PAX2 is reactivated in urinary tract obstruction and partially protects collecting ducts from programmed cell death

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Cohen T, Loutochin O, Amin M, Capolicchio J-P, Goodyer P, Jednak R. PAX2 is reactivated in urinary tract obstruction and partially protects collecting ducts from programmed cell death. Am J Physiol Renal Physiol 292: F1267–F1273, 2007. First published December 12, 2006; doi:10.1152/ajprenal.00281.2006.—Obstruction of the urinary tract activates apoptotic pathways in collecting duct cells and leads to loss of renal parenchyma before surgical intervention. It has been suggested that developmental pathways may be reactivated to offset acute organ damage. One such molecule, PAX2, is expressed throughout the fetal collecting duct and was recently shown to suppress apoptosis during kidney development. We hypothesized that acute unilateral urinary tract obstruction (UUO) reactivates PAX2 expression in the mature kidney and partially suppresses apoptosis. If so, animals with PAX2 mutations should have increased susceptibility to parenchymal damage. Wild-type and heterozygous Pax2 mutant (C3H/Pax2¹Neu) mice underwent unilateral ureteric ligation or sham operation at 6 wk of age; kidneys were examined after 5, 10, and 15 days. Whereas PAX2 protein levels fell to low levels in the first weeks of life, it was sharply reactivated by day 10 in collecting duct cells of wild-type but not in Pax2¹Neu mutant mice with UUO. Wild-type mice with UUO had marked TUNEL and cleaved spectrin staining in tubular cells and reduced kidney weight after 10–15 days. Mutant mice had exaggerated increases in markers of apoptosis and exaggerated loss of renal parenchymal loss in the obstructed kidney. These observations suggest that PAX2 is rapidly reactivated in UUO and that mice with genetically limited PAX2 expression have heightened susceptibility to apoptosis.

Hydronephrosis; obstructive nephropathy; kidney; ¹Neu

OBSTRUCTIVE NEPHROPATHY is an important cause of renal failure in infants and children. Acute obstruction of the urinary tract initiates a complex series of pathological events which lead to programmed cell death in tubular cells and rapid loss of renal parenchyma (2, 18). Tubular atrophy is accompanied by a macrophage infiltrate in the interstitium and profound peritubular fibrosis (14, 21). In rodents, tubular apoptosis is dramatically increased within ~5 days after unilateral ureteral obstruction (UUO) and remains high for at least 2 wk (25). Apoptosis then subsides until about day 20, when a secondary wave of programmed cell death begins (25, 26).

UUO appears to induce programmed cell death in renal tubular cells via activation of proapoptotic stimuli such as TNF-α (17) and TGF-β (16). This is accompanied by suppression of Bcl2 (4), dephosphorylation of BAD (15), activation of DAP kinase, and increased expression of the caspase cascade genes (26). Interestingly, the pathways of programmed cell death can be activated by mechanical stretch, alone. Caspase 3-mediated apoptosis is induced by applying gentle stretch to the substratum on which monolayers of human kidney cells are cultured (20). Similarly, mechanical strain was shown to activate BAD dephosphorylation in cultured proximal tubular cells (15). In neonatal mice with UUO, stretch-induced apoptosis predominates in the collecting ducts, where dilatation is maximal (3). Conversely, in the proximal tubule, vasoconstriction tends to cause ischemic necrosis (3).

During fetal life, cells of the collecting duct lineage are relatively protected from programmed cell death. In large part, this resistance to apoptosis is afforded by the “paired-box” transcription factor, PAX2. Mice bearing heterozygous mutations of the PAX2 genes exhibit a marked increase in ureteric bud (UB) cell apoptosis during fetal life (19). As a consequence, UB branching is suboptimal and mice are born with reduced nephron number (19). Renal hypoplasia can be rescued by treating the pregnant mice with caspase-inhibitory drugs which replace the missing antiapoptotic function of PAX2 in mutant fetal kidney (6). Conversely, when endogenous PAX2 levels in cultured collecting duct cells are reduced when transfected with an antisense Pax2 cDNA, apoptosis is increased (23).

