A role for Wnt-4 in renal fibrosis

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Wnt4 activation induced by injury is limited to collecting ducts, with initial activation in the collecting duct epithelium followed by activation in fibrotic lesions surrounding the collecting ducts. The highest cellular Wnt4 expression is in interstitial fibroblasts in the fibrotic lesions that also express high levels of collagen-α1(I) mRNA and α-smooth muscle actin. In support of a functional role for Wnt-4 in these activated myofibroblasts, Wnt-4 induces stabilization of cytosolic β-catenin in a cultured myofibroblast cell line. Furthermore, Wnt-4-producing fibroblasts placed under the renal capsule of adult mice induce lesions with tubular epithelial destruction. These observations suggest a role for Wnt-4 in the pathogenesis of renal fibrosis.

tubulointerstitial disease; interstitial fibroblast; β-catenin; kidney

DESTRUCTION OF RENAL TUBULAR epithelial cells as a result of interstitial fibrosis is a common component of the major diseases that lead to end-stage renal failure, including glomerulosclerosis and diabetic nephropathy (4, 5). The extent of tubulointerstitial fibrosis correlates directly with the decline in renal function in these diseases (28). Any tubular injury results in a typical sequence of inflammation and fibrosis (4), in which fibroblasts and extracellular matrix proteins accumulate in the interstitium and ultimately disrupt the tubulointerstitial architecture so that renal function is lost (11). This pathophysiological process is termed tubulointerstitial disease. Notable among the profibrotic secreted mediators critical in this process is transforming growth factor (TGF)-β1, which is synthesized during renal fibrosis (54) and can activate conversion of fibroblasts to myofibroblasts (48). Myofibroblasts are interstitial cells with the characteristics of fibroblasts but that also express α-smooth muscle actin (42). Myofibroblasts actively secrete extracellular matrix proteins (33, 57), and myofibroblast presence in chronic renal disease parallels the decline in renal function (1) as well as predicts prognosis (32). Renal tubular epithelial cells can also acquire α-smooth muscle actin expression after damage (29). Nephrogenesis involves modulation of the type and quantity of extracellular matrix proteins, suggesting the possibility that molecules regulating nephrogenesis are also involved in the pathophysiology of tubulointerstitial disease.

Wnt-4 is a secreted glycoprotein required for nephrogenesis (21, 47), and metanephric mesenchymal condensation does not occur in mice deficient in Wnt-4. We explored the potential role of Wnt-4 in tubulointerstitial disease. Others have found that Wnt4 was inactive in the adult kidney and reactivated after unilateral ureteral obstruction (UUO; Ref. 30). Ureteral obstruction has been used to study the molecular basis of tubulointerstitial disease (9). In contrast to Wnt4, Wnt7b and Wnt11 are expressed in the developing kidney but are not activated after UUO (30). The Wnt4 gene is also transcriptionally activated in the early stages of dermal wound healing in mice, whereas Wnt1, Wnt3a, and Wnt7b are not (24). Neither the identity of the cells expressing Wnt4 after renal or dermal injury nor the function of Wnt4 in these models was reported.

In the canonical Wnt signal transduction pathway, Wnt family ligands bind to frizzled family receptors, and intracellular signaling is mediated by stabilization of cytosolic β-catenin (26). In the absence of Wnt signaling, cytosolic β-catenin is targeted for degradation by glycogen synthase kinase 3-β. In the presence of Wnt signaling, glycogen synthase kinase 3-β activity and cytosolic β-catenin degradation are inhibited, resulting in migration of β-catenin to the nucleus and regulation of target genes through interaction of β-catenin with high-mobility group box family transcription factors. The ability of a Wnt family member to stabilize cytosolic β-catenin depends on the cell type. Wnt-4, Wnt-3a, and Wnt-1 stabilize cytosolic β-catenin levels in embryonic stem cells, whereas four other Wnts do not (39). However, Wnt-1 and three other Wnts stabilize cytosolic β-catenin in C57MG mammary epithelial cells, whereas Wnt-4 and five other Wnts do not (45).
In this study we examined the expression pattern of Wnt4 in four different murine models of renal damage that result in tubulointerstitial disruption. Renal damage by UUO and folic acid administration is accomplished by blockage of the flow of luminal fluid through the nephrons (36, 51); the increased tubular pressure results in tubular epithelial damage. Blockage in UUO is achieved by ligation of the ureter, whereas blockage after folic acid administration is achieved through temporary deposition of folic acid crystals in the distal segment of some, but not all, nephrons (14). After tubular damage, there is an increase in macrophages and other inflammatory cells, expansion of interstitial fibroblasts, and development of fibrotic lesions characteristic of tubulointerstitial disease. Irreversible blockage of the ureter in UUO results in a continuous progression of fibrosis in all nephrons, which ultimately involves the entire kidney. In contrast, temporary blockage of some, but not all, nephrons by folic acid results in more limited fibrotic lesions. Tubulointerstitial lesions can also be initiated by direct disruption of the renal tissue with a needle. Finally, an alternative model of tubulointerstitial disease is genetic renal cystic disease, in which pericytic fibrosis and inflammation occur (52). Juvenile cystic kidney (jck) mice contain an unknown genetic defect that results in development of progressive renal epithelial cysts after birth (2). In all four models of renal disease, Wnt4 was activated in areas with tubulointerstitial disruption.

