Prevention of Acute Ischemic Renal Failure by Targeted Delivery of Growth Factors to the Proximal Tubule in Transgenic Mice: The Efficacy of Parathyroid Hormone-Related Protein and Hepatocyte Growth Factor

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Abstract. Treatment of acute renal failure (ARF) would be enhanced by identification of factors that accelerate renal recovery from injury. Parathyroid hormone–related protein (PTHrP) and hepatocyte growth factor (HGF) have been shown to stimulate proliferation in proximal nephron-derived cells. For studying the pathophysiologic roles and therapeutic potential of these two factors in ARF, transgenic mice overexpressing PTHrP or HGF in the proximal tubule under the direction of the γ-glutamyl transpeptidase-I promoter were developed. These mice display (1) abundant expression of the respective transgenes in the kidney; (2) similar PTH type I receptor and HGF receptor (c-met) expression levels in the proximal tubule compared with control littermates; and (3) normal renal morphology, function, and tubule cell proliferation under basal conditions. However, in contrast to control mice, when acute ischemic renal injury was induced, renal function rapidly and dramatically recovered in HGF-overexpressing mice. In addition, 48 h after ischemia, HGF-overexpressing transgenic mice displayed a fourfold increase in tubule cell proliferation and a threefold decrease in apoptotic tubule cell death compared with control mice. In contrast, PTHrP-overexpressing mice responded to either ischemic or folic acid–induced renal damage similarly to control mice. These studies demonstrate that overexpression of PTHrP in the proximal nephron of mice does not seem to provide protection against acute renal injury. In marked contrast, HGF overexpression results in dramatic protection from ischemia-induced ARF, without inducing any apparent alteration in the physiology of the kidney under normal conditions. These studies suggest that HGF, when targeted specifically to the proximal tubule, may have therapeutic potential in providing protection against ischemia-induced renal failure.
CELERATES RECOVERY FROM ACUTE RENAL INJURY IN RODENTS BY ENHANCING TUBULAR EPITHELIAL CELL PROLIFERATION AND PROTECTION AGAINST RENAL CELL DEATH (9, 10, 27). HOWEVER, THE EFFECTS OF SYSTEMIC HGF ADMINISTRATION ON OTHER PERIPHERAL TISSUES, MANY OF WHICH EXPRESS c-MET (28), ARE UNKNOWN. USING THE METALLOTHIONEIN (MT) PROMOTER, TAKAYAMA ET AL. (29) CREATED A TRANSGENIC MOUSE WITH HIGH LEVELS OF CIRCULATING AS WELL AS RENAL HGF. THESE MICE DISPLAY MANY ADVERSE MORPHOLOGIC AND FUNCTIONAL RENAL ALTERATIONS (GLOMERULOSCLEROSIS, RENAL TUBULAR HYPERPLASIA, AND POLYCYSTIC DISEASE) THAT MAKE THIS MODEL UNSUITABLE FOR EVALUATING THE THERAPEUTIC UTILITY OF HGF IN RENAL REGENERATION AFTER KIDNEY DAMAGE.

IN THE PRESENT STUDY, WE GENERATED TRANSGENIC MICE TO ANALYZE THE EFFECT OF SPECIFIC PROXIMAL TUBULAR OVEREXPRESS OF PTHrP OR HGF: (1) IN THE NORMAL GROWTH AND FUNCTION OF THE KIDNEY AND (2) IN THE RESPONSE TO ARF. OUR RESULTS INDICATE THAT OVEREXPRESS OF PTHrP AND HGF IN THE PROXIMAL TUBE DOES NOT AFFECT THE NORMAL GROWTH AND FUNCTION OF THE MOUSE KIDNEY. HOWEVER, HGF OVEREXPRESS IN THE PROXIMAL TUBE QUICKLY AND DRAMATICALLY AMELIORATES RENAL FAILURE INDUCED BY ISCHEMIA. PTHrP OVEREXPRESS, HOWEVER, DESPITE PROMISING OBSERVATIONS IN VITRO, HAS NO APPARENT SALUTARY EFFECT IN ARF IN VIVO.

OUR RESULTS SUGGEST THAT GENE DELIVERY OF HGF TO THE PROXIMAL TUBULE MAY HAVE PROMISING THERAPEUTIC APPLICATIONS IN RENAL FAILURE WITHOUT INDUCTION OF TUMORIGENESIS.

MATERIALS AND METHODS
CONSTRUCTION OF TRANSGENES AND GENERATION OF TRANSGENIC MICE

The proximal convoluted tubule (PCT) specificity was conferred by the γ-glutamyl transpeptidase-I (GGT-I) promoter, which is expressed almost exclusively in the PCT of fetal and adult kidney (30, 31). The tetracycline-controlled transactivator strategy, which creates a reversible switch “off” for gene expression, was used, allowing for temporal control of gene activity. Construction of the transgenes was performed as described in Figure 1. First, a 2.2-kb GGT-I promoter fragment, supplied by Dr. M.W. Lieberman (Baylor College of Medicine, Houston, TX) (31), was inserted upstream of sequences encoding a tet transactivator fusion protein (tTA) that functions as a strong transcription activator (construct A) (32, 33) (provided by Dr. D. Schatz, Yale University, New Haven, CT). Mice generated with the construct A are referred to as “GGT-tTA” transgenic mice. Second, a human PTHrP (1-141) cDNA was placed under the control of a hybrid regulatory element consisting of a heptamerized tetracycline operator (TetoX7) fused to a minimal human cytomegalovirus promoter element (32), which itself is inactive, followed by 3’ untranslated sequences of the hGH gene (construct B), as previously reported (34). Mice generated with the construct B are referred to as “Teto-PTHrP” transgenic mice. Third, in the above construct, the hPTHrP cDNA was removed and replaced by a 2.2-kb mouse HGF cDNA (construct C) (35). Mice generated with the construct C are referred to as “Teto-HGF” transgenic mice. Transgenic founders were identified by PCR analysis of tail DNA, using primers for tTA (5’-AACAAACGCCTCTAGCT and 3’-ATGATTTCCGGCACTTTCC), hGH gene, and human β-globin sequences.

