Systemic administration of naked plasmid encoding HGF attenuates puromycin aminonucleoside-induced damage of murine glomerular podocytes

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Am J Physiol Renal Physiol 301: F784–F792, 2011. First published July 20, 2011; doi:10.1152/ajprenal.00210.2011.—Podocyte injury is considered to play important roles in the pathogenesis of human glomerular disease. There is accumulating evidence suggesting that hepatocyte growth factor (HGF) elicits preventive activity for glomerular cells in animal models of chronic renal diseases. In this study, we demonstrated that delivery of a naked plasmid vector encoding the human HGF gene into mice by a hydrodynamic-based in vivo gene transfection approach markedly reduced proteinuria and attenuated podocyte injury in a mouse model induced by puromycin aminonucleoside (PAN) injection. Systemic administration by rapid injection via the tail vein of a naked plasmid containing HGF cDNA driven under a cytomegalovirus promoter (pCMV-HGF) produced a remarkable level of human HGF protein in the circulation. Tissue distribution studies suggested that the kidney expressed a high level of the HGF transgene. Meanwhile, compared with tubules and interstitium, a higher level of exogenous HGF protein was detected in theglomeruli. Administration of pCMV-HGF dramatically abated the urinary albumin excretion and podocyte injury in PAN nephropathy in mice. Exogenous expression of HGF produced evidently beneficial effects, leading to restoration of Wilms’ tumor-1 (WT1) and α-actinin-4 expression and attenuation of ultrastructural damage of the podocytes. In vitro, HGF not only restored WT1 and α-actinin-4 expression but also inhibited albumin leakage of podocytes incubated with PAN in a Transwell culture chamber. These results suggest that HGF might provide a novel strategy for amelioration of podocyte injury.

hepatocyte growth factor; proteinuria; puromycin aminonucleoside

THE GLOMERULAR CAPILLARY WALL comprises a tripartite structure including podocytes (epithelial cells) on the urinary aspect, fenestrated glomerular endothelial cells on the luminal aspect, and the highly negatively charged glomerular basement membrane (GBM) between. Numerous evidence suggests that podocyte injury is a common feature of many forms of renal disease, including minimal-change disease, focal and segmental glomerulosclerosis (FSGS), IgA nephropathy, and diabetic nephropathy (DN), which is largely due to podocyte’s detachment, and apoptosis in response to injurious stimuli, which depends on the severity and duration of the injury (5, 14, 19, 23, 24, 26). Due to its limited proliferate capacity, the detachment from GBM or apoptosis of the podocyte could inevitably impair the structural integrity of the glomerulus, which could consequently lead to altered glomerular filtration and production of proteinuria. Injection of puromycin aminonucleoside (PAN) into mice produces severe proteinuria, which mimics the lesions of minimal-change disease (9, 17, 28, 37). It is known that PAN specifically targets podocytes by eliminating their foot processes, deforming the actin cytoskeleton, and abating expression and distribution of slit diaphragm proteins such as nephrin and podocin. All these destructive effects resemble a unique model of podocyte injury (17).

Despite tremendous efforts having been made to unravel the pathogenesis of podocyte diseases during the past decades, no effective treatment is currently available, and the proteinuria remains. In light of the fact that certain renotropic growth factors exhibit capacities to promote recovery of renal functions, it is reasonably conceivable that these factors might be candidates as therapeutic agents for podocyte injury and proteinuria.

Hepatocyte growth factor (HGF) was originally characterized as a potent mitogen for hepatocytes. It was then indicated to exert mitogenic, motogenic, tubulogenic, and antiapoptotic activities in a wide range of target cells, including renal tubular epithelial cells. Its pleiotropic properties render HGF specifically suited for promoting tissue repair, regeneration, and recovery after injury. Previous studies have demonstrated that HGF is rapidly upregulated both in blood circulation and in multiple organs, where it leads to acceleration of renal regeneration after renal injury (6, 27, 31, 33, 34).