High levels of PAX2 protein are evident in the UB as it emerges from the nephric duct and expression is sustained throughout the collecting system as it arborizes during kidney development. Low levels of PAX2 in metanephric mesenchyme activate GDNF, a trophic factor for the ureteric bud (2). Higher levels of Pax2 protein also appear in condensing mesenchymal cells as they cluster to form individual nephrons (8, 9). Once nephrogenesis is complete, however, the transcription factor is downregulated and the level of expression is very low in mature kidney (8, 9, 11, 24).

Recent evidence suggests that some forms of postnatal renal injury may reactivate expression of fetal genes. Following acute tubular necrosis proximal tubular cells have been shown to reexpress PAX2 during the process of regeneration (13). Given its powerful antiapoptotic role during fetal life, we hypothesized that PAX2 might be reactivated during urinary tract obstruction and might serve to limit apoptotic damage. Here we demonstrate that, following UUO, apoptosis, tubular atrophy, and parenchymal loss are heightened in the heterozygous Pax2¹Neu mouse with suboptimal reactivation of PAX2. Thus reactivation of PAX2

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shown.

Fig. 1. Unilateral left ureteral obstruction. Filled arrow, ligature on left ureter; open arrow, dilated renal pelvis following 15 days of obstruction.

normally plays an important protective role during the renal response to urinary tract obstruction.

MATERIALS AND METHODS

Breeding of litters for Pax2<sup>1Neu</sup> mutant and wild-type mice. C3H mice were obtained from Charles River Laboratories (St. Constant, Quebec). Heterozygous Pax2<sup>1Neu</sup> mutant male mice bearing a single base-pair G insertion within the DNA-binding paired domain were crossed with wild-type (C3H) females as previously described (6, 19). Offspring were genotyped before weaning using specially designed PCR primers to create a unique restriction site for XmnI in the presence of the mutation as reported elsewhere (6).

The sense primer was 5'-GTGTGAAACCAGCTCggGGTG-3', and the antisense primer was 5'-GCCAGGATTTTGTGAACAGC-3'.

Wild-type mice displayed one band at 166 base pairs, while heterozygous 1Neu mice showed two bands, one at 166 base pairs and another at 151 base pairs. Samples of DNA used for genotyping were obtained using a Wizard DNA Purification Kit (Promega).

Surgical procedure and tissue preparation. Pax2<sup>1Neu</sup> and C3H mice were subjected to complete UUO for 5, 10, or 15 days (5 animals/group in each time period). Five control mice for each group were sham operated and the kidneys harvested on day 0. General anesthesia was induced using 4% halothane/0.5% oxygen at 1 l/min and maintained intraoperatively using 2% halothane/1% oxygen at 1 l/min. The abdomen was entered using a upper paramedian incision.

Longitudinal exposure of the left ureter was established by mediolaterally reflecting the colon. The proximal ureter was then ligated using two 5-0 silk sutures. The muscles of anterior abdominal wall were closed using 5-0 Vicryl and the skin reapproximated using 2-0 silk. The incision was covered with collodion.

On postoperative days 5, 10, and 15, the animals were anesthetized as above, both kidneys were removed and the animals were killed by an overdose of anesthetic. The kidneys were decapsulated, decompressed, and weighed. Each kidney was bisected along the sagittal plane. Half the kidney was fixed in 10% formalin for 24 h and then stored in 70% ethanol and embedded in paraffin for histological analyses, immunohistochemistry, and Tdt-mediated uridine-nick-end-labeling (TUNEL) assay. The remaining half of the kidney was snap-frozen and stored at −70°C for Western immunoblot analysis.