**EXPERIMENTAL PROCEDURES**

**Murine renal damage models.** Approval was obtained from the Washington University Institutional Animal Care and Use Committee for all experiments involving animals. Four models of renal damage were utilized in these studies. UUO was performed in female FVB/N mice aged 6–10 wk and was accomplished by surgical cautery of the left renal ureter 15 mm from the renal pelvis. Renal puncture injury was performed by passing a sterile 32-gauge needle completely through the surgically exposed left kidney of female FVB/N mice aged 6–10 wk. Punctures were made parallel with the sagittal plane in each kidney at five separate locations. Folic acid damage studies were performed with female FVB/N mice aged 6–10 wk at the start of the experiment. Renal damage induced by folic acid occurs subsequent to temporary deposition of folic acid crystals in the distal segment of some, but not all, nephrons (14). At the dose of folic acid utilized (250 mg/kg in 150 mM sodium carbonate ip), some animals escaped significant crystallization and damage. All animals utilized in this study had sufficient renal damage to induce a blood urea nitrogen level of >100 mg/dl at 24 h after folic acid administration. For mice utilized before 24 h, only those animals with sufficient damage to induce at least a fivefold induction in renal steady-state clusterin mRNA levels were analyzed further. Clusterin mRNA levels were determined by Northern blotting (46) with a radiolabeled probe synthesized from the EcoRI/Xhol fragment of the clusterin sequence in the IMAGE consortium murine EST clone 516894 (GenBank accession no. AA062416). Clusterin is a lipoprotein elevated in response to pathological stress (49) that is rapidly induced after renal damage (50). Mice carrying the jck mutation on the C57BL/6J background were obtained from the Jackson Laboratory. Kidneys from all mice were harvested and snap-frozen in liquid nitrogen for isolation of total RNA or frozen in optimum cutting temperature embedding medium for in situ analysis or immunohistochemistry.

**RNase protection assays.** Total RNA was prepared from frozen kidney samples using a commercial method (Qiagen RNeasy kit). Wnt4 cDNA was a gift from Andy McMahon, and the ribosomal protein L32 gene (rpl32) template was a gift from John Hassell. RNA from each sample (5 μg) in 10 μl hybridization solution (40 mM PIPES, pH = 6.4; 0.4 M NaCl; 2 mM EDTA, pH = 8.0; 80% formamide) were hybridized with gel-purified 32P-radiolabeled riboprobes. After overnight hybridization at 45°C, 300 μl of RNase digestion buffer [3.6 μg RNase A (Roche); 150 U RNase T1 (Roche); 10 mM Tris, pH = 7.5; 5 mM EDTA, pH = 8.0; 350 μM NaCl] were added and the reactions were incubated at 37°C for 30 min. Proteinase K (10 μl of 10 mg/ml) and 20 μl 10% SDS were then added to each sample for an additional 15 min at 37°C. Undigested probes were isolated by phenol/chloroform extraction followed by isopropanol precipitation and separation by electrophoresis on denaturing polyacrylamide gels (5% acrylamide, 8 M urea). Radioactivity was visualized by exposure of the dried gels to the Molecular Dynamics storage phosphorimaging system.

**In situ hybridization.** Frozen sections (4–6 μm) were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Sections were washed once for 5 min in PBS at three times the normal concentration of all salts. Sections were then washed three times in PBS for 5 min; once in water for 2 min; once in 0.1 M triethanolamine, pH 8.0, for 10 min; twice for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine, pH = 8.0; and twice for 2 min in 2× standard sodium citrate (SSC; 1× SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH = 7.0). The sections were then dehydrated through graded ethanols, followed by air drying for 5 min and vacuum drying for 1 h at room temperature. Each slide was then hybridized for 18 h at 60°C with 106 counts/min of 32P-radiolabeled riboprobe in 80 μl hybridization mixture (50% formamide; 2% Denhardt’s solution; 10% dextran sulfate; 0.3 M NaCl; 10 mM Tris, pH = 8.0; 2 mM EDTA, 0.25 g/ml tRNA). The collagen-α1(I) (Col1a1) probe was derived from an IMAGE consortium EST clone (accession no. AA792230). Slides were then treated as follows: 4× SSC for 20 min at room temperature; four times 4× SSC for 5 min at room temperature; RNase A (20 μg/ml) for 30 min at 37°C in 0.5 M NaCl, 0.01 M Tris, pH = 8.0, and 1 mM EDTA; twice 2× SSC for 5 min at room temperature; 1× SSC for 10 min at room temperature; 0.5× SSC for 10 min at room temperature; and 0.1× SSC for 30 min at 60°C. The sections were then dehydrated using increasing concentrations of ethanol followed by vacuum desiccation for 30 min. Slides were dipped in liquid photographic emulsion (Kodak NTB2) and exposed for 1 wk at 4°C. After development, slides were counterstained with hematoxylin and eosin.