To address the question of whether HGF has protective roles in podocytes, we applied a classic approach of systemic administration of a naked HGF plasmid vector, through which to introduce exogenous HGF gene expression and evaluate the protective efficacy of HGF on PAN-induced podocytopathy. In addition, we performed an in vitro study using immortalized podocytes to confirm the direct protective effects of HGF on podocyte injuries.

MATERIALS AND METHODS

Cell culture and treatment. Mice podocytes were acquired from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. To propagate podocytes, cells were cultured at 33°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY) and 10 U/ml mouse recombinant interferon-γ (R&D Systems, Minneapolis, MN) to enhance the expression of a thermosensitive T antigen. To induce differentiation, podo-
cytes were grown under nonpermissive conditions at 37°C in the absence of interferon-γ for 14 days. Podocytes were treated under differentiating conditions. For PAN (Sigma) treatment, podocytes were seeded at 80% confluence in a complete medium containing 10% fetal bovine serum. Twenty-four hours later, the cells were changed to a serum-free medium and incubated for 16 h. Thereafter, PAN was added at different concentrations for various periods of time as indicated. The cells were then collected at different time points for further characterization.

Animals. Male BALB/c mice, weighing 21–23 g, were obtained from the Shanghai Experimental Animal Center. They were housed in the animal facilities of the Kidney Disease Center of the 2nd Affiliated Hospital of Nanjing Medical University, with free access to food and water. Animals were treated humanely using approved protocols in accordance with the guidelines of the Institutional Animal Care and Use Committee of National Institutes of Health at Nanjing Medical University. PAN nephrosis was induced by a single intravenous injection of PAN at 250 mg/kg body wt dissolved in 150 mM sodium bicarbonate (vehicle). The control group was injected with vehicle only in an identical manner.

Plasmid injection. The recombinant human HGF expression plasmid (pCMV-HGF) that contains full-length human HGF cDNA driven under a human cytomegalovirus promoter was kindly provided by Dr. Youhua Liu (Univ. of Pittsburgh, Pittsburgh, PA). The empty expression plasmid vector pcDNA3 was purchased from Invitrogen. Plasmid DNA was administered into mice by a hydrodynamic-based gene transfer technique via rapid injection of a large volume of DNA solution through the tail vein. Briefly, 20 μg of plasmid DNA was diluted in 1.6 ml of saline and injected via the tail vein into the circulation within 5–10 s. Mice from the control group were injected with 20 μg of empty plasmid vector pcDNA3 in 1.6 ml of saline in an identical fashion. Mice were randomly divided into three groups: 1) normal control group; 2) PAN/control group, in which mice were preadministered empty pcDNA3 plasmid 24 h before PAN injection; and 3) PAN/preadministration of pCMV-HGF plasmid group, in which mice were preadministered pCMV-HGF plasmid 24 h before PAN injection. Six mice from each group were euthanized at different time points as indicated after injection of PAN, except the normal control group, in which six mice were euthanized at day 1 only. Serum was collected at the time of death. One part of the kidney was processed for tissue histology. Another part was snap-frozen in Tissue-Tek O.C.T. compound for subsequent cryosectioning. The remaining kidney was immediately frozen in liquid nitrogen and stored at −80°C for tissue lysate preparation.

