Intravenous Administration of Hepatocyte Growth Factor Gene Ameliorates Diabetic Nephropathy in Mice

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Abstract. Diabetic nephropathy is characterized by progressive loss of renal function, persistent proteinuria, and relentless accumulation of extracellular matrix leading to glomerulosclerosis and interstitial fibrosis. This study investigated the potential effects of long-term expression of exogenous hepatocyte growth factor (HGF) on normal and diabetic kidneys. Intravenous injection of human HGF gene via naked plasmid vector resulted in abundant HGF protein specifically localized in renal glomeruli, despite an extremely low level of transgene mRNA in the kidney. In uninephrectomized mice made diabetic with streptozotocin, delivery of exogenous HGF gene ameliorated the progression of diabetic nephropathy. HGF attenuated urine albumin and total protein excretion in diabetic mice. Exogenous HGF also mitigated glomerular mesangial expansion, reduced fibronectin and type I collagen deposition, and prevented interstitial myofibroblast activation. In addition, HGF prevented kidney cells from apoptotic death in the glomeruli and tubulointerstitium. Moreover, expression of HGF inhibited renal expression of TGF-β1 and reduced urine level of TGF-β1 protein. Therefore, despite the effects of HGF on diabetic nephropathy being controversial, these observations suggest that supplementation of HGF is beneficial in ameliorating diabetic renal insufficiency in mice.

Diabetic nephropathy (DN) is the leading cause of chronic kidney diseases (CKD) that ultimately progress to end-stage renal failure (1,2). The number of patients who are afflicted with DN is steadily increasing worldwide, mainly as a result of a growing population of type 2 diabetes. The pathogenesis of DN is characterized by progressive loss of renal function, persistent proteinuria, and accumulation of extracellular matrix (ECM) that leads to glomerulosclerosis and tubulointerstitial fibrosis (3). Current therapy only slows but does not inhibit the progression of diabetic renal insufficiency in clinical settings (4–6). Therefore, new therapeutic approaches are needed to halt the progressive loss of renal function in patients who have this devastating illness.

Hepatocyte growth factor (HGF) has recently emerged as a potent antifibrogenic factor that prevents the onset and progression of type 2 diabetes. The pathogenesis of DN is characterized by progressive loss of renal function, persistent proteinuria, and accumulation of extracellular matrix leading to glomerulosclerosis and interstitial fibrosis. Current therapy only slows but does not inhibit the progression of diabetic renal insufficiency in clinical settings (4–6). Therefore, new therapeutic approaches are needed to halt the progressive loss of renal function in patients who have this devastating illness.

In this study, we investigated the potential role of exogenous HGF in normal and diabetic kidneys in mice by intravenously administrating naked HGF plasmid. Our results indicate that the antifibrotic actions of HGF are primarily mediated by specifically interrupting TGF-β1/Smad signal transduction (15,18,19). In view of a causative role of hyperactive TGF-β1 signaling in the initiation and development of DN (1,20), it is conceivable to speculate that HGF might be effective in ameliorating diabetic fibrotic lesions and kidney dysfunction.

Paradoxically, an earlier report by Lapin et al. (21) indicated that administration of HGF into genetically obese db diabetic mice reduces creatinine clearance and increases microalbuminuria, although fibronectin mRNA and renal pathology are unaltered. This directly contradicts many observations that implicate HGF as a major antifibrotic cytokine (19,22). It remains unclear, however, whether the detrimental effects of HGF in db diabetic mice are associated with the particular genetic background and/or specific gene mutations of these mice or have a general implication for the role of HGF in diabetic renal disease.

In this study, we investigated the potential role of exogenous HGF in normal and diabetic kidneys in mice by intravenously administrating naked HGF plasmid. Our results indicate that long-term expression of exogenous HGF has no adverse effects on normal kidney structure and function. Furthermore, delivery of HGF significantly ameliorates renal proteinuria, glomerular mesangial expansion, cell apoptosis, and renal TGF-β1 expression in uninephrectomized diabetic mice. Hence, our results are consistent with the notion that HGF is beneficial in attenuating diabetic renal insufficiency in vivo.

Materials and Methods

Animals

Male CD-1 mice that weighed ~18 to 22 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). They were housed in the...
animal facilities of the University of Pittsburgh Medical Center with free access to food and water. Two sets of animal experiments were carried out. For studying the effects of HGF on normal kidney, groups of mice were administered the human HGF expression plasmid (pCMV-HGF) or empty vector (pcDNA3) by intravenous injection, as described previously (17,23). Mice underwent UNx 1 wk before streptozotocin (STZ; Sigma, St. Louis, MO) injection (day −7). At day 0, mice received an intravenous injection of STZ at 150 mg/kg body wt. One week after STZ (day 7), mice were randomized on glucose levels into two groups. One group of mice received an injection of HGF plasmid at 1 mg/kg body wt at day 7 initially and every other week (biweekly) thereafter, and another group received an injection of pcDNA3 in an identical manner. An additional group of mice that underwent sham operation at day −7 served as normal controls. Urine was collected for total protein and albumin determination at 4, 8, and 12 wk after STZ. At 4 and 12 wk after STZ, mice were killed, serum, urine, and kidney were analyzed as described below.

Determination of Human HGF mRNA Expression by Northern Blot and Reverse Transcriptase–PCR Analyses

Total RNA isolation and Northern blot analysis was carried out by the procedures described previously (26). For detecting low levels of exogenous human HGF expression, a more sensitive reverse transcriptase–PCR (RT-PCR) approach was used. For distinguishing transgene expression (human) from endogenous mouse HGF, a primer pair (forward, 5′-GGCAACTTATCCAAAAACAGG-3′; reverse, 5′-GGCAATCCACGAGCAGAATG-3′) was designed to generate a PCR product that spans the cDNA region in which an EcoR I restriction site resides in human but not in mouse HGF. After RT-PCR, the product was digested with EcoR I and then separated on agarose gels.

Determination of Human HGF Protein Expression by ELISA and Immunofluorescence Staining

Human HGF protein expression was determined quantitatively by a specific ELISA, as described previously (23). This ELISA detects only human but not mouse HGF protein. For measuring HGF protein in different tissue compartments, renal glomeruli and tubules were separated by differential sieving technique, according to an established procedure (27). Localization of exogenous HGF protein was determined by an indirect immunofluorescence staining using a specific monoclonal antibody (Clone H14) against human HGF, as described elsewhere (23).

Western Blot Analysis

Protein expression in kidney tissue or isolated glomeruli was analyzed by Western blot analysis according to the procedures described elsewhere (10). The primary antibodies used were as follows: anti-phospho-specific extracellular signal-regulated kinase-1 and -2 (Erk-1/2; Cell Signaling Technology, Beverly, MA), anti-Erk-1/2 and anti-α-smooth muscle actin (α-SMA; Sigma), and antiﬁbronectin (clone 10; Transduction Laboratories, Lexington, KY), anti-type I collagen (Calbiochem, La Jolla, CA), and anti-actin (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA).

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick-End Labeling Staining

Apoptotic cell death was determined by using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining with Apoptosis Detection System (Promega, Madison, WI) (28). Apoptotic cells were counted in 40 to 90 glomeruli for each mouse and expressed as apoptotic cells per glomerulus. Apoptosis in tubulointerstitial area was accounted in at least 10 randomly chosen nonoverlapping high-power (×400) fields for each mouse and expressed as apoptotic cells per field.

Morphologic Studies

Kidney sections were prepared at 3-μm thickness by a routine procedure. Sections were stained with periodic acid–Schiff reagents by standard protocol. Collagen deposition was evaluated by Masson-Trichrome staining, as described previously (29). Renal glomerular lesions and collagen deposition were determined and scored on periodic acid-Schiff and Masson-Trichrome–stained slides, respectively. Glomerular injury characterized by mesangial expansion was graded on the basis of the extent of mesangial involvement on a scale of 1 to 3 as follows: 1, normal; 2, mild; 3, moderate to severe. Approximately 43 to 82 glomeruli were examined in the cortical region from each individual animal. All kidneys from the surviving animals in each group were evaluated (n = 6 to 8). All analyses were made in a blinded manner; values were obtained from the average of two independent assessments by two individuals. Kidney cryosections were immunostained with primary antibodies against ﬁbronectin and collagen I, respectively. As a negative control, the primary antibody was replaced with nonimmune IgG, and no staining occurred. Slides were viewed with a Nikon Eclipse E600 Epi-fluorescence microscope equipped with a digital camera (Melville, NY). For colocalization of α-SMA and the proximal tubular marker in the kidney, cryosections were stained for α-SMA using the Vector M.O.M. Immunodetection kit (Vector Laboratories, Burlingame, CA) (26). The slides were then stained with fluorescein-conjugated lectin from Tetragonolobus purpurascens (Sigma).

Determination of TGF-β1 Levels by ELISA

Serum and urine TGF-β1 levels were determined by using a commercial Quantikine TGF-β1 ELISA kit in accordance with the protocol specified by the manufacturer (R&D Systems, Minneapolis, MN). This kit detects active TGF-β1 protein that binds to its soluble type II receptor precoated onto a microplate. Serum and urine TGF-β1 levels were expressed as pg/ml and ng/mg creatinine, respectively.

Serum Biochemistry

Serum concentrations of total protein, albumin, alanine aminotransferase, blood urea nitrogen, and serum creatinine were measured by automated analyzer in the clinical chemistry laboratory at the Presbyterian Hospital of the University of Pittsburgh Medical Center.

Determination of Blood Glucose, Creatinine, and Albumin

The levels of blood glucose were determined by using the Accu-Chek Active glucometer and test strips (Roche Diagnostic, Indianapolis, IN). Serum and urine creatinine were determined by a routine procedure as described previously (28). Total protein levels were
determined using a bicinchoninic acid–based protein assay kit (Sigma) with BSA as a standard. Urine albumin was measured by using a mouse Albumin ELISA Quantitation kit, according to the protocol suggested by the manufacturer (Bethyl Laboratories, Inc., Montgomery, TX).

**Statistical Analyses**

Statistical analysis of the data was performed by using SigmaStat software (Jandel Scientific, San Rafael, CA). Comparison between groups was made using one-way ANOVA followed by the Student-Newman-Keuls test. \( P < 0.05 \) was considered significant.

**Results**

**Renal Expression of Exogenous HGF Protein**

Intravenous injection of naked plasmid encoding human HGF resulted in high level of HGF protein in the circulation, as well as in liver and kidney (23,28). To investigate whether renal exogenous HGF is derived from *in situ* expression of transgene, we examined human HGF mRNA expression in liver and kidney after intravenous injection of naked plasmid. As shown in Figure 1A, no human HGF transgene mRNA with the corresponding size of 2.3 kb was detected in the kidney by Northern blot analysis. Under the identical conditions, robust HGF transcript was found in the liver. To increase the sensitivity of the mRNA measurement, we used an RT-PCR approach. By designing the primer pairs to generate a PCR product that contained an EcoRI restriction site in human but not in mouse HGF, we were able to distinguish transgene expression (human) from endogenous mouse HGF (Figure 1B). Figure 1C shows the results derived from different ratios of human and mouse RNA mixture. After digestion with EcoRI, the abundance of human HGF PCR product (445 bp) was roughly proportional to the amount of corresponding RNA in the samples. By using this strategy, we found that kidney did express a low level of human HGF mRNA after intravenous injection of plasmid vector (Figure 1D). However, the magnitude of *in situ* expression of transgene in the kidney was much lower than that in liver.

Despite the low level of HGF transgene mRNA expression, there was abundant exogenous HGF protein in the kidney after intravenous injection of plasmid vector. Figure 2A shows the human HGF protein levels in renal glomeruli and tubules at different time points after a single plasmid injection, as detected by ELISA using specific antibody against human HGF. Consistent with a previous report (28), exogenous HGF was predominantly localized in the renal glomeruli (data not shown). However, both renal glomeruli and tubules clearly

![Figure 1](image_url)

**Figure 1.** Expression of human hepatocyte growth factor (HGF) mRNA in mouse kidney after intravenous injection of naked plasmid. (A) Northern blot shows liver as the predominant site for human HGF transgene expression after intravenous injection of naked plasmid vector. Total RNA was isolated from the liver and kidney at 24 h after injection of either control pcDNA3 or pCMV-HGF plasmid and subjected to Northern blot analysis with HGF and 18S RNA probes, respectively. Human HGF gene transcript that was 2.3 kb in size is indicated. (B) Diagram illustrates a PCR strategy to differentiate exogenous (human) and endogenous (mouse) HGF mRNA expression. P1 and P2 denote the forward and reverse primers, respectively. (C) Validation of the PCR strategy. Total RNA from human and mouse origin was mixed at different ratios as indicated. Reverse transcriptase–PCR (RT-PCR) products were separated on agarose gels before (top) or after (bottom) EcoRI digestion. (D) RT-PCR shows low level of human HGF mRNA in the mouse kidney after intravenous injection of naked plasmid. (Top) Before EcoRI digestion. (Middle) After EcoRI digestion. (Bottom) Actin.
responded to and were activated by exogenous HGF expression. Figure 2, B and C, shows the Erk-1/2 activation in renal glomeruli and tubules, respectively, after a single injection of HGF plasmid.

Effects of Sustained HGF Expression on Normal Kidney

We examined the potential effects of sustained expression of exogenous HGF on normal kidneys. To achieve long-term expression, we repeatedly administered pCMV-HGF plasmid by weekly injections. Hence, two groups of mice received injections of either pcDNA3 or pCMV-HGF once a week for 8 wk. Consistent with a previous report (23), there was no gross and microscopic alteration in kidney morphology between these two groups at the end of 8 wk. Table 1 shows the data on serum biochemistry, urine protein excretion, and renal TGF-β1 levels in mice that received weekly plasmid injections for 8 wk. No significant difference in blood urea nitrogen, creatinine, and urine protein excretion was found between these two groups (P > 0.05, n = 6). Furthermore, renal TGF-β1 was similar in mice that received either pcDNA3 or pCMV-HGF injections for 8 wk (Table 1).

HGF Ameliorates Proteinuria in Diabetic Nephropathy

We next investigated the effect of exogenous HGF on the progression of DN. Table 2 shows general characteristics of UNx-diabetic mice at the end of 4 and 12 wk after STZ. Three of nine mice died over the 12 wk period in the UNx-diabetic group that received pcDNA3 injection (Table 2). The exact causes of these deaths were unknown. Substantial reduction in weight gain was noticed in the UNx-diabetic mice over the 12 wk period, when compared with the normal controls (Table 2). Intravenous injection of HGF plasmid did not significantly affect blood glucose levels in the surviving animals when administered at 1 wk after STZ (Table 2). Diabetic kidney at 4 and 12 wk after injection of STZ displayed a marked enlargement in size, and HGF significantly reduced it (Table 2). However, serum creatinine levels only slightly increased in diabetic mice at 12 wk after STZ when compared with that in normal controls (Table 2).

Expression of exogenous HGF resulted in a substantial alleviation of proteinuria in diabetic mice. Figure 3A shows urine albumin levels at different time points in diabetic mice. At 12 wk after STZ, the urine albumin level reached 1.5 mg/mg creatinine. However, injection of HGF plasmid reduced albumin excretion by >70% (Figure 3A). Likewise, HGF expression reduced total urine protein excretion in diabetic animals (Figure 3B).

HGF Attenuates Mesangial Expansion and Matrix Deposition in the Glomeruli

Figure 4 demonstrates that HGF attenuated mesangial expansion and glomerular injury in diabetic kidneys. Immunofluorescence staining exhibited an induction of α-SMA in the mesangium of diabetic glomeruli, and exogenous HGF largely inhibited it (data not shown). Compared with normal controls, diabetic kidneys displayed enlarged glomeruli in mice that received pcDNA3. Delivery of HGF significantly reduced glomerular size (Figure 4G). Diabetic glomeruli exhibited expanded mesangial area and narrowed capillary lumen, as well as an increased collagen deposition in renal glomeruli at 12 wk. These morphologic injuries were attenuated in diabetic mice that received pCMV-HGF injections (Figure 4H). Of note, three of six mice showed focal and segmental glomerulosclerosis (FSGS) in ~1 to 4% of the glomeruli in the pcDNA3
Table 1. Serum biochemistry, urine protein excretion, and kidney TGF-β1 levels in mice that received weekly plasmid injections for 8 weeksa

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<th>Total protein (g/dl)</th>
<th>Albumin (g/dl)</th>
<th>ALT (U/dl)</th>
<th>BUN (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
<th>Urine Protein Excretion (mg/mg Cr)</th>
<th>Kidney TGF-β1 (pg/mg Protein)</th>
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<td>pcDNA3</td>
<td>4.87 ± 0.11</td>
<td>2.13 ± 0.03</td>
<td>107 ± 29</td>
<td>22.3 ± 1.1</td>
<td>0.17 ± 0.03</td>
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<td>9.82 ± 2.58, 6.01 ± 2.03</td>
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<tr>
<td>pCMV-HGF</td>
<td>5.16 ± 0.23</td>
<td>2.33 ± 0.07</td>
<td>114 ± 23</td>
<td>22.0 ± 0.6</td>
<td>0.20 ± 0.01</td>
<td>95 ± 7.0</td>
<td>8.98 ± 3.68, 4.62 ± 1.69</td>
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a ALT, alanine aminotransferase; BUN, blood urea nitrogen; Cr, creatinine. There was no statistical difference (P > 0.05, n = 6) in all parameters between these two groups.

The present study was undertaken to address two specific issues: The effects of long-term expression of exogenous HGF on normal kidney structure and function and the potential impacts of exogenous HGF on DN. By using naked plasmid vector as a simple gene delivery system, we have shown that sustained expression of exogenous HGF in the kidney did not cause adverse side effects on normal renal structure and function. Furthermore, expression of exogenous HGF gene prevented the onset and progression of DN, resulting in reduced proteinuria, attenuated glomerulosclerotic lesions, and decreased renal TGF-β1 expression. These observations are consistent with many recent reports demonstrating HGF as an endogenous antifibrotic factor in a wide variety of CKD (7–14).

**Group**, whereas only one of eight kidneys displayed the similar FSGS in the pCMV-HGF group.

Figure 4 also illustrates the deposition of fibronectin and type I collagen in renal glomeruli at 12 wk after STZ. To determine quantitatively the effect of exogenous HGF on matrix deposition in glomeruli, we analyzed the fibronectin and type I collagen expression in the isolated glomeruli by the differential sieving technique. As presented in Figure 4I, Western blot analysis demonstrated that exogenous HGF suppressed the accumulation of fibronectin and type I collagen in the glomeruli of diabetic kidneys. Quantitative analyses revealed that HGF suppressed glomerular fibronectin and type I collagen by 37 and 43%, respectively. Similar results were obtained that HGF suppressed glomerular fibronectin and type I collagen expression in the isolated glomeruli by the differential sieving technique. As presented in Figure 4I, Western blot demonstrated that HGF suppressed fibronectin and type I collagen expression in the isolated glomeruli by the differential sieving technique.

**HGF Inhibits TGF-β1 Expression in DN**

We examined the expression of TGF-β1 in diabetic mice that received either control vector or HGF plasmid. Renal production of TGF-β1 was determined by measuring TGF-β1 excretion in the urine using a specific ELISA. As illustrated in Figure 7A, urine TGF-β1 protein levels increased in a time-dependent manner. At 12 wk after diabetes, TGF-β1 protein level in urine reached 25 ng/mg creatinine (Figure 7A). HGF reduced the urine TGF-β1 level by ~50% at different time points. Of note, neither diabetic condition nor HGF significantly modulated serum TGF-β1 levels, which presumably represent the sum of the circulating TGF-β1 and that released from platelet (Figure 7B). These results suggest that the increase in urine TGF-β1 in DN is primarily attributable to local production in the kidney.

Figure 7, C through H, shows the representative micrographs of TGF-β1 staining in the glomeruli and tubulointerstitium of diabetic kidneys. Compared with normal controls, TGF-β1 staining was increased in renal glomeruli and tubules of diabetic mice at 12 wk after the onset of diabetes. HGF gene therapy also inhibited TGF-β1 expression in diabetic kidney (Figure 7, E and H).

**Discussion**

The effect of exogenous HGF on cell apoptosis in diabetic kidneys is illustrated in Figure 6. There was marked increase in glomerular cell apoptosis at 12 wk after STZ, although the absolute number of apoptotic cells was still very low (Figure 6G). Expression of human HGF dramatically prevented glomerular cell death. Quantitative determination revealed that the numbers of apoptotic cells in diabetic kidneys were comparable to that in normal controls (Figure 6G). HGF also prevented cell death in the tubulointerstitium of the diabetic kidney (Figure 6, F and H).

**HGF Prevents Kidney Cells from Apoptosis in Diabetic Nephropathy**

The effect of exogenous HGF on cell apoptosis in diabetic kidneys is illustrated in Figure 6. There was marked increase in glomerular cell apoptosis at 12 wk after STZ, although the absolute number of apoptotic cells was still very low (Figure 6G). Expression of human HGF dramatically prevented glomerular cell death. Quantitative determination revealed that the numbers of apoptotic cells in diabetic kidneys were comparable to that in normal controls (Figure 6G). HGF also prevented cell death in the tubulointerstitium of the diabetic kidney (Figure 6, F and H).
and suggest that supplementation of exogenous HGF may be an effective strategy for the prevention and treatment of DN.

The findings presented in this study contradict an earlier report by Laping et al. (21), who demonstrated a detrimental effect of exogenous HGF in genetically obese db diabetic mice. The reason behind these inconsistent observations remains unknown. One potential explanation is attributable to different genetic backgrounds and/or specific mutation of physiologically important genes such as leptin receptor, which have been shown to exhibit strong influence on the pathogenesis of DN (30–32). Another possible interpretation accounting for the discrepancy could be due to variations in the dose, duration, and route of exogenous HGF used in different studies. Laping et al. used a very low dose of HGF (160 ng/mouse per d for 18 d) via subcutaneous administration by osmotic pumps (21). Although not determined, the circulating HGF level may be low in that study, given that the half-life of exogenous HGF in the circulation is <15 min (33). In addition, it remains unclear whether exogenous HGF is still biologically active after a long incubation (up to 18 d) at body temperature by osmotic pump delivery (21). However, our present study used an efficient gene delivery approach that yielded a high level of HGF gene expression in vivo. The peak level of circulating exogenous HGF can exceed 8 ng/ml (23), and a high level of HGF protein

![Figure 3. HGF ameliorates proteinuria in diabetic mice. Urine albumin (A) and total protein (B) were determined at different time points as indicated in diabetic mice that received either control pcDNA3 or pCMV-HGF plasmid injections. Data are expressed as mg/mg creatinine and presented as means ± SEM of five to eight animals per group. *P < 0.05 versus control pcDNA3 group.](image-url)

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<th>Table 2. General characteristics of mice among different treatment groups a</th>
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<td><strong>Animal Numbers</strong></td>
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a KW, kidney weight; BW, body weight; UNx, uninephrectomized; STZ, streptozotocin; nt, not tested.

b One week after STZ injection.

c P < 0.05 versus control pcDNA3 group.

d P < 0.05 versus normal controls.

> KW, kidney weight; BW, body weight; UNx, uninephrectomized; STZ, streptozotocin; nt, not tested.
is confirmed in the kidney (Figure 2). Finally, different sources and particular preparations of recombinant HGF may exhibit dissimilar biologic potency. These issues are essentially eliminated by virtue of the utilization of a gene therapy approach in the present study. Of interest, our findings that HGF ameliorates DN are independently confirmed by two recent reports with a similar conclusion (34,35).

The present study used a UNx-diabetic model in an effort to accelerate the development and progression of DN in mice (24,25). In our pilot experiments, no significant renal fibrosis was observed after 6 mo in a standard STZ diabetic mouse model (data not shown). Although the clinical relevance of the UNx-STZ mice to diabetic patients is uncertain, this model essentially recapitulates major pathologic features of DN, including proteinuria, mesangial expansion, TGF-β1 overexpression, cell apoptosis, and glomerulosclerotic lesions. Of note, possibly still in the early stage of DN, tubulointerstitial lesions are generally mild and serum creatinine level rises only moderately at 12 wk after STZ in UNx mice.

Delivery of HGF gene seems to show a tendency of improving survival of the unmanaged diabetic mice, although the sample size is small in this study. The exact causes of animal death in the pcDNA3 control group were unknown; however, it is very unlikely that they died of kidney disease, given the mild manifestation of renal dysfunction at this stage. Diabetic mice most likely died of ketoacidosis and/or dehydration, as a result of severe hyperglycemia. HGF is known to protect pancreatic β cells from apoptosis and promote their proliferation when it is given before STZ administration (36). In addition, HGF can increase β cell function (37). It therefore is possible that the beneficial effect of HGF in diabetic mice may be partially mediated by its actions on surviving pancreatic β cells after STZ, which has been largely concealed by the glucose level assessment as a result of the selection effect from animal loss in the control group. Such action of HGF in β cells may account for a potential improvement of animal survival in this study. Nevertheless, it should be emphasized that the effect of HGF on DN is unlikely mediated by ameliorating hyper-

Figure 4. HGF reduces mesangial expansion and attenuates glomerular matrix accumulation in diabetic nephropathy. Representative micrographs demonstrate mesangial expansion and morphologic injury (periodic acid-Schiff staining; A through C) and glomerular collagen deposition (Masson-Trichrome staining; D through F) in normal control mice (A and D), diabetic mice that received pcDNA3 (B and E), and diabetic mice that received pCMV-HGF (C and F). Arrows in B and E indicate injured glomeruli. Scale bar = 100 μm. Data on glomerular size (G) and injury scores (H) in different groups are also presented. Data are presented as mean ± SEM of six to eight animals per group. *P < 0.01 versus normal controls; **P < 0.05 versus pcDNA3 group. (I) Western blot analyses demonstrate the effects of HGF on fibronectin and type I collagen expression in diabetic glomeruli. Renal glomeruli were isolated by a differential sieving technique from a portion of the kidney (pooled from six to eight animals per group) and subjected to Western blot analysis using antibodies against fibronectin, type I collagen, and actin, respectively.
glycemia, because the blood glucose levels in the surviving animals for the control and HGF groups are similar (Table 2).

The mechanism by which HGF ameliorates DN is not completely understood. In diabetic glomeruli, mesangial cell activation characterized by α-SMA induction is regarded as a central event leading to matrix accumulation and glomerulosclerotic lesions. Although copious factors can induce the phenotypic transformation of mesangial cells, TGF-β1 is believed to be the most potent one (38–41). In this regard, HGF inhibition of TGF-β1 expression in the diabetic kidney may provide a mechanistic explanation for its therapeutic efficacy. It seems that suppression of TGF-β1 expression in the diabetic kidney may occur in a kidney-specific manner, as serum TGF-β1 levels are not altered (Figure 7B). In addition, HGF inhibition of TGF-β1 expression takes place exclusively in disease states but not in normal kidney (Table 1). Consistently, recent in vitro studies indicate that HGF solely suppresses high glucose–induced TGF-β1 in cultured mesangial cells but not at basal conditions (34). Collectively, it is conceivable that HGF suppression of TGF-β1 expression is primarily mediated by selective blockade of its induction under pathologic conditions, rather than by directly inhibiting its expression per se. Recently, we obtained evidence that HGF upregulates Smad transcriptional co-repressor TGIF in mesangial cells and in diabetic glomeruli (18). Such action would lead to suppression of TGF-β1/Smad-mediated gene transcription (18). Because TGF-β1 induces its own expression by a positive autocrine loop, antagonizing Smad action by HGF-induced TGIF would disrupt the TGF-β1–positive regulatory loop, thereby indirectly suppressing its induction.

Renal glomeruli consist mainly of three types of cells: mesangial cells, endothelial cells, and podocytes. Earlier studies indicated that all three types of glomerular cells express functional c-met receptor and respond to HGF stimulation to activate prosurvival Akt phosphorylation in vitro (42). Hence, HGF may act on all glomerular cells to preserve their phenotypes in diabetic kidney. As HGF has been demonstrated to protect podocytes and endothelial cells from apoptosis (43,44), this action may explain its advantageous effect on mitigation of proteinuria, an early pathologic feature not only manifesting glomerular injury but also being linked to secondary tubulointerstitial lesion (45). Consistently, a dramatic attenuation of glomerular cell apoptosis by HGF is observed in the diabetic kidney (Figure 7).

DN is associated with tubulointerstitial fibrosis at the advanced stage. Although interstitial fibrotic lesions in DN may be initiated by the same injurious factors as glomerular sclerosis, it is secondarily influenced and accelerated by factors that originated from glomerular lesions, such as proteinuria as a result of podocyte injury and hypoxia as a result of vascular capillary collapse. Of interest, interstitial lesions are usually the best predictor of declined renal function (3,46). In this regard, DN is similar to other renal diseases in which progressive loss...
of renal function correlates with advancing interstitial fibrosis. Although tubulointerstitial lesions are generally mild at this early stage of DN, exogenous HGF clearly reduces α-SMA expression and blocks matrix-producing myofibroblast activation (Figure 6), an early event followed by interstitial matrix accumulation and deposition. In agreement with in vivo results, HGF has been shown to suppress myofibroblast activation from interstitial fibroblasts and to block tubular epithelial to mesenchymal transition (10,15,47). Therefore, HGF may exert its antifibrotic actions through multiple mechanisms by acting on a broad spectrum of kidney cells.

In light of the antifibrotic effects of HGF in a variety of animal models of CKD, it is plausible to speculate that supplementation of exogenous HGF or its gene may be an effective strategy for combating chronic renal fibrosis in a clinical setting. However, concerns are also raised on the utility of HGF as a therapeutic agent, because a transgenic mouse strain overexpressing HGF under metallothionein promoter manifested renal tubular hyperplasia, polycystic disease, and glomerulosclerosis (48). The relevance of the phenotype of that particular strain of transgenic mouse to the actions of HGF in clinical renal disease, however, remains uncertain. One plausible explanation for the phenotypes of this transgenic mouse strain could be attributable to the inherent nature of de novo ubiquitous HGF expression under metallothionein promoter and/or random chromosomal insertions associated with a transgenic model. This view is supported by the observation that there is no correlation between kidney pathology and HGF levels in these mice (48). Furthermore, no renal lesion is reported in other HGF transgenic mouse lines under different promoters (49–53). In particular, transgenic mice with targeted overexpression of exogenous HGF in renal proximal tubules display normal kidney morphology and function, with no renal tumors, cysts, or glomerular abnormalities (53). Consistently, sustained expression of HGF for 8 wk does not induce any side effects in adult animals (Table 1).

In summary, we have shown herein that long-term expression of HGF is safe without causing adverse side effects on normal kidney structure and function. Moreover, we demonstrated that delivery of exogenous HGF attenuates diabetic nephropathy, a devastating illness with great morbidity and mortality. However, in view of the controversy pertaining to the effects of HGF on DN in the literature, more studies are needed in this area before considering any therapeutic utilization of HGF for combating diabetic renal insufficiency in a clinical setting.

Acknowledgments

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References