Figure 1. Schematic representation of the different transgenes used to generate the transgenic mice. Construct A, containing sequences for the γ-glutamyl transpeptidase-I (GGT-I) promoter, cDNA sequences for the tTA protein, and the SV-40 T antigen 3’ UTR, was used to generate GGT-tTA transgenic mice, resulting in specific expression of the tTA protein in the renal proximal tubule cells. Constructs B and C, containing sequences for a hybrid regulatory element composed of a heptamerized tetracycline operator (TetoX7) fused to a minimal human cytomegalovirus promoter element, the human growth hormone (hGH) 3’ UTR, and either hPTHrP (1-141) or mHGF cDNA sequences, were used to generate Teto-PTHrP and Teto-HGF transgenic mice, respectively. Hemizygous mice bearing the construct A were bred with construct B- or construct C-bearing hemizygous mice to induce expression of parathyroid hormone–related protein (PTHrP) or hepatocyte growth factor (HGF) in the renal proximal tubule cells. (Inset) RNase protection assay analysis of total RNA isolated from kidneys of adult GGT-tTA and normal littermate mice. Two lines (GGT-tTA 1 and GGT-tTA 2) showed the highest level of transgene expression.
glyceraldehyde-3-phosphate dehydrogenase as a housekeeping gene (34). Transgene-bearing founders were continuously outbred to normal CD-1 mice to generate hemizygotes.

To induce the expression of PTHrP or HGF in the PCT, we bred hemizygote mice for construct B or C to construct A-bearing hemizygote mice, and hemizygote mice double transgenic (DT) for GGT-tTA and either PTHrP or HGF were generated. These are referred to as DT-PTHrP and DT-HGF mice, respectively. In the experiments described below, adult mice (4 to 8 mo) were used. The results obtained with DT mice were compared with the results obtained with control littermates (GGT-tTA, Teto-PTHrP, and/or Teto-HGF).

All studies were performed with the approval of and in accordance with guidelines established by Institutional Animal Care and Use Committees at the University of Pittsburgh, Yale University, and Fundación Jiménez Díaz, when appropriate.

**RNA Expression Analysis**

RNAse protection analysis (RPA) was performed as described previously (34) using 30 µg of total RNA and different cRNA probes corresponding to sequences of the following genes: mPTHrP (349 bp), hPTHrP (309 bp), mHGF (467 bp), tTA (444 bp), mPTHr1 (283 bp), mouse c-met (407 bp), and mouse cyclophilin (103 bp).

**Immunohistochemical Analysis**

Kidneys from DT and control (Teto-PTHrP, Teto-HGF, or GGT-tTA) mice were dissected; fixed overnight in Bouin’s solution or formaldehyde; embedded; and stained using a rabbit affinity-purified antibody against hPTHrP that also recognizes mPTHrP (Ab-2; Oncogene, Uniondale, NY), a rabbit polyclonal antibody against hHGF (Santa Cruz, Santa Cruz, CA) that also recognizes mHGF, a rabbit affinity-purified antibody against the extracellular domain of the rat PTH type I receptor (PTH1R) that also reacts against mPTHr1R (AbVII; Babco, Richmond, CA), or a rabbit affinity-purified antibody against murine c-met receptor (Santa Cruz). Nonspecific staining was assessed in DT kidney sections incubated with the same concentration of normal rabbit IgG instead of primary antibody. Visualization of staining was accomplished using biotinylated anti-rabbit secondary antiserum and the avidin-biotin-peroxidase complex (Vector, Burlingame, CA), as described previously (16). Immunofluorescence staining was performed in kidney sections incubated with FITC-phalloidin (green; Sigma, St. Louis, MO), an actin marker that stains mainly proximal tubules, and the corresponding primary antibody followed by a rhodamine-conjugated secondary antibody (red), and mounted with mounting media-containing DAPI to detect nuclei (blue).

**Renal PTHrP Content**

Kidneys from DT-PTHrP and control (Teto-PTHrP, Teto-HGF, or GGT-tTA) mice were dissected, snap-frozen, and homogenized in RIPA buffer (PBS with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na3VO4, 100 µM/µl PMSF, and 5.7 µg/ml aprotinin), as described previously (19). Protein content in the extracts was determined by the Micro BCA assay (Pierce, Rockford, IL). Equal amounts of protein extract were diluted in immunoradiometric assay (IRMA) buffer and assayed for PTHrP (1-36) IRMA as described (36). Because human and mouse PTHrP (1-36) sequences are identical, PTHrP (1-36) IRMA recognizes PTHrP (1-36)-containing fragments from both species with equal affinity (15).

**Western Blot Analysis**

Analysis of HGF protein expression was performed in protein extracts from whole kidneys of DT-HGF and Teto-HGF mice made with RIPA buffer, as described above. Protein extracts (100 µg) were boiled in SDS-PAGE sample buffer containing β-mercaptoethanol, and then the samples were resolved on a 10% SDS–polyacrylamide gel.