Western blot analysis of Pax2 expression. Kidneys were homogenized in lysis buffer consisting of 62.5 mM Tris, pH 6.8, 10% glycerol, 6 M urea, 2% SDS, and 5% β-mercaptoethanol. Loading dye was added and the samples were boiled at 100°C for 5 min, spun, and submitted to electrophoresis on a 10% polyacrylamide gel at 100 V for 2 h. Proteins were then transferred electrophoretically to a nitrocellulose membrane (Hybond) for 1 h at 100 V. The blot was blocked with PBS-Tween (0.1%) plus 5% milk at room temperature for 2 h and then probed with rabbit polyclonal Pax2 antibody (1:250, Zymed) for 2 h. The blot was washed with PBS-T (0.1%), reprobed with secondary antibody (1:1,000 anti-rabbit IgG), and washed 3 × 5 min with PBS-T (0.1%). Immunoreactive bands were detected by enhanced chemiluminescence. The blots were then stripped using 62.5 mM Tris, pH 6.7, 2% SDS, 100 mM β-mercaptoethanol, and distilled water, heated to 50°C. The blots were then reprobed with β-actin antibody as above.

Intensity of immunoreactive Pax2 and β-actin bands was quantified using MCID-M5 4.0 image-analysis software; spatial measures for the number of pixels contained in each band of Western blots were computed. Intensities from the Pax2 bands were divided by the β-actin band intensities to normalize the Pax2 signal intensity for loading variation.

Immunohistochemistry. For Pax2 immunohistochemistry, 5-μm sections were heated to 60°C for 1 h, deparaffinized in xylene, and rehydrated. Endogenous peroxidases were blocked with 3% H<sub>2</sub>O<sub>2</sub> for 30 min at room temperature. The tissue sections were then incubated in blocking serum (Vectastain Universal Kit) for 20 min and exposed to primary anti-PAX2 polyclonal antibody (Zymed) diluted in buffer (1:50 dilution) for 30 min at 37°C. The sections were washed in buffer and then incubated for 30 min in diluted biotinylated secondary antibody with avidin-tagged horseradish peroxidase (Vectastain Uni-

Fig. 2. Apoptosis of collecting duct cells in wild-type mice with unilateral ureteral obstruction (UUO). Tdt-mediated uridine-nick-end-labeling (TUNEL) staining (×400) at days 0 (A), 5 (B), and 10 (C) of obstruction is shown. D: high-power (×1,000) view showing nuclear TUNEL staining of apoptotic cells. Widespread apoptosis is seen in a subset of medullary tubules as demonstrated by immunohistochemical staining for cleaved spectrin (×200) in wild-type mouse kidney at day 0 (E) and after 10 days of UUO (F).
PBS (2 sections incubated for 1 min at room temperature, washed with graded ethanol (70, 95, 100%) After rinsing in tap water, the slides were dehydrated through mounting with Permount (Fisher Scientific).

As a sensitive indicator of early stages of apoptotic caspase activity, we assessed cleaved spectrin (p120 neoepitope) immunohistochemistry. Rehydrated sections were exposed three times to PBS + Triton (0.03%) for 5 min. The sections were then digested with trypsin (0.05%) at 37°C, washed in PBS + Triton (0.03%), and endogenous peroxidases were blocked with 3% H2O2 for 30 min at room temperature before incubation with primary anti-spectrin p120 neoepitope antibody (1:100 dilution) overnight at room temperature. The affinity-purified anti-Spectrin p120 neoepitope antibody (SVEALIK-KLH; 24 mg/ml, no. G0021805K) was obtained from Merck-Frosst (Montreal, Quebec). The sections were washed in buffer and then incubated for 30 min in biotinylated secondary antibody with avidin-tagged horse radish peroxidase (Vectastain Universal Kit) at 37°C. Immunoreactive spectrin 120 neoepitope was detected with the Vectastain kit as above and counterstained with methyl green dye.

TUNEL immunohistochemistry was performed with a TdT-mediated uridine-nick-end-labeling kit (Roche Diagnostics, Mississauga, Ontario). Rehydrated sections were treated with proteinase K (5 mg/ml) (Invitrogen, Burlington, Ontario) in 10 mM Tris·HCl, pH 7.5, for 10 min at room temperature and the sections washed in PBS (2 × 5 min). Endogenous peroxidase was quenched with 3% hydrogen peroxide (Sigma) in methanol for 30 min. TdT reaction buffer was added, and the sections were covered with paraffin and incubated in a humidified chamber at 37°C for 60 min. The slides were washed in PBS (2 × 5 min), incubated in the presence of peroxidase converter for 30 min at 37°C, and washed again in PBS (2 × 5 min). DAB substrate (Vector Laboratories) was added, the sections incubated for 1 min at room temperature, washed with PBS (2 × 5 min), and counterstained with 0.5% methylene green.