**Kidney capsule cell transfer.** RatB1a fibroblasts expressing hemagglutinin-tagged Wnt-4 (RatB1a-Wnt4) and RatB1a fibroblasts transfected with only the pLNCTX vector (RatB1a-LNCTX) were generously provided by Jan Kitajewski (45). Cells were grown in DMEM plus 10% fetal bovine serum and nonessential amino acids. RatB1a fibroblasts were treated with 1 mM sodium butyrate for 16 h and then resuspended in cold PBS (2 × 105 cells/μl). The right kidney of an anesthetized female FVB/N mouse, aged 6–8 wk, was surgically exposed, and a small incision was made in the kidney capsule. One million RatB1a-Wnt4 or RatB1a-LNCTX cells were transferred under the kidney capsule through this incision with a glass capillary pipette. The identities of the Wnt-4 and
control cells were masked from the investigator performing the transfers. Kidneys were harvested at times up to 72 h after cell transfer and either frozen in OCT embedding medium or fixed in 10% buffered formalin and embedded in paraffin. Cytosolic β-catenin was detected by Western blotting of fractionated cell extracts (56). Detection of β-actin with a specific antibody (Santa Cruz Biotechnology) was utilized as a loading control in the Western blotting analysis.

Immunohistochemistry. Primary antibodies utilized were rabbit anti-aquaporin-3 antibody (a gift from Mark A. Knepper) at 11.5 ng IgG/ml, rabbit anti-fibronectin antibody (Sigma) at 1 μg/ml, and mouse monoclonal anti-α-smooth muscle actin (Sigma) at 1.5 μg IgG/ml. Frozen sections (4–6 μm) were fixed in acetone for 15 min at –20°C; air dried 10 min at room temperature; immersed 10 min in water; washed 5 min in PBS three times; treated 1 min in 0.03% H2O2; and blocked 10 min in PBS containing 1% bovine serum albumin, 2% normal donkey serum, 0.2% nonfat dry milk, and 0.3% Triton X-100. For the mouse anti-α-smooth muscle actin antibody, 1.6 μg/ml of donkey anti-mouse IgG F(ab)2 fragment (Jackson ImmunoResearch) was added to the blocking buffer. Primary antibodies were incubated with the sections in blocking buffer overnight at 4°C. The sections were washed 5 min in PBS three times and incubated 1 h at room temperature with 1 μg/ml horseradish peroxidase-conjugated secondary antibodies raised in donkeys against the appropriate species (Jackson ImmunoResearch). Sections were then washed 5 min in PBS three times and developed for 30 s with diaminobenzidine-nickel substrate (Pierce). Sections were counterstained with hematoxylin.

RESULTS

Wnt4 mRNA expression is induced in murine models of renal tubulointerstitial disease. RNase protection assays (RPA) were utilized to determine that Wnt4 is activated during three forms of renal damage (Fig. 1). Wnt4 mRNA from mice at 16.5 days of gestation (E16.5) was included as a positive control because Wnt4 is expressed in the condensing metanephric mesenchymal cells throughout nephrogenesis (22, 47). Very low levels of Wnt4 mRNA were detected in the normal adult mouse kidney (Fig. 1, A–C), but a protected fragment was readily apparent in the control E16.5 renal sample (Fig. 1, A and C). Control animals were treated with sodium carbonate vehicle instead of folic acid and did not develop renal damage or exhibit increased Wnt4 mRNA levels. Wnt4 mRNA was detected in kidneys from adult folic acid-treated animals beginning on the third day after treatment (Fig. 1A). Levels of renal Wnt4 mRNA were increased by varying amounts above levels in undamaged kidneys for up to 12 wk after folic acid treatment. Renal Wnt4 transcript levels were assayed for at least two animals at each time point up to 2 wk. All samples were assayed twice, with no significant difference in relative levels of Wnt4 mRNA. Wnt4 mRNA levels were higher in all kidneys derived from animals 1–2 wk after damage than from animals at earlier times after damage. Wnt4 mRNA levels in kidneys from animals >2 wk after folic acid treatment were variable but higher than Wnt4 mRNA levels in undamaged kidneys.

