Regression of Advanced Diabetic Nephropathy by Hepatocyte Growth Factor Gene Therapy in Rats

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Diabetic nephropathy is the main cause of end-stage renal disease requiring dialysis in developed countries. In this study, we demonstrated the therapeutic effect of hepatocyte growth factor (HGF) on advanced rather than early diabetic nephropathy using a rat model of streptozotocin-induced diabetes. Early diabetic nephropathy (16 weeks after induction of diabetes) was characterized by albuminuria, hyperfiltration, and glomerular hypertrophy, whereas advanced diabetic nephropathy showed prominent transforming growth factor (TGF)-β1 upregulation, mesangial expansion, and glomerulosclerosis. An SP1017-formulated human HGF (hHGF) plasmid was administered by intramuscular injection combined with electroporation over a 30-day follow-up in rats with early and advanced diabetic nephropathy. hHGF gene therapy upregulated endogenous rat HGF in the diabetic kidney (rat HGF by RT-PCR was threefold higher than in diabetic rats without therapy). hHGF gene therapy did not improve functional or morphologic abnormalities in early diabetic nephropathy. hHGF gene therapy reduced albuminuria and induced strong regression of mesangial expansion and glomerulosclerosis in advanced diabetic nephropathy. These findings were associated with suppression of renal TGF-β1 and mesangial connective tissue growth factor (CTGF) upregulation, inhibition of renal tissue inhibitor of metalloproteinase (TIMP)-1 expression, and reduction of renal interstitial myofibroblasts. In conclusion, our results suggest that hHGF gene therapy may be considered as an innovative therapeutic strategy to treat advanced diabetic nephropathy. *Diabetes* 53: 1119–1127, 2004

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CTGF, connective tissue growth factor; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; HGF, hepatocyte growth factor; hHGF, human HGF; MGV, mean glomerular volume; MMP, metalloproteinase; SMA, smooth muscle actin; STZ, streptozotocin; TGF, transforming growth factor; TIMP, tissue inhibitor of MMP.

Diabetic nephropathy is the main cause of chronic renal failure and end-stage renal disease requiring dialysis in developed countries. Despite recent advances in renoprotection, incidence of diabetic nephropathy as a cause of end-stage renal disease is on the increase (1). Moreover, in a significant number of patients, diabetic nephropathy is diagnosed in the later stages, which are considered incurable. Hyperglycemia generates a cascade of events in the kidney, which finally result in mesangial extracellular matrix (ECM) accumulation (2). Growth factors, such as transforming growth factor (TGF)-β1 and connective tissue growth factor (CTGF), play an important role in the pathogenesis of diabetic nephropathy by increasing expression of ECM proteins as well as suppressing ECM degradation (3–5).

Hepatocyte growth factor (HGF) is a mesenchyme-derived polypeptide that has antifibrogenic and regenerative properties in a variety of chronic nephropathies (6–8). Actions of HGF are mediated by c-met receptor, a member of the tyrosine kinase receptor superfamily (7). Renal HGF is produced by mesangial cells, endothelial cells, and interstitial fibroblasts, whereas c-met receptor expression is ubiquitous (8). Local production of HGF stimulates growth in epithelial and endothelial cells but not in mesangial cells (9). Also, the supplementation of HGF to mesangial cells in culture produces an increase in HGF synthesis, whereas addition of angiotensin II or TGF-β1 inhibits HGF production (9).

The role of HGF in diabetic nephropathy remains controversial. Morishita et al. (10) described that elevated levels of D-glucose induce reduction of the renal production of HGF by increasing TGF-β. Liu et al. (11) showed in vitro and in vivo that hyperglycemia increases renal expression of HGF and c-met, suggesting that HGF has a role in the renal hypertrophy of diabetes. However, in a mouse model of type 2 diabetes, Nakamura et al. (12) found a decrease in circulating and renal HGF levels. Recently, Laping et al. (13) showed that administration of HGF into genetically obese diabetic mice reduced renal function and increased microalbuminuria, although HGF treatment did not exacerbate histological lesions. All those discrepancies may be merely a reflection of the changing HGF expression throughout the evolution of diabetic nephropathy. In fact, Mizuno et al. (14) demonstrated that renal HGF declined in parallel with the progression of renal damage and, overall, that renal HGF inversely correlated

with both TGF-β and ECM protein production. It is well documented that TGF-β1 and HGF inhibit the synthesis of each other (15) and that HGF also downregulates the expression of TGF-β1 receptor 1 in vivo (16). Thus, some authors (17–19) described a reduction of TGF-β1 by HGF supplementation in several models of organ fibrosis. Recently, Inoue et al. (20) performed 5/6 nephrectomy in TGF-β1 transgenic mice and reported that HGF counteracts TGF-β1 through attenuation of CTGF induction, which in turn influenced signaling by TGF-β1 via Smad inhibition.

In this study, we used streptozotocin (STZ)-induced diabetic rats with early and advanced diabetic nephropathy to assess the effect of human HGF (hHGF) gene therapy at both stages. Our findings demonstrated that hHGF gene therapy resulted in supraphysiological levels of circulating HGF and induced endogenous rat HGF upregulation in the damaged kidney. Although hHGF gene therapy had no effect on early diabetic nephropathy, it clearly reversed glomerular damage in advanced diabetic nephropathy by reducing the profibrogenic growth factors, TGF-β1 and CTGF, and protease inhibitors, such as tissue inhibitor of metalloproteinase (TIMP)-1.

RESEARCH DESIGN AND METHODS

The experiments were carried out in accordance with the current legislation on animal experiments in the European Union and approved by our institution's Ethics Committee for Investigation with Animals. Young (4- to 8-week-old) male Sprague-Dawley rats (200–250 g body wt) were purchased from Harlam Iberica (Sant Feliu de Codina, Spain) and housed under controlled environmental conditions (temperature 22°C, 12-h darkness period). Animals were given free access to water and standard laboratory diet.

Induction of diabetes and insulin administration. Diabetes was induced by intravenous injection of STZ (Sigma, Madrid, Spain) 60 mg/kg body wt in 0.01 mol/l citrate buffer (pH 4.5) after 12 h of food deprivation. Three days after STZ administration and twice a week thereafter, the animals were weighed and tail vein blood glucose was determined by Glucocard (Menarini, Barcelona, Spain). Insulin (Insulatard NPH; NovoNordisk Madrid, Spain) (1–5 units/day, subcutaneously) was initiated 7 days after administration of STZ after having checked that all of the animals had blood glucose levels >400 mg/dl. Insulin was administered daily to maintain blood glucose between 300 and 400 mg/dl and to avoid ketosis.

Gene therapy. We have recently reported (21) an efficient gene therapy approach for the systemic production of therapeutic proteins. In this work, we demonstrated that the intramuscular injection of hHGF plasmid DNA formulated with the nonionic carrier SP1017 (Supratek-Pharma, Laval, Canada) followed by electroporation enhanced hHGF expression in both plasma and peripheral organs of control rats. The developed methodology has been applied in the present study. Briefly, equal volumes of plasmid DNA and SP1017 (0.02%) were gently mixed to get a final concentration of DNA of 2 μg/μl and 0.01% wt/vol of SP1017. Plasmid was injected (200 μl) into the tibialis anterior muscle. Then, an electrical field was applied to the area around the injection. Muscles were held by caliper electrodes composed of two 1.5-cm^2 steel plates, and eight pulses of 20 ms each at a frequency of 2 Hz were delivered (voltage, 175 volts/cm) by pulse generator (BTX ECM830 electroporator; Genetronics, San Diego, CA). To improve plasmid DNA diffusion, 25 units of bovine hyaluronidase (Sigma) in 60 μl saline were injected into the muscle 2 h before the administration of plasmid DNA (22). Therapeutic interventions with hHGF were performed at 16 (early) or 32 weeks (advanced) of diabetic nephropathy. The process was repeated 15 days after the first dose.

Study groups. We determined renal HGF and TGF-β1 at 1, 8, 16, 24, and 32 weeks after induction of diabetes in rats. We used appropriate age-matched nondiabetic animals as controls. Animals were killed and tissue samples processed and stored as needed. There were five rats per group (diabetic and nondiabetic) and time point. Therapeutic intervention with hHGF gene therapy was carried out on early (16) and advanced (32 weeks) diabetic nephropathy. Thus, 16 or 32 weeks after diabetes induction, the rats were randomly allocated to different groups: early diabetic nephropathy (D1, n = 7), early diabetic nephropathy with hHGF gene therapy (D1-HGF, n = 14), advanced diabetic nephropathy (D2, n = 7), and advanced diabetic nephropathy with hHGF gene therapy (D2-HGF, n = 10). There were early (ND1, n = 6) and advanced (ND2, n = 6) nondiabetic, age-matched control rats. After a follow-up of 30 days, animals were killed and tissue samples processed and stored as needed.

Serum and urine chemistry. Rats were placed in metabolic cages to collect 24-h urine specimens on day 0 (before therapeutic intervention) and at the end of the study period (on day 30). Blood was obtained from the tail vein. Serum and urine creatinine (in milligrams per deciliter) levels were determined on an autoanalyzer (Beckman Instruments, Palo Alto, CA), and creatinine clearance (in microliters per minute per 100 g body wt) was calculated. The urinary albumin excretion was determined by an immunoturbidimetric method in a Nefelometer II (Dade Behring, Barcelona, Spain).

Determination of renal TGF-β1 levels. Pieces of kidney cortex were homogenized in 10 mmol/l Tris-HCl buffered solution (pH 7.4) containing 2 mol/l NaCl, 1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l EDTA, and 0.01% Tween-80. The samples were centrifuged at 19,000g for 30 min, and the supernatant was aliquoted and stored at −80°C until analyzed. The total protein concentration was measured using the Bradford protein assay (Bio-Rad, Hercules, CA). TGF-β1 levels were determined by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine Kit; Research & Diagnostics Systems, Minneapolis, MN). The levels of TGF-β1 were expressed as nanograms per milligram total protein.

Determination of rat and human plasma HGF. Blood samples were collected into EDTA tubes, centrifuged at 900g for 30 min at 4°C, and kept in polypropylene vials. Human plasma HGF was measured using a commercially available ELISA kit (Quantikine kit). Rat HGF was determined by another specific commercially available ELISA kit (Rat HGF-EIA; Institute of Immunology, Tokyo, Japan). This rat HGF antibody does not cross-react with hHGF (18).

Determination of rat kidney HGF. Kidneys were homogenized in the HGF extraction buffer containing 20 mmol/l Tris-HCl, pH 7.5, 2 mol/l NaCl, 0.1% Tween-80, 1 mmol/l EDTA, and 1 mmol/l phenylmethylsulfonyl fluoride, as previously described (23). The homogenates were centrifuged at 19,000g for 30 min at 4°C, and the supernatant was recovered. The determination of HGF was done using a specific commercially available ELISA kit (Rat HGF-EIA). This rat HGF antibody does not cross-react with hHGF (18). Renal HGF concentration was expressed in nanograms per milligram protein.

Renal TIMP-1 quantification. Renal tissue was homogenized in 10 vol of cold lysis buffer (50 mmol/l Tris-HCl, 150 mmol/l NaCl, 5 mmol/l $CaCl_2$, 0.05% BRIJ-35, 0.02% sodium azide, 1% Triton X-100, pH 7.6) (24). The homogenates were centrifuged at 12,500g at 4°C for 5 min. TIMP-1 levels in tissue homogenate supernatants were determined using a commercially available ELISA kit (Quantikine kit). The levels were expressed as picograms per milligram protein.

Histological studies. Tissue sections (3–4 μm thick) were placed in 4% formaldehyde for paraffin embedding and subsequent staining with periodic acid schiff and silver methenamine. Masson's trichrome staining was used to demonstrate collagen deposition. All of the samples were evaluated by a pathologist blinded to the group assignment. For glomerulosclerosis quantification (as a percentage), the segmental and global sclerosed glomeruli of each kidney section were counted and divided by the total number of glomeruli. Mesangial expansion was evaluated in periodic acid silver methenamine–stained sections as 0 (absent), 1 (mild), 2 (moderate), 3 (severe), and 4 (severe plus glomerular sclerosis). The mean glomerular volume (MGV, $\mu^3 \times 10^6$) was evaluated in periodic acid schiff sections according to the formula of Weibel (25) as previously described (26). Interstitial area (as a percentage) was quantified by morphometric analysis on periodic acid silver methenamine–stained slices examined as previously described (27).

Immunohistochemical analyses. As primary antibodies, we used a 1:100 diluted monoclonal mouse anti-rat α-smooth muscle actin (α-SMA) antibody for myofibroblasts (Neomarkers, Fremont, CA), 1:20 diluted rabbit polyclonal-IgG for CTGF (Santa Cruz Biotechnology, Santa Cruz CA), and 1:20 diluted mouse monoclonal-IgG for collagen IV (Dako, Glostrup, Denmark). For secondary antibodies, we used purified rat-adsorbed anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA) and anti-rabbit IgG (1:200; Vectastain ABC kit; Vector Laboratories), respectively (27). α-SMA staining was evaluated as 0 (absent), 1 (mild), 2 (moderate), and 3 (severe). Positive CTGF-stained mesangial cells were counted in at least 10 glomeruli per sample. All the samples were evaluated in a blinded fashion.

Quantification of renal HGF by real-time PCR. RNA extraction and reverse transcription was performed in a total volume of 40 μl as previously described (27). Tissue cDNA-HGF was amplified and quantified by real-time PCR (ABI Prism 7700; Applied Biosystems, Madrid, Spain) using the comparative C_T (Threshold Cycle) method. We validated the method for our pair of amplicons (rat HGF and rat rRNA 18S) (results not shown) to ensure that their amplifying efficiencies were similar. So, the comparative C_T method could be

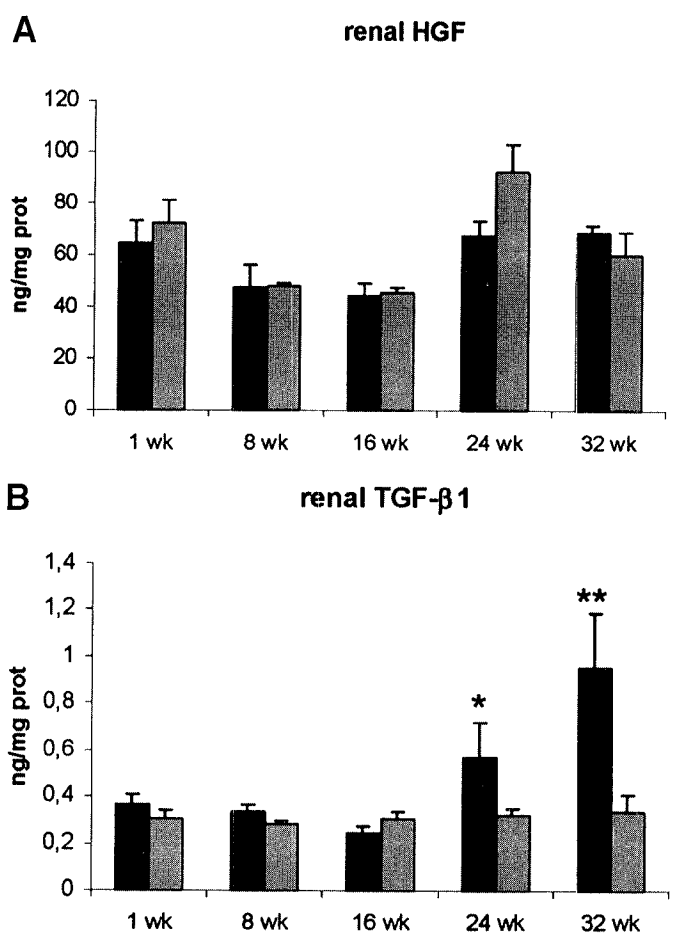

FIG. 1. Sequential analysis of renal HGF and TGF-β1 protein after diabetes induction. There was no significant variation of renal HGF (*A*). Renal TGF-β1 (*B*) increased in diabetic kidneys beyond the 24th week (P* = 0.06), reaching statistical significance with respect to control at 32 weeks (***P* = 0.02). ■, diabetic; ▒, nondiabetic.**

used. Previously, we had optimized the concentration of rat HGF primers and probe. For rat 18S PCR, 2 μl of each cDNA sample was mixed with 2× TaqMan Universal PCR Master Mix (12.5 μl) + 20× Target Primers and Probe (1.25 μl) in a total reaction volume of 25 μl. For rat HGF-PCR, 2 μl of each cDNA sample was mixed with primers and probe in a total reaction volume of 25 μl to reach a final concentration of 900 nmol/l for both forward and reverse primers and 200 nmol/l for the probe. Amplification was performed as previously described (27). Values of normal kidneys were pooled and used as the reference value. Results were expressed as "many fold of the unknown sample" with respect to the reference value.

Statistical analysis. All data are presented as means ± SE. A Student's *t* test or ANOVA for parametric values and Mann-Whitney *U* test or Kruskal-Wallis for nonparametric values were used to compare group means. All *P* values were two tailed, and a *P* value <0.05 was considered statistically significant.

RESULTS

Sequential analysis of renal HGF and TGF-β1 protein after diabetes induction. We measured renal content of rat HGF and TGF-β1 protein at 1, 8, 16, 24, and 32 weeks after induction of diabetes and at the same time points in age-matched nondiabetic controls. There was no significant variation of renal HGF but beyond the 24th week, diabetic kidneys overexpressed TGF-β1, which reached statistical significance with respect to control at 32 weeks (Fig. 1). Thus, by the 24th week, the balance between renal HGF and TGF-β1 shifted to TGF-β1.

hHGF gene therapy: pharmacokinetic and pharmacodynamic profile. We measured circulating hHGF because it is the final product of our therapy. As Fig. 2 illustrates, peak levels were reached at 20 days (5 days after the second injection). We also measured endogenous rat HGF protein in plasma and kidney (Figs. 3 and 4*A*). Basal plasma rat HGF at 16 weeks was slightly higher in diabetic animals (D1 and D1-HGF) than in controls (ND-1), but this finding vanished at 32 weeks. The content of HGF in the kidney was similar in diabetic and nondiabetic animals (Figs. 1*A* and 4*A*), but it was aiming at lower levels in early diabetic nephropathy ($P = 0.07$, D1 vs. ND-1). hHGF gene therapy induced overexpression of endogenous plasma (Fig. 3) and renal rat HGF (Fig. 4*A*). This positive feedback occurred in the kidney at the gene transcription level since rat HGF-mRNA increased significantly after hHGF gene therapy (Fig. 4*B*).

Effect of hHGF gene therapy on early diabetic nephropathy in rats. Sixteen-week diabetic rats had higher diuresis and lower body weight than nondiabetic rats. There were no differences between D1 and D1-HGF in glycemic control, body weight, and diuresis, either before hHGF gene therapy or after 30-day follow-up (Table 1), suggesting that HGF did not improve control of diabetes and did not induce excess weight gain. Functionally, early diabetic nephropathy was characterized by increasing creatinine clearance and microalbuminuria (Table 1). Before HGF administration, creatinine clearance and microalbuminuria were similar in D1 and D1-HGF. hHGF gene therapy did not modify functional abnormalities observed in early diabetic nephropathy after 30 days of treatment. Pathologically, early diabetic nephropathy was characterized by renal and glomerular hypertrophy (higher kidney weight and MGV), whereas mesangial expansion and glomeruloesclerosis were negligible (Tables 1 and 2). Again, hHGF gene therapy did not modify these pathologic abnormalities (Tables 1 and 2). Actually, kidney weight and MGV were similar in D1 and D1-HGF after the 30-day follow-up. In early diabetic nephropathy, there was upregulation of renal TIMP-1, but interstitial myofibroblast staining, renal TGF-β1, and mesangial CTGF staining were similar to ND1. hHGF gene therapy reduced TIMP-1 without modifying α-SMA, TGF-β1, or CTGF (Table 2).

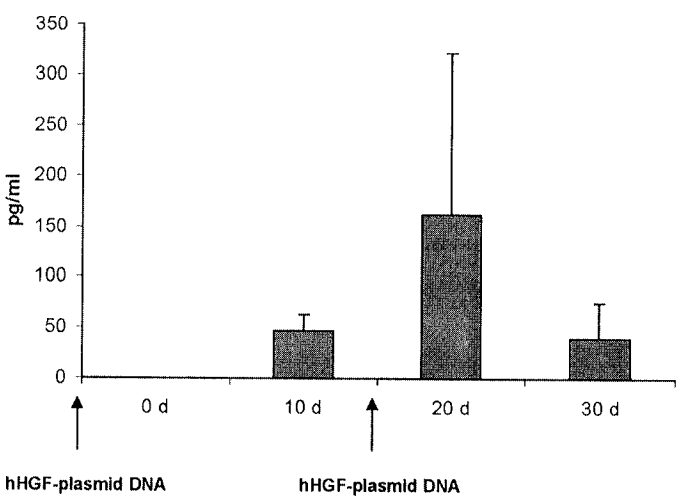

FIG. 2. Plasma hHGF in diabetic-treated rats. Intramuscular injection of hHGF plasmid DNA combined with electroporation was performed twice on days 0 and 15 (*arrows*). hHGF peak levels were reached 5 days after the second dose.

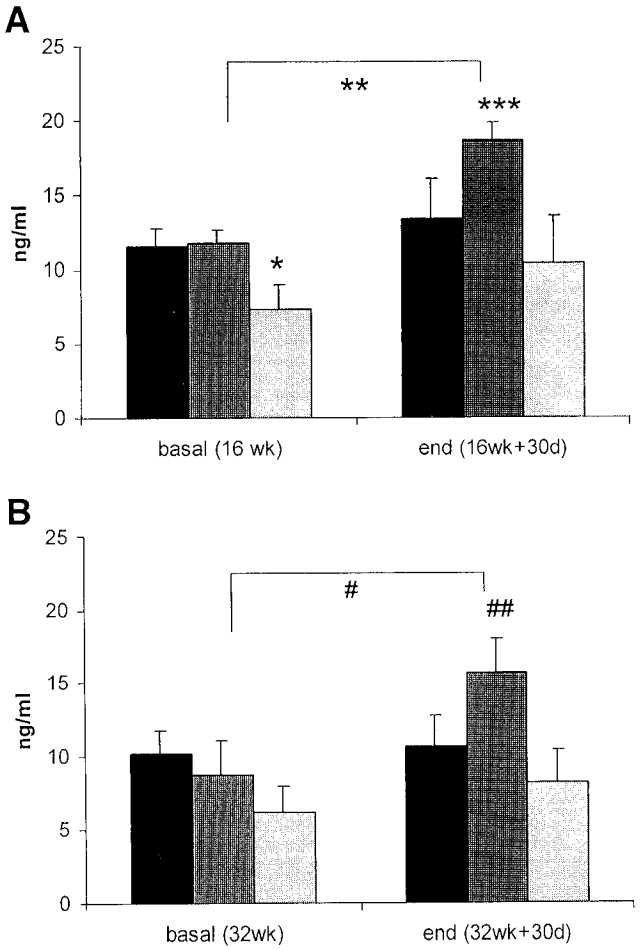

FIG. 3. Plasma rat HGF in early and advanced diabetic nephropathy: the effect of hHGF gene therapy. *A*: In early diabetic nephropathy (16 weeks after diabetes induction), diabetic animals showed higher rat HGF than controls (*P < 0.05, basal ND-1 versus basal D1 and D1-HGF). hHGF gene therapy induced overexpression of circulating rat HGF (P = 0.01, basal D1-HGF versus end D1-HGF; ***P < 0.05, end D1-HGF versus end D1 and ND-1). ■, D1; ▨, D1-HGF; ▩, ND-1. *B*: In advanced diabetic nephropathy (32 weeks after diabetes induction), diabetic and nondiabetic animals showed similar rat HGF. hHGF gene therapy induced overexpression of circulating rat HGF (#P = 0.01, basal D2-HGF versus end D2-HGF; ##P < 0.05, end D2-HGF versus end D2 and ND-2). ■, D2; ▨ D2-HGF; ▩ ND-2.**

Effect of hHGF gene therapy on advanced diabetic nephropathy in rats. Thirty-two-week diabetic rats had higher diuresis and lower body weight than nondiabetic rats (Table 1). There were no differences between D2 and HGF-D2 in glycemic control, body weight, and diuresis, suggesting again that in our experiment HGF did not improve control of diabetes and did not induce excess weight gain.

As is shown in Table 1, advanced diabetic nephropathy is characterized by substantial and progressive albuminuria, whereas creatinine clearance became similar to ND2 and trended to decline. Functional data were similar between D2 and D2-HGF before treatment. hHGF gene therapy reduced albuminuria and preserved creatinine clearance (Table 1). Pathologic findings were mesangial expansion, collagen deposition, and glomerulosclerosis (Fig. 5). D2 kidney weight and MGV became similar to ND2, probably due to the age-related renal hypertrophy in rats (Tables 1 and 2). Remarkably, hHGF gene therapy reversed the mesangial expansion and glomerulosclerosis that characterized advanced diabetic nephropathy (P < 0.05, D2-HGF vs. 32 weeks diabetes and D2) (Fig. 5 and Table 2). We also measured renal interstitial surface as a marker of tubulointerstitial injury in diabetic nephropathy. We observed that renal interstitial fibrosis was nearly absent after 36 weeks diabetes induction. However, there was upregulation of interstitial myofibroblasts staining (Fig. 6 and Table 2), thus suggesting that mechanisms for developing interstitial fibrosis had been initiated at this stage. Renal TIMP-1 remained elevated. In contrast to early diabetic nephropathy, there was upregulation of renal TGF-β1 (P = 0.04 D2 vs. ND2) (Table 2), interstitial α-SMA, and glomerular CTGF (Table 2 and Fig. 6). hHGF gene therapy induced notable reduction of renal TGF-β1 (P = 0.03 D2 vs. D2-HGF) and mesangial CTGF, as well as renal TIMP-1 and interstitial α-SMA (Table 2 and Fig. 6).

DISCUSSION

We assessed the changes in renal HGF and TGF-β1 to provide further insights into the natural history of those growth factors in experimental diabetic nephropathy. Our findings showed that renal content of HGF ran in parallel in diabetic and nondiabetic animals, whereas TGF-β1 gradually increased only in diabetic animals. In the pro-

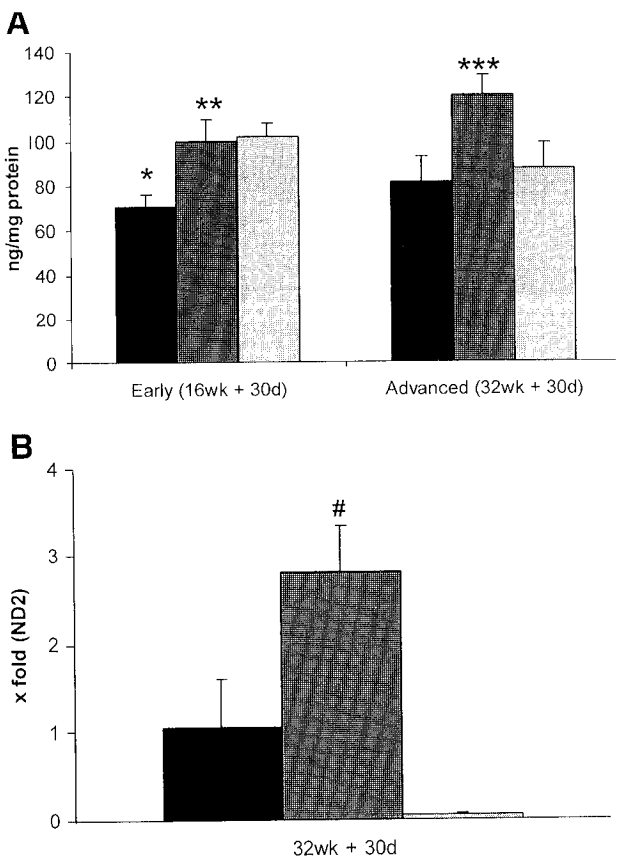

FIG. 4. Kidney rat HGF in early and advanced diabetic nephropathy and rat HGF mRNA in advanced diabetic nephropathy: the effect of hHGF gene therapy. *A*: At the early stage, diabetic kidneys trended to show lower renal rat HGF than controls (*P = 0.07, D1 versus ND-1). HGF gene therapy induced overexpression of renal rat HGF in both early (P = 0.02, D1-HGF versus D1) and advanced (***P = 0.01, D2-HGF versus D2; P = 0.05, D2-HGF versus ND-2) diabetic nephropathy. ■, D; ▨, D-HGF; ▩, ND. *B*: There was induction of kidney rat mRNA HGF after hHGF gene therapy (#P = 0.04, D2-HGF versus D2). ■, D2; ▨, D2-HGF; ▩, ND-2.**

TABLE 1
Physiological, functional, and kidney weight determinations in early and advanced diabetic nephropathy: influence of hHGF gene therapy

	Early diabetic nephropathy				Advanced diabetic nephropathy			
	D1	D1-HGF	ND-1	*P*	D2	D2-HGF	ND2	*P*
Basal (before treatment)								
Body weight (g)	354 ± 13	338 ± 10	543 ± 66*	0.02	404 ± 18	408 ± 27	678 ± 44†	<0.001
Diuresis (ml/d)	82 ± 15	82 ± 13	15 ± 3*	0.008	83 ± 14	101 ± 14	18 ± 4†	0.002
Glycemia (mg/dl)	391 ± 43	379 ± 35	60 ± 5*	<0.001	361 ± 43	369 ± 55	60 ± 5†	<0.001
Albuminuria (μg/mg creatinine)	140 ± 34	114 ± 27	6 ± 3*	0.02	211 ± 41	193 ± 43	13 ± 6†	0.03
Creatinine clearance (ml · min^{-1} · 100 g body wt $^{-1}$)	397 ± 13	421 ± 10	311 ± 27*	0.007	396 ± 33	398 ± 13	317 ± 16†	0.04
After 30-day follow-up								
Body weight (g)	375 ± 6	353 ± 9	574 ± 69*	0.02	410 ± 17	414 ± 32	716 ± 56†	<0.001
Diuresis (ml/d)	75 ± 11	87 ± 11	17 ± 3*	0.001	62 ± 9	84 ± 13	21 ± 3†	0.006
Glycemia (mg/dl)	392 ± 31	385 ± 22	63 ± 5*	<0.001	392 ± 21	394 ± 44	63 ± 5†	<0.001
Albuminuria (μg/mg creatinine)	139 ± 29	112 ± 32	10 ± 12*	0.03	412 ± 89‡,§	145 ± 39§	20 ± 5	0.006
Creatinine clearance (ml · min^{-1} · 100 g body wt $^{-1}$)	387 ± 10	387 ± 14	309 ± 10*	0.04	331 ± 13§	387 ± 22‖	293 ± 21	0.01
Kidney weight (g)	3.2 ± 0.1	2.9 ± 0.1	2.5 ± 0.3¶	0.04	3.9 ± 0.2	4.1 ± 0.2	3.6 ± 0.1	NS

Data are means ± SE. *$P < 0.05$, ND-1 vs. D1- and D1-HGF; †$P < 0.05$, ND-2 vs. D2 and D2-HGF; ‡$P < 0.05$, D-2 vs. ND2 and D2-HGF; §$P < 0.05$, basal vs. after 30 days; ‖$P < 0.05$, D2-HGF vs. ND2; ¶$P < 0.05$, ND-1 vs. D1.

gression of renal fibrosis there is clear evidence that HGF decreases in a reciprocal manner to the increase of TGF-β1 (7,8,14,28), independent of the primary insult. It is already known that the decrease of HGF is related to the destruction of tubular epithelial cells (14), so it is not surprising that the renal synthesis of HGF remained unchanged. In fact, tubulointerstitial damage was not already apparent since we illustrated by morphometrical quantification of the interstitial area in advanced diabetic nephropathy. Nevertheless, the balance between HGF and TGF-β1 during the evolution of diabetic nephropathy changed in a similar way to other chronic nephropathies (14,19,29), mainly due to the gradual TGF-β1 upregulation. Thus, HGF supplementation at supraphysiological levels (3,30) may exert a therapeutic action in diabetic nephropathy. On the other hand, transgenic mice overexpressing HGF displayed tubular cystic disease, glomerulosclerosis, renal disease (31), and increased incidence of tumors (32), which is probably related to the extremely high systemic levels of HGF. Therefore, as suggested by Vargas et al. (33), the window between HGF-induced organ regeneration and disease induction must be narrow.

Administration of exogenous HGF has provided therapeutic benefit in chronic nephropathies (17,19,34,35). Despite this, its clinical application is controversial because HGF is extremely unstable in blood circulation, with a half-life of 3–5 min (36). Intramuscular gene transfer may help to overcome this limitation (37,38). As we recently reported (21), intramuscular injection of SP1017-formulated hHGF plasmid followed by electroporation induces supraphysiological serum levels of HGF in rats. The delivery of HGF into the blood stream is maintained for 2 weeks, providing a feasible, efficient, and well-tolerated therapeutic strategy. We administered hHGF gene therapy twice, on day 0 and 15, to encompass a period of time long enough to observe a therapeutic effect. Likewise, we assessed the pharmacokinetic and pharmacodynamic effect of hHGF gene therapy. After gene therapy, diabetic rats had circulating hHGF during the whole follow-up, with a peak at 5 days after the second dose, thus confirm-

TABLE 2
Quantification of MGV, interstitial area, renal content of TIMP-1, interstitial α-SMA, TGF-β1, and CTGF: determinations in early (16-week) and advanced (32-week) diabetic nephropathy, the influence of hHGF gene therapy

	Early diabetic nephropathy					Advanced diabetic nephropathy				
	16-week diabetes	D1	D1-HGF	ND-1	*P*	32-week diabetes	D2	D2-HGF	ND2	*P*
MGV ($\mu^3 \times 10^6$)	4.2 ± 0.4	4.6 ± 0.4	4.5 ± 0.3	3.3 ± 0.2*	0.03	4.7 ± 0.5	4.9 ± 0.3	4.4 ± 0.2	4.3 ± 0.4	NS
Glomerulosclerosis (%)	—	—	—	—	—	42 ± 1.7	51.6 ± 6.7†	30.3 ± 4.4‡	23.9 ± 4.4	0.006
Mesangial expansion (0, 4)	0.50 ± 0.2	0.57 ± 0.2	0.50 ± 0.3	0.4 ± 0.2	NS	2.4 ± 0.24	3.0 ± 0.44†	1.3 ± 0.30‡	0.6 ± 0.24	<0.001
Interstitial area (%)	—	—	—	—	—	6.0 ± 0.3	6.1 ± 0.5	6.0 ± 0.4	5.7 ± 0.3	NS
TIMP-1 (pg/mg protein)		1.1 ± 0.1§	0.59 ± 0.1	0.57 ± 0.1	0.02	—	1.82 ± 0.3†	1.28 ± 0.1	1.23 ± 0.1	0.04
α-SMA (0, 3)	—	0.6 ± 0.2	0.5 ± 0.3	0.3 ± 0.2	NS	—	2.3 ± 0.3†	0.7 ± 0.2	0.4 ± 0.3	<0.001
TGF-β1 (ng/mg protein)	0.57 ± 0.1	0.31 ± 0.04	0.35 ± 0.02	0.27 ± 0.02	NS	0.95 ± 0.24	0.67 ± 0.1†	0.28 ± 0.1‡	0.35 ± 0.02	<0.001
CTGF (+ cell × glomeruli)	—	3.5 ± 0.2	3.7 ± 1.0	3.7 ± 0.7	NS	—	13.4 ± 0.5†	7.2 ± 0.5	9.7 ± 0.6‖	<0.001

Data are means ± SE. *$P < 0.05$, ND-1 vs. D1, 16-week diabetes, and D1-HGF; †$P < 0.05$, D-2 vs. ND2 and D2-HGF; ‡$P < 0.05$, D2-HGF vs. 32-week diabetes; §$P = 0.02$, D1 vs. D1-HGF and ND1; ‖$P = 0.003$, ND2 vs. D2-HGF.

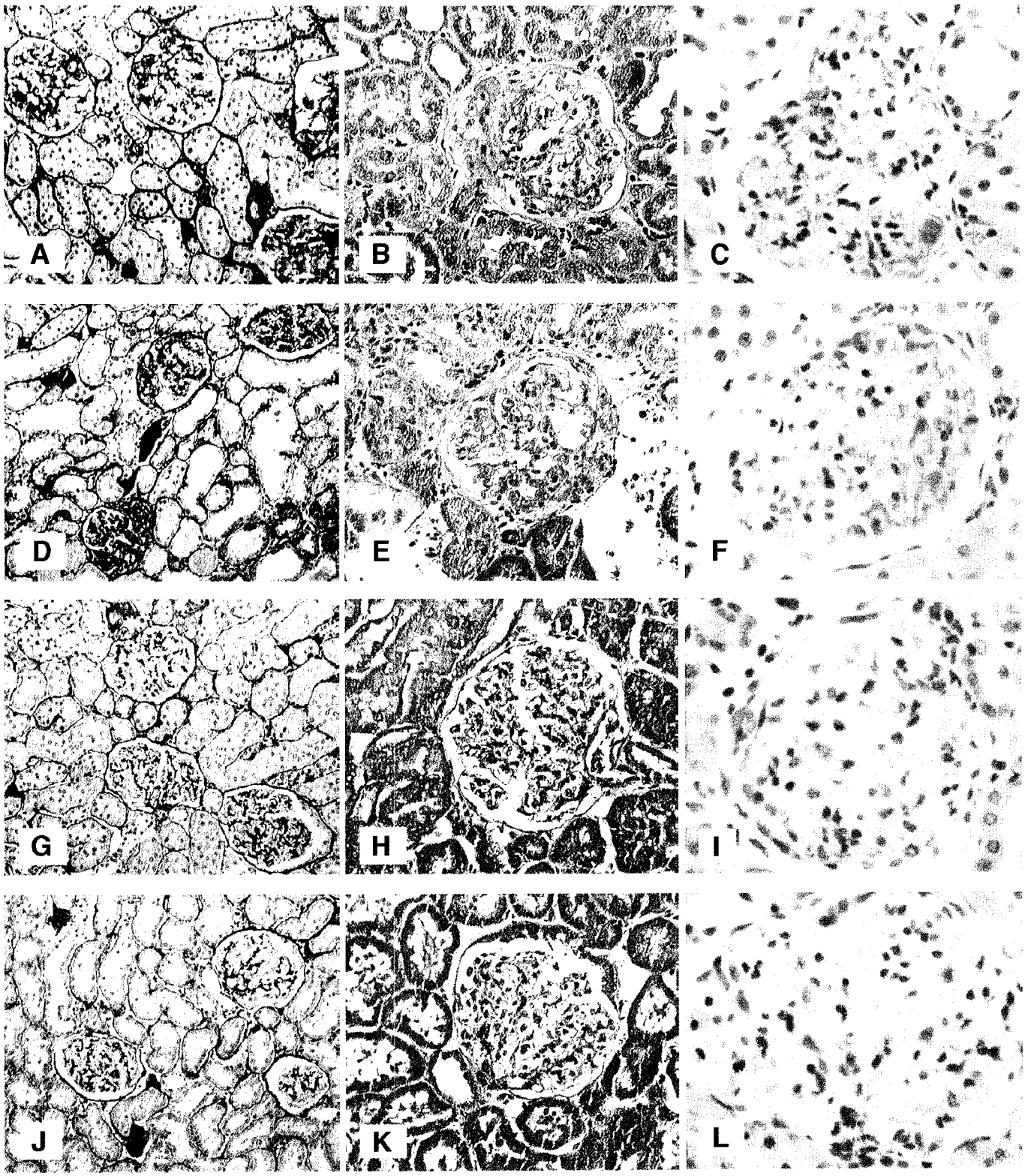

FIG. 5. Effect of hHGF gene therapy on glomerular lesions in advanced diabetic nephropathy. Sections were stained with silver methenamine (*A*, *D*, *G*, and *J*, magnification ×200) and Masson's trichrome (*B*, *E*, *H*, and *K*, magnification ×400) and immunostained for collagen IV (*C*, *F*, *I*, and *L*, magnification ×630). Diabetic animals killed at 32 weeks (*A–C*) as well as D2 (*D–F*) showed prominent glomerulosclerosis, mesangial expansion, and collagen deposition with respect to ND-2 (*J–L*). hHGF gene therapy (*G–I*) clearly reduced the glomerulosclerosis, mesangial expansion, and collagen deposition observed in advanced diabetic nephropathy. See also Table 2.

ing the value of the two electrotransfer schema. Pharmacodynamic monitoring was carried out by measuring endogenous HGF because it is well known that hHGF induces endogenous rat HGF (18). We found that hHGF gene therapy induced overexpression of circulating and renal endogenous HGF. Furthermore, induction of HGF in the diabetic kidney was at the transcription gene level because we observed enhancement of rat HGF mRNA. So,

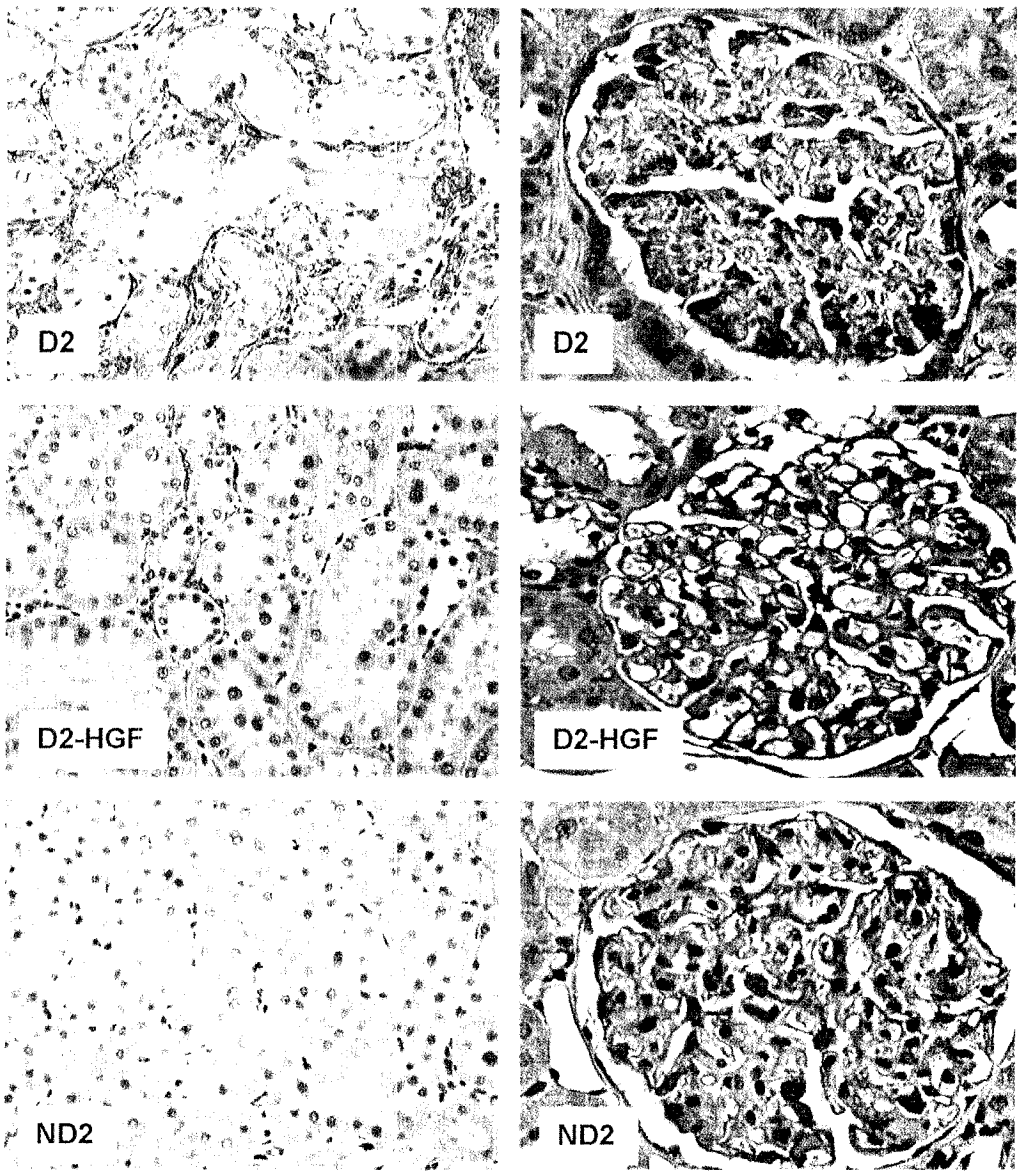

FIG. 6. Kidney rat interstitial myofibroblast and glomerular CTGF staining in advanced diabetic nephropathy: the effect of hHGF gene therapy. There was an enhanced interstitial α-SMA staining in diabetic kidneys (magnification ×400). There were an increased number of CTGF-positive mesangial cells in incompletely sclerosed diabetic glomeruli (magnification ×630). hHGF gene therapy reduced the number of CTGF-positive mesangial cells. See also Table 2.

the potential benefit of hIIGF gene therapy may be by either the circulating hHGF and/or the induction of endogenous rat HGF into the damaged diabetic kidney.

Glomerular hypertrophy and basement membrane thickening are the earliest pathologic alterations in diabetic nephropathy (39,40). In a recent review, Mason and Wahab (2) proposed that hyperglycemia, advanced glycation end products, and angiotensin II generate reactive oxygen species and stimulate TGF-β1, which in turn upregulates CTGF, also induced by high glucose. All of these factors upregulate the transcription of matrix genes and repress those of matrix degradation leading to glomerulosclerosis. There are some therapeutic approaches to provide renoprotection in diabetes. ACE inhibitors and angiotensin II receptor antagonists are clinically used as renoprotective agents (41,42). These drugs reverse diabetic nephropathy in the very early stages and slow (but do not avoid) progression of renal damage in established diabetic nephropathy. As a consequence, the development of novel therapeutic antifibrotic strategies is needed to reverse advanced diabetic nephropathy (43).

hHGF gene therapy arises as one of those new therapeutic approaches to ameliorate diabetic nephropathy. It was previously reported (33) that HGF supplementation has regenerative properties on several models of cell damage. Dai et al. (44) recently showed that HGF prevents β-cells from destructive depletion and promotes their proliferation in STZ-induced diabetic mice, thus mitigating hyperglycemia. On the other hand, our study on hHGF gene therapy was begun several months after induction of diabetes, when β-cell mass was completely destroyed. Thus, the therapeutic benefit of HGF on diabetic nephropathy did not depend on improving glycemic control.

Notably, hHGF gene therapy resulted in a clear benefit in advanced diabetic nephropathy rather than in early stages, suggesting that HGF exerted its therapeutic action

when TGF-β1 was upregulated. Several stimuli of gene transcription for ECM are present in diabetic nephropathy, and hyperglycemia is the main factor that initiates these renal changes (2,45). However, TGF-β1 and CTGF are crucial for further induction of ECM genes (2). It has been reported (46) that TGF-β1 expression increased in STZ-diabetic rats 24 h after the onset of hyperglycemia, but it was transient. The sustained renal elevated expression of TGF-β1 occurred after 24 weeks. Since induction of CTGF depends on TGF-β1 (2,20), there was no mesangial CTGF upregulation in early diabetic nephropathy. According to these findings, mesangial expansion was not present at this time. hHGF gene therapy did not modify the functional and pathologic alterations that define nephropathy at this time, namely microalbuminuria and glomerular hypertrophy. However, it reduced the renal expression of TIMP-1, inactivating a potential mechanism of glomerular ECM accumulation in diabetes.

Physiological turnover of mesangial matrix proteins in diabetic nephropathy is compromised by decreased expression of metalloproteinase (MMP) genes and increased expression of endogenous MMP inhibitors, such as TIMP-1. The suppressive effect of HGF on TIMP-1 and other renal matrix degradation pathways was previously investigated by Liu et al. (47). These authors demonstrated in vitro that HGF enhanced MMP and decreased TIMP-1 as well. Discordant results were reported by other authors (13) in SV-40–transformed mouse mesangial cells and rabbit tubular epithelial cells. They found that HGF increased fibronectin and collagen α1 (IV) in a concentration-dependent manner. However, HGF had no additional effect on glomerular fibronectin expression and renal histology in 5-month-old lean and obese db mice. Our results, using a model of type 1 diabetes, were partly similar to those reported by Laping et al. (13) since hHGF gene therapy administered 16 weeks after induction of diabetes did not modify renal histology.

We found a consistent therapeutic effect in advanced diabetic nephropathy wherein upregulation of TGF-β1 and glomerular CTGF had actually happened. Mesangial expansion, ECM accumulation, and glomerulosclerosis were observed at this late stage. Scarce tubulointerstitial lesions were found, although mechanisms responsible for interstitial fibrosis were triggered, as was demonstrated by the enhanced staining for interstitial myofibroblasts. In this way, Wang et al. (4) suggested that CTGF acts downstream of TGF-β1 and contributes to chronic interstitial fibrosis in diabetic nephropathy. Our results showed that hHGF gene therapy administered on advanced diabetic nephropathy normalized renal TGF-β1 and mesangial expression of CTGF and reduced renal TIMP-1. All of these changes resulted in a dramatic regression of mesangial expansion and glomerulosclerosis, which ultimately reduced albuminuria and preserved renal function. Furthermore, hHGF gene therapy inhibited accumulation of myofibroblasts in the interstitium, thus blocking a key event in the development of interstitial fibrosis in diabetic nephropathy.

In conclusion, hHGF gene therapy was effective to treat late stages of diabetic nephropathy in the diabetic rat when prominent mesangial expansion and ECM accumulation is observed. So, HGF emerges as an innovative approach to revert advanced diabetic nephropathy.

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