Hepatocyte growth factor-induced amelioration in renal interstitial fibrosis is associated with reduced expression of α-smooth muscle actin and transforming growth factor-β1

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Received 06 April 2011; revised 27 August 2011

Several studies have shown that hepatocyte growth factor (HGF) ameliorates renal interstitial fibrosis, but the mechanism is not fully clear. This study was designed to examine whether HGF can relieve renal interstitial injury in 5/6 nephrectomized rats, and to confirm whether this function was associated with decrease in α-smooth muscle actin (α-SMA) and transforming growth factor-beta1 (TGF-β1) expression. The animals were randomized into 8 groups comprising 6 animals (n = 6) each: control (group I), PCI-neo (group II, 900 µg), Sham-operation (group III, no nephrectomy), model or 5/6 nephrectomy group (group IV), lotensin group (an angiotensin converting enzyme inhibitor, group V, 0.6 mg/100 g/day for 5 weeks), low-dose PCI-neo-HGF group (group VI, 690 µg), high-dose PCI-neo-HGF group (group VII, 1380 µg) and lotensin + high-dose PCI-neo-HGF group (group VIII, 0.6 mg/100 g/day for 5 weeks, 1380 µg). The animals were sacrificed in the 5th week after 5/6 nephrectomy. The specimens of kidneys were used for pathological examination (hematoxylin-eosin staining), detection of α-SMA and TGF-β1 mRNA (Reverse transcriptase-polymerase chain reaction) and protein (Western blot and immunohistochemistry) expression. The results showed that in 5/6 nephrectomized rats blood urea nitrogen (BUN), serum creatinine (CRE) and 24 h urinary albumin excretion (UAE) were increased, renal interstitium was injured seriously and α-SMA, TGF-β1 mRNA and protein expression were elevated compared with those of control. The above changes were ameliorated and α-SMA and TGF-β1 expression was reduced by both PCI-neo-HGF and lotensin. The lotensin + high-dose PCI-neo-HGF group rats exhibited the most significant therapeutic effect both in decreasing the BUN, CRE and 24 h UAE and in relieving renal interstitial injury. In conclusion, the study demonstrated that HGF can relieve renal interstitial injury and this protection was associated with down-regulation of α-SMA and TGF-β1 expressions.

Keywords: PCI-neo-hepatocyte growth factor; 5/6 Nephrectomized rats; Transforming growth factor-β1; α-Smooth muscle actin

Renal interstitial injury plays an important role in the progression of chronic renal failure (CRF). Hepatocyte growth factor (HGF) is a pleiotropic cytokine which has multiple biological functions, such as promoting caryomitosis, accelerating cell locomotion and antiapoptosis. It has renoprotective effects in many animal models, such as acute renal failure and diabetic nephropathy models. It can also restrain renal interstitial fibrosis. Up-regulation of TGF-β1 is one of the key factors responsible for the increased fibrotic changes and the decrease in its expression slows the progression of renal interstitial fibrosis.

In renal interstitial fibrosis, tubular epithelial cells (TEC) are activated and differentiated into myofibroblasts which is called tubular epithelial-myofibroblast transdifferentiation (TEMT). HGF might inhibit TGF-β1 function and prevent the transdifferentiation of renal TEC. α-Smooth muscle actin (α-SMA) is a characteristic protein of smooth

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Abbreviations: ACEI, angiotensin converting enzyme inhibitors; ALB, plasma albumin; Ang, angiotensin; BUN, blood urea nitrogen; CRE, serum creatinine; CRF, chronic renal failure; ECM, extracellular matrix; HGB, hemoglobin; HGF, hepatocyte growth factor; RAS, renin-angiotensin system; α-SMA, α-smooth muscle actin; TEC, tubular epithelial cells; TEMT, transforming growth tubular epithelial-myofibroblast transdifferentiation; TGF-β1, transforming growth factor-beta 1; UPE, urinary protein excretion; VSMC, vascular smooth muscle cell.
muscle cells and myofibroblasts and its high expression in kidney has been suggested as the sign of TEMT \(^8\). In addition, TGF-\(\beta\) has been shown to activate glomerular mesangial cells, leading to accumulation of extracellular matrix (ECM) and glomerulosclerosis \(^9,10\).