Determination of HGF levels by ELISA. For measurement of serum and tissue HGF level, collected serum or the kidney was homogenized in the HGF extraction buffer containing 20 mM Tris-HCl (pH 7.5), 2 M NaCl, 0.1% Tween 80, 1 mM EDTA, and 1 mM PMSF, as described elsewhere (33). After centrifugation at 19,000 g for 20 min at 4°C, the supernatant was recovered for determination of HGF by use of the ELISA method. This assay uses a sandwich method that consists of three steps of antigen antibody reactions. Briefly, the 96-well microtiter plates were incubated with 50 μl of uncoupled monoclonal anti-HGF antibody (H14)/well diluted in 50 mM Tris-HCl (pH 8.0) at a final concentration of 1.5 μg/ml at room temperature for 16 h. H14 anti-human HGF antibody was prepared by use of a standard protocol of hybridoma technology. This antibody could detect human HGF protein but not rodent HGF in an ELISA or in immunohistochemical staining. The plates were washed three times with PBS that contained 0.05% Tween 20 (pH 7.4) and were blocked with 200 μl of blocker solution (PBS with 1% bovine serum albumin) at room temperature for 2 h. The plates were extensively washed five times with PBS containing 0.05% Tween 20 and kept at 4°C. Fifty-microliter aliquots of standard human HGF solution or tissue samples were added to the wells and incubated for 2 h at room temperature. After extensive washing, a 100-μl aliquot of biotinylated goat anti-human HGF polyclonal antibody (R&D Systems) at a dilution of 1:2,000 was added, and the plates were incubated for another 2 h. After being washed, they were then incubated with 10 μl of horse radish peroxidase-conjugated streptavidin (Zymed Laboratories) at a dilution of 1:20,000 and subsequently with an enzyme substrate solution that contained 0.1 mg/ml of tetramethylbenzidine and 0.006% H2O2 in 0.1 M sodium citrate (pH 6.0). The plates were allowed to stand for 30 min at room temperature, and the reaction was stopped by addition of 50 μl 4N H2SO4. Absorbance was read at 405 nm in an automatic Emax Precision Microplate Reader (Molecular Devices). Samples were diluted if necessary to give an optical density that was within the linear portion of the standard curve. Total protein levels were determined by use of a bicinconinic-acid protein assay kit (Sigma) with BSA as a standard. The concentration of HGF in kidneys was expressed as nanograms per milligram total protein.

Western immunoblot analysis. For analysis of human HGF transgenic expression, total kidney extracts as described above for ELISA were used for Western immunoblotting. For detection of other proteins, kidney tissues were homogenized with a polytron homogenizer (Brinkmann) in RIPA lysis buffer (1% NP-40, 0.1% SDS, 100 μg/ml PMSF, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml antipain, and 2 μg/ml leupeptin in PBS) on ice. The supernatants were collected after centrifugation at 13,000 g at 4°C for 20 min. Protein concentration was determined by use of a bicinconinic-acid protein assay kit (Sigma), and tissue lysates were mixed with an equal amount of 2× SDS loading buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, and 0.2% bromophenol blue), as described elsewhere (36). Samples were heated at 100°C for 5–10 min before loading and were separated on precast 10 or 15% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA). The proteins were electrophoresed to a nitrocellulose membrane (Amersham, Arlington Heights, IL) in transfer buffer that contained 48 mM Tris-HCl, 39 mM glycine, 0.037% SDS, and 20% methanol at 4°C for 1 h. Nonspecific binding to the membrane was blocked for 1 h at room temperature with 5% Carnation nonfat dry milk in TBS buffer (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20). The membranes were then incubated for 16 h at 4°C with various primary antibodies in blocking buffer that contained 5% milk. Monoclonal anti-human HGF antibody (H14) was kindly provided by Dr. Youhua Liu. The Wilms’ tumour-1 (WT1) and α-actinin-4 antibody were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). After extensive washing three times, the membranes were then incubated with horse radish peroxidase-conjugated secondary antibody (Bio-Rad) for 1 h at room temperature in 1% nonfat dry milk. The signals were visualized with the enhanced chemiluminescence system (Amersham). Quantitation was performed by measuring the intensity of the hybridization signals with the aid of National Institutes of Health Image analysis software.