The temporal expression pattern of Wnt4 correlates best with the appearance of fibrotic lesions, among the many pathophysiological processes after folic acid-induced renal injury. Folic acid-induced tubular damage results from folate crystallization in the distal tubule and is characterized by extensive epithelial cell death.
within 24 h, followed by cellular dedifferentiation and proliferation throughout the nephron, peaking 48–72 h after injection (3, 7). Interstitial cell accumulation begins by 4 days (38), with frank fibrotic lesions apparent at 7–14 days (data not shown). The extent of the fibrotic lesions in any individual kidney is dependent on the amount of initial damage caused by folic acid crystallization, which varies from animal to animal. Fibrotic lesions characterized by increased interstitial cell accumulation and extracellular matrix protein deposition increase up to 14 days after injury. The first detectable increase in Wnt4 expression occurs at the peak of epithelial cell proliferation, but the time of maximum Wnt4 induction at 10–14 days correlates best temporally with the peak of development of fibrotic lesions. Fibrotic lesions in kidneys from folic acid-treated mice do not progress after 14 days but are still evident for at least 12 wk (data not shown), and the levels of renal Wnt4 mRNA do not increase after 14 days after injury. The temporal relationship between Wnt4 mRNA levels during fibrotic progression is consistent with involvement of Wnt4 in the fibrotic process. A different renal damage model better suited for investigating renal fibrosis was utilized to explore the relationship between Wnt4 expression and fibrotic progression: UUO.

The UUO model of tubulointerstitial disease with complete renal blockage consistently results in fibrosis involving essentially the entire kidney and has been widely used to study molecular mechanisms of renal fibrosis (9). Wnt4 expression was measured in the UUO model of tubulointerstitial fibrosis. Wnt4 message is elevated 48 h after obstruction, and levels increase steadily for 4 wk (Fig. 1B), at which time the renal cellular architecture is largely destroyed. Wnt4 expression was determined in obstructed kidneys from at least two different animals at each time point. All samples were assayed at least twice, with no relative difference in the amount of Wnt4 transcript detected. Similar relative amounts of Wnt4 mRNA were detected between different animals at each time point in this damage model, reflecting the reproducible progression of fibrosis after ureteral obstruction. Duplicate samples are shown for 7 and 14 days after obstruction to illustrate this point. Fibrotic lesions increase for 4 wk after UUO, suggesting a correlation between Wnt4 expression and fibrosis. No increase in Wnt4 message occurred in the UUO contralateral kidneys, which undergo compensatory hypertrophy but not fibrotic or inflammatory changes (data not shown). Because the time course of Wnt4 activation matched that of fibrosis in both the UUO and the folic acid obstructive damage models, we examined Wnt4 expression in a nonobstructive renal disease model: polycystic kidney disease. Renal cysts due to genetic polycystic kidney disease are accompanied by pericytic fibrosis and inflammation (52). Wnt4 was activated in jck mice (Fig. 1C), which develop progressive renal epithelial cysts after birth (2).

Wnt4 is activated in collecting duct epithelial cells and adjacent interstitial cells during renal damage. In situ analysis for Wnt4 transcript was performed to determine the cellular localization of Wnt4 expression during renal damage (Fig. 2). Renal Wnt4 expression in the normal adult mouse is limited to the papillary collecting ducts (Fig. 2A). One week after UUO, strong and diffuse Wnt4 expression is observed throughout the papillary region and also the inner medulla (Fig. 2B). Expression is also activated in the outer medulla in distinct rays. Papillary Wnt4 expression in the uninjured mouse kidney is limited to epithelial cells (data not shown). One week after UUO, expression in the papillary and inner medullary regions occurs in both the epithelial cells and the numerous interstitial cells that accumulate after injury (Fig. 2C). Close examination of the outermost medullary rays revealed that this expression pattern is derived from activation of Wnt4 in collecting duct epithelial cells (Fig. 2D). To verify that Wnt4 expression was limited to the region of the collecting ducts, immunohistochemical staining for aquaporin-3 was performed on sections adjacent to sections hybridized with the Wnt4 probe (Fig. 2E). Aquaporin-3 is expressed in collecting duct epithelial cells of the cortex and inner and outer medulla (10). Wnt4 was activated in not only collecting duct epithelial cells in the outermost outer medulla but also some interstitial cells surrounding the innermost outer medullary collecting ducts 1 wk after UUO (Fig. 2, F and G). This expression pattern is consistent with initial activation of Wnt4 in the collecting duct epithelium, followed by activation in interstitial cells surrounding the collecting ducts. Furthermore, Wnt4 activation after UUO expands from the normal site of expression in the most distal papillary collecting ducts to collecting ducts throughout the medulla and into the cortex (Fig. 2, A and B, and 4A).

