Hepatocyte Growth Factor Ameliorates Renal Interstitial Inflammation in Rat Remnant Kidney by Modulating Tubular Expression of Macrophage Chemoattractant Protein-1 and RANTES

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Abstract. Hepatocyte growth factor (HGF) has been shown to reduce renal injury in a variety of animal models of chronic renal disease. Suggested mechanisms to explain this action include prevention of tubular cell apoptosis, blocking epithelial-to-mesenchymal transition, and promotion of extracellular matrix degradation. Inflammation is another common finding in kidneys that progress to end-stage renal failure; however, the effect of HGF on inflammation has hardly been investigated.

For examining this issue, beginning 2 wk after subtotal nephrectomy, rats received a continuous infusion of recombinant HGF, neutralization of endogenous HGF by daily injection of an anti-HGF antibody, or preimmune IgG for an additional 2 wk. HGF infusion halted the progression of proteinuria and decreased renal collagen accumulation. Renal inflammation in both glomeruli and tubulointerstitium was significantly attenuated, associated with reductions in the tubular expression of the chemokines macrophage chemoattractant protein-1 (MCP-1) and RANTES (regulated upon expression normal T cell expressed and secreted). In contrast, HGF neutralization worsened renal fibrosis, aggravated renal inflammation, and enhanced tubular expression of MCP-1 and RANTES. In vitro, HGF suppressed basal and TNF-α–induced expression of these chemokines at both the mRNA and protein levels in a time- and dose-dependent manner in proximal tubular epithelial cells. HGF also blunted TNF-α–induced nuclear translocation and activation of NF-κB, a pivotal transcription factor that regulates chemokine expression. Immunohistochemistry showed that activated NF-κB was evident in tubules in remnant kidneys and increased remarkably with anti-HGF treatment. HGF infusion markedly suppressed expression of activated NF-κB in remnant kidneys. These findings suggest that the beneficial effect of HGF in chronic renal disease is attributable, at least in part, to a direct anti-inflammatory action, likely via NF-κB, on tubular epithelial cells.

Influx of inflammatory cells into interstitium is an invariable pathologic finding in almost all forms of chronic renal disease regardless of the original cause (1,2). Infiltrating cells promote renal scarring and loss of function by several mechanisms. They secrete extracellular matrix components (3,4) that directly contribute to matrix accumulation. Leukocytes generate radical oxygen species (5), lipid mediators, and proinflammatory cytokines (6) that promote tissue injury. Mononuclear cells, including lymphocytes and macrophages, are important sources of profibrotic molecules such as TGF-β1, basic fibroblast growth factor, and PDGF (6). These factors in turn activate resident fibroblasts (7,8) and promote epithelial-to-mesenchymal transition (9). Heterogeneous fibroblasts proliferate and generate excessive extracellular matrix, resulting in renal scarring. Consistently, the extent of inflammation is well correlated with kidney function and predicts long-term prognosis in a number of clinical settings (10). Suppression of renal inflammation by administration of immunosuppressive drugs is beneficial even in nonimmune models of renal disease (11) such as remnant kidney (12) and diabetic nephropathy (13).

Inflammatory infiltrates in chronic renal disease are composed of heterogeneous cell types (14). Although some cells are derived from proliferation of resident macrophages (15), the majority enter the kidney from the circulation. Chemokines activate leukocytes and mediate selective leukocyte trafficking at multiple stages, attracting specific populations to the site of injury (16–18). In the kidney, tubular epithelial cells (TEC) are considered to be a prominent source of chemokines (19). In vitro, protein overload (20) as well as proinflammatory cytokines (19) such as IL-1, TNF-α, and interferon-γ, markedly induce TEC expression of macrophage chemoattractant protein-1 (MCP-1) and RANTES (regulated upon expression normal T cell expressed and secreted) (19), key chemokines that recruit mononuclear cells to the kidney (16,17).

HGF is a multifunctional, pleiotropic growth factor (21) that
ameliorates chronic renal injury in a variety of models, including remnant kidney (22–24), unilateral ureteral obstruction (25,26), and diabetic nephropathy (27). TEC are a major target for HGF in the kidney, and several mechanisms have been proposed to explain this beneficial action, including antiapoptosis (21,25,26), promoting TEC proliferation (21,25,26), prevention of epithelial-to-mesenchymal transition (9,25,26), and increased activity of matrix degradation pathways (23,24). In many models, inflammatory infiltration subsides as well (28); however, the potential anti-inflammatory effects of HGF have been largely overlooked. In the present study, we examined the suppressive effects of HGF on renal inflammation by modulating HGF activity in remnant kidney rats by infusing recombinant HGF or by neutralizing endogenous HGF using an anti-HGF antibody. The mechanisms of this effect were also explored by examining the actions of HGF on TEC expression of chemokines in vivo and in vitro.