Frozen sectioning and HGF staining. Cryosections were prepared at 5 μm by routine procedures and fixed in cold methanol for 10 min at −20°C. Immunostaining of human HGF protein was performed by use of a Vector mouse on mouse (M.O.M.) immunodetection kit, according to the protocol specified by the manufacturer (Vector Laboratories, Burlingame, CA). Briefly, cryosections were first incubated with M.O.M. blocking reagent for 1 h at room temperature to block endogenous mouse immunoglobulins in the tissue and to reduce background staining. After being washed with PBS, sections were incubated with primary anti-HGF monoclonal antibody (H14) at a 1:50 dilution in PBS containing 1% BSA overnight at 4°C. Sections were then incubated for 1 h with secondary anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:200 in PBS containing 1% BSA. After being thoroughly rinsed with PBS, sections were mounted with antifade mounting media (Vector Laboratories) and examined under a Nikon (Melville, NY) Eclipse E600 Epi-fluorescence microscope equipped with a digital camera. The negative control sections were processed in exactly the same way as described above, except the primary antibody was omitted.

AJP-Renal Physiol. • VOL 301 • OCTOBER 2011 • www.ajprenal.org
Histology and immunohistochemical staining. For light microscopy, kidney tissue was fixed in formalin and embedded in paraffin. Tissue sections were stained with hematoxylin/eosin for histopathological examination. For electron microscopy, the kidney tissue was fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) and postfixed with 1% osmium tetroxide. Fixed tissue blocks were then dehydrated and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined under a transmission electron microscope. Immunohistochemical staining was performed by use of a Vector M.O.M. immunodetection kit, as described above (Vector Laboratories). The primary antibody used was anti-syntrophin (Santa Cruz Biochemicals). As a negative control, the primary antibody was replaced with nonimmune normal IgG.

RT-PCR analysis. Total RNA was prepared using a TRIzol RNA isolation system according to the instructions specified by the manufacturer (Invitrogen). The first strand of cDNA was synthesized using 1 µg of RNA in 20 µl of reaction buffer using Moloney leukemia virus-RT (Promega, Madison, WI) and random primers at 42°C for 30 min. PCR was performed using a standard PCR kit on 1 µl aliquots of cDNA and HotStarTaq polymerase (Promega) with specific primer pairs. The sequences of primer pairs were as follows: WT1 (forward 5′-GGC ATG CTA GGC TAG GAA AAG-3′ and (reverse) 5′-GAG AGT CAG ACT TAA CAG T-3′; and β-actin (forward 5′-CAT CTC AGA GGA AAA TCG TG-3′ and (reverse) 5′-CGT TGC CAA TAG TGA TCA CC-3′). The PCR products were size fractionated on a 1.0% agarose gel and detected by NA-green (D0133, Beyotime) staining.

Immunofluorescence staining. Indirect immunofluorescence staining was performed using an established procedure (36). Briefly, cells cultured on coverslips were washed twice with cold PBS and fixed with cold methanol/acetone (1:1) for 10 min at −20°C. Following three extensive washings with PBS, the cells were blocked with 0.1% Triton X-100 and 2% normal donkey serum in PBS buffer for 40 min at room temperature and then incubated with the specific primary anti-F-actin antibody (Santa Cruz Biochemicals), followed by staining with TRITC-conjugated secondary antibody. Kidney cryosections at 5-µm thickness were prepared and fixed in cold methanol/acetone (1:1) for 10 min. After being blocked with 20% normal donkey serum in PBS for 40 min, the sections were incubated with anti-WT1 or anti-α-actinin-4. As a negative control, the primary antibody was replaced with nonimmune IgG. Cells and the kidney cryosections were double stained with 4,6-diamidino-2-phenylindole to visualize the nuclei. Slides were viewed with a Nikon Eclipse 80i Epi-fluorescence microscope equipped with a digital camera (DS-Ri, Nikon). Immunofluorescence images were captured under identical light exposure times and aperture settings.