Analysis of PTH1R protein expression was performed in whole-kidney extracts from DT-PTHrP and Teto-PTHrP mice prepared in ice-cold extraction buffer (5% sorbitol, 0.5 mM Na3EDTA, 5 mM histidine-imidazole buffer containing 0.2 mM PMSF, and 1.4 µM aprotinin [pH 7.5]) and centrifuged at 2000 × g for 10 min. The supernatant, containing total membranes and soluble proteins, was pelleted by centrifugation at 100,000 × g for 90 min and resuspended in extraction buffer. Protein extracts (10 µg) were resolved on a 7.5% SDS–polyacrylamide gel as mentioned above.

In both cases, proteins were transferred to either Immobilon P membranes (Millipore, Bedford, MA) or nitrocellulose membranes (Trans-Blot; Bio-Rad, Hercules, CA) using standard techniques. Membranes were blocked with blocking solution (either 5% nonfat milk or 5% BSA in PBS with 0.1% Tween-20) and then incubated with an affinity-purified antibody against the PTH1R (AbIV; Babco), a rabbit polyclonal antibody against HGF-α (Santa Cruz), a rabbit anti-PTHr1R antiserum (C6) that recognizes mouse and human PTHrP (5,22), or a goat polyclonal antibody against actin (Santa Cruz) for 16 h at 4°C. Subsequently, membranes were incubated with the corresponding peroxidase-conjugated antibody and developed using enhanced chemiluminescence reagent (ECL; Amersham Pharmacia Biotech). Gel-loading equivalence was assessed by staining of the membranes with Ponceau’s solution.

**Renal Morphology, Histology, and DNA Labeling Index**

Kidneys from DT and control (Teto-PTHrP, Teto-HGF, or GGT-tTA) mice were fixed in paraformaldehyde-lysine-sodium periodate, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Before fixation, the renal wet weight (absolute and % of body weight) in DT and control littermates was determined. In addition, the DNA labeling index in tubule cells was assessed by immunohistochemical detection of 5-bromo-2′-deoxyuridine (BrdU) incorporation into tubule cell nuclei, as previously reported (35). Briefly, 6 h after intraperitoneal injection of BrdU (Cell proliferation Kit; Amersham Pharmacia Biotech), mice were killed and their kidneys were removed and immediately fixed in Bouin’s solution for 16 h. Sections were then stained with the anti-BrdU antibody and counterstained with Meyer’s hematoxylin. Four randomly selected 400× high-power fields (approximately 500 tubule cells per field), that contained at least one glomerulus to localize the field to renal cortex, were counted to determine the number of BrdU-labeled nuclei.

**Renal Ischemia Procedure**

Mice were anesthetized (ketamine 45 mg/kg and xylazine 5 mg/kg body wt, intraperitoneally) and placed on a heating pad to maintain body temperature. After a midline laparotomy incision, both left and right renal arteries and veins were isolated and occluded with a clamp for 30 min. Kidneys were inspected for immediate color change indicating successful clamping. After clamp removal, kidneys were checked for a change in color within 1 min to ensure reperfusion. Forty-eight hours after surgery was performed, mice received an intraperitoneal injection of BrdU, and 6 h later, blood samples were obtained, mice were killed, and kidneys were harvested.

Renal ischemia/reperfusion was also performed in a different set of DT-HGF and Teto-HGF mice supplemented for 7 d presurgery and during the reperfusion period with doxycycline hydrochloride (20 mg/L; Figure 1) dissolved in 5% sucrose in the drinking water (37).
Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick-End Labeling Staining

Apoptotic cell death was detected by enzymatic in situ labeling of DNA strand breaks using the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) method in kidney sections from control (Teto-HGF and GGT-tTA) and DT-HGF mice obtained 48 h after renal ischemia. TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. Four randomly selected 400× high-power fields (approximately 500 tubule cells per field) that contained at least one glomerulus to localize the field to renal cortex were counted to determine the number of apoptotic nuclei.

Folic Acid–Induced ARF

Control (Teto-PTHrP and GGT-tTA) and DT-PTHrP mice received a single intraperitoneal injection of 250 mg/kg folic acid (FA; Sigma) in 300 mM sodium bicarbonate. At 24 and 72 h after FA injection, blood samples were obtained, mice were killed, and kidneys were removed.

Plasma Biochemical Evaluation

Plasma creatinine, blood urea nitrogen (BUN), phosphate, and calcium were measured in DT and control (Teto-PTHrP, Teto-HGF, or GGT-tTA) mice. In the renal ischemia and FA experiments, the recovery of the kidney function was determined by measuring creatinine and BUN in plasma samples obtained after the intervention. These measurements were performed using standard autoanalyzer methods in the Clinical Chemistry Laboratories at the Yale-New Haven Hospital, Fundación Jiménez Díaz (Madrid), and the Central Animal Facility in the University of Pittsburgh.

Statistical Analyses

Results are expressed as means ± SEM. Statistical analysis was performed using the unpaired t test. P < 0.05 was considered statistically significant. Animal survival was assessed using Fisher exact test (38).

Results

Generation of DT-PTHrP Mice Expressing hPTHrP in the PCT

Three GGT-tTA (construct A; Figure 1) transgenic lines derived from three GGT-tTA founders capable of germline transmission were studied. Tet transactivator mRNA expression in the kidney of these three transgenic lines was analyzed by RPA (Figure 1, inset). GGT-tTA 1 and GGT-tTA 2 lines showed the highest level of expression, whereas GGT-tTA 3 expressed the transgene at lower levels. Thus, the GGT-tTA 1 and GGT-tTA 2 transgenic mouse lines were selected for breeding with construct B– or C–bearing mice (Figure 1).