The 5/6 nephrectomized rat is a common model in the studies of renal diseases. After 5/6 nephrectomy, number of nephrons are reduced, causing glomerular high infusion, high pressure and high filtration, leading to tubular atrophy, glomerular sclerosis and renal interstitial fibrosis. These changes resemble the pathological progression of renal fibrosis in human \(^11,12\).

Lotensin, one of angiotensin converting enzyme inhibitors (ACEI) confers substantial renal benefits in patients with advanced renal insufficiency, in particular in the patients having increased urinary albumin excretion (UAE) \(^13,14\). At present, lotensin is being used to treat renal diseases with satisfactory curative effects.

In this study, we examined whether HGF can improve renal function, lessen urinary protein excretion and relieve renal interstitial injury in the chronic interstitial fibrosis and also assessed whether the above functions of HGF are associated with decrease in \(\alpha\)-SMA and TGF-\(\beta\)1 mRNA and protein expression. We have used lotensin as a positive control and investigated its synergetic effect with HGF on renal interstitial injury. As the half-life of HGF is short, it is difficult to make it continuously available in the blood \(^15\), therefore, we have utilized PCI-neo plasmid carrier which can express stably \(^16\).

Materials and Methods

Materials

PCI-neo (a mammalian expression plasmid without any gene) was obtained from Promega Co. (USA). PCI-neo-HGF (a mammalian expression plasmid with HGF gene) was a gift from Prof NU Jun-qi of the First Hospital of Jilin University. TGF-\(\beta\)1, \(\alpha\)-SMA and \(\beta\)-actin antibodies were obtained from Boster Biology Technique Co. (Wuhan, China).

Animals and experimental protocol

All animal experiments described in this article were conducted in accordance with ‘Animal Centre of Jilin University Guide for the Care and Use of Laboratory Animals’. Clean male rats weighting 210 to 250 g were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the study period.

\(E.\ coli\) JM109 of PCI-neo-HGF and PCI-neo were amplified and the plasmids were prepared with the kit (V-gene Biotechnology Ltd. Co.). Plasmids and aluminum hydroxide gel were blended (ratio, 1:4) in advance. The animals were randomized into 8 groups comprising 6 animals (\(n = 6\)) each: Group I, control; group II, PCI-neo; group III, sham-operated; group IV, model (5/6 nephrectomy); group V, lotensin; group VI, low-dose PCI-neo-HGF; group VII, high-dose PCI-neo-HGF; and group VIII, lotensin + high-dose PCI-neo-HGF. We performed two surgeries where rats were anesthetized with 2% nembutal by intraperitoneal injection. In the first surgery, the upper pole and under pole (1/3 kidney, respectively) of the left kidney were cut-off. The 2\(^{nd}\) surgery was done on the 7\(^{th}\) day after the first operation and the right kidney was extirpated. These surgeries were done in groups IV-VIII rats. The group III rats were operated only by opening the skin to expose kidneys and closing the skin, and the kidneys were not cut out. PCI-neo-HGF was given to groups VI (230 \(\mu\)g per week), VII (460 \(\mu\)g per week) and VIII (460 \(\mu\)g per week) rats by intramuscular injection (inner muscles of right hind) three-times on the 2\(^{nd}\), 3\(^{rd}\) and 4\(^{th}\) week after the 2\(^{nd}\) surgery. Group II rats were treated with PCI-neo (300 \(\mu\)g per week) at the same time. Lotensin (0.6 mg/100g/day) was given to groups V and VIII rats in drinking water (20 ml) from the day after the 2\(^{nd}\) surgery and continued for 5 weeks.

