American Journal of Pathology, Vol. 152, No. 5, May 1998

Short-Term Urinary Flow Impairment Deregulates PAX2 and PCNA Expression and Cell Survival in Fetal Sheep Kidneys

Rehab Attar,* Feargal Quinn,* Paul J.D. Winyard,* Pierre D.E. Mouriquand,* Peta Foxall,† Mark A. Hanson,‡ and Adrian S. Woolf*

From the Nephrourology Unit, Institute of Child Health, and the Institute of Urology and Nephrology,† Middlesex Hospital, London, United Kingdom and the Department of Obstetrics and Gynaecology,‡ University College Hospital, University College London Medical School, London, United Kingdom*

Renal malformations account for most children with chronic renal failure and are often associated with urinary tract anatomical obstruction. We examined cellular and molecular events after experimental urinary flow impairment in fetal sheep. Ovine gestation lasts 144 to 150 days with the metanephros appearing at 27 to 30 days. We generated complete unilateral ureteric anatomical obstruction at 90 days when a few layers of glomeruli had formed. After 10 days, we recorded ureteric and pelvic dilatation with renal parenchymal weight greater than contralateral organs or those from unoperated fetuses. The nephrogenic cortex was replaced by disorganized cells separated by edema and prominent vascular spaces. Cortical histology was dominated by cysts associated with malformed glomerular tufts. Cystic epithelia expressed PAX2, a growth-stimulating transcription factor down-regulated during normal maturation, and proliferating cell nuclear antigen, a surrogate marker of cycling cells. Detection of apoptosis using propidium iodide and *in situ* end labeling showed a significant increase of the point prevalence of death in the obstructed cortex. Hence, PAX2 and proliferating cell nuclear antigen expression as well as death were deregulated, as we previously reported in human kidney malformations. Medullary collecting ducts and loops of Henle were also disrupted, correlating with impaired urinary dilution and sodium reabsorption. Therefore, complex aberrations of morphogenesis, gene expression, cell turnover, and urine composition occur relatively early after experimental impairment of fetal urinary flow. *(Am J Pathol 1998, 152:1225–1235)*

Renal malformations account for most cases of chronic renal failure in early childhood[1] and are often associated with anatomically obstructed urinary tracts.[2,3] As examples, both cystic dysplastic and hydronephrotic kidneys are associated with posterior urethral valves in males and with ureterocoeles in females, whereas multicystic dysplastic kidneys are attached to atretic, obstructed ureters.[2] Bilateral obstructive nephropathy is associated with oligohydramnios with lung hypoplasia, and newborns often die from respiratory or renal failure. Although prenatal surgical decompression is feasible, controlled clinical studies to assess potential benefits on lung and kidney development are lacking.[3]

Renal malformations can be generated by experimental anatomical obstruction of the fetal urinary tract.[4] The ovine metanephros appears at 27 to 30 days of gestation whereas term is at 145 days,[5] and this species constitutes a good model because the urinary tract can be manipulated *in vivo* relatively early in gestation to generate pathologies resembling human malformations.[6–8] Urinary flow impairment caused by complete ureteric obstruction at 90 days gestation generated hydronephrosis and subcapsular cysts whereas the same maneuver at 45 to 70 days caused growth failure and dysplasia.[6–8] The more dramatic effects in early gestation are perhaps explained by a relative structural immaturity and higher growth rate.[9]

Nephrogenesis is controlled by genes that enhance or inhibit growth by affecting cell survival, proliferation, differentiation, and morphogenesis.[10] For example, when renal mesenchymal cells begin to differentiate into nephron tubules, they aggregate into condensates and undergo a burst of proliferation with increased expression of PAX2 transcription factor; later in nephrogenesis, both proliferation and PAX2 expression are down-regulated.[11,12] Metanephric growth is impaired in mice with kidneys genetically engineered to lack PAX2 protein[13,14] whereas transgenic overexpression of PAX2 generates renal cysts.[15] Furthermore, humans with heterozygous,

Presented at the UK Renal Association, London, October 1997.

Supported by the National Kidney Research Fund project grant R11/2/96 and Action Research project grant S/P/3187.

Accepted for publication February 16, 1998.

Address reprint requests to Dr. Adrian S. Woolf, Nephrourology Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK. E-mail: a.woolf@ich.ucl.ac.uk.

inactivating PAX2 mutations are born with hypoplastic kidneys.[16] Although these studies link PAX2 expression to growth and proliferation, it is also established that programmed cell death, or apoptosis, is involved in normal nephrogenesis.[17–19] A limited degree of cell suicide is probably required to regulate nephron number and morphogenesis, but excess apoptosis, as occurs in mice deficient in BCL2 survival factor,[20,21] results in malformed organs with few nephrons.

Little is known about the effects of urinary flow impairment on metanephric cell turnover and gene expression. In this study, we caused complete unilateral ureteric obstruction in fetal sheep and correlated anatomical renal parenchymal perturbations with the expression patterns of PAX2 protein and proliferating cell nuclear antigen (PCNA), a surrogate marker of mitosis.[22] Apoptosis was also assessed by detection of pyknotic nuclei[18] and *in situ* end labeling.[23]

Materials and Methods

Unless otherwise stated, chemicals were obtained from Sigma Chemical Co. (Poole, UK).

Surgery

Operations, approved by the Home Office, were performed at 90 days gestation. Sheep were of the Mule crossbreed purchased from R. White (Oxfordshire, UK). Pregnant ewes were fasted for a day, anesthetized with sodium pentothal, and intubated with a size 14 endotracheal tube. Anesthesia was maintained with halothane and oxygen. The abdomen was incised in the midline, the uterus entered with diathermy, and the fetal hindquarters delivered. Diathermy was used to make a lumbotomy incision through skin and muscle, and the fetal ureter was dissected with cotton buds and tightly ligated with 4.0 silk. The muscle layers and uterus were closed with vicryl sutures. Gentamicin and penicillin were injected into the uterine cavity, and the mother's skin was sutured with 2.0 silk. In the presence of a twin, the second fetus was left as a control. Surgery lasted 45 minutes, and mothers recovered from anesthesia by 2 hours when they were fed. Intramuscular streptomycin was administered for 5 days postoperatively. Two fetuses were sacrificed at 90 days, and their four kidneys were collected for documentation of weight and histology. Obstructed and contralateral kidneys of six operated fetuses were harvested 10 days after surgery at 100 days gestation (obstructed and contralateral groups). As human and sheep kidneys can hypertrophy *in utero* when opposite a malformed or absent organ,[24–26] we harvested six kidneys from three intact twins and eight kidneys of four fetuses from separate pregnancies in which ewes and fetuses received no surgery (100-day control groups 1 and 2). An additional three obstructed organs were harvested 1 day after surgery at 91 days for limited histological analysis.