Renal Wnt4 activation after folic acid damage also occurred in the collecting duct epithelium and surrounding interstitial cells and was limited to regions with fibrotic lesions (data not shown). In adult jck mice, Wnt4 was activated in the renal cysts (Fig. 2H), with expression primarily in the numerous surrounding interstitial cells (Fig. 2I). In situ analysis of Wnt4 expression during cyst development revealed that Wnt4 was activated in a minority of cysts aged 3 wk, and the proportion of expressing cysts increased until essentially all cysts were involved (Fig. 2I). Furthermore, initial expression was primarily in the cystic epithelium at earlier ages (Fig. 2J), with a shift to the pericyctic interstitial cells at later ages (Fig. 2I).

Wnt4 activation during tubulointerstitial disease is limited to damaged collecting ducts and surrounding interstitial cells. Wnt4 activation is initiated in the collecting ducts and surrounding interstitial cells in the three models of renal injury examined. The UUO and folic acid damage models rely on distal obstruction to induce tubular damage, and the damage is initiated distally and may be more severe in collecting ducts than more proximal nephron segments (55). Immunohistochemical analysis revealed that >90% of cysts in jck mice aged 2 wk were aquaporin-3 immunoreactive, indicating origination in the collecting duct epithelium.
Fig. 2. Wnt4 is activated in the collecting duct epithelium and adjacent cells during renal damage. A: in situ hybridization with an antisense probe to Wnt4 reveals expression only in the renal papillae of the uninjured adult murine kidney (arrow). No transcript is detected in the medulla (md) or cortex (cx). B: Wnt4 in situ hybridization with a section from a kidney analyzed 1 wk after UUO. Wnt4 expression is maintained but is more widespread in the papillae (large arrow). This widespread expression extends throughout the inner medulla (arrowhead). In the outer medulla, Wnt4 expression occurs in stripes (small arrow). C: high-magnification brightfield view of Wnt4 transcript in the inner medulla. Wnt4 (dark grains) is activated in both tubular epithelium (arrow) and interstitial cells (arrowhead). Expression is relatively higher in interstitial cells compared with epithelial cells. D: high-magnification brightfield view of renal Wnt4 expression in an outer medullary stripe 1 wk after UUO reveals that expression is limited to collecting duct epithelial cells (arrows). E: immunohistochemical detection of aquaporin-3 is visualized in the collecting duct epithelium as brown peroxidase development in a section adjacent to that shown in D. F: Wnt4 expression in an outer medullary stripe closer to the medulla 1 wk after UUO occurs in both collecting duct epithelial cells (arrows) and adjacent interstitial cells (arrowheads). G: aquaporin-3 immunostaining in the collecting duct epithelium in a section adjacent to that shown in F. H: Wnt4 is expressed in the renal papillae (arrow) and around cysts (arrowheads) in jck mice aged 16 wk. I: brightfield view of Wnt4 expression in the cystic kidney of a jck mouse aged 16 wk reveals that expression is predominantly in interstitial cells adjacent to the cystic epithelium (arrowheads) and to a lesser extent in the epithelial cells (arrows). J: high-power view of a renal cyst in a jck mouse aged 6 wk demonstrates that Wnt4 expression is mainly in the cystic epithelial cells (arrows). *, Cyst lumen.
A fourth renal damage model was employed to determine if collecting duct injury was required for Wnt4 activation. Needle puncture was used to induce fibrotic lesions in both the renal cortex (where collecting ducts are relatively sparse) and the renal inner medullary region (where collecting ducts are relatively numerous). Fibrotic lesions were observed in both medulla (Fig. 3C) and cortex (Fig. 3A) 1 wk after needle puncture, but Wnt4 was activated only in the medullary lesions (Fig. 3D). Aquaporin-3 immunoreactivity clearly defined abundant collecting ducts in the regions surrounding the medullary lesion but not in the medullary tissue involved in the fibrotic lesion (Fig. 3E). Loss of cellular differentiation after renal damage has been reported, including loss of expression of aquaporin genes (15, 20). We observed that aquaporin-3 immunostaining is also lost in severe UUO-induced fibrotic lesions (data not shown). Close examination of medullary lesions after needle puncture revealed that some damaged tubules in the fibrotic lesion still retained weak aquaporin-3 immunoreactivity, indicating collecting duct origin (Fig. 3F). The lack of Wnt4 transcript in the cortical lesions provides evidence that Wnt4 is not activated in all renal regions involved in fibrosis. Furthermore, the presence of collecting ducts in the medullary lesions where Wnt4 mRNA is detected is consistent with initiation of Wnt4 activation being limited to injured collecting ducts.