Tissue sections were examined for apoptotic nuclei in tubular cells (no interstitial apoptosis was seen) at ×400 magnification by a blinded investigator. TUNEL-positive tubular cells were counted in randomly selected fields from cortex (n = 10) and medulla (n = 10), and the data were pooled; five kidneys were evaluated for each group. Data are expressed as the mean number of TUNEL-positive tubular cells per high-power field ± SE in each experimental group.

Detection of tubular atrophy. Tissue sections (5 μm) fixed in formalin were deparaffinized and hydrated in graded alcohols and stained with periodic acid–Schiff reagents. The slides were then washed with water for 5 min, stained with Harris hematoxylin for 5 min, washed, and treated with ammonia water. Following a final wash, the sections were dehydrated in graded alcohols, mounted, and examined by light microscopy at ×400 magnification.

Five sections were examined for each kidney. Ten randomly selected fields (2/section) were used to count both normal and atrophic tubules by the point-counting method described by Sharma et al. (21). The average number of normal and atrophic tubules was calculated from 10 fields examined. The number of atrophic tubules as a percentage of the total number of tubules was determined for each kidney. Tubules were considered atrophic if there was thickening or duplication of the basement membrane according to criteria described by Chevalier et al. (5).

Statistical analysis. Statistical analysis was performed using ANOVA and a post hoc independent Student’s t-test using SPSS software. Data are reported as means ± SE.

RESULTS

UUO in young adult wild-type mice. The basal effects of UUO (Fig. 1) were characterized in normal 6-wk-old C57 mice over 15 days. Apoptosis, as reflected by DNA fragmentation (an increase in TUNEL-positive cells), was evident by day 5 and was maximal by day 10 of obstruction (Fig. 2, A–C). At higher power, the intense TUNEL staining is clearly localized to nuclei of cells undergoing programmed cell death (Fig. 3). Early stages of apoptosis, as reflected by cleavage of cytoskeletal caspase targets, were tracked by immunohistochemical staining for cleaved spectrin. The spectrin neoepitope was detected with a mouse monoclonal antibody against spectrin (clone 4B11; Sigma–Aldrich). Tubular atrophy (Fig. 4) was characterized by thickening of basement membranes and expressed as a percentage of the total number of tubules (magnification ×400; C).

Fig. 3. Tubular atrophy (periodic acid-Schiff stain) in wild-type mice at day 0 (A) and after UUO for 15 days (B). Tubular atrophy was quantified by identifying tubules with reduplication or thickening of basement membranes and expressed as a percentage of the total number of tubules (magnification ×400; C).

Fig. 4. Loss of renal parenchyma following UUO in wild-type mice. A: low-power image of left hemikidney after 15 days of UUO showing scalloping of renal medulla vs. hypertrophied contralateral unobstructed right kidney. B: ratio of obstructed left kidney weight to right unobstructed kidney weight in wild-type mice on day 0 and following 5, 10, and 15 days of obstruction. *P < 0.05 vs. control group at day 0 of obstruction.
was detected primarily in collecting ducts of the obstructed kidney (Fig. 2F) but not at baseline (Fig. 2E) or in the contralateral unobstructed right kidney (a).

Atrophic tubules were identified by their thickened, irregular and sometimes duplicated tubular basement membrane according to published criteria (5). Following UUO, progressive tubular atrophy was seen in wild-type C3H mice from 5 to 15 days of UUO (Fig. 3, A–C). By day 15, striking loss of renal parenchyma was evident after decompression of the hydronephrosis. The obstructed kidneys were markedly smaller than the unobstructed contralateral kidney and showed prominent scalloping of the renal medulla (Fig. 4A). Loss of parenchyma in the obstructed kidney was tracked by comparing left to right kidney weights at each time point (Fig. 4B).