Three Teto-PTHrP founders (construct B; Figure 1), which displayed germline transmission, were also obtained. When Teto-PTHrP mouse lines were bred with the GGT-tTA mice, double-transgenic mice that did express hPTHrP mRNA in the kidney, as assessed by RPA, were generated (DT-PTHrP 1, DT-PTHrP 2, and DT-PTHrP 3; Figure 2A). Overexpression of hPTHrP mRNA in DT-PTHrP mice did not alter the level of expression of endogenous mPTHrP mRNA as compared with control mice (Figure 2B). Because of the similar phenotypes in the different lines of DT-PTHrP mice, data from these lines were pooled in subsequent studies.

For confirming that DT-PTHrP mice also overexpress PTHrP at the protein level, PTHrP content in whole-kidney extracts of DT-PTHrP mice was examined using a IRMA for PTHrP (1-36) and Western blot analysis. The level of PTHrP in kidney extracts from DT-PTHrP mice, expressed in picomoles of PTHrP (1-36) per milligram of protein, was significantly (P < 0.01) higher than in renal extracts from control (Teto-PTHrP and GGT-tTA) littermates (4.3 ± 0.5 [n = 8] versus 1.3 ± 0.3 [n = 12], respectively). Given that expression is targeted to the tubules (see below) and PTHrP levels are based on whole-kidney extracts, one may infer that local levels of PTHrP in the proximal tubule are still higher. This increase in the PTHrP expression levels in DT-PTHrP mice was confirmed by Western blot analysis of kidney extracts from these mice and control (GGT-tTA and Teto-PTHrP) littermates (Figure 3A). Immunohistochemical analysis of PTHrP expression in kidney sections from DT-PTHrP and Teto-PTHrP littermates was also performed. As shown in Figure 2C, proximal tubules from DT-PTHrP mice display increased intensity of staining for PTHrP as compared with sections from Teto-PTHrP littermates. Plasma concentrations of PTHrP in both DT-PTHrP and Teto-PTHrP mice were below the level of detection of the PTHrP (1-36) IRMA (0.2 pm).

RPA analysis of PTH1R mRNA expression revealed that the level of PTH1R mRNA in GGT-tTA, Teto-PTHrP, and DT-PTHrP transgenic mice was similar (Figure 2A). In addition, Western blot analysis of PTH1R expression in whole-kidney extracts reveals that PTH1R levels are similar in Teto-PTHrP and DT-PTHrP mice (Figure 3, B and C). Furthermore, immunohistochemical analysis of PTH1R protein expression in renal sections from DT-PTHrP and Teto-PTHrP mice reveals that the localization and intensity of staining for PTH1R is similar in sections from both types of mice (Figure 2D). These findings suggest that PTH1R expression is not influenced by PTHrP overexpression in this system.

Generation of DT-HGF Mice Overexpressing mHGF in the PCT

Double-transgenic HGF mice were generated by breeding the GGT-tTA mice with the four founder Teto-HGF transgenic mice that transmitted the transgene to their progeny. RPA analysis of mHGF mRNA expression in whole kidney revealed that the four lines of DT-HGF mice displayed increased expression of mHGF mRNA (4- to 25-fold; Figure 4A), when compared with the expression in Teto-HGF mice. In addition, at the protein level, HGF was overexpressed in kidneys from DT-HGF mice as determined by Western blot analysis of whole-kidney extracts (Figure 4B). Immunofluorescence analysis of kidney sections from DT-HGF and Teto-HGF mice revealed that HGF is overexpressed in the proximal tubule of DT-HGF mice compared with the expression observed in Teto-HGF mouse kidney sections (Figure 4C). Because of their similar phenotypes, data obtained from these four DT-HGF lines were pooled in the studies described below.

DT-HGF mouse kidneys displayed similar levels of c-met


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mRNA expression to those observed in control mice as assessed by RPA (Figure 4D). In addition, no differences were observed in the localization or intensity of c-met staining in DT-HGF mouse kidney sections when compared with Teto-HGF littermates (Figure 4E). These findings suggest that c-met expression is not influenced by HGF overexpression in this system.

Renal Morphology and Function in DT-PTHrP or DT-HGF Transgenic Mice under Normal Physiologic Conditions

Analysis of kidney wet weights (absolute and % of body wt) and BrdU incorporation in tubule cells in DT-PTHrP and DT-HGF mice at 4 mo of age revealed no significant differences when compared with control (Teto-PTHrP, Teto-HGF, or GGT-tTA) mice at the same age (data not shown). In addition, detailed microscopic analysis of hematoxylin- and eosin-stained kidney sections obtained from DT-PTHrP or DT-HGF mice did not reveal any morphologic difference from the sections obtained from control (Teto-PTHrP, Teto-HGF, or GGT-tTA) mice (Figure 5A, top).

Circulating markers of kidney function, BUN, and plasma creatinine concentrations were not significantly different in DT-PTHrP or DT-HGF mice as compared with control (Teto-PTHrP, Teto-HGF, or GGT-tTA) mouse values. BUN values (mg/dl) were as follows: 32.3 ± 2.6 (n = 9; DT-PTHrP), 37.6...
bilateral ischemia in DT-HGF mice are shown in Figure 5, B and C. As expected, plasma creatinine and BUN were markedly and progressively increased in control (Teto-PTHrP, Teto-HGF, or GGT-tTA) mice at 24 and 48 h after ischemia/reperfusion compared with preoperative values. However, in marked contrast, when ischemia was performed in DT-HGF mice, plasma creatinine and BUN levels remained reduced compared with control mice, returning to similar preinjury levels by 48 h after the procedure was performed (Figure 5, B and C). The survival rate 48 h after ischemia/reperfusion was also dramatically and significantly increased in DT-HGF mice compared with the survival rates in control (Teto-PTHrP, Teto-HGF, or GGT-tTA) (Figure 5D).