Each fetal kidney was separated from its ureter, the renal pelvis drained, and the wet weight measured. One-half of each freshly removed fetal kidney was fixed in 2% paraformaldehyde and embedded in paraffin wax whereas the symmetrical other half was snap-frozen in liquid nitrogen and stored at −70°C. In a limited set of fetuses (n = 3 for each group), fluid for electrolyte analysis was aspirated from the pelvis of obstructed kidneys, from the bladder (ie, contralateral kidney urine), and from the amniotic cavity.

Immunohistochemistry

We used a rabbit polyclonal antibody (Ab) raised against amino acids 188 to 385 in the PAX2 carboxy terminal[27]; this sequence does not include the highly conserved paired domain in the amino terminal of full-length PAX2.[11] Using PAX2-, PAX5-, and PAX8-transfected cells, there is only appreciable reactivity to PAX2, and using PAX2 deletion mutants, this Ab recognizes epitopes between amino acids 270 and 338.[28] On Western blot of mouse and human metanephros, the Ab recognizes a single major doublet (46 to 48 kd[12,27]). Mouse monoclonal Ab to PCNA, a DNA-polymerase-δ-associated protein expressed at high levels in S phase,[22] was obtained from Oncogene Science (Cambridge, MA; PCNA Ab-1). Immunohistochemistry was performed as described.[12] Six-micron sections were dewaxed through Histo-Clear (National Diagnostics, Atlanta, GA), rehydrated through 100% to 30% alcohols, washed in phosphate-buffered saline (PBS, pH 7.4), immersed in citric acid buffer (2.1 g/L, pH 6.0), and microwaved for 10 minutes. They were cooled, washed in PBS, and incubated in 3% hydrogen peroxide to quench endogenous peroxidase, and nonspecific Ab binding was blocked with 10% fetal calf serum (FCS)/PBS. Primary Abs were applied for 1 hour at 37°C (PCNA Ab at 1/50 dilution and PAX2 Ab at 10 mg/L) and were detected using a streptavidin-biotin peroxidase system followed by diaminobenzidine (ABC kit, Dako, High Wycombe, UK). In control experiments, primary Abs were omitted. Sections were counterstained with methyl green or hematoxylin, mounted in DPX (BDH, Poole, UK), and examined on a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Western Blots

Organs were homogenized in lysis buffer (50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (1 μg/ml sodium orthovanadate, 100 μg/ml phenylmethylsulfonyl fluoride, and 30 μl/ml aprotinin).[29] Samples were electrophoresed through a 5% stacking and 12% resolving Tris-glycine SDS-polyacrylamide gel (Protogel, National Diagnostics, Atlanta, GA), and proteins were electrophoretically transferred to membranes (Hybond-C extra, Amersham, Amersham, UK) by semi-dry blotting. Filters were placed in block solution overnight (10% marvel, 0.05% Tween in PBS) and incubated for 1 hour with PAX2 or PCNA Abs at a dilution of 1:500 in block solution. In control experiments, these Abs were omitted. Membranes were washed three times for 10 minutes in block and incubated for 2 hours with a goat anti-rabbit peroxidase-conjugated Ab (Dako). After washing in PBS, sig-

nals were detected with enhanced chemiluminesence reagent (Amersham).

Apoptosis

Apoptotic nuclei stained with propidium iodide (PI) can be identified under fluorescence microscopy as small, fragmented nuclei that stain brightly. We used the technique of Coles et al[18] with modifications.[19] Paraffin sections were dewaxed, hydrated, and incubated in PI (4 mg/L) with RNAse A (100 mg/L; Unipath, Basingstoke, UK) in PBS at 37°C for 30 minutes, mounted in Citifluor (Chemical Labs, University of Kent, Canterbury, UK), and examined at 568 nm on a Zeiss Axiophot microscope. During apoptosis, DNA is digested by endonucleases leaving free 3′ ends; using terminal deoxytransferase, labeled nucleotides can be added and visualized on tissue sections.[23] We used the *in situ* cell death detection kit (Boehringer Mannheim, Mannheim, Germany). Paraffin sections were dewaxed, rehydrated, and exposed to terminal deoxytransferase and fluorescein isothiocyanate (FITC)-conjugated UTP according to the manufacturer's instructions. Labeled nuclei were detected using a Leica confocal laser scanning microscope (Aristoplan-Leica, Heidelberg, Germany). Preliminary experiments with both PI and *in situ* end-labeled specimens showed a good correlation. To measure apoptosis, we photographed eight random fields from the cortex of each metanephros. The mean number of apoptotic nuclei per eight fields represented the point prevalence.

Comparisons of weights and apoptosis were made using Wilcox rank sum tests with $P < 0.05$ considered significant.

Results

Anatomy and Histology

We ligated ureters of metanephroi at 90 days gestation when there was a prominent nephrogenic zone overlying three layers of maturing glomeruli (Figure 1A). The median (range) kidney wet weight was 5.2 (4.1 to 5.3) g (n = 4). By 100 days, weight of organs from unoperated fetuses increased to 5.6 (4.1 to 6.3) g in control group 1 (n = 6) and 6.1 (5.0 to 6.9) g in control group 2 (n = 8). No differences were noted in weight or histology of left *versus* right organs. After 10 days of urinary flow impairment, the six kidneys became swollen with pelvic dilatation, and a loss of distinction between cortex and medulla was observed (Figure 1B). None spontaneously decompressed. Contralateral organs weighed 6.3 (5.0 to 6.7) g (n = 6), significantly less ($P < 0.05$) than obstructed metanephroi (8.6 (7.8 to 9.7) g, n = 6) but not different from the 100-day controls.

At 100 days, the cortex of controls still had a nephrogenic zone and an additional glomerular layer had formed (Figure 1C), with similar observations in contralateral organs. Renal mesenchymal cells below the capsule were densely packed, and pyknotic nuclei were rare (Figure 1D). In organs obstructed from 90 to 100 days gestation, the cortex was grossly abnormal, with a uniform pattern in the six specimens (Figure 1, E and F). Histology was similar in the two left kidneys and four right kidneys that had been obstructed. Only sparse mesenchymal condensates were discerned, and no normal S-shaped bodies (nephron precursors) were apparent (Figure 1E). Instead, cortical architecture was dominated by cysts up to 0.5 mm across, which often contained malformed glomerular tufts (Figure 1E). There was interstitial edema in the outer cortex with prominent vascular spaces; these may have been venules or lymphatics, in view of the single layer of cells surrounding their lumens (Figure 1F). In addition, pyknotic nuclei were noted in interstitial areas as well as in the lining and lumens of cysts (Figure 1F).

PCNA and PAX2 Expression

PCNA and PAX2 Abs, respectively, detected major species at approximately 35 kd (Figure 2A) and 43 kd (Figure 2B) in Western blots. Moreover, omission of primary Abs in blots and immunohistochemistry produced no signal (data not shown), suggesting that the immunostaining patterns, below, were specific. Immunohistochemistry of control and contralateral groups harvested at 100 days were similar, with PAX2 prominent in the nephrogenic zone and confined to collecting ducts in deeper cortex (Figure 3A). Renal mesenchyme was negative for PAX2, but the protein was up-regulated during nephron formation within condensates and S-shaped bodies where primitive proximal tubules, visceral glomerular epithelia (podocytes), and parietal epithelia (Bowman's capsule) were immunoreactive (Figure 3C). As nephrons matured, proximal tubules and podocytes lost PAX2 staining with a very faint signal detected in Bowman's capsule (Figure 3E). Ureteric bud branch tips (Figure 3A) and cortical and medullary collecting ducts (Figure 3, E and G) expressed PAX2, whereas maturing loops of Henle did not (Figure 3G). As expected for a transcription factor, staining was predominantly nuclear.

Figure 3 represents PAX2 staining in kidneys obstructed from 90 to 100 days; in Figure 3, B, D, F, and H fields were at comparable anatomical levels to controls in Figure 3, A, C, E, and G. It was evident that there were few PAX2-staining condensates, but epithelia lining cysts were uniformly reactive with PAX2 Ab (Figure 3, D and F). In contrast, malformed glomerular tufts attached to cyst walls did not express PAX2 (Figure 3D). In the medulla of the obstructed kidney, collecting ducts were mildly distended and their cells were vacuolated with faint nuclear and cytoplasmic PAX2 staining (Figure 3H). Few normal loops of Henle could be discerned (Figure 3H).

Figure 4, A, C, and E, illustrates PCNA immunoreactivity in control kidneys, with similar patterns noted in contralateral organs (data not shown). In the nephrogenic cortex, most nuclei in S-shaped bodies and ureteric bud branch tips stained (Figure 4A). Deeper in the cortex, as nephrons matured, positive nuclei were generally confined to interstitial cells, collecting ducts, and cells in the center of glomerular tufts, which we speculate were en-

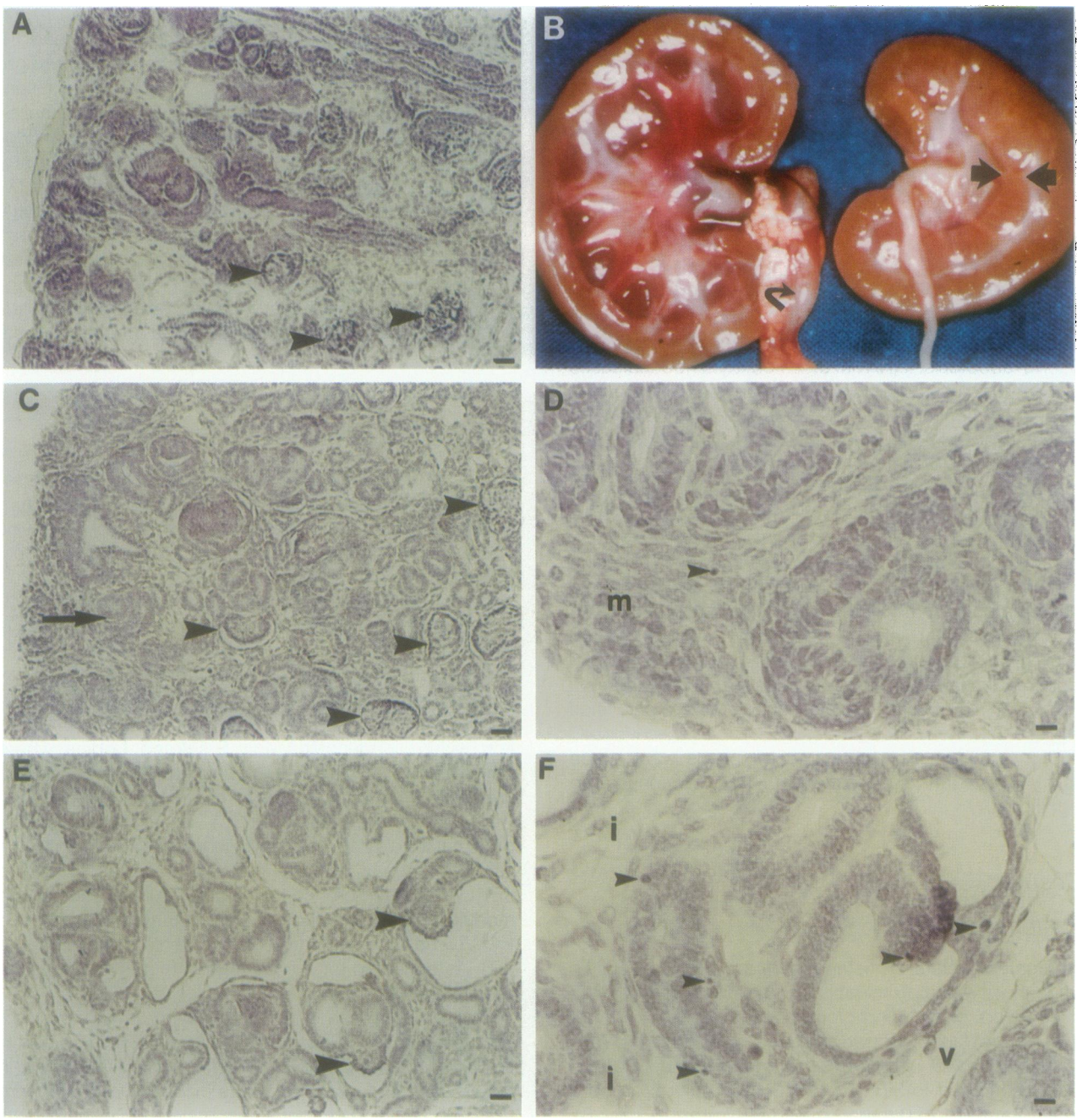


Figure 1. Gross anatomy and histology. **A:** Metanephros at 90 days gestation with nephrogenic zone on left and three layers of glomeruli (**arrowheads**) deeper in the cortex. **B:** The ureter of the kidney on the left was ligated between 90 and 100 days. Note ureteric (**curved arrow**) and pelvic dilatation with poor definition of corticomedullary junction. The contralateral organ is on the right, with **arrows** indicating the medulla. **C:** Cortex of control kidney at 100 days. Note four layers of glomeruli (**arrowheads**) and minimal dilatation of ureteric bud branch tips. An S-shaped body is indicated by an **arrow**. **D:** Nephrogenic cortex of 100-day control with compact mesenchyme (m) and a pyknotic nucleus (**arrowhead**). **E:** Cortex of 100-day organ after 10 days of ureteric ligation. Note predominance of cysts, some containing glomerular tufts (**arrowheads**). **F:** High-power field of **E** with edematous interstitium (i), prominent vascular channels (v), and numerous pyknotic nuclei (**arrowheads**). Bars, 34 μm (**A**, **C**, and **E**) and 8 μm (**D** and **F**); **B** is three times life size.

dothelia or mesangial cells.[30] PCNA was expressed by all epithelial cells lining cysts (Figure 4, D and F), whereas the differentiating epithelial cells from Bowman's capsule (arrowheads in Figure 4C) and proximal tubules (p in Figure 4E) in control kidneys expressed PCNA in fewer than 30% of their nuclei. Views from fetal kidneys obstructed from 90 to 100 days gestation (Figures 4, B, D, and F) demonstrated that epithelia lining cysts in the superficial and deep cortex contained cells that uniformly expressed PCNA, a similar pattern to that described for PAX2, above.

Apoptosis

The apparent increase in pyknotic nuclei in the cortex of obstructed *versus* control organs (compare Figure 1, F

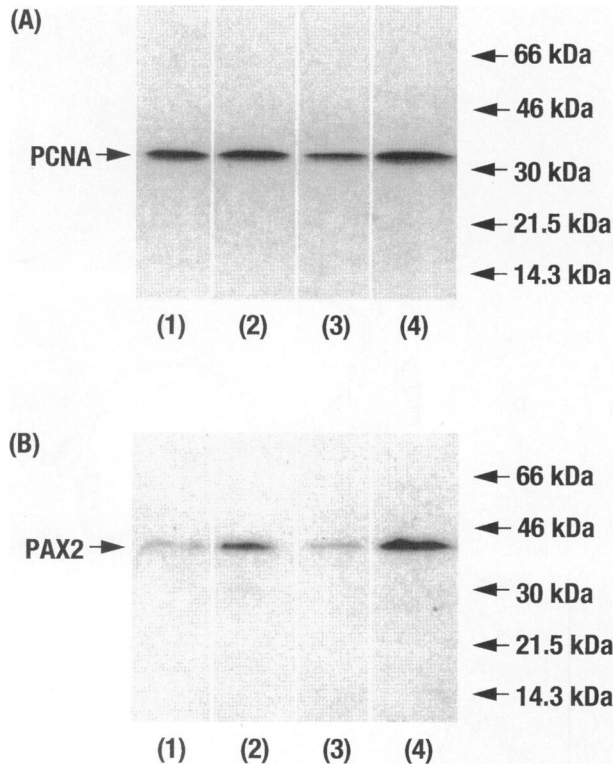


Figure 2. Western blot. **A:** PCNA. **B:** PAX2. Lanes were loaded with ~20 of μg protein. **Lane 1**, 90 days gestation metanephros; **lanes 2** to **4**, 100-day-gestation organs obstructed for 10 days, a contralateral organ, and an unoperated control, respectively. Molecular weight markers are on right. No signal was obtained on omission of primary Abs. The blot demonstrates Ab specificity but is not quantitative.

with D) was consistent with enhanced apoptosis. We therefore examined cell death by PI staining and *in situ* end labeling, which, in our hands, gave similar spatial distributions of positive nuclei. As demonstrated in Figure 5, A and B, occasional apoptotic nuclei were detected in the cortex of control and contralateral organs at 100 days gestation. The cortex of obstructed organs contained labeled cells in the interstitium, cyst walls, and cyst lumens, where they sometimes formed clusters (Figure 5, C and D). When the point prevalence of apoptosis was measured, the medians (ranges) were as follows: 3.7 (3.0 to 4.2) in six obstructed organs; 1.6 (1.2 to 1.8) in six contralateral organs; 1.9 (1.5 to 2.1) in six organs from control group 1; and 1.6 (1.2 to 1.8) in six organs from control group 2. Apoptosis in the obstructed group was increased *versus* other groups ($P < 0.05$).

Urine Electrolytes

We found increased urinary concentrations of sodium and osmolality in the urine aspirated from the dilated renal pelvis of organs with ligated ureters when compared with contralateral urine and urine from unoperated fetuses (Figure 6). This demonstrated impaired ability of urinary dilution and Na^+ reabsorption, consistent with the anatomical damage in loops of Henle and collecting ducts (Figure 3H). Potassium concentrations were similar in all groups.

Analysis after 1 Day of Urinary Flow Impairment

Three organs obstructed for 1 day only (ie, between 90 and 91 days gestation) demonstrated a uniform histological picture of minor dilatation of S-shaped bodies; the epithelia lining these dilated primitive tubules expressed PAX2 (Figure 7A) and PCNA (Figure 7B). Of note, the ureteric bud branch tips were not dilated. Hence, consistent with the data from 10 days, in the nephrogenic cortex, urinary flow impairment affects the morphology of the developing proximal nephron, rather than elements that will mature into collecting ducts. The point prevalence of apoptosis in the cortex of these three obstructed samples (2.2, 2.5, and 3.2) tended to be higher than contralateral organs (1.4, 1.5, and 1.9) or those harvested from unoperated twins (1.5, 1.8, and 1.7), but the absolute differences are small.

Discussion

We hypothesized that prenatal urinary flow impairment would induce a defined biological program that would disrupt nephrogenesis. Our data demonstrate that aberrations of morphogenesis, gene expression, and cell turnover occur after only 10 days of anatomical obstruction of the ureter. How can we relate these observations to human kidney malformations and other models of obstructive nephropathy, and what are the mechanisms by which urinary flow impairment might initiate these profound effects?

Human multicystic dysplastic kidneys are associated with atretic ureters; hence, obstruction has been invoked in their pathogenesis. Dysplastic epithelia are malformed branching tubules, often terminating in cysts,[2] observations consistent with the hypothesis that they are malformed collecting ducts. Other cysts in dysplastic kidneys may arise from glomeruli, based on findings of glomerular tufts attached to cyst walls,[31] and other tubule segments.[32] Human dysplastic kidney cysts express PAX2 and PCNA, and malformations harvested postnatally show persistent patterns of fetal gene expression whereas normal maturing kidneys down-regulate proliferation and PAX2.[12] As transgenic PAX2 expression causes renal cysts,[15] persistent expression in human renal epithelia may drive proliferation. Other studies confirm the association of PAX2 with pathological growth. Wilms' tumors[33] and renal carcinomas[34] express the gene, and PAX2 transforms murine cells[35] and also inhibits the promoter of p53, a tumor suppressor.[36] Undifferentiated cells around dysplastic kidney tubules have a high point prevalence of apoptosis,[19,37] perhaps partly explaining a tendency for some of these organs to regress.[38] Hence, cell proliferation and apoptosis are enhanced in certain human kidney malformations.

Although these observations are intriguing, it can be argued that cyst formation and apoptosis are unspecific effects after exposure to diverse primary insults, includ-

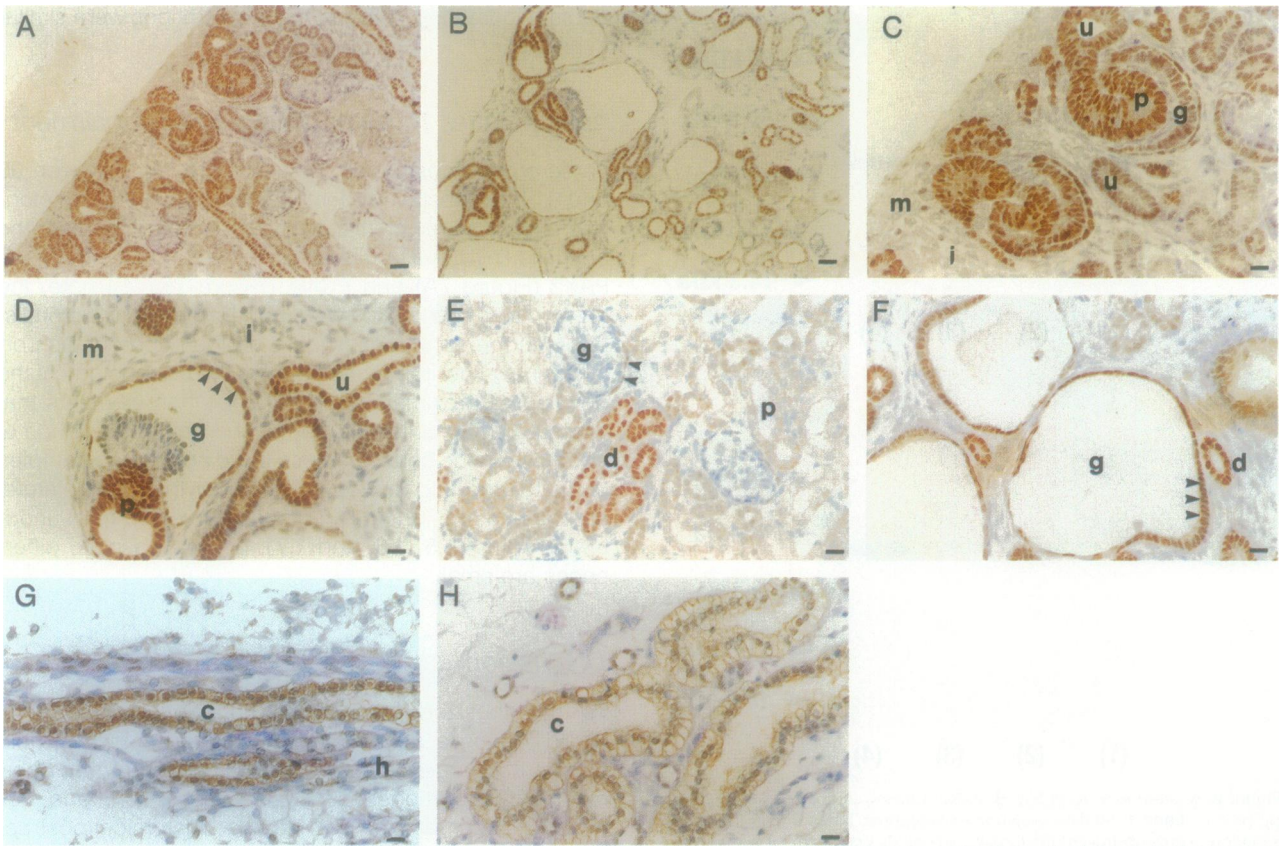


Figure 3. PAX2 immunostaining. Positive nuclei are brown. No signal was noted on omission of primary Ab (not shown). **A:** Cortex of control kidney, 100 days gestation. Most immunoreactivity is in the nephrogenic zone, on left, whereas deeper collecting ducts also express PAX2. **B:** Cortex of 100-day organ obstructed for 10 days. **C:** Higher-power field of control cortex. The mesenchyme (m) and interstitium (i) are unreactive whereas PAX2 appears in mesenchymal condensates and persists in S-shaped bodies; primitive proximal tubule (p) and the double crescent of cells (g) will differentiate into visceral and parietal glomerular epithelia. Ureteric bud branches (u) also express PAX2. **D:** High-power field of obstructed outer cortex. Mesenchymal condensates are sparse, and S-shaped bodies are absent. **Arrowheads** indicate PAX2-positive epithelia lining a cyst containing a malformed glomerular tuft (g). **E:** Deeper in the control cortex, glomerular podocytes (g) and proximal tubules (p) are maturing and have lost PAX2 immunoreactivity. Minimal PAX2 staining is noted in Bowman's capsule (**arrowheads**) whereas distal tubules (d) continue to express the gene. **F:** Deeper in the obstructed cortex, glomerular cysts (g) are lined by PAX2-expressing epithelia (**arrowheads**). **G:** Control medulla at 100 days. PAX2 staining is in collecting ducts (c) but not in thinner tubules, which are loops of Henle (h). **H:** The medulla of the obstructed metanephros is edematous with dilated, vacuolated collecting ducts; intact loops of Henle cannot be discerned. Bars, 34 μm (**A** and **B**), 17 μm (**C** to **E**), and 12 μm **G** and **H**).

ing mutations or teratogens as well as urinary flow impairment.[10] For example, polymorphisms of the angiotensin II receptor type 2, a gene implicated in apoptosis,[39] are associated with human kidney malformations,[40] and glomerular cysts can be generated by genetic mechanisms, as in oral-facial-digital syndrome type 1.[41,42] Furthermore, teratogens can cause enhanced death in the developing murine urinary tract.[43] Hence, it becomes critical to determine the biological changes in reproducible models of kidney obstruction.

Many experiments have addressed the effects of urinary flow impairment on mature kidneys where ureteric ligation leads to an early fall of glomerular filtration rate and renal blood flow fall.[44] Tubules with high oxygen requirements, notably the straight portion of the proximal tubule and thick ascending loop of Henle, are particularly vulnerable to reduced blood flow.[45] After a brief proliferative response, the pathology is dominated by epithelial apoptosis, tubular atrophy,[46] interstitial fibrosis, and transforming growth factor (TGF)-β1 expression.[47] Obstruction of neonatal murine kidneys, which will develop 80% of their nephrons in the subsequent 2 weeks,[48] causes apoptosis, with aberrant expression of BCL2, TGF-β1, angiotensin II, and epidermal growth factor.[49] Berman and Maizels obstructed the ureter of the avian metanephros engrafted onto the chorioallantoic membrane; although a vascular organ developed, only mild pelvic distension was generated.[50] However, glomerular capillary morphogenesis in this milieu is rudimentary,[30] and hence, plasma ultrafiltration may not favor the production of enough urine to generate a damaging pressure signal in the urinary tract.

Although these models provide some biological insights, they do not develop dysplastic and subcortical cystic changes commonly observed in human kidneys associated with prenatal urinary tract obstruction.[2] Other studies have addressed effects on sheep metanephroi. In 1971, Beck reported that complete ureteric obstruction "after approximately 80 days of gestation" produced "hydronephrosis (and) parenchymal atropy" with "slightly increased size of Bowman's spaces" when examined in the last month of gestation.[6] Obstruction at 60 days produced a small, dysplastic organ whereas the addition of contralateral nephrectomy resulted in excess growth with

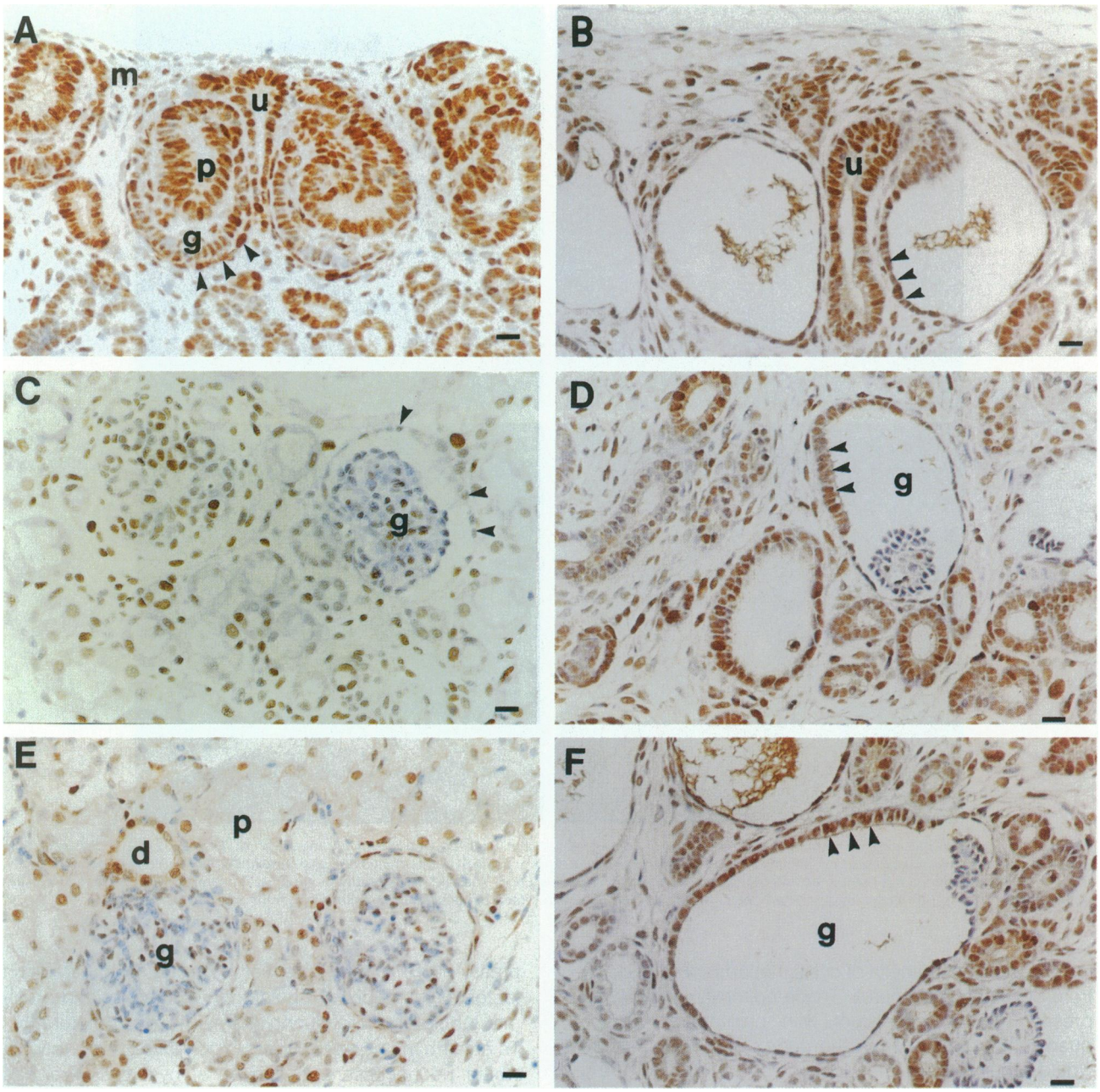


Figure 4. PCNA immunostaining. **A:** Most cells in the nephrogenic cortex of the 100-day control kidney are immunoreactive. m, mesenchyme; u, ureteric bud branch tip; p and g, primitive proximal and glomerular epithelia, respectively, in S-shaped body. **Arrowheads** indicate primitive parietal glomerular epithelium. **B:** In the obstructed organ, a terminal ureteric bud branch (u) is flanked by two PCNA-staining cysts (**arrowheads**). **C** and **E:** Deeper areas of control cortex. PCNA expression is down-regulated in Bowman's capsule (**arrowheads** in **C**) and proximal tubules (p in **E**). Some positive nuclei are apparent in interstitium and in distal tubules (d). **D** and **F:** Corresponding areas of deeper cortex in obstructed kidneys showing glomerular cysts (g) lined by PCNA-positive epithelia (**arrowheads**). Bars, 34 μm.

multiple subcortical cysts.[6] Tanagho[7] reported the effects of partial unilateral ureteric obstruction at 70 to 75 days gestation, which later "became complete"; kidneys harvested at term showed hydronephrosis, parenchymal atrophy, and "massive distension of Bowman's capsules." Others have confirmed and elaborated these anatomical observations, documenting effects of complete and partial urinary obstruction.[4,8]

Most of the above studies, however, documented anatomical changes weeks or months after impairment of urine flow. In the current study, we focused on the cellular consequences relatively soon after complete unilateral ureteric obstruction, reasoning that any effects would be more likely to be of primary importance to the disease process rather than unspecific, secondary reactions. Our data demonstrate that complex aberrations of morphogenesis, gene expression, cell turnover, and urine composition occur after only 10 days of fetal ureteric obstruction. In particular, the expression of PAX2, a gene associated with enhanced metanephric growth, was up-

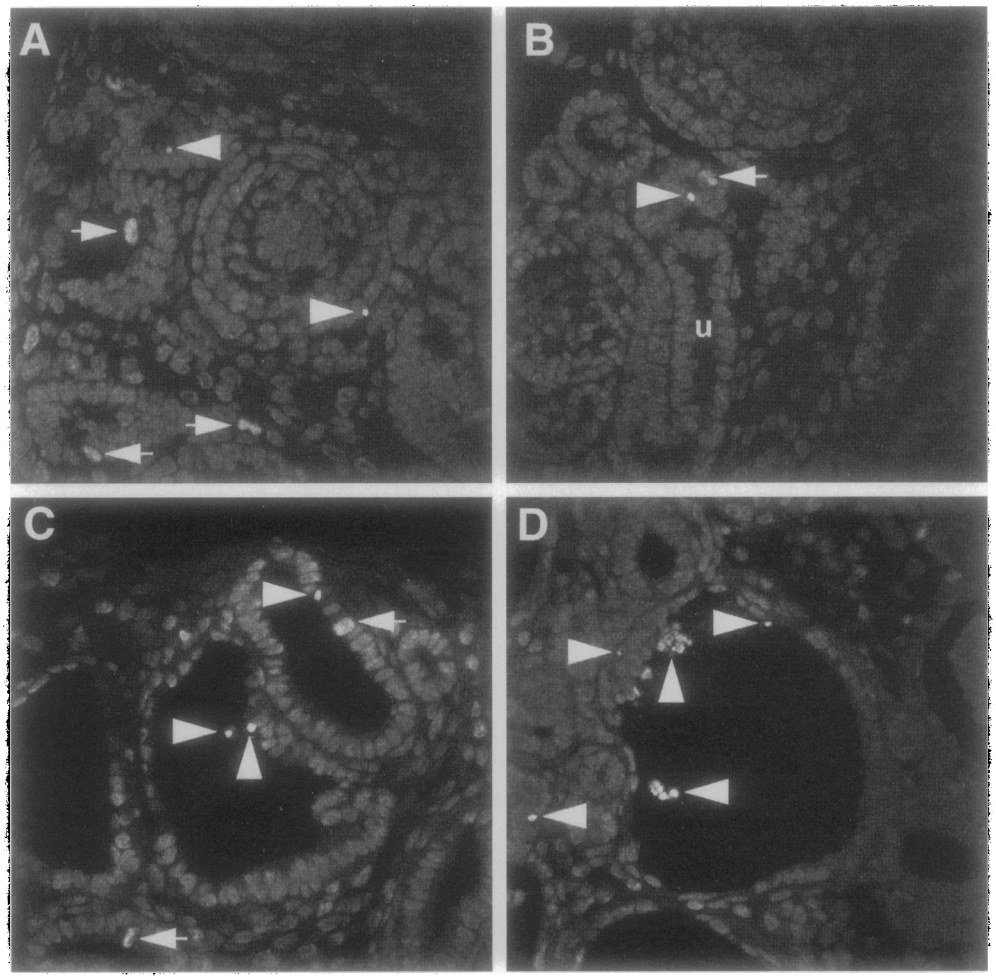
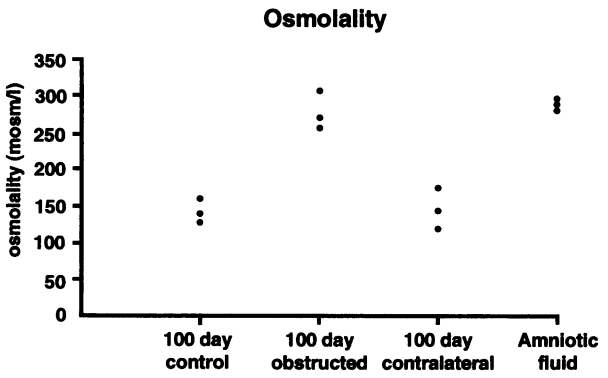
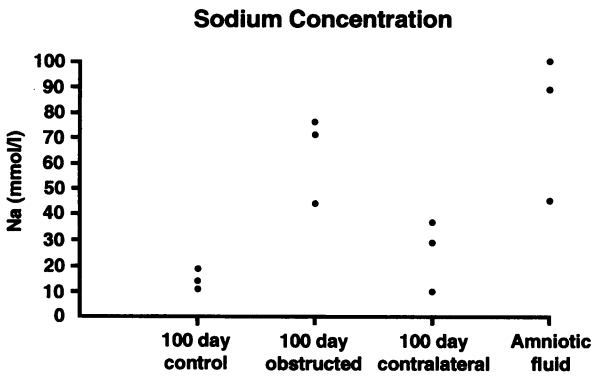


Figure 5. Apoptosis in metanephric cortex. High-power fields of control (**A**), contralateral (**B**), and obstructed metanephroi (**C** and **D**) at 100 days gestation. Apoptotic nuclei, assessed by *in situ* end labeling and a FITC detection system, appear as small points of intense signal (**arrowheads**). Larger, nonapoptotic nuclei, some of which are undergoing mitosis (**arrows**), show weak, nonspecific fluorescence. Note the relative rarity of apoptosis in control and contralateral kidneys *versus* increased point prevalence in obstructed organs where labeled nuclei are noted in interstitium as well as cyst walls and lumens.

regulated in the epithelia of cysts that appeared to derive from developing glomeruli. These epithelia uniformly expressed PCNA, consistent with proliferation. Formal quantitation of mitoses in the cortex of the fetal kidney would require assessment of bromodeoxyuridine or tritiated thymidine incorporation, but these experiments are beyond the scope of the current study. At the same time, the point prevalence of apoptosis was increased as assessed by PI and *in situ* end labeling. Although compensatory growth of the contralateral kidney has been reported in another study,[26] this process takes many weeks and was not notable in our short-term experiments.

Impairment of fetal urinary flow causes an increased hydrostatic pressure in the renal pelvis.[4] We hypothesize that this signal may be transmitted to the developing nephrons where it would stretch primitive epithelia and elicit an enhanced growth response. There is evidence, based on *in vitro* experiments using Madin Darby canine kidney cells in collagen gels,[51] that enhanced pressure within cyst lumens causes increases proliferation. Our data also suggest that the PCNA expression, a surrogate marker of cell proliferation, is temporally linked with expression of PAX2, a potentially oncogenic transcription factor, which is down-regulated in glomerular and proximal tubule epithelia during normal nephron maturation. To confirm the hypothesis that PAX2 drives proliferation it would be necessary to show that cyst pressure was increased *in vivo* and to demonstrate that reduction of PAX2 protein, for example by antisense oligonucleotides,[13] could prevent morphological epithelial overgrowth and reduce mitosis as assessed by formal measures such as tritiated thymidine or bromodeoxyuridine incorporation. Certainly, proliferation is a feature of renal cyst formation in diverse animal models and human diseases.[52,53] In autosomal dominant polycystic kidney disease, it has been proposed that active solute excretion into cyst lumens[54,55] and *de novo* somatic mutations[56] are also required for cyst growth. It would seem unlikely that the latter event could make a uniform contribution to cyst formation in the 10-day experimental period in our study. Furthermore, the interstitium of obstructed kidneys was markedly edematous, consistent with a net fluid flux out of the lumens of developing nephrons.





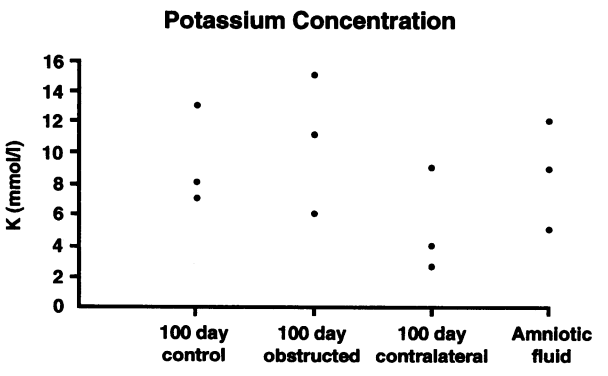


Figure 6. Urine electrolytes. Values shown for sodium, osmolality, and potassium for urine harvested from the bladder of 100-day control fetuses, from the obstructed urinary system at 100 days, from bladder urine produced by contralateral kidneys at 100 days, and from amniotic fluid of manipulated fetuses (n = 3 for each group).

Even though cysts dominated the cortical histology of obstructed kidneys, it was notable that the nephrogenic zone was disrupted, with no evidence of S-shaped bodies. This implied block in *de novo* nephron formation

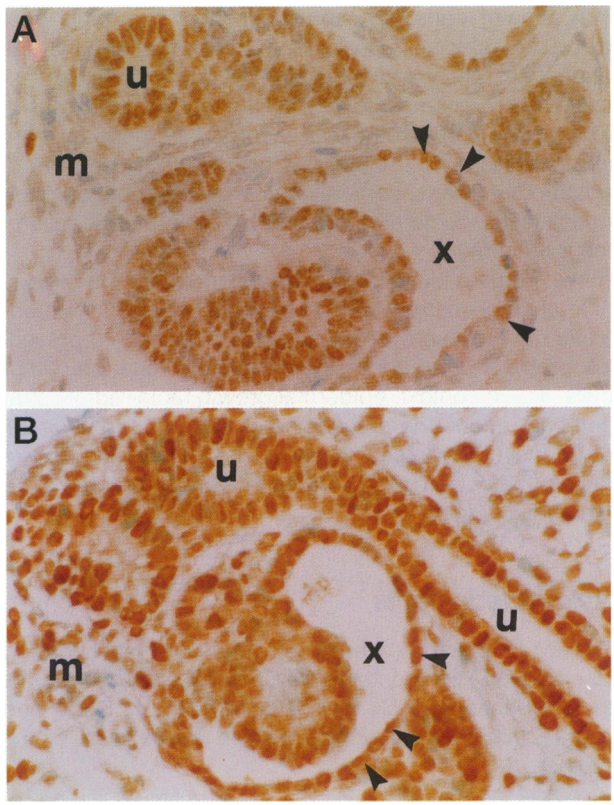


Figure 7. Organs obstructed from 90 to 91 days gestation. There is minor dilatation (x) of the proximal limbs of S-shaped bodies corresponding to the primitive Bowman's spaces. The epithelial cells lining these dilated primitive tubules (**arrowheads**) expressed PAX2 (**A**) and PCNA (**B**). Ureteric bud branch tips (u) were not dilated. m, nephrogenic mesenchyme. Magnification, ×150.

remains unexplained, but we suggest an answer may be provided by an imbalance of levels of nephron-promoting and -inhibiting growth factors.[57] In particular, TGF-β1 inhibits nephron formation in organ culture[58] and is up-regulated in obstructed neonatal and adult kidneys[47,49]; if this factor were also up-regulated in obstructed fetal sheep kidneys, perhaps the major effect would be to inhibit the formation of nephrons rather than eliciting scarring as occurs in adults. In future, it would be interesting to measure TGF-β1 in our current model.

In addition, we documented increased apoptosis in the obstructed kidney. If the clearance of apoptotic cells is assumed to be similar in obstructed and normal organs, this implies that the rate of programmed cell death is significantly enhanced by 10 days of ureteric obstruction. Although it is possible that a more prolonged period of apoptosis might lead to the atrophy or involution of renal tissue, the contribution of apoptosis to the observed loss of the nephrogenic zone is unknown. It is, however, interesting to note that enhanced apoptosis often coexists with cystic proliferation in human and animal kidney malformations and also in polycystic kidney diseases.[12,19,20,59]

Ultrasound scanning of the human fetus to detect malformations is common obstetric practice.[3] The distended urinary tract associated with obstruction can be accessed and human fetal urine analyzed to provide an

indication of kidney function.[3] Indicators of a good renal outcome include urinary sodium concentrations that fall during gestation and the ability to dilute urine, as assessed by a urine osmolality less than that of plasma.[3] It was therefore of note that we recorded increased urinary concentrations of sodium and osmolality in the urine of obstructed organs, correlating with microscopic damage to collecting ducts and loops of Henle in the medulla of the metanephros.

We suggest that a better understanding of the biology of fetal renal obstruction may eventually lead to the modulation of disease by administration of agents to prevent apoptosis and enhance differentiation of renal precursor cells. In this context, epidermal growth factor administration can reduce the prevalence of apoptosis in neonatal rat kidneys[18] whereas the administration of insulin-like growth factor abrogates fibrosis in obstructed, immature marsupial kidneys.[60]

Acknowledgments

The PAX2 antibody was a gift from Gregory Dressler, University of Michigan Medical Center, Ann Arbor, MI. We thank Charles Rodeck, Philip Ransley, Patrick Duffy, and Jeeta Dhillon for helpful discussion at the start of the project.

References

1. McEnery PT, Stablein DM, Arbus G, Tejani A: Renal transplantation in children. N Engl J Med 1992, 326:1727–1732
2. Potter EL: Normal and Abnormal Development of the Kidney. Chicago, Year Book Medical Publishers, 1972
3. Coplen DE: Prenatal intervention for hydronephrosis. J Urol 1997, 157:2270–2277
4. Peters CA: Obstruction of the fetal urinary tract