PAX2 expression in postnatal mouse kidney. To characterize the ontogeny of PAX2 expression in normal postnatal mouse kidney, we quantified the level of PAX2 protein in extracts of whole kidney by Western immunoblotting (normalized for /H9252-actin). PAX2 expression was rapidly downregulated in the first 2 wk of life, approaching barely detectable levels characteristic of adult kidney by 4–6 wk of age (Fig. 5).

On ligation of the left ureter at 6 wk of age, PAX2 protein rose rapidly in the obstructed kidney but not in the contralateral kidney (Fig. 5). By 15 days of UUO, renal PAX2 expression approximated the level seen in fetal kidney (Fig. 5). Reactivation of PAX2 was noted primarily in collecting ducts of the obstructed kidney (Fig. 6).

To examine the effects of UUO on animals lacking this normal PAX2 expression response, we also performed unilateral ureteral ligation in 6-wk-old heterozygous Pax21Neu mutant mice. This mouse strain transmits a null Pax2 allele containing a frame-shift mutation in the second exon (12). In previous studies, we found that these mice express about half the level PAX2 protein during fetal kidney development compared with wild-type littermates (19). As seen in Fig. 7, the normal reactivation of renal PAX2 expression seen in wild-type mice with UUO is severely blunted in Pax21Neu mutants (Fig. 7).

Comparative effects of UUO in Pax21Neu vs. wild-type mice. The effect of UUO on collecting duct cell apoptosis was compared in Pax21Neu mice vs. control animals. Both groups underwent UUO at 6 wk of age. At baseline, TUNEL-positive cells were rarely seen in kidneys from either the mutant or wild-type mice (Fig. 8A). However, by day 10 of UUO, TUNEL staining was much more striking in the mutant animals than in wild-type (Fig. 8, C and D). This effect was quantified by counting the number of TUNEL-positive tubular cells/high-power field in the obstructed kidney of each group. The level of renal apoptosis was nearly doubled (P < 0.05) in the Pax21Neu group compared with the wild-type group at both 5 and 10 days of UUO (Fig. 8E).

We also compared the degree of tubular atrophy induced by UUO in the Pax21Neu mice to that in wild-type animals. Tubules in representative sagittal sections were scored as atrophic or normal using the published criteria of Chevalier et al. (5) as above. In Pax21Neu mice with UUO, the percentage of tubules showing evidence of atrophy was nearly twice that of obstructed wild-type animals at days 5 and 10 (P < 0.05) (Fig. 9). The contralateral kidney showed no evidence of tubular atrophy in either group (data not shown).
Increased tubular cell death resulted in loss of renal parenchyma as predicted. This was greater in the mutant than in wild-type mice (Fig. 10, A and B). To quantify the effect of UUO on parenchymal loss by wild-type vs. Pax2^{1Neu} mice (with modest renal hypoplasia at baseline), we calculated the ratio of left (obstructed) to right (contralateral) kidney weight for each mouse. In the heterozygous Pax2^{1Neu} mutant mouse, the ratio of left to right kidney weight fell from baseline (1.0) to 0.55 after 15 days of UUO (Fig. 10C). However, in the wild-type mouse, the left to right kidney weight ratio fell only to 0.70 ($P < 0.05$) (Fig. 10C). This effect is also seen if left kidney weight is compared with total body weight for each group. On day 0, the left kidney weighed ~1% of body weight for both groups (Fig. 11). However, the obstructed kidney weight of wild-type animals fell to 0.7% of body weight by day 15 vs. 0.5% of body weight in the mutant mice ($P < 0.05$) (Fig. 11).

**DISCUSSION**

The Pax genes encode a family of evolutionarily conserved transcription factors with central roles in embryonic development (7). The characteristic feature is a “paired box” DNA-binding domain which activates transcription of target genes by binding to specific motifs in their promoter regions. Of the nine human PAX genes, only PAX2 and PAX8 are expressed in kidney. Although PAX8 may contribute to development of the nephric duct (1), its function is not critical and PAX8 knockout mice have no obvious renal phenotype; in contrast, homozygous PAX2 mutants are anephric (24).