Wnt4-expressing interstitial cells are myofibroblasts active in fibrotic matrix deposition. Wnt4 mRNA is substantially increased 2 wk after UUO compared with 1 wk after UUO (Fig. 1B), and expression remains primarily confined to the collecting duct epithelium and surrounding interstitial cells (Fig. 4, A and D). The increased expression is localized primarily in interstitial cells (Fig. 4, D, G, and J), which are greatly increased in number after UUO. Epithelial expression appears relatively lower in later lesions where Wnt4-producing interstitial cells are present (Figs. 2, C and F, and 4D). Renal interstitial fibroblasts produce increased amounts of collagen I, collagen III, and fibronectin after UUO (43). Col1a1 mRNA expression is restricted to perivascular cells in the normal kidney (Fig. 4, C and F). In situ assays on adjacent sections from a kidney 2 wk after ureteral obstruction revealed that Col1a1 mRNA has the same overall expression pattern as Wnt4 mRNA (Fig. 4, A and B). The steady-state level of Col1a1 transcript in the damaged kidney is significantly greater than that of the Wnt4 tran-

Fig. 3. Wnt4 expression is induced in medullary, but not cortical, fibrotic lesions after needle puncture. A and B: renal cortex 1 wk after needle puncture. C and D: renal medulla 1 wk after needle puncture. Sections shown in A and C have been stained with hematoxylin and eosin and are adjacent to the sections shown in B and D, respectively. Sections in B and D were hybridized with a Wnt4 antisense riboprobe. Arrows in A–D indicate the margin of the fibrotic lesions induced by the needle puncture, and arrows in adjacent sections are in the same relative locations. E and F: medullary lesion with increased Wnt4 expression contains damaged collecting duct tubules with evident weak aquaporin-3 expression (brown stain). The arrows in E designate aquaporin-3-positive collecting ducts in the tissue surrounding the medullary fibrotic lesion. The arrows in F indicate weak aquaporin-3 staining present in a damaged collecting duct in the fibrotic lesion. *, Lumen of the same damaged tubule (C, E, and F).
Calyx has expanded because of the cell death that follows ureteral obstruction. Wnt4 mRNA expression is identical to that of adjacent sections from a kidney 2 wk after ureteral ligation. Wnt4 is expressed at high levels in the inner medulla (A, arrow) and in stripes that extend from the inner medulla into the cortex (arrowhead). The pattern of Col1a1 expression is predominantly in interstitial cells in areas of tubulointerstitial fibrosis. A and B: in situ hybridization localizes Wnt4 (A) and collagen-α1(I) (Col1a1) mRNA (B) expression in adjacent sections from a kidney 2 wk after ureteral ligation. Wnt4 is expressed at high levels in the inner medulla (A, arrow) and in stripes that extend from the inner medulla into the cortex (arrowhead). The pattern of Col1a1 mRNA expression is identical to that of Wnt4, but Col1a1 expression is significantly more robust (B). The renal calyx has expanded because of the cell death that follows ureteral obstruction (*). C: Col1a1 mRNA expression in a normal kidney occurs in a punctate pattern (arrow) coincident with the location of the renal arteries. D and E: high-magnification brightfield images of a stripe of Wnt4 expression reveal that Wnt4-expressing interstitial cells (arrows in D) also express Col1a1 mRNA (arrows in E). F: cells containing Col1a1 mRNA in the normal kidney are located adjacent to arteries (arrows). G and H: transverse view of collecting ducts on adjacent sections. Wnt4-expressing interstitial cells (arrow in G) are embedded in extensive fibronectin deposits (brown color in H, arrow). Fibronectin was visualized by immunohistochemistry with peroxidase development. I: peritubular fibronectin is apparent in the normal adult kidney (arrow). J and K: collecting ducts visualized on adjacent sections reveal that Wnt4-expressing interstitial cells (arrows in J) also express α-smooth muscle actin (brown color in K, arrow). L: arterial smooth muscle cells (arrow) are the only site of α-smooth muscle actin expression in the uninjured adult kidney.

