by both casein zymography and the PA plaque assay. In the absence of changes in expression, alterations in u-PA activity most likely result from variations in levels of the u-PAR and/or PAI-1, which may form a PAI-1–u-PA–u-PAR complex that is removed by endocytosis (26). This hypothesis was indirectly supported by slight downregulation of u-PAR in cells that were treated with TGF-β1, without a transcriptional change. Of note, we also found that HGF and TGF-β1 had additive effects in inducing t-PA activity. Most researchers have suggested that the major function of t-PA is to maintain fibrin homeostasis (26), and its role in matrix degradation is still largely unknown.

HGF is beneficial in several animal models of chronic renal disease (12–21,36); however, in most previous studies (13–18,20,21), HGF was administered beginning before or simultaneously with the onset of injury. These studies demonstrate that HGF can prevent injury but not necessarily ameliorate established renal disease. Therefore, the finding here that HGF suppresses injury even when treatment is delayed is noteworthy. To understand better its antifibrotic effects, we examined remnant kidney rats in which HGF activity was either augmented or blocked. Consistent with in vitro data, HGF infusion ameliorated collagen accumulation, decreased expression of the profibrotic molecules PAI-1 and TIMP-2, and increased expression of the MMP-9, generating an antifibrotic environment that was corroborated by fluorescence in situ zymography. Conversely, blocking endogenous HGF produced opposite changes in these molecules, a profibrotic milieu, and worsened injury.

Similar to observations in murine UUO (33), renal ablation suppressed TIMP-3 expression. HGF blocking enhanced this suppression, whereas HGF infusion restored TIMP-3 expression. Surprising is that the effects of HGF treatment on TIMP-3 expression in HKC cells were opposite. This apparent contradiction might be due to the discrepancies in responses in normal versus transformed cells. In addition, although TIMP-1 and -2 are soluble, TIMP-3 is found exclusively bound to the ECM (44), suggesting that its major role may be to prevent excessive matrix remodeling (45). Of note, decreased TIMP-3 expression has been associated with transition from an epithelial to fibroblast-like cell phenotype (40), suggesting that increased TIMP-3 expression after HGF might actually be beneficial, tending to block transdifferentiation. In contrast, TIMP-1 deficiency does not prevent renal injury in mice (33,46), and HGF was not associated with decrements in TIMP-1 expression in our rats.

A major finding in this study is that HGF upregulated expression of MMP-9 (Figure 6). Heightened MMP-9 expression correlated with enhanced proteolytic activity that was most evident in HGF-treated animals as demonstrated by zymography. It was suggested recently that increased activity of MMP can be detrimental, causing tubular base membrane

Figure 8. Representative pictures show that HGF infusion increases, whereas HGF antibody suppresses, the in situ gelatinolytic activity in remnant kidneys, mainly located in peritubular interstitium. Fluorescence in situ gelatin zymography was performed on the frozen kidney sections from NCtrl group (A to C), Rem group (D), α-HGF group (E), and HGF (F). (A) EDTA (100 mM) was added to verify the specificity of the method. (C) PMSF was depleted from the whole system to reveal the contribution of serine proteases to the gelatinolytic activity in rat kidney. Magnification, ×200.
damage, enhanced EMT, and interstitial fibrosis (47–49). However, the expression of TIMP and PAI-1 were not evaluated in these studies. Furthermore, increments in EMT were not observed in HGF-treated animals in either the murine UUO model (36) or our remnant kidney (50) model. Importantly, our MMP-9 data are in agreement with the findings of Kim et al. (33) demonstrating that downregulation of MMP-9 is associated with interstitial fibrosis.

In summary, HGF markedly attenuates TGF-β1–mediated inhibition of matrix collagen catabolism in renal tubular epi-

Figure 9. HGF stimulates the PA/plasmin proteolytic activity in remnant kidney via modulating the tubular expression of PAI-1. (A to E) Representative pictures of immunohistochemistry staining of PAI-1 (arrowheads and asterisks) in sections from NCtrl group (A and B), Rem group (C), α-HGF group (D), and HGF group (E). (A) Negative control. (F) Semiquantitative scores for PAI-1 for each group; *p < 0.01 versus Rem and α-HGF; #p < 0.01 versus all other groups. (G) Western immunoblot of PAI-1 on kidney homogenates and (H) arbitrary unit of the relative abundance of PAI-1 in immunoblot expressed as densitometric ratios of PAI-1/actin as folds of normal controls; *p < 0.05 versus Rem and α-HGF; #p < 0.01 versus all other groups. (I) Casein zymography on kidney homogenates (80 μg) reveals the bands for u-PA. (J) Arbitrary unit of the relative abundance of u-PA in casein zymography expressed as densitometric folds of normal control; *p < 0.05 versus Rem. (G and I) Representative pictures of experiments on three individual animals (1 to 3) randomly selected from each group. (H, J, and K) n = 6 in each group. Magnification, ×400 in A to E.
thelial cells and, similarly, prevents collagen accumulation and renal injury in remnant kidneys. HGF antagonizes the profibrotic effects of TGF-β1 by increasing the activity of both the MMP and PA/plasmin proteolytic pathways that account for the bulk of ECM degradation.

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