Materials and Methods

Animal Experimental Design

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) that initially weighed ~180 to 210 g were housed in an approved animal care facility and fed standard diet. Five-sixths renal ablation was performed as described previously (23,24). Two weeks after surgery, rats were randomly assigned to one of three groups (n = 7): (1) HGF-treated group (HGF): rats received recombinant human HGF (Genentech, South San Francisco, CA) as a continuous intravenous infusion via an implanted osmotic minipump into the jugular vein at a dose of 100 μg/kg per d for 14 d; (2) anti-HGF antibody–treated group (α-HGF): rats received daily injections of a high titer, rabbit polyclonal anti-HGF neutralizing antibody intravenously through the tail vein for 14 d; (3) Preimmune IgG–treated group (Ctrl-IgG): rats received a continuous intravenous infusion of saline via minipump as well as daily tail-vein injections of rabbit preimmune IgG. Sham-operated rats (Sham) with no renal ablation served as normal controls (n = 7). Two weeks after treatment, animals were killed. Kidneys were perfused with iced saline and excised. One portion of the kidney was fixed in 10% phosphate-buffered formalin for morphologic and immunohistochemistry analyses, and another part was frozen immediately for cryostat sectioning. The remainder was snap-frozen in liquid nitrogen and stored at −80°C for RNA and protein extraction.

Animal Studies

At the end of weeks 2 and 4, rats were weighed and placed in metabolic cages, and urine was collected for 24 h. Urine volume was measured, protein concentration was determined by precipitation with 3% sulfosalicylic acid, and turbidity was assessed by detecting absorbance at 595 nm with a spectrophotometer. Urinary protein excretion was calculated as milligrams of protein per 24 h. Blood was obtained from the tail vein. Creatinine levels in serum and urine were measured using a semiautomatic creatinine analyzer (Beckman Instruments, Brea, CA). Creatinine clearance was calculated using the standard formula and factored for body weight. On the day that the rats were killed, they were anesthetized and placed on a heated table to maintain constant body temperature. A polyethylene catheter (PE-50) was inserted into the left femoral artery, and mean arterial pressure (MAP) was measured by a pressure transducer connected to a computer.
ELISA of Chemokines

The contents of MCP-1 and RANTES in kidney homogenates that contained equal amounts of total protein were determined using specific sandwich enzyme immunoassay kits for rat MCP-1 (Assay Design, Ann Arbor, MI) and rat RANTES (Biosource International, Camarillo, CA). The contents of MCP-1 and RANTES in conditioned media from HKC cells were determined by specific Quantikine sandwich ELISA kits for human MCP-1 and RANTES (R&D Systems). The results were normalized by total protein content in kidney homogenates or by cell numbers in culture.

Semiquantitative Reverse Transcription–PCR

Total RNA was extracted from ~2 × 10⁶ cells. Reverse transcription–PCR (RT-PCR) was performed as described previously (23) using primers listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase served as an internal normalizing gene for RT-PCR. PCR products resolved in ~1.5 to 2% agarose gels were photographed under ultraviolet light.

Immunofluorescent Analyses of NF-κB Translocation

HKC cells were plated on chamber slides for 24 h, then deprived of serum for another 24 h. Quiescent cells were treated with 20 ng/ml HGF, 2 ng/ml hTNF-α, or the combination for 30, 60, and 120 min. Nontreated cells in serum-free medium served as controls. Cells were rinsed with PBS buffer and fixed in 4% paraformaldehyde for 20 min, followed by incubation with 1% Triton X-100. Cells were then sequentially incubated with a polyclonal antibody to NF-κB p65 (Cell Signaling) and a FITC-labeled donkey anti-rabbit antibody (Santa Cruz Biotechnology). Results were interpreted using a fluorescence microscope.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay

Quiescent HKC cells were treated with HGF or hTNF-α for the indicated times. Nuclear and cytoplasmic extracts were prepared using the NE-PER extraction kit (Pierce, Rockford, IL). Protein concentration was measured using the BCA reagent (Sigma). For quantifying the nuclear translocation of NF-κB, nuclear extracts were immuno-blotted for p65. Electrophoretic mobility shift assay was conducted using LightShift EMSA kits (Pierce). Briefly, binding reactions that contained 5 μg of nuclear protein, 10 mM Tris, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM MgCl₂, 0.05% Nonidet P-40, and 15 pmol of biotinylated NF-κB consensus double stranded oligonucleotide probe (5′-agttgaggggactttcccaggc-3′) were incubated in a final volume of 20 μl for 20 min at room temperature. Specific binding was confirmed by adding 100-fold excess unlabeled probe as a specific competitor. After incubation, samples were separated by 5% polyacrylamide nondenaturing gel electrophoresis, transferred to nylon membranes via electroblotting, and UV cross-linked. The DNA-protein complexes were visualized with a streptavidin-horseradish peroxidase followed by chemiluminescent detection. For supershift assay, the binding reaction was carried out in the presence of a rabbit polyclonal anti-p65 antibody (Santa Cruz Biotechnology).