To confirm at the histologic level what was observed at the functional level, we examined DT-HGF and control (Teto-PTHrP, Teto-HGF, or GGT-tTA) mice histologically at 48 h after ischemia. Control kidneys after 48 h of reperfusion showed widespread and severe damage in the tubules located primarily in the inner cortex region, with tubular necrosis and sloughing of tubules with obstructing casts in the lumen (Figure 5A, bottom). In contrast, kidney sections from DT-HGF mice showed markedly reduced tubular damage with fewer obstructing casts (Figure 5A, bottom). To determine whether this protective effect of HGF overexpression in DT-HGF mice might correlate with an increase in the expression of c-met in renal cortical tubules in these mice, we performed immunofluorescence analysis of c-met expression in sections of DT-HGF mouse kidneys obtained at 48 h after ischemia/reperfusion and at baseline conditions. As shown in Figure 5E, the intensity of c-met expression was increased in tubules of DT-HGF mice 48 h after ischemia compared with the expression in DT-HGF mice in baseline conditions. Strong staining was also observed in kidney sections from control (Teto-HGF) mice obtained at 48 h after ischemia/reperfusion. However, this staining was preferentially localized to damaged tubules and sloughed cells, similar to what has been previously reported (26). These results suggest that upregulation of c-met could mediate the protective effects of HGF in DT-HGF mice after ischemia/reperfusion.

For confirming that HGF overexpression in the PCT was responsible for the beneficial effects in the recovery of the renal function in DT-HGF mice after ischemia/reperfusion, HGF overexpression was reversed using the tetracycline down-regulatable system (Figure 1). As shown in Figure 6A, 20 mg/L doxycycline added to the drinking water time-dependently reduced renal mHGF mRNA expression in DT-HGF mice. At 7 d after the administration, renal mHGF mRNA levels in DT-HGF mice were downregulated to levels indistinguishable from control (Teto-HGF and GGT-tTA) kidneys (Figure 6A). On the basis of these results, 20 mg/L doxycycline was added to the drinking water of DT-HGF and control (Teto-HGF and GGT-tTA) mice for 7 d presurgery and also during the reperfusion period in the renal ischemia/reperfusion studies. Forty-eight hours after ischemia/reperfusion, plasma creatinine was significantly increased in doxycycline-treated DT-HGF mice compared with their presurgery values (Figure 6B) and with the values in untreated DT-HGF mice (Figure 5B). Importantly, at 48 h after ischemia/reperfusion, plasma creatinine in
doxycycline-treated DT-HGF mice was similar to that observed in doxycycline-treated (Figure 6B) or untreated control (Teto-HGF and GGT-tTA) mice (Figure 5B). In addition, the survival rate after ischemia/reperfusion in doxycycline-treated DT-HGF mice was dramatically reduced, reaching similar levels to doxycycline-treated (Figure 6C) or untreated control (Teto-HGF and GGT-tTA) mice (Figure 5D). Severe and comparable tubular damage to that shown in Teto-HGF mice (Figure 5, bottom) was observed in kidney sections from doxycycline-treated DT-HGF and doxycycline-treated control (Teto-HGF and GGT-tTA) mice obtained at 48 h after ischemia (data not shown). These findings confirm that the previously ob-

Figure 4. Expression levels of HGF and c-met in kidneys of adult Teto-HGF and DT-HGF littermate mice. (A) Thirty micrograms of total kidney RNA was analyzed by RNase protection assay. RNA samples were hybridized with mouse HGF and mouse cyclophilin (internal control) cRNA probes as previously reported (35) to detect transgene expression in the kidney of these DT-HGF mice. (B) Western blot analysis of HGF levels in total kidney extracts obtained from control (Teto-HGF) and DT-HGF mice showing increased HGF levels in the kidney of DT-HGF mice compared with control littermates. Similar HGF levels were observed in DT-HGF mouse kidneys after ischemia/reperfusion and at baseline conditions. (C) Detection of HGF in DT-HGF and control (Teto-HGF) littermates by immunofluorescence. Both control and DT-HGF sections were processed identically and simultaneously as described in Materials and Methods. Upper left photomicrograph shows a representative DT-HGF kidney section stained with FITC-phalloidin (bright green) to identify proximal tubules (46). Upper right photomicrograph shows the same section stained with the anti-HGF antibody and a rhodamine-conjugated secondary antibody (bright red). Lower left section photomicrograph shows a representative control (Teto-HGF) kidney section stained with FITC-phalloidin, and lower right photomicrograph shows the same section stained with the anti-HGF antibody and a rhodamine-conjugated secondary antibody. The pattern of staining is consistent with the overexpression of HGF mainly in proximal tubules. Similar level of staining was observed in the different DT-HGF mouse lines. Nuclei were stained with DAPI (blue). (D) Expression levels of c-met mRNA in kidneys of DT-HGF and control (Teto-HGF) littermates. RNA samples were hybridized with mouse c-met and mouse cyclophilin cRNA probes to detect changes in the endogenous levels of c-met receptor expression in the kidney of these DT-HGF mice. (E) Immunohistochemical detection of c-met in the kidneys of DT-HGF and control (Teto-HGF) littermates. Both transgenic and double transgenic sections were processed identically and simultaneously as described in Materials and Methods. The pattern and intensity of c-met staining were similar in sections from Teto-HGF and DT-HGF mice.
Figure 5. (A) Morphology of Teto-HGF, DT-PTHrP, and DT-HGF kidneys in baseline condition (top) and 48 h after ischemia/reperfusion (bottom). Ischemia induced severe damage in Teto-HGF and DT-PTHrP mouse kidneys with tubular necrosis and sloughing of tubules with obstructing casts in the lumen. Kidneys from DT-HGF showed highly reduced tubular damage with fewer obstructing casts. Hematoxylin and eosin staining. (B) Plasma creatinine and (C) blood urea nitrogen (BUN) levels after bilateral renal ischemia in control (Teto-HGF, Teto-PTHrP,
served protective effect of HGF overexpression in DT-HGF mice was indeed HGF-specific and HGF-dependent.