Transfer of fibroblasts producing Wnt-4 into the normal kidney disrupts the tubular epithelium and promotes interstitial cell accumulation. Wnt4 is activated in many of the interstitial fibroblasts that accumulate during tubulointerstitial disease. Cultured fibroblasts that express Wnt-4 were transferred to normal murine kidneys to determine the potential roles of Wnt4-expressing myofibroblasts in renal cell function after injury. The cultured cells used as a model of myofibroblasts are RatB1a cells that have been stably transfected with a construct engineered to constitutively produce Wnt-4 (RatB1a-Wnt-4 cells; Ref. 45). In culture, these cells constitutively produce high levels of Col1a1 and α-smooth muscle actin (data not shown). RatB1a cells stably transfected with a vector that lacks the Wnt-4 open reading frame were utilized as a control (RatB1a-LNCX cells). Lesions were subsequently observed in 10 of 14 kidneys that were harvested 3 days after transfer of Wnt-4-expressing fibroblasts (Fig. 5). No lesions were observed in any of the 10 kidneys harvested after transfer of control fibroblasts. The lesions occurred adjacent to the transferred fibroblasts...
producing Wnt-4 (Fig. 5, B and D) but not adjacent to the control fibroblasts (Fig. 5, A and C). These lesions are characterized by disrupted tubular structures and an increased number of interstitial cells (Fig. 5D). In-situ analysis of Wnt4 expression was performed on sections prepared from kidneys 24 h after transfer of the cultured fibroblasts. Strong Wnt4 expression was observed in the transferred RatB1a-Wnt-4 cells but not in control RatB1a-LNCX cells (data not shown). Wnt4 expression was not detected in murine renal cells, nor was migration of the RatB1a cells into the kidney observed. Immunohistochemistry for β-catenin was performed on sections from kidneys after cellular transfer, but no changes in cellular β-catenin distribution were observed in the murine renal cells adjacent to the transferred RatB1a cells (data not shown). In addition, the expression of the genes for fibronectin or matrilysin, known targets of Wnt/β-catenin signaling (8, 17), was not induced in murine renal cells adjacent to the transferred RatB1a cells (data not shown).

Wnt-4 stabilizes cytosolic β-catenin in cultured fibroblasts. The lesions observed after transfer of Wnt-4-producing RatB1a fibroblasts could be the direct result of Wnt-4 action on renal cells. Alternatively, damage could result indirectly as a result of Wnt-4 autocrine action on the RatB1a fibroblasts to induce a separate process. Cytosolic β-catenin levels in Wnt-4-producing and control RatB1a fibroblasts were assayed to determine whether RatB1a cells could respond to Wnt-4. The half-life of cytosolic β-catenin is increased by activation of the canonical Wnt signaling pathway (26). Steady-state cytosolic β-catenin levels in RatB1a cells expressing Wnt-4 were markedly increased compared with control cells (Fig. 5E). This result indicates that the lesions caused by transfer of RatB1a fibroblasts under the kidney capsule may be due to autocrine activation of these cells by Wnt-4.

DISCUSSION

Wnt4 activation in the four examined renal injury models was limited to the collecting duct epithelium and adjacent interstitial cells. Cysts in the jck mice form primarily in the collecting duct epithelium, a location presumably defined by the (unknown) genetic defect. Tubular damage and tubular cell proliferation occur throughout the entire nephron in both UUO and folic acid damage (23, 27), although injury and fibrosis after UUO are more severe in the distal nephron (16, 55). Wnt4 activation after needle puncture is limited to collecting ducts and surrounding interstitial cells, despite similar injury and fibrotic response to different nephron segments. Collecting duct epithelial cells are the initial cells in which Wnt4 is activated after renal injury as well as the only nephron segment in which activation occurs. Moreover, diverse types of original injury lead to collecting duct epithelial Wnt4 activation. Wnt4 expression is also detected in the most distal collecting duct epithelial cells of the uninjured adult kidney. Thus Wnt-4 may play a unique role in the normal biology and response to injury of the collecting duct epithelium.

It is interesting that, early in the fibrotic progression, Wnt4 is activated in interstitial fibroblasts immediately surrounding the collecting ducts that also ex-