Albumin influx assay. A simple albumin influx assay was adapted to evaluate the filtration barrier function of podocyte monolayers, as described previously (13). Briefly, podocytes (5 × 104) were seeded onto the collagen-coated Transwell filters (3-µm thickness) and cultured under differentiating conditions. After 10 days, podocytes were serum-starved overnight and treated as indicated for 48 h. Cells were washed twice with PBS supplemented with 1 mM MgCl2 and 1 mM CaCl2 to preserve the cadherin-based junctions. The top chamber was then refilled with 0.15 ml of RPMI 1640 and the bottom chamber with 1 ml of RPMI 1640 supplemented with 40 mg/ml of bovine serum albumin and incubated at 37°C. A small aliquot of media from the top chamber was collected at different time points, and the albumin concentration was determined using a bicinchoninic acid protein assay kit (Sigma).

Statistical analysis. Animals were randomly assigned to control and treatment groups (with 6 mice/group). Computed data are expressed as means ± SE. Each experiment was repeated three times independently. Statistical analysis was performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA). Comparisons between groups were made using one-way ANOVA, followed by a t-test. P < 0.05 was considered significant.

RESULTS

Renal expression of exogenous HGF after systemic administration of naked plasmid vector. Systemic administration of pCMV-HGF produced a substantial level of exogenous HGF protein in the serum and kidneys. Quantitation of human HGF protein by a specific ELISA revealed ~4 ng of exogenous HGF/ml of serum at day 1 after a single injection of 20 µg of pCMV-HGF/mouse (Fig. 1A). ELISA analysis also revealed a significant increase in exogenous HGF per milligram of total tissue protein in the kidneys at hour 3 after a single injection of 20 µg of pCMV-HGF/mouse. Under the same conditions, exogenous HGF was undetectable in the kidneys at any given time points when mice were injected with empty pcDNA3 plasmid in an identical manner (Fig. 1A). The expression of the exogenous HGF transgene in the kidneys was sustained for at least 3 days. Although the renal human HGF level tended to decline thereafter, a significant level of exogenous HGF (~8 ng/mg protein) was still detected in the kidneys at day 7 after the initial delivery of the human HGF gene (Fig. 1B), which suggests that systemic administration of a naked plasmid vector provides a highly efficient way to deliver exogenous HGF protein to the kidneys in vivo.

The mature form of HGF is a heterodimeric glycoprotein, consisting of a 69-kDa α-chain and a 34-kDa β-chain held together by a disulfide bond (15). The robust expression of exogenous HGF in the kidneys after intravenous injection of a naked plasmid was independently confirmed by Western blot analyses of whole-kidney lysates using a monoclonal anti-human HGF antibody (provided by Dr. Youhua Liu). As shown in Fig. 1C, human HGF protein in the kidneys after administration of the naked plasmid vector was detected as an ~90 kDa band under nonreducing conditions. Direct injection of pCMV-HGF plasmid yielded a strong band in the Western blot of whole-kidney lysates at day 3.

Immunofluorescence staining revealed that HGF transgene expression was largely limited to renal glomeruli in the kidneys (Fig. 1E). More than 80% of glomeruli were positively stained for human HGF protein, whereas tubular epithelium was essentially negative for human HGF transgene expression. As expected, no staining was observed in the kidneys from mice that received the empty vector pcDNA3 injection (Fig. 1D).

Expression of exogenous HGF gene prevents PAN-induced albuminuria in mice. Administration of PAN in mice induced temporal podocyte injury characterized by albuminuria. Figure 2 shows the excretion levels of albuminuria of 24 h, an indicative marker for podocyte dysfunctions, at different time points after mice were preadministered either empty pcDNA3 or pCMV-HGF plasmid 24 h before PAN injection. Mice that received the empty pcDNA3 vector displayed a rapid and marked increase in urine albumin excretion as early as 1 day after injection of PAN. Urine albumin excretion levels remained significantly elevated continuously for up to 3 days and started to decline toward the baseline level thereafter (Fig. 2). This dynamic pattern of urine albumin excretion levels manifests the course of altered podocyte functions typically seen in this model of temporal renal injury. In contrast, single preadministration of pCMV-HGF plasmid dramatically prevented PAN-
caused podocyte dysfunctions. Urine albumin excretion remained relatively constant at the baseline levels during the entire experimental scheme (Fig. 2), which indicates that pre-administration of the exogenous HGF gene essentially precludes temporal podocyte injury induced by PAN.