To determine whether the markedly reduced damage observed in the kidneys of DT-HGF mice after ischemia was related to an increase in tubular cell proliferation and/or a decrease in tubule cell apoptosis, we determined the number of BrdU- or TUNEL-positive nuclei in tubule cells of DT-HGF mice 48 h after ischemia (Figure 7). BrdU incorporation rates in DT-HGF mice were significantly higher than the proliferation rates observed in control (Teto-HGF and GGT-tTA) mice 48 h after reperfusion (Figure 7, A and B). In addition, the number of TUNEL-positive nuclei was significantly lower in DT-HGF mice than in control mice at 48 h after reperfusion (Figure 7, C and D). These results indicate that after ischemia, HGF overexpression in the proximal tubule promotes cell proliferation and prevents apoptosis of tubule cells in DT-HGF mice.

**Induction of Renal Damage by Ischemia/Reperfusion in DT-PTHrP Mice**

In contrast to observations in the DT-HGF mice, ischemia induced severe renal failure in DT-PTHrP mice, indistinguishable from that observed in control (Teto-PTHrP, Teto-HGF, and GGT-tTA) mice. Plasma creatinine, BUN, and renal damage were similar in DT-PTHrP and control mice after ischemia/reperfusion (Figure 5, A through C). Similar to control mice, DT-PTHrP mice had a high mortality rate at 24 to 48 h after the operation (Figure 5D).

To confirm independently the apparent absence of ameliorative effect of PTHrP overexpression in ARF, we analyzed the response of DT-PTHrP mice to a second, distinct model of induction of ARF by injecting intraperitoneally a high dose of FA. DT-PTHrP and control (Teto-PTHrP and GGT-tTA) mice showed similar plasma creatinine and BUN at different time points after FA administration. Plasma creatinine (mg/dl) was as follows: 3.0 ± 0.1 (DT-PTHrP mice; n = 5) versus 2.6 ± 0.2 (control mice; n = 11) at 24 h and 3.0 ± 0.4 (DT-PTHrP mice; n = 8) versus 3.1 ± 0.3 (control mice; n = 9) at 72 h after FA. BUN (mg/dl) values were as follows: 176 ± 4 (DT-PTHrP mice) versus 170 ± 8 (control mice) and 228 ± 38 (DT-PTHrP mice) versus 247 ± 22 (control mice) at 24 and 72 h, respectively, after FA. To determine whether a delayed protective response from overexpressed PTHrP could take place, we measured plasma creatinine and BUN 7 d after FA injection in control (GGT-tTA and Teto-PTHrP) mice and DT-PTHrP mice. No differences in plasma creatinine or BUN levels were observed at 7 d after FA injection in both types of mice. Plasma creatinine was as follows: 1.0 ± 0.2 (DT-PTHrP; n = 5) versus 1.0 ± 0.5 (control; n = 5). BUN was as follows: 96 ± 8 (DT-PTHrP; n = 5) versus 109 ± 8 (control; n = 5).

The apparent absence of ameliorative effect of PTHrP in DT-PTHrP mice after renal ischemic injury could be related in theory to downregulation of either PTHrP or PTH1R expression in the kidney. Analysis of PTHrP expression in DT-PTHrP kidney extracts by Western blot revealed that the level of PTHrP at 24 or 48 h after ischemia/reperfusion remained increased compared with the level in control mice at baseline conditions (Figure 3A). Similarly, HGF expression levels in DT-HGF mice did not decrease after ischemia/reperfusion in these mice (Figure 4B). Rapid and transient downregulation of PTH1R mRNA after ischemia/reperfusion in the rat has been previously reported (16). To assess whether downregulation of the PTH1R was also an event occurring in the DT-PTHrP mice after either ischemia/reperfusion or FA injection, we performed Western blot analysis of kidney extracts from these mice at different times after induction of ARF in both experimental models. Although no decrease in PTH1R protein levels was observed in DT-PTHrP transgenic mouse kidneys at 24 h, a profound decrease (40%) in these levels was detected at 48 h after ischemia/reperfusion (Figure 3B). Moreover, PTH1R protein levels were significantly decreased (50%) at 72 h after FA in DT-PTHrP mice (Figure 3C). Collectively, these results indicate that acute renal injury but not constitutive PTHrP overexpression in the proximal tubule downregulates PTH1R in the kidney.