Fig. 5. A–D: transfer of Wnt-4-producing fibroblasts into the adult mouse kidney causes disruption of tubular structures. Hematoxylin and eosin-stained sections from adult murine kidneys 3 days after injection of RatB1a cells under the kidney capsule are shown. Control RatB1a-LNCX cells that do not produce Wnt-4 are shown in A and C. Wnt-4-producing RatB1a-Wnt-4 cells are shown in B and D. The arrows in A–C indicate RatB1a cells that have remained in a compact layer under the kidney capsule. The arrowheads in B indicate a V-shaped lesion adjacent to the injected Wnt-4-producing cells. Arrow in D points to a normal tubule adjacent to the lesion marked by loss of tubular architecture, increased interstitial cells, and severely damaged tubules (arrowhead). E: steady-state levels of cytosolic β-catenin are elevated in RatB1a cells expressing Wnt-4. Western blotting of the cytosolic fraction of RatB1a cells was performed simultaneously with antibodies against β-catenin and β-actin. Each lane contains the fractionated extract from a separate flask of cells. Control lanes are extracts from RatB1a-LNCX cells that do not express Wnt-4.
press Wnt4. As the fibrotic lesion and interstitial cell number grows, interstitial cells more distant from collecting ducts also express Wnt4. Fibroblasts constitute only a small percentage of the cells in the normal kidney (25), and the large numbers of interstitial fibroblasts that accumulate after renal damage likely have diverse origins. One hypothesis is that interstitial fibroblasts derive from transdifferentiation of tubular epithelial cells (48). The timing and cellular location of Wnt4 activation are consistent with this possibility. Expression is initially detected in the collecting duct epithelium, then later in the interstitial cells with a decrease in epithelial cell expression. Wnt4 expression is autoactivated in the condensing metanephric mesenchyme (21) and might be retained during renal damage in the cells in which expression is initiated, despite the transformation. Conversion of mammary epithelial cells to a mesenchymal phenotype by oncogene activation involves stimulation of the β-catenin/lymphoid enhancer binding factor (LEF)-1 pathway (12), and stimulation of the β-catenin pathway is critical for inducing an epithelial-to-mesenchymal transformation of cultured Madin-Darby canine kidney cells (37). An alternate possibility is that Wnt4 expression in the collecting duct epithelium may independently activate Wnt4 expression in the adjacent interstitial cells, because Wnt-4 activates its own expression. Autocrine activation in the fibroblasts would maintain expression and induce expression in neighboring interstitial cells. Another possibility is that the same signal may initiate Wnt4 activation in both cell types. Resident interstitial cells in the normal kidney are derived from metanephric mesenchyme (13). A population of medullary stromal cells retains Wnt4 expression in the developing kidney (22), and these cells may retain the competence to express Wnt4 and be the source of the Wnt4-expressing medullary interstitial fibroblasts that appear in the adult kidney after UUO.

Wnt4 is activated in most of the interstitial myofibroblasts that accumulate near collecting ducts during tubulointerstitial disease. Cultured myofibroblasts producing Wnt-4 cause wedge-shaped lesions in the renal cortex, with evident tubular destruction. Lesion formation may be mediated by the direct action of Wnt-4 on the adjacent renal cells and/or indirectly through autocrine action of Wnt-4 on the RatB1a fibroblasts to stimulate another pathway. We have not yet detected evidence of Wnt-4-induced signaling or target gene activation in renal cells adjacent to the transplanted RatB1a-Wnt-4 cells. Furthermore, the formation of lesions extending many cell diameters from the transferred cells argues against a direct action of Wnt-4 on the normal renal constituents to produce the entire lesion, because Wnt-4 action is likely to be localized in the immediate vicinity of the producing cells. Wnts bind tightly to the extracellular matrix and do not readily translocate (6, 34). Transferred RatB1a cells were observed only in their original location at the cortical margin, and in situ analysis of Wnt4 transcript expression showed no migration of Wnt-4-producing cells from the original location under the capsule. Direct evidence for Wnt-4 autocrine stimulation of fibroblasts is provided by the stabilization of cytosolic β-catenin in the Wnt-4-producing cells compared with control cells. Stabilization of cytosolic β-catenin activates gene expression through the T cell factor/LEF transcription factor family, and genes activated by this pathway include potential inflammatory/fibrotic mediators such as fibronectin (17), matrilysin (8), cyclooxygenase-2 (19), and members of the connective tissue growth factor family (35, 53). The increased fibronectin secretion by interstitial Wnt-4-producing interstitial myofibroblasts (Fig. 4H) may derive in part from this pathway. The cellular pathways activated by Wnt-4 in the fibroblast could induce attraction and/or activation of inflammatory cells with concomitant destruction of epithelial cells. Synovial fibroblasts isolated from joints with active rheumatoid arthritis produce Wnt-5a, whereas fibroblasts from normal joints do not (41). Transfection of cultured synovial fibroblasts with an expression vector for Wnt-5a induces these cells to produce the proinflammatory cytokines interleukin (IL)-6, IL-8, and IL-15 (41), and inhibition of Wnt signaling in these cells inhibits cytokine production (40). Thus it is possible that the inflammatory lesions observed after transfer of Wnt-4-producing RatB1a cells are caused by autocrine induction of inflammatory mediators in the RatB1a cells.

A role for Wnt-4 in fibroblast activation is also suggested by interaction between Wnt and TGF-β signaling pathways. TGF-β1 is a central regulator of fibroblast activation and transformation to the myofibroblast phenotype (48). TGF-β1 signaling is mediated through transcriptional regulators of the SMAD family, notably SMAD4 (18). LEF1/T cell factor and β-catenin were found to form a complex with SMAD4 and synergistically activate the target gene twin through interaction with the twin promoter (31). Thus Wnt-4 and TGF-β1 may both contribute to fibroblast activation by combinatorial stimulation of gene expression. TGF-β1 has an important role in renal tubulointerstitial disease (44), with production in the medullary tubules after UUO in a similar time frame as Wnt4 induction (51). It will be of interest to define the target genes of Wnt-4 in myofibroblasts alone and in combination with TGF-β1.

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