Rats were put separately in clean metabolic cages to collected 24 h urine before sacrificing them. The animals were sacrificed in the 5\(^{th}\) week after the 2\(^{nd}\) surgery. Blood samples were collected during execution. A part of left kidney’s remnant was preserved in liquid nitrogen and the rest of left kidney’s remnant was fastened in 10% formalin for the later experiments.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using with RNA-STAT60 reagent according to the manufacturer's instructions. cDNA was synthesized with 1 \(\mu\)g of RNA using murine leukemia virus reverse transcriptase and random hexamers. RNA was similarly reverse transcribed. The RT-PCR was performed as described previously \(^17\). The RT-PCR products were separated by electrophoresis using 1% agarose gels and DNA
band intensities were quantified using Quantitation One software (Hercules, USA) and normalized to β-actin. The primers used are summarized in Table 1.

**Western blot analysis**

Isolated renal tissue samples were lysed in a sodium dodecyl sulfate (SDS) sample buffer [2% SDS, 10 mmol/L Tris-HCl, pH 6.8, 10% (vol/vol) glycerol]. The lysates were centrifuged at 12,000 × g for 15 min at 4°C and the supernatants were stored at -70°C. Protein concentrations were determined using a Bio-Rad kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Fifty micrograms of protein were loaded per lane, separated in a 10% SDS-PAGE gel and transferred on to a nitrocellulose membrane and immunoblotted with antibodies to α-SMA and TGF-β1 (1:1000). After washing, the membranes were incubated for 1 h with a horseradish peroxidase-linked anti-rabbit secondary antibody (1:2000) and immunoreactive proteins were detected by Supersignal chemiluminescence reagent. The blots were stripped and then reprobed with an antibody to β-actin (1:5000). The immunoblots were scanned using a densitometer and protein bands were quantified with Quantitation One software (Bio-Rad Laboratories, Hercules, CA).

**Histology (hematoxylin-eosin staining) and immunohistochemistry**

The renal tissues were fixed in 10% formalin, embedded in paraffin, cut into 2 µm thick slices and stained with hematoxylin-eosin (HE) by standard method for histological examination. We observed 10 no-repeat renal tubulointerstitial regions randomly in each specimen under light microscope (×100) and detected the area of renal tubular atrophy, interstitial inflammation and interstitial fibrosis and then graded: 1 point, none; 2 points, injury area <25%; 3 points, injury area between 25 to 49%; and 4 points, injury area >50%. All histological examinations were performed by the experienced renal pathologist.

Renal tissues for immunohistochemical staining were fixed in 10% neutral buffered formalin and paraffin embedded by standard technique and 5 micron sections were cut. TGF-β1 and α-SMA staining was performed using a commercial kit (Dako Corporation, Carpinteria, CA, USA) according to the manufacturer's instructions and as described previously. The sections were examined with a Leica DMRE light microscope. We observed 2 specimens per sample under light microscope (×200) and graded according to the intensity of staining and total stained area: 0 point, none or intensity of staining was lower; 1 point, stained areas <25% or intensity of staining was low; 2 points, stained area between 25 to 49% or intensity of staining was high; 3 points, stained area between 50 to 75% or intensity of staining was higher; and 4 points, stained areas >75% or intensity of staining was the highest. The sections were examined by the experienced renal pathologist.

**Statistical analysis**

Results were expressed as the mean ± standard error (SE). Factor analysis of variance was used to assess the differences between multiple groups. SPSS13.0 was used in data analysis. A p-value <0.05 was used as the criterion for a statistically significant difference.

**Results**

**Biochemical parameters**

Table 2 shows the data of clinical parameters in the 5th week after the second surgery, including red blood cell (RBC), hemoglobin (HGB), plasma albumin (ALB), BUN, CRE and 24 h UAE. No significant differences were observed for all the above parameters in the groups I, II and III. Compared with group I, BUN, CRE and 24 h UAE levels were significantly higher in group IV rats. Compared with

<table>
<thead>
<tr>
<th>Table 1—Primer sequences and extending conditions</th>
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<tr>
<td><strong>Target</strong></td>
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<td>α-SMA Sense</td>
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<td>β-Actin Sense</td>
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group IV, BUN, CRE and 24 h UAE levels were lower in the groups V to VIII rats. The levels in the group VIII were the lowest among them. There was statistical difference between groups V and VIII.