**Discussion**

Using mouse genetic approaches, we have developed transgenic mice with tetracycline-controlled HGF or PTHrP overexpression in the proximal tubule using the GGT-I promoter. This promoter has been shown to be effective in inducing the expression of several transgenes in the proximal tubule in mice (31,39). To our surprise, despite its promising in vitro observations, PTHrP overexpression in the proximal tubule in vivo did not result in any significant functional or morphologic improvement in the acutely injured kidney after either ischemia or FA injection, and it did not induce changes in renal function (but a discrete hypophosphatemia) or morphology under baseline conditions. In marked contrast, our studies convincingly demonstrate that HGF overexpression in the proximal tubule is
able dramatically to ameliorate damage induced by bilateral ischemia/reperfusion in the mouse. Importantly, despite the protective effect in the setting of renal injury, HGF-transgenic mice do not display any renal morphologic or functional abnormality under basal physiologic conditions.

HGF has been shown to have renostrophic activity, being capable of stimulating renal regeneration after ARF. Thus, subcutaneous or intravenous HGF administration accelerates the recovery from acute renal injury in rodents (9,10,40). However, the effects of high levels of circulating HGF on other peripheral tissues have not been studied carefully. Transgenic mice with specific overexpression of HGF in a variety of different tissues have been reported (35,41,42), but none that targets HGF specifically to the proximal tubule cell has been described. Importantly, a transgenic mouse model of generalized overexpression of HGF in all tissues by using an MT promoter has been reported (29). MT-HGF transgenic mice display very high circulating levels of HGF and also markedly increased diffuse HGF expression within the kidney, resulting in numerous adverse morphologic and functional alterations in the kidney. These include glomerulosclerosis, renal tubular hyperplasia, and polycystic disease. These phenotypic characteristics make diffuse overexpression or systemic administration of HGF at high levels worrisome. To avoid these concerns, we developed transgenic mice with tetracycline-reversible HGF overexpression in the proximal tubule. Using this tetracycline-reversible system, we had planned to block HGF overexpression and its undesired renal effects until adult mice were ready to undergo ischemia/reperfusion experiments. To our surprise, when DT-HGF transgenic mice were generated overexpressing HGF specifically in the proximal tubule, we found that these mice displayed normal plasma creatinine and BUN, normal tubule cell proliferation, and normal renal morphology. Renal tumors, cysts, or glomerular abnormalities were not detected. The differences observed in the phenotype between DT-HGF mice and the previously reported MT-HGF mice (29) are most likely the result of more specific targeting of HGF to proximal tubule cells and perhaps to lower levels of HGF expression in the DT-HGF mouse compared with the MT-HGF mouse.

The most remarkable observation in the current report is that overexpression of HGF in the proximal tubule, although having no apparent effect on renal development and none on basal renal physiology or morphology in adult animals, has a striking protective effect against ischemia-induced ARF. Indeed, DT-HGF mice displayed normal plasma creatinine and BUN within 48 h after renal ischemia. This recovery was specific for these mice, because normal, single-transgenic and DT-PTHrP mice displayed markedly elevated levels of plasma creatinine and BUN 48 h after ischemia. These biochemical findings were corroborated by the histologic changes found in these mice after renal damage. An explanation for the different effects of overexpressed HGF in basal and ischemic conditions might be related to an early, persistent, and dramatic upregulation of renal c-met levels after renal ischemia and injury, as previously reported in the rat (24–26). In the last study, immunohistochemical analysis demonstrated that the increased c-met immunoreactivity occurred principally in the most severely damaged nephron segments in the cortex and the outer medulla (26). In the present study, basal levels of c-met mRNA expression and c-met immunoreactivity were similar in control and DT-HGF mice. Interestingly, an increase in c-met staining was observed in DT-HGF mice at 48 h after ischemia/reperfusion compared with the levels in DT-HGF mouse kidneys at baseline conditions. Moreover, in a different set of experiments, when doxycycline was used to inhibit the expression of mHGF transgene in the DT-HGF mouse, recovery 48 h after renal ischemia/reperfusion was completely blunted. Taken together, these findings suggest that overexpression of HGF potentially may be an ideal “stealth” protective agent for the kidney, remaining unnoticed until ischemic injury occurs.

Previously documented biologic effects of HGF in renal tubule cells include increased proliferation in vivo and in vitro (9,10,43). Recent evidence suggests an antitubule cell death effect in both situations as well (27,44). In our studies, accord-

![Figure 6. Effect of doxycycline treatment on mHGF mRNA expression in DT-HGF mice and renal function and survival rates after bilateral ischemia in control mice and DT-HGF mice. (A) RNase protection assay was performed as mentioned in Figures 2 and 4 with total kidney RNA from control (Teto-HGF), DT-HGF, and doxycycline-treated DT-HGF and control (Teto-HGF) littermates. Doxycycline (20 mg/L) dissolved in 5% sucrose was added to the drinking water for the period of time described in the figure. Murine HGF mRNA expression levels in the kidney of DT-HGF mice decrease time-dependently after 7 d of doxycycline administration, reaching basal expression levels similar to control mice. (B) Doxycycline (20 mg/L) was added to the drinking water for 7 d presurgery and during the reperfusion period in the renal ischemia studies. Creatinine was measured in plasma obtained from doxycycline-treated control (Teto-HGF) and DT-HGF mice before the surgery and 48 h after bilateral ischemia. Plasma creatinine in doxycycline-treated DT-HGF mice 48 h after ischemia/reperfusion was significantly increased (*P < 0.05) compared with the presurgery values and was similar to the level in doxycycline-treated control mice. (C) The survival rate after the ischemia/reperfusion process was identical in doxycycline-treated DT-HGF mice (n = 8) and in the doxycycline-treated control (Teto-HGF) mice (n = 12).
ingly, we found that BrdU incorporation in tubule cells of DT-HGF mice was markedly increased at 48 h after ischemia/reperfusion as compared with the levels found in control mouse kidneys under similar conditions. Evidence of a stimulatory effect of intravenously or subcutaneously injected HGF on tubule cell proliferation in ARF has been reported previously (9,10). In addition, we found a decrease in the number of apoptotic tubule cells in DT-HGF mice at 48 h after ischemia.