Statistical Analyses

One investigator in a blinded manner performed counting of ED-1–positive cells as well as histologic scoring. For immunoblot analysis and RT-PCR, bands were scanned and the integrated pixel density was determined using a densitometer and the NIH image analysis program. All data are expressed as mean ± SD. Statistical analysis of the data from multiple groups was performed by ANOVA followed by Student-Newman-Keuls tests. Data from two groups were compared by t test. Linear regression analysis was applied to examine possible relationships between two parameters. P < 0.05 was considered significant.

Results

HGF Halts the Progression of Proteinuria and Attenuates Renal Fibrosis in Rat Remnant Kidneys

In rats with remnant kidneys, serum creatinine increased and proteinuria gradually worsened during the 2-wk treatment period. Continuous intravenous infusion of HGF significantly slowed progression of proteinuria without altering MAP (Table 2). Renal fibrosis was also attenuated as demonstrated by a reduction in total kidney collagen content and type I collagen immunofluorescent staining (Figure 1). Semiquantitative morphometric studies showed that HGF also decreased fibrosis score in both glomeruli and tubulointerstitium. In contrast, neutralizing the endogenous HGF with the anti-HGF antibody significantly elevated the MAP, impaired renal function, and enhanced fibrosis.

HGF Ameliorates Renal Inflammation in Rat Remnant Kidney

Four weeks after renal ablation, an inflammatory infiltrate consisting primarily of macrophages was prominent in both glomeruli and interstitium in the kidney of the preimmune IgG-treated remnant control group (Figure 2B) compared with

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Molecules</th>
<th>Forward Primer (5′-3′)</th>
<th>Reverse Primer (5′-3′)</th>
<th>Amplimer Size (bp)</th>
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<tr>
<td>Human</td>
<td>MCP-1</td>
<td>aactgaaagctgcactctcg</td>
<td>tcaacagatctcttgcc</td>
<td>258</td>
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<td></td>
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<td>tgacaatggtctgtgaggca</td>
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<tr>
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<td>atcagcttgaatcccccctc</td>
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<td>332</td>
</tr>
<tr>
<td></td>
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<td>caatccttcaacgccatc</td>
<td>gttcatctcaaatgttg</td>
<td>257</td>
</tr>
<tr>
<td></td>
<td>GAPDH</td>
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<td>gcccaggtgcccttgtgaggccctc</td>
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* RT-PCR, reverse transcription–PCR; MCP-1, macrophage chemoattractant protein-1; RANTES, regulated upon expression normal T cell expressed and secreted; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
sham-operated rats (Figure 2A). In addition to increasing tubular atrophy and interstitial and glomerular sclerosis, blockade of endogenous HGF significantly exacerbated the macrophage infiltration (Figure 2C). In contrast, HGF supplementation significantly attenuated renal inflammation, and this was associated with improvement in kidney morphology (Figure 2D). Counting the absolute number of ED-1–positive cells revealed a marked increase in macrophage infiltration after HGF blockade and a significant reduction in response to exogenous HGF in both glomeruli and interstitium (Figure 2E). In addition, the extent of interstitial macrophage infiltration was highly correlated with total kidney collagen content (Figure 2F). Because

Table 2. HGF attenuates progression of proteinuria and renal fibrosis in rat remnant kidney models