HGF gene expression protects kidney morphology integrity and prevents podocyte foot process effacement in PAN nephrosis mice. Concomitant with rapidly increased urine albumin excretion levels, cellular morphology of the kidney underwent changes during PAN-induced podocyte injury. Figure 3, A–C, shows a representative micrograph of the kidney’s general morphology, while Fig. 3, D–F, shows a representative electronic micrograph of cellular morphology of the kidney typically seen at day 3 after the injection of PAN. Compared with the sham-operated group (Fig. 3D), the PAN injection group exhibited typical foot process effacement (Fig. 3E). Preadministration of the HGF plasmid largely prevented the morphological injuries after podocyte dysfunction caused by PAN. The morphology of these kidneys was almost indistinguishable from that of normal controls, which suggests that HGF gene therapy did preserve the cellular integrity of kidney and prevented foot process effacement.

Exogenous HGF attenuates synaptopodin protein loss caused by PAN-induced podocyte injury. Synaptopodin is an actin-associated protein expressed in renal podocytes and appears to play a role in actin-based plasticity. Synaptopodin localizes to the foot processes of podocytes. It is absent in the sclerotic glomeruli in idiopathic nephrotic syndrome. We next examined the effects of expression of HGF transgene on the expression of synaptopodin in the PAN-injured kidneys. Figure 4 shows the immunohistochemistry staining of synaptopodin in the kidneys 3 days after intravenous injections of PAN with either PCMV-HGF or empty plasmid vector pretreatment. Podocyte injury, characterized by loss expression of foot process marker synaptopodin, occurred in the early stage after PAN injection (Fig. 4B). Under the same conditions, expression of the HGF transgene significantly attenuated these injuries in the kidneys (Fig. 4C).

Exogenous HGF restores expression and distribution of WT1 and α-actinin-4 in PAN nephrosis mice. The WT1 gene (WT1) is expressed in the podocyte throughout life. Reduced expression levels of WT1 result in either crescentic glomerulonephritis or mesangial sclerosis. α-Actinin-4 is involved in the adhesion of cells to the extracellular matrix, which has been shown to link actin to integrin in the plasma membrane through interactions with the vinculin and talin complex or by a direct interaction with integrin. We further examined the expression and deposition of WT1 and α-actinin-4, two markers of podocyte structure and function. Figure 5 shows the results of WT1 in the kidneys of PAN...
nephrosis mice as determined by RT-PCR and indirect immunofluorescence staining. As expected, the expression level of WT1 mRNA was markedly decreased in the kidneys 3 days following PAN injection (Fig. 5A). However, substantial restoration of WT1 expression was evident in the PAN-injured kidneys pretransferred with HGF gene (Fig. 5, A and E). Similar results were seen for renal α-actinin-4 expression (Fig. 6). Both Western blot analyses with whole kidney lysates (Fig. 6A) and immunofluorescence staining (Fig. 6, C–E) exhibited a marked restoration of α-actinin-4 expression in the kidneys 3 days following PAN injection with the HGF gene pretransferred, which is consistent with the notion that exogenous HGF restored the expression and distribution of WT1 and α-actinin-4 in PAN nephrosis mice.

HGF restores expression of WT1 depressed by PAN in podocytes. In an effort to provide mechanistic insight into understanding the actions of HGF on an established podocytopathy, we investigated the effects of HGF on the depression of podocyte marker WT1 expression induced by PAN using an in vitro cell culture system. A scheme was designed to mimic in vivo situations. As shown in Fig. 7, A and B, incubation of podocytes with different amounts of PAN for various time periods induced podocyte injury, as evidenced by depressed WT1 mRNA expression in a PAN dose- and time-dependent manner. Along with this treatment, which is analogous to the onset of podocytopathy in vivo, HGF was added to the cultures with fresh medium in the continuous presence of PAN for 24 (Fig. 7C) and 48 (Fig. 7D) h, respectively. As shown in Fig. 7, C and D, exogenous HGF restores the depression of WT1 mRNA and protein expression in podocytes. These results suggest that HGF effectively prevented the podocyte injury induced by PAN.