Although Pax2 appears to have a number of roles during renal development, a key function is to suppress programmed cell death in UB cells, thereby optimizing the rate of branching nephrogenesis (6, 10, 19, 22). Heterozygous Pax2 mutant mice display increased apoptosis of UB cells without any change in proliferative rate (19, 23). Since the mechanical stress of acute urinary tract obstruction is known to induce rapid apoptotic death in collecting duct cells (derived from the UB lineage), we were particularly interested to know whether Pax2 might be reactivated and serve a protective role during the renal response to this form of injury. When we examined the ontogeny of PAX2 protein expression in mouse kidney, we observed that the transcription factor is gradually downregulated during the early postnatal period. By 6 wk of age, renal PAX2 protein approaches the adult nadir of <10% of the fetal level. We decided to induce acute UUO at that age and observed that PAX2 was rapidly increased, approximating the fetal range within 10–15 days. Interestingly, PAX2 reactivation in the obstructed kidney of mutant Pax2^{1Neu} mice was sharply blunted. This is somewhat surprising, since the heterozygous mutant animals retain one wild-type allele. Conceivably, PAX2 reactivation involves a positive feedback loop in which an increase in PAX2 amplifies its own expression.
In normal mice, acute UUO at 6 wk of age induced a wave of renal apoptosis detected by TUNEL staining of fragmented DNA. Increased nuclear TUNEL staining was first noted by day 5, becoming maximal by day 10. This was shortly followed by evidence of tubular atrophy and parenchymal loss by day 15. A similar pattern has been reported by others in adult rodents (25). As a second measure of programmed cell death, we assessed the accumulation of cleaved spectrin. Spectrin and other cytoskeletal elements are degraded by caspase 3 during programmed cell death. The 120-kDa spectrin cleavage fragment can be identified immunohistochemically with an antibody, which specifically recognizes a neoepitope in the degraded protein. Intense staining for cleaved spectrin was particularly strong in a large subset of the medullary tubules and nearly absent in others. Cachat et al. (3) have reported selective induction of apoptosis in the collecting duct of obstructed kidneys. Although the proximal tubule may be susceptible to ischemic injury due to the hemodynamic changes induced by UUO (3), it is conceivable that the pathways of programmed cell death are more responsive to mechanical stretch (15) applied to the tight intercellular junctions of the collecting duct rather than the leakier proximal tubular segments. In our study, parenchymal loss was particularly prominent in the renal medulla, leading to a scalloped appearance of the obstructed kidney after decompression. Interestingly, cleaved spectrin was evident in all cells of the affected medullary tubules, whereas only some cells were TUNEL positive. Conceivably, caspase cleavage of spectrin may precede the irreversible stages of programmed cell death marked by DNA fragmentation, allowing the potential for cellular recovery.

To assess the role of PAX2 reactivation as a normal protective response to UUO, we performed parallel experiments in heterozygous Pax2<sup>2/neu</sup> mutant mice and in wild-type animals at 6 wk of age. Although the mutant mice retain one functional Pax2 allele, we noted that reexpression of renal PAX2 protein in response to UUO was markedly blunted. Accordingly, we observed that Pax2<sup>2/neu</sup> mice exhibit heightened susceptibility to apoptosis, tubular atrophy, and parenchymal loss compared with wild-type. These data strongly support the hypothesis that, in normal adult animals, reactivation of PAX2 partially protects renal collecting duct cells from apoptosis initiated by obstructive injury. It is unknown whether reactivation of PAX2 by UUO varies among mouse strains or whether there is interindividual variation in the PAX2 response among humans. However, it is conceivable that the extent of renal parenchymal loss is modulated by the PAX2 response. We recently found that the caspase inhibitor Z-VAD-fmk can substitute for the loss of PAX2 antiapoptotic function during renal development (6). It is tempting to speculate that caspase-inhibitory drugs might ameliorate renal parenchymal loss during acute urinary tract obstruction when endogenous mechanisms are overwhelmed. If so, there might be a role for use of these drugs during the period between discovery of urinary tract obstruction and surgical intervention.
PAX2 PROTECTS RENAL COLLECTING DUCT CELLS FROM APOPTOSIS

GRANTS

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