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ctrl-IgG (n = 7)</th>
<th>α-HGF (n = 7)</th>
<th>HGF (n = 7)</th>
<th>P Value (by ANOVA)</th>
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<tr>
<td>Body weight (g)</td>
<td>268.14 ± 13.14</td>
<td>276.57 ± 8.84</td>
<td>286.86 ± 16.66</td>
<td>NS</td>
</tr>
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<td>MAP (mmHg)</td>
<td>172.86 ± 9.40</td>
<td>204.71 ± 6.28b</td>
<td>169.57 ± 6.89</td>
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<td>Scr (mg/ml)</td>
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<td></td>
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<td>pretreatment</td>
<td>1.01 ± 0.11</td>
<td>0.89 ± 0.04</td>
<td>1.06 ± 0.13</td>
<td>NS</td>
</tr>
<tr>
<td>posttreatment</td>
<td>1.21 ± 0.14c</td>
<td>1.09 ± 0.11</td>
<td>1.00 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td>ΔScr</td>
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<td>0.20 ± 0.12</td>
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<td>pretreatment</td>
<td>19.36 ± 5.35</td>
<td>27.71 ± 7.31</td>
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<td>NS</td>
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<tr>
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<td>ΔProteinuria</td>
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<td>Ccr (mg/min per 100 g)</td>
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<td>0.086 ± 0.007b</td>
<td>0.100 ± 0.019</td>
<td>0.037</td>
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<tr>
<td>Total kidney collagen content (µg/mg kidney)</td>
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<td>12.89 ± 1.52</td>
<td>5.80 ± 0.50</td>
<td>0.002</td>
</tr>
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<td>Glomerulosclerosis score</td>
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<td>1.37 ± 0.53k</td>
<td>0.55 ± 0.29l</td>
<td>0.002</td>
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<td>Interstitial fibrosis score</td>
<td>1.51 ± 0.56</td>
<td>2.16 ± 0.46m</td>
<td>0.98 ± 0.31n</td>
<td>0.001</td>
</tr>
</tbody>
</table>

a HGF, hepatocyte growth factor; MAP, mean arterial pressure; Scr, serum creatinine; Ccr, creatinine clearance; NS, not significant.
b P = 0.009 versus Ctrl-IgG, P = 0.012 versus HGF.
c P = 0.018 versus pretreatment.
d P < 0.05 versus Ctrl-IgG or α-HGF.
e P = 0.035 versus HGF.
f P = 0.003 versus HGF.
g P = 0.008 versus Ctrl-IgG, P < 0.001 versus α-HGF.
h P = 0.035 versus Ctrl-IgG.
i P = 0.046 versus Ctrl-IgG, P = 0.001 versus HGF.
j P = 0.049 versus Ctrl-IgG.
k P = 0.039 versus Ctrl-IgG, P = 0.001 versus HGF.
l P = 0.049 versus Ctrl-IgG.
m P = 0.02 versus Ctrl-IgG, P < 0.001 versus HGF.
n P = 0.04 versus Ctrl-IgG, by Student-Newman-Keuls test after ANOVA.

Figure 1. Continuous intravenous infusion of hepatocyte growth factor (HGF) decreases interstitial type I collagen deposition in rat remnant kidney, whereas blocking the endogenous HGF increases its accumulation. Representative photomicrographs of type I collagen immunofluorescent staining in frozen kidney sections from sham-operated control rats (A and B) and nephrectomized rats that were treated with preimmune IgG (C), HGF (D), or anti-HGF antibody (E). As negative controls (A) for immunostaining, sections were processed with nonimmune serum from the same species as the primary antibody. All sections were counterstained with Evans’ blue. Magnification, ×200.
Macrophages were not uniformly distributed in remnant kidneys and for avoiding bias in selecting sections for counting, immunoblot analysis of ED-1 protein was performed on whole-kidney homogenates. These studies confirmed that kidney ED-1 content was modulated by HGF (Figure 2, G and H).

HGF-Induced Remission of Renal Inflammation Is Associated with Suppression of Tubular Expression of MCP-1 and RANTES

Immunohistochemistry staining for MCP-1 showed very weak expression in normal kidney (Figure 3A). In remnant kidney, renal expression of MCP-1 increased focally primarily in tubular cells and, to a lesser extent, in glomeruli tufts (Figure 3B). Administration of HGF antibody for 2 wk significantly enhanced tubular staining (Figure 3C) and increased MCP-1 content in kidney homogenates determined by sandwich ELISA as compared with the preimmune IgG-treated group or sham-operated rats (Figure 3E). Conversely, continuous HGF infusion markedly reduced MCP-1 staining and decreased its absolute quantity in kidney homogenates (Figure 3, D and E). Consistent with its chemokine properties, MCP-1 content was strongly correlated with the interstitial macrophage infiltration (Figure 3F).

Normal rat kidney showed a mild constitutive immunostaining for RANTES in both tubules and in glomerular mesangium (Figure 4A). Renal ablation increased staining focally, most obviously in tubules (Figure 4B). Neutralization of endogenous HGF enhanced staining and increased the number of positive tubules (Figure 4C). Conversely, HGF infusion suppressed RANTES to almost normal levels, with only sporadic staining noted in a few tubular cells (Figure 4D). Absolute content of RANTES (Figure 4E) determined by ELISA on tissue homogenates was increased in remnant as compared with normal kidney. The level was further increased in antibody-treated rats and markedly suppressed by HGF infusion. Linear regression analysis showed a strong correlation between RANTES expression and the number of interstitial macrophages in individual rats (Figure 4F).
HGF Suppresses Constitutive and TNF-α Induced Expression of MCP-1 and RANTES in TEC Cells In Vitro

To examine further the effects of HGF on chemokine expression, we exposed HKC cells to HGF at different dosages and for different intervals. HGF treatment for 24 h markedly suppressed constitutive (Figure 5A) as well as TNF-α-induced (Figure 5B) MCP-1 mRNA expression in a dose-dependent manner. The greatest inhibitory effect was
observed at concentrations between 10 and 80 ng/ml. HGF-induced (20 ng/ml) suppression of MCP-1 mRNA levels in the presence (Figure 5D) or absence (Figure 5C) of TNF-α/H9251 was time dependent, peaking at 12 to 24 h and declining at 48 h of exposure. MCP-1 content in the conditioned media from cultured cells as determined by ELISA revealed a similar pattern of responses to HGF (Figure 6). At 24 h, maximal suppression of MCP-1 levels was observed with

Figure 5. HGF suppresses constitutive or TNF-α–induced MCP-1 expression in HKC cells in a dose- and time-dependent manner. Representative pictures for semiquantitative reverse transcription–PCR (RT-PCR) studies in HKC cells that were treated by HGF with different dosage (ng/ml) for 24 h (A and C) or by HGF (20 ng/ml) for different time (B and D) in the presence (C and D) or absence (A and B) of hTNF-α (2 ng/ml). Representative gels are shown at the top, and the results of densitometry are shown at the bottom. Values in the graph are expressed as densitometric ratios as folds over control from three separate experiments. In A and C, *P < 0.05 versus other treatment; in B and D, *P < 0.05 versus non-HGF treatment at the same time point.

Figure 6. Dose-dependent and temporal effect of HGF on the constitutive and the TNF-α–induced expression of MCP-1 in HKC cells. HKC cells were treated in triplicate with HGF and/or hTNF-α at different dosages and for different times. Conditioned media were harvested and subjected to ELISA analysis of MCP-1. (A) HKC cells were treated with HGF of indicated concentrations for 24 h; P < 0.001 by ANOVA; *P < 0.01 versus other groups with HGF, *P = 0.078 versus nontreated cells. (B) HKC cells were treated with HGF of indicated concentrations in the presence of TNF-α (2 ng/ml) for 24 h; P < 0.001 by ANOVA, *P < 0.05 versus other groups with HGF. (C) HKC cells were treated with 20 ng/ml HGF in the presence or absence of 2 ng/ml TNF-α for different indicated times; *P = 0.037, bP = 0.006, cP = 0.002 versus TNF-α + HGF; dP = 0.012, eP < 0.001, fP = 0.002 versus HGF alone. For all values in the graph, n = 3.
HGF at concentrations between 10 and 80 ng/ml (Figure 6A). The greatest inhibition of TNF-α–induced MCP-1 production was observed at the concentration of 20 ng/ml (Figure 6B). HGF treatment significantly reduced MCP-1 production as early as 24 h and produced a >25% decrement in MCP-1 content at 48 h in HKC cells in the presence or absence of TNF-α (Figure 6C).

HGF also suppressed expression of RANTES in HKC cells at both mRNA and protein levels in a time- and dose-dependent manner. At 24 h, HGF at concentrations from 10 to 80 ng/ml HGF produced greater suppression of RANTES mRNA levels than 5 ng/ml HGF (Figure 7A). In the presence of TNF-α, HGF in concentrations from 10 to 80 ng/ml suppressed the mRNA expression of RANTES to almost basal levels (Figure 7B). Temporal studies showed that 20 ng/ml HGF inhibited RANTES expression beginning at 12 h with the maximal effect seen at 48 h (Figure 7, C and D). RANTES protein levels were modulated in a similar pattern. At 24 h, HGF inhibited the RANTES content in a dose-dependent pattern with the greatest effect at 20 ng/ml (Figure 8A). In the presence of TNF-α, HGF reduced RANTES content to the level produced by nontreated cells (Figure 8B). HGF also temporally inhibited the RANTES protein levels. TNF-α–induced RANTES production was significantly decreased after 24 h by HGF to a level comparable to that of nontreated cells. Maximal suppression of RANTES production was observed at 48 h and was >50% for constitutive expression and >60% for TNF-α–induced expression (Figure 8C).

The effect of HGF on expression of MCP-1 and RANTES was also examined in IRPTC. Although IRPTC were less responsive to TNF-α stimulation than HKC, the results in IRPTC were remarkably similar. HGF downregulated the expression of MCP-1 and RANTES in IRPTC (Figure 9) in both the presence and the absence of TNF-α.

**Influence of HGF on NF-κB–Mediated Expression of MCP-1 and RANTES in TEC**

To determine whether MCP-1 and RANTES induction in HKC by TNF-α is regulated by NF-κB activation, we examined the effects of two known inhibitors of NF-κB, TPCK and PDTC. Both inhibitors decreased the expression of MCP-1 and RANTES (Figure 10A) at concentrations that did not affect the HKC cell viability (Figure 10, B and C).

Nuclear translocation is required for NF-κB to mediate its proinflammatory events (30,31). Exposure of HKC to TNF-α induced intense nuclear staining for NF-κB, suggesting that nuclear translocation was promoted (Figure 11C). In contrast, NF-κB was totally sequestered in the cytoplasm, and translocation was not observed at any time point in normal cells or cells that were treated with HGF alone (Figure 11, A and B). TNF-α–induced translocation of NF-κB was suppressed by HGF, as revealed by weak nuclear staining in some cells (Figure 11D). HGF treatment for 30 or 120 min also partially blocked TNF-α–dependent nuclear translocation of NF-κB (data not shown). These immunocytochemistry findings were corroborated by immunoblot analysis of nuclear extracts for NF-κB p65 (Figure 11F). Quality of the nuclear extraction was ensured by absence of cytoplasmic actin in nuclear extracts (Figure 11E) compared with an equal amount of total protein of cytoplasmic extract. Densitometric analysis indicated that TNF-α induced nuclear translocation of NF-κB, which was markedly decreased by HGF at 60 min and 2 h (Figure 11F). Consistently, gel shift assay showed that TNF-α–induced DNA binding by NF-κB was abol-
HGF suppresses TNF-α-induced NF-κB translocation and activation, thereby inhibiting MCP-1 and RANTES expression in TEC.

**HGF Modulates the Activation of NF-κB In Vivo**

Phosphorylation of NF-κB is a prerequisite of NF-κB activation (32). We probed NF-κB activation in vivo using a specific monoclonal antibody against phosphorylated NF-κB p65 (p-p65). Normal kidneys from sham-operated rats had weak staining for activated NF-κB, mainly located in nuclei of tubular epithelial cells (Figure 12A). This staining pattern was markedly increased after renal ablation (Figure 12B) and was further significantly elevated by HGF blockade with antibody (Figure 12C). In response to HGF supplement, NF-κB activation was suppressed to almost basal levels (Figure 12D). Immunoblots of kidney homogenates revealed attenuated or enhanced expression of p-p65 after HGF infusion or blockade, respectively (Figure 12, E and F), corroborating the morphology findings. In addition, the ratio of activated p65 (p-p65) to total p65 was markedly regulated by HGF, although the difference in total p65 expression did not reach a statistical significance after HGF infusion or blockade (Figure 12F).

**Discussion**

Renal inflammation is an important factor contributing to progression of chronic renal disease (1,2,11). In the present study, HGF suppressed renal inflammation and MCP-1 and RANTES expression in TEC of the rat remnant kidney, attenuating renal fibrosis, and similarly inhibited chemokine expression in HKC and IRPTC in vitro. Conversely, neutralization of endogenous HGF by an anti-HGF antibody aggravated renal inflammation and enhanced tubular expression of chemokines, promoting tubular and glomerular injury and fibrosis.

Several recent reviews (33–35) emphasized the antifibrogenic effects of HGF as its major beneficial action in models of...
renal injury. Our data provide three lines of experimental evidence for another novel beneficial effect of HGF, suppressing renal inflammation. Thus, HGF treatment inhibited macrophage infiltration in the remnant kidney model (Figure 2), suppressed expression of proinflammatory chemokines MCP-1 and RANTES, and inhibited the activation of the NF-κB transcription factor, the primary signaling pathway of inflammation both in vivo and in vitro (30,31).

Although the precise mechanism whereby inflammatory cells cause fibrosis is uncertain, most data suggest that macrophages promote renal fibrosis (1,2,11,36,37). However, two notable studies (38,39) seem to contradict this hypothesis. One (38) reported exacerbation in renal interstitial fibrosis associated with less macrophage infiltration at 14 d after unilateral ureteral obstruction (UUO) in lethally irradiated mice that received a transplant of angiotensin receptor type 1a−deficient (Agtr1−/−) bone marrow cells, as compared with Agtr1+/+ bone marrow cell recipients. However, both groups failed to show the florid histopathologic changes and renal dysfunction that are characteristic of the standard UUO model. In contrast, similar studies by Satoh et al. (40) and by Esteban et al. (41) demonstrated the essential role of macrophage and NF-κB activation in the UUO model in wild-type (Agtr1+/+) mice. Consistently, Anders et al. (42) demonstrated that chemokine receptor-1 blockade reduced cellular infiltration and prevented renal fibrosis in the UUO model.

In evaluating the role of urokinase receptor (uPAR) in regulating the phenotypic composition of interstitial cells (macrophage, myofibroblasts, and capillaries) in the UUO model, Zhang et al. (39) also noted decreased macrophage infiltration with increased interstitial myofibroblasts and endothelial cells associated with more renal fibrosis. Although Zhang et al. clearly demonstrated that the uPAR deficiency decreased renal macrophage recruitment, they emphasized that the absence of this scavenger receptor is the main perpetrator of the fibrogenic response. Thus, they postulated that uPAR-deficient macrophages delayed the clearance of angiogenic/profibrotic molecules such as plasminogen activator inhibitor-1. This may have resulted in aggressive myofibroblastic response and neovascularization with enhanced renal fibrosis. Thus, their findings underscore the phenotype of the macrophage as an important factor in interstitial injury that has been recently reviewed (36). Because of lack of specific reagents and inability to similarly manipulate the genome of the experimental rat model, we cannot ascertain the functional phenotype of the macrophage in the remnant kidney.

Chemokines are crucial in recruiting inflammatory cells from the intravascular compartment to the renal parenchyma (16,17). Our data suggest that the effect of HGF to downregulate MCP-1 and RANTES expression in the tubulointerstitial compartment was largely mediated by TEC (Figures 3 and 4). Alternatively, infiltrating leukocytes may serve as a source of chemokines. For example, in a rat model of LPS-induced renal inflammation, Haberstroh et al. (43) demonstrated cross-talk between glomerular resident cells and monocytes/macrophages, whereby MCP-1 released by glomerular resident cells regulated monocyte/macrophage infiltration that then enhanced glomerular RANTES expression. Accordingly, we examined HGF modulation of chemokine production in rat alveolar macrophages (CRL-2192, ATCC). HGF failed to modulate the constitutive expression of MCP-1 and RANTES in this cell line (data not shown), consistent with our view that effects on TEC predominate. However, further studies are necessary to resolve the question completely.

To model the proinflammatory milieu in damaged kidneys, we exposed TEC cells to the potent cytokine TNF-α, which has been implicated in the pathogenesis of renal inflammation and injury in the remnant kidney model (44). As predicted, TNF-α induced expression of MCP-1 and RANTES in both HKC cells and rat TEC cells, and this was attenuated by HGF at both the mRNA and protein levels. However, there were noticeable differences in responses at the mRNA and protein levels. Thus,
marked inhibition of MCP-1 mRNA expression (Figure 5) by HGF was not well correlated with the statistically significant but marginal decreases in secreted MCP-1 protein (Figure 6). This discrepancy might be due to several factors, including (1) inherent differences in the turnover rates of mRNA and protein, (2) the technical nature of the assay whereby the cumulative levels of MCP-1 protein are compared with the mRNA transcription levels at a single-timed point, and (3) the lagged effect of HGF on NF-κB inhibition (Figure 11F). This may have allowed for a window of significant MCP-1 mRNA production in response to TNF-α, resulting in the steep slope of MCP-1 protein levels during the first 12 h.

Cellular regulation of the expression of MCP-1 and RANTES is complex; however, the transcription factor NF-κB is thought to play a critical role in this process (30,31). More specific, TNF-α has been shown to induce proinflammatory cytokines and chemokines via NF-κB activation (45). We found that HGF mitigates TNF-α-provoked NF-κB translocation and activation in TEC. In vivo, activated NF-κB p65 was evident in tubules. HGF infusion inhibited whereas HGF blocking enhanced NF-κB activation. The mechanism for HGF inhibition of NF-κB activation is largely unknown. Although HGF clearly inhibited NF-κB nuclear translocation at 60 min, we observed lack of inhibition at the earlier time period of 15 min. This may be due to the nature of the test system whereby both the activator (TNF-α) and the inhibitor (HGF) were added simultaneously with the kinetics of the activator initially superseding that of HGF action. Alternatively, the initial lag period may reflect HGF’s exerting an indirect inhibitory effect on NF-κB translocation through end products of other signaling pathways activated by the HGF/c-Met axis. Detailed investigation is necessary to identify the mediators involved in the interaction of HGF and NF-κB in tubular epithelial cells.

Another important finding in the present study is that HGF halted the progression of proteinuria and ameliorated glomerulosclerosis in remnant kidneys, demonstrating that HGF has beneficial effects on glomerular as well as interstitial injury (Table 2). Previously, we demonstrated that continuous infusion of HGF for 1 wk decreased glomerular capillary pressure (24) in remnant rats, and this might account for the antiproteinuric effect of HGF. Our findings are also consistent with recent reports demonstrating beneficial effects of HGF on

Figure 11. HGF blunts the TNF-α–induced NF-κB activation in tubular epithelial cells. (A through D) HGF mitigates the TNF-α–activated NF-κB translocation in HKC cells. Quiescent cells were treated with HGF (20 ng/ml; B), hTNF-α (2 ng/ml; C), or both (D) for 1 h, then were immunofluorescently stained for NF-κB p65. Cells that were treated with serum-free media (A) served as normal control. (E and F) HKC cells were treated with HGF, TNF-α, or both for different times before the nuclear and cytoplasmic extract was prepared. (E) The quality of the nuclear extract was ensured by the absence of the cytoplasmic actin on immunoblot compared with an equal amount of total protein of cytoplasmic extract. 1 through 4 represent four different experiments. (F) For quantifying the translocation of NF-κB, nucleus extracts with equal amounts of total protein were immunoblotted with anti-p65 antibody. Representative blots are shown at the top, and the results of densitometry are shown at the bottom. Values in the graph are expressed as fold increase over cells at time 0 from three separate experiments. *P < 0.05 versus TNF-α treatment alone at the same time point. (G) Gel shift assay reveals that HGF blunts the TNF-α–activated DNA binding activity of NF-κB. HKC cells were treated with HGF, TNF-α, or both for 1 h before the nuclear extracts were prepared. Nucleus extracts with equal amounts of total protein were incubated with biotin-labeled oligonucleotides with NF-κB consensus sequence, then subjected to gel shift assay in the presence or absence of anti-p65 antibody. ss, super shift band.
glomerular injury in models of mesangial damage (46) and in diabetic glomerulopathy (27).

Alternatively, HGF may have reduced glomerular injury by attenuating glomerular macrophage infiltration (Figure 2E). Although absolute macrophage number was much lower in glomeruli than in interstitium, HGF administration substantially inhibited and HGF blocking markedly aggravated glomerular infiltration. The primary target cell for HGF action in the glomerulus is still unclear. Mesangial cells seem to play a pivotal role in regulating glomerular infiltration; however, basal expression of c-met is extremely low (47) in these cells, making them an unlikely target cell for HGF. Consistent with this view, basal and TNF-α-induced expressions of chemokines were not regulated by HGF in cultured rat mesangial cells (CRL-2573, ATCC; data not shown). Nevertheless, data from this (47) and other laboratories (48) demonstrate that cytokines such as IL-6 and PDGF can induce expression of c-met in mesangial cells. It follows that increased production of these cytokines in diseased glomeruli might render these cells susceptible to modulation by HGF.

In conclusion, HGF reduces glomerular and tubulointerstitial inflammation and ameliorates proteinuria and renal fibrosis in the rat remnant kidney model. Attenuation of interstitial inflammation is associated with parallel reductions in tubular expression of MCP-1 and RANTES as well as blunted NF-κB activation in tubules. In vitro, HGF suppresses basal and stimulated expression of these chemokines and inhibits NF-κB activation in TEC. Our findings suggest that HGF exerts its beneficial effects on progression of chronic renal disease at least in part by its anti-inflammatory actions, likely via the NF-κB pathway.

Acknowledgments

This work was supported by National Institutes of Health Grant R01-DK-52314 to L.D.D. R.G. was supported by a postdoctoral research fellowship from the Department of Medicine, Brown Medical School.

We are grateful to Dr. Ingelfinger for providing the IRPTC cell line.

References


Figure 12. HGF supplement suppresses whereas HGF blockade enhances the NF-κB activation, mainly located in tubules, in rat remnant kidneys. (A through D) Representative micrographs of phosphorylated NF-κB p65 immunofluorescent staining in frozen kidney sections from sham-operated rats (A) and nephrectomized rats that were treated with preimmune IgG (Ctrl-IgG; B), anti-HGF antibody (α-HGF; C), or HGF (D). (E) Representative blots of Western immunoblot analysis of phosphorylated NF-κB p65 (p-p65) and total p65 in kidney homogenates. (F) Arbitrary units of phosphorylated NF-κB p65 and p65 abundance in immunoblot expressed as densitometric ratios over actin in Ctrl-IgG (blue bar), HGF (maroon bar), and α-HGF (grey bar) groups as folds of sham-operated group (white bar). Ratios between phosphorylated NF-κB p65 and p65 were also expressed as folds of sham-operated group. *P < 0.05 versus other remnant groups; #P < 0.05 versus other groups (n = 7 for each group). Magnification, ×200 in A through D.