HGF protects podocytes against PAN-induced cytoskeleton disruption. F-actin filaments in cultured podocytes were distributed as stress fiber-like bundles along the axis or into the process of cells. PAN caused podocyte cytoskeleton reorganization. As shown in Fig. 8B, treatment with PAN for 48 h resulted in cell retraction and remarkable loss of actin stress-fiber organization. However, when cells were preincubated with HGF before exposure to PAN, podocyte avoided the above changes (Fig. 8D).

HGF attenuates PAN-induced filtration barrier dysfunction of podocytes. To assess the functional consequence of podocyte injury, we examined the filtration barrier function of podocytes by using a paracellular permeability influx assay. As depicted in Fig. 9A, this simple assay measured the albumin flux rate across the differentiated podocyte monolayer. Differentiated podocytes pretreated with or without HGF were incubated with PAN for 48 h to induce podocyte injury and then subjected to an albumin influx assay. As shown in Fig. 9B, compared with the controls, PAN treat-
ment resulted in a greater albumin influx across the podocyte monolayer, while preincubation with HGF significantly attenuated PAN-induced albumin influx. These results indicate that HGF attenuated PAN-induced filtration barrier dysfunction of the podocyte monolayer.

DISCUSSION

Podocyte dysfunction is the hallmark of a range of proteinuric kidney diseases. It has already been proved that podocytes play a major role in the development of proteinuria and progression of glomerulosclerosis (11, 16, 18, 29). Despite the
recognition of the pathogenesis of podocyte diseases, no effective therapeutic strategy is currently available. Hence, a strategy that has the potential to preserve the structure integrity of glomeruli and/or ameliorate the prognosis of podocyte dysfunction would be greatly beneficial to the patients with proteinuria frequently seen in clinical settings.

The purpose of this study was to investigate whether exogenous HGF, which has been proved to be capable of protecting and retarding the onset of renal fibrosis and kidney dysfunction (6, 31–35), also has protective effects on protecting podocyte injuries. Dai and colleagues (4) recently demonstrated that conditioned knockout of HGF receptor c-Met aggravated albuminuria and podocyte injury in adriamycin-induced FSGS-like glomerulopathy. Our results demonstrate that HGF markedly reduced proteinuria and ameliorated podocyte foot process effacement in PAN nephrotic mice. Recovery from podocyte injury was confirmed by efficiently restoration of podocyte-specific proteins WT1 and α-actinin-4 expression, which were downregulated in PAN. In vitro studies further validated that HGF protected PAN-induced cytoskeleton disruption, WT1 depression, as well as filtration barrier dysfunction.

Although the pathological mechanism underlying proteinuric kidney diseases is not yet completely elucidated, clearly podocyte dysfunction plays critical roles in ultimately resulting in the loss of the structural and functional integrity of the kidney (8, 13, 22, 25). Unlike other podocytopathy models, such as diabetic nephropathy, in which it can take months to establish proteinuria, the measurable proteinuria in our study was initiated at a very early stage after PAN treatment in our mouse model, and the urine albumin excretion level was kept significantly elevated for up to 3 days. Thereafter, the level declined toward the baseline level, which manifests the course of altered podocyte functions typically seen in the model of minimal-change renal disease. Single-injection PAN-induced nephrosis is perhaps an exceptionally aggressive and temporal form of podocytopathy. In accordance with this, a 40% depression of WT1 and 50% of α-actinin-4 expression are observed in the injured kidneys at 3 days after PAN injection compared with the sham control group. An earlier study shows that significant foot process enfacement and proteinuria take place in the kidneys as early as 5 days after PAN treatment in rats (37). Thus major hallmarks for podocyte lesions can be shown at day 3 after PAN injection in our mouse model.
In this study, we established a minimal-change disease model induced by PAN, which was morphologically confirmed at both the cellular and ultrastuctural levels by light and electron microscopy. After PAN injection, proteinuria peaked at 3 days in nephrotic mice. During the same period of time, podocyte foot process effacement was the most evident along with remarkable decreased expression and reduction of WT1 and α-actinin-4. The changes inevitably resulted in massive proteinuria manifested in acute podocyte injury. In comparison, at 3 days after HGF administration of PAN to nephrotic mice, it was found that proteinuria was reduced significantly, accompanied by improvement of podocyte foot process effacement and restoration of both WT1 and α-actinin-4 expression. These findings indicated that the antiproteinuric effect of HGF was obviously associated with recovery from podocyte injury.