Figure 7. Tubule cell proliferation (A) and tubule cell apoptotic rates (C) in postischemic kidneys from control (Teto-HGF and GGT-tTA) and DT-HGF mice obtained 48 h after ischemia/reperfusion. Kidney sections from control (n = 8) and DT-HGF (n = 7) mice were stained for 5-bromo-2′-deoxyuridine (BrdU) to detect cell proliferation and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) to detect cell apoptosis. After counterstaining with hematoxylin, approximately 2000 tubule cell nuclei in four fields containing a glomerulus to localize the field to renal cortex were counted. The data are represented as percentage of either replicating tubule cells (A) or apoptotic tubule cells (C). *P < 0.02 and **P < 0.01. Representative photomicrographs of kidney sections from control (Teto-HGF) and DT-HGF mice obtained 48 h after ischemia and stained for BrdU and hematoxylin (B) or TUNEL and hematoxylin (D). The number of black-stained nuclei (BrdU-positive nuclei) was highly increased in the DT-HGF mouse kidney section compared with the number in the Teto-HGF section. However, the number of blue stained nuclei (TUNEL-positive nuclei) was highly increased in the Teto-HGF mouse kidney section compared with the section of the DT-HGF mouse. In the Teto-HGF kidney sections, a field in which tubule cells and their nuclei were more intact was chosen for comparative purposes.
Again, previous evidence of an inhibitory activity of subcutaneously injected HGF on apoptosis in the setting of renal ischemic injury in rats has been reported (27). Taken together, these results suggest that HGF overexpressed within the proximal tubule may ameliorate renal damage induced by ischemia/reperfusion by at least two different processes: enhancement of tubule cell proliferation and protection against cell death.

Using an identical approach, we also developed transgenic mice overexpressing PTHrP in the proximal tubule using the GGT-I promoter. PTHrP also has been postulated to be a renotrophic factor that might have a potential therapeutic role in renal function recovery after kidney damage (5–8,16,18–22). In our studies, increased proximal tubule expression of PTHrP in DT-PTHrP mice did not induce any morphologic change in the kidney of these mice. Moreover, plasma calcium, creatinine, and BUN were not different between control and DT-PTHrP mice. It is important to note that circulating levels of PTHrP were undetectable in these mice, as previously reported for other transgenic mouse models with tissue-specific PTHrP overexpression (34,45).

The absence of phenotypic changes in DT-PTHrP mice might have been related to homologous downregulation of PTH1R in the kidney of these transgenic mice. Indeed, it has been shown that overexpression of PTHrP is accompanied by PTH1R downregulation in several renal pathologic situations (8,16,21). However, this is unlikely, because DT-PTHrP mice displayed normal levels of PTH1R mRNA and protein as assessed by RPA, Western blot, and immunohistochemical analysis. The absence of phenotypic changes also might have been related to the use of human PTHrP instead of mouse PTHrP for the generation of these transgenic mice. However, this seems unlikely for two reasons: (1) the amino-terminal part of both human and mouse PTHrP is almost identical (2 different amino acids out of 111), suggesting that they both should bind the PTH1R with similar affinity (15), and (2) the same hPTHrP transgene but driven by different promoters has been used to overexpress hPTHrP in different cell types in transgenic mice, resulting in profound phenotypic changes (15,34,45). Furthermore, DT-PTHrP mice showed significantly lower plasma phosphate levels than control mice, suggesting that the expected physiologic local inhibition of phosphate reabsorption occurred in the proximal tubule as a result of overexpressed PTHrP. This finding also indirectly confirms that the overexpressed PTHrP was biologically active. However, whether a higher level of PTHrP overexpression would be necessary to observe strong phenotypic alterations in the growth and function of the kidney is unknown.

In striking contrast to HGF, overexpression of PTHrP in the renal proximal tubule did not prevent the acute deterioration in renal function after ischemia/reperfusion or FA injection. Although basal levels of PTH1R in DT-PTHrP mice seemed to be normal, downregulation of the receptor after acute renal injury, as previously reported (8,16), could account for the absence of effect of overexpressed PTHrP in renal recovery after acute kidney damage. As shown in the Western blot experiments (Figure 3), PTHrP transgene expression is not significantly altered by ischemia/reperfusion. However, PTH1R protein levels profoundly decreased in DT-PTHrP mouse kidneys at 48 or 72 h after ischemia/reperfusion or FA injection. Additional studies are necessary to clarify the role, if any, of PTH1R downregulation on the absence of an effect of PTHrP in the renal recovery process after ARF in DT-PTHrP mice.

In summary, our studies indicate that overexpression of HGF but not of PTHrP (at least under the conditions studied) in the proximal tubule dramatically improves the recovery of kidney function and markedly decreases the acute renal damage induced by ischemia/reperfusion. Surprisingly, neither overexpression of HGF nor PTHrP in the proximal tubule was accompanied by baseline renal morphologic changes or basal renal functional alterations in transgenic mice. We conclude that gene delivery of HGF to the proximal tubule may be an interesting approach in designing future strategies aimed at the treatment and prevention of ARF.

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