In case of ALB, compared with group I, the ALB level in group IV rats was significantly decreased, while in groups V to VIII level was higher than group IV rats; the level in group VIII was the highest. There was no statistical difference between groups V and VIII.

**Histology**

Hematoxylin-eosin (H-E) staining showed that renal glomeruli, tubule and interstitium were normal in groups I, II and III rats. In group IV rats, interstitial areas had big focal lesions and there was excessive accumulation of fibrous tissue and tubular atrophy. Group V rats had small renal interstitial focal lesions and the pathological changes were less severe than group IV rats; the level in group VIII was the highest. There was no statistical difference between groups V and VIII.

**Table 2**—Effect of PCI-neo-HGF on RBC, HGB, ALB, BUN, CRE and UAE

<table>
<thead>
<tr>
<th>Groups</th>
<th>RBC (× 10^9/L)</th>
<th>HGB (g/L)</th>
<th>ALB (g/L)</th>
<th>BUN (mmol/L)</th>
<th>CRE (umol/L)</th>
<th>UAE (mg/d)</th>
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<tr>
<td>I</td>
<td>6.37 ± 0.90</td>
<td>117.25 ± 9.34</td>
<td>34.00 ± 3.02</td>
<td>8.28 ± 1.45</td>
<td>35.75 ± 3.35</td>
<td>5.25 ± 1.95</td>
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<tr>
<td>II</td>
<td>6.32 ± 0.38</td>
<td>116.50 ± 5.97</td>
<td>35.60 ± 3.78</td>
<td>8.10 ± 1.20</td>
<td>33.93 ± 3.62</td>
<td>5.79 ± 2.14</td>
</tr>
<tr>
<td>III</td>
<td>6.51 ± 0.80</td>
<td>115.00 ± 10.13</td>
<td>35.25 ± 3.04</td>
<td>8.19 ± 1.81</td>
<td>36.95 ± 2.74</td>
<td>5.55 ± 2.22</td>
</tr>
<tr>
<td>IV</td>
<td>5.84 ± 0.38</td>
<td>113.08 ± 4.32</td>
<td>27.90 ± 2.36 $^a$</td>
<td>13.53 ± 3.67 $^a$</td>
<td>57.95 ± 6.70 $^a$</td>
<td>16.17 ± 2.57 $^a$</td>
</tr>
<tr>
<td>V</td>
<td>5.90 ± 0.68</td>
<td>114.75 ± 9.88</td>
<td>29.17 ± 3.03</td>
<td>11.20 ± 2.16 $^*$</td>
<td>52.78 ± 5.97 $^*$</td>
<td>11.35 ± 2.08 $**$</td>
</tr>
<tr>
<td>VI</td>
<td>6.51 ± 0.42</td>
<td>110.51 ± 6.95</td>
<td>28.13 ± 3.62</td>
<td>12.29 ± 2.44</td>
<td>55.10 ± 6.64</td>
<td>13.16 ± 2.78 $^*$</td>
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<tr>
<td>VII</td>
<td>6.14 ± 0.70</td>
<td>114.25 ± 8.22</td>
<td>30.85 ± 2.89 $^*$</td>
<td>10.77 ± 1.74 $^*$</td>
<td>53.24 ± 4.89 $^*$</td>
<td>12.24 ± 2.34 $^*$</td>
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<tr>
<td>VIII</td>
<td>6.50 ± 0.54</td>
<td>113.50 ± 6.86</td>
<td>32.05 ± 2.88 $^*$</td>
<td>8.94 ± 1.41 $^*$</td>
<td>44.23 ± 3.89 $^*$</td>
<td>9.85 ± 1.98 $^*$</td>
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</table>

Group I, control group; Group II, PCI-neo group; Group III, sham-operation group; Group IV, model (5/6 nephrectomy) group; Group V, lotensin group; Group VI, low-dose PCI-neo-HGF group; Group VII, high-dose PCI-neo-HGF group; Group VIII, lotensin + high-dose PCI-neo-HGF group. $^a$$P<0.05$ vs. Group I; $^*$$P<0.05$, $**$$P<0.01$ vs. Group IV; $^#$$P<0.05$ vs. Group V. RBC, red blood cell; HGB, hemoglobin; ALB, plasma-albumin; BUN, blood urea nitrogen; CRE, serum creatinine; UAE, 24 h urinary albumin excretion.

Expression of $\alpha$-SMA mRNA and protein

No statistical differences were observed in $\alpha$-SMA mRNA expression in the groups I, II and III rats, indicating that PCI-neo and sham-operation had no influence on expression of $\alpha$-SMA. The $\alpha$-SMA mRNA expression in group IV rats increased compared to group I, while in groups V to VIII decreased compared to group IV rats; the expression was weakest in group VIII rats. There was significant difference between groups V and VIII (Fig. 1). The trend of $\alpha$-SMA protein expression was in accordance with that of mRNA expression (Fig. 3).

Immunohistochemical analysis for $\alpha$-SMA protein

The major expression of $\alpha$-SMA was in smooth muscles near tubule and renal interstitium in the injured section. The $\alpha$-SMA expression in group IV rats was the strongest. Among the groups V to VIII, the expression in group VIII rats was the weakest (Fig. 4).

Expression of TGF-β1 mRNA and protein

There were also no statistical differences in TGF-β1 mRNA expression in groups I, II and III rats. The TGF-β1 mRNA expression in group IV rats was elevated compared with group IV, while in groups V to VIII was lower compared with group IV rats; the expression was weakest in
Fig. 1—Effect of PCI-neo-HGF (A) on pathology (H-E staining, 200X) and (B) on injury grade of renal tubulointerstitium [Group I, control; Group II, PCI-neo group; Group III, sham-operation group; Group IV, model (5/6 nephrectomy); Group V, lotensin group; Group VI, low-dose PCI-neo-HGF group; Group VII, high-dose PCI-neo-HGF group; and Group VIII, lotensin + high-dose PCI-neo-HGF group. *P<0.05 vs. Group I; *P<0.05 vs. Group IV; $P<0.05 vs. Group V. Excessive accumulation of fibrous tissue (a): tubular atrophy (b): interstitial inflammation (c): vacuolar degeneration of TEC (d)].

Fig. 2—Effect of PCI-neo-HGF on α-SMA mRNA expression [(A): A representative RT-PCR; and (B) Values of densitometric readings of α-SMA mRNA corrected for β-actin mRNA. Groups were same as indicated in Fig. 1. #P<0.05 vs. Group I; *P<0.05 vs. Group IV; $P<0.05 vs. Group V].

Fig. 3—Effect of PCI-neo-HGF on α-SMA protein expression [(A): A representative Western blot; and (B) Values of densitometric readings of α-SMA protein corrected for β-actin protein. Groups were same as indicated in Fig. 1. #P<0.05 vs. Group I; *P<0.05 vs. Group IV; $P<0.05 vs. Group V].

Fig. 4—Effect of PCI-neo-HGF on α-SMA protein expression by immunohistochemistry [(A) α-SMA expression (200X); and (B) Quantification of α-SMA expression. α-SMA expression in Group IV rats was the strongest and in Group VIII rats the weakest. Groups were same as indicated in Fig. 1. #P<0.05 vs. Group I; *P<0.05 vs. Group IV; $P<0.05 vs. Group V].
There was statistical difference between groups V and VIII (Fig. 5). The trend of TGF-β1 protein expression was also in accordance with that of mRNA expression (Fig. 6).