Because of hydrodynamic and anatomic characteristics, expressed HGF protein is predominantly limited to glomeruli in the kidney, whose structure has the tendency to trap the HGF protein in circulation. Because of the presence of endothelial and GBM, whether the injected HGF protein could directly affect podocyte remains unclear. However, HGF is a secreted protein and previous studies have shown the protective role of HGF in tubular epithelial cells (6, 31, 33–35). In this study, immunofluorescence staining confirmed the presence of human HGF protein, expressed by hepatocytes after plasmid injection, in the glomeruli (Fig. 1E), which also has the potential to induce Erk1/2 phosphorylation (data not shown). It is reasonable to postulate that exogenous HGF mimics an endocrine and paracrine mechanism in podocytes, which needs further investigation. Although the protective efficacy of exogenous HGF on established podocyte injury is discernible in our study, there remains a long road toward clinical administration. HGF stimulates motility and morphogenesis in a wide range of cellular targets during development and tissue regeneration (1, 21). As in this study, HGF incubation might probably affect podocyte morphology and cell-cell junctions, which resulted in increased albumin leakage (Fig. 9). However, compared with the control group, HGF-induced albumin leakage was not significantly increased and preincubation with HGF significantly attenuated PAN induced albumin influx. These results confirm the protective effect of HGF on podocytes. In this study, HGF plasmid was injected before PAN in mice and HGF was incubated before PAN in podocytes. The prevention of podocyte injury by HGF is discernable in this study. However, preadministra-

Fig. 8. HGF protected podocytes against PAN-induced cytoskeleton (F-actin) disruption. (A–D) Immunofluorescence staining is shown for F-actin (red) in podocytes after various treatments. D: control. E: PAN. F: HGF. G: PAN plus HGF. Cell nuclei were stained with DAPI (blue).

Fig. 9. HGF attenuated PAN-induced filtration barrier dysfunction of the podocyte monolayer. A: schematic depiction of the paracellular permeability influx assay. Podocyte monolayer on collagen-coated Transwell filters was incubated without or with PAN plus HGF for 48 h, and albumin permeability across podocyte monolayer was then determined. B: graphic presentation of the albumin influx across the podocyte monolayer. Duration of albumin incubation is shown on x-axis. Values are means ± SE; n = 6. *P < 0.05 vs. control. ‡P < 0.05 vs. PAN group.
tion of HGF seems unsuitable in clinical settings. The therapeutic effect of HGF after the evidence of podocyte injury remains to be determined.

In summary, such remarkable protective efforts of HGF will undoubtedly lead to amelioration of foot process effacement, restoration of podocyte marker expression, and ultimately prevention of albuminuria, thereby limiting podocyte loss and dysfunction in kidneys. In view of the efficacy of HGF in preventing proteinuria and podocyte injury in PAN nephropathy, and the recent study confirming the protective role of HGF in adriamycin-induced nephrosis (4), it is plausible to speculate that a supplement of HGF may be a novel therapeutic strategy for halting the progression of podocyte injury in a clinical setting.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