**Immunohistochemical analysis for TGF-β1 protein**

The major expression of TGF-β1 was in TEC and renal interstitium in the injured section. The TGF-β1 expression in group IV rats was the strongest. Among the groups V to VIII, the expression was weakest in group VIII rats (Fig. 7).

**Discussion**

It has been shown that HGF represses renal interstitial fibrosis. HGF and TGF-β1 are in dynamic balance in the normal tissues, but the balance is lost in the morbid tissues and if the balance is in favor of HGF, it helps in repair of damaged tissues, otherwise, the tissues develop fibrous changes. As for the relationship between HGF and α-SMA, it has been observed that HGF ameliorates TEMT, while high expression of α-SMA in kidney has been shown as the sign of TEMT.
Our study showed that BUN, CRE and 24 h UAE were increased, while ALB was decreased in the 5th week after the second surgery in the 5/6 nephrectomized rats. H-E staining showed big focal lesion in interstitial region and interstitial inflammation, excessive accumulation of fibrous tissue, tubular atrophy and vacuolar degeneration of TEC. The levels of BUN and CRE indicated that the renal function of the 5/6 nephrectomized rats group was in the azotemia stage, which was consistent with the previous report. Compared with 5/6 nephrectomized rats, BUN, CRE and 24 h UAE were decreased and ALB was increased in the PCI-neo-HGF-treated groups. The interstitial inflammation, deposition of fibrous tissue and tubular atrophy were all alleviated in the PCI-neo-HGF-treated groups. The decreasing of BUN, CRE and 24 h UAE and the relieving renal interstitial injury were more significant in high-dose PCI-neo-HGF group than low-dose group. In lotensin-treated rats, there was also improvement both in decreasing of BUN, CRE and 24 h UAE and in relieving renal interstitial injury compared to model group. Lotensin plus high-dose PCI-neo-HGF group exhibited the most significant therapeutic effect both in decreasing of BUN, CRE and 24 h UAE and in relieving renal interstitial injury.

Further, we noted that the mRNA and protein expression of α-SMA and TGF-β1 were increased in the 5th week after 5/6 nephrectomy which were in accordance with the biochemical and pathological changes, while PCI-neo-HGF and lotensin reduced mRNA and protein expression of α-SMA and TGF-β1. The inhibition for α-SMA and TGF-β1 expression reached to the best level when lotensin and high-dose PCI-neo-HGF were used together, which was also in accordance with the biochemical and pathological changes. Thus, HGF could relieve renal interstitial injury and this protection was associated with down-regulation of α-SMA and TGF-β1 expression.

Angiotensin (Ang) II as a part of renin-angiotensin system (RAS) plays an important role in several of renal diseases. Ang II-induced renal injury is mediated by its systemic effect through blood pressure regulation and/or by its regulatory effect on TGF-β1 levels. Experimental animal and in vitro studies have demonstrated that ACE inhibitors may also decrease the synthesis and secretion of renal TGF-β1. HGF and Ang II are in the dynamic balance and in vascular smooth muscle cell (VSMC) Ang II could restrain production of HGF in dose-dependent manner. Lotensin is an ACE inhibitor which can inhibit Ang II production. The renal protection by lotensin is well known. It is used in the clinical treatment actively and decreases UPE and stabilizes renal function in the early stage of CRF. Reno-protective effects of lotensin are reported to be due to the up-regulation of HGF and downregulation of TGF-β1. Thus, in this study, the synergistic effect of lotensin and HGF could be due to upregulatory effect lotensin on HGF expression and its concurrent downregulatory effect on TGF-β1 expression. In addition, blood pressure lowering effect of lotensin could also be contributing to the synergistic reno-protection.

In conclusion, the study demonstrated that PCI-neo-HGF and lotensin when used together was more effective for the protection of kidney.

Acknowledgement
The study was supported by Jilin Provincial Department of Health (No. 2009ZC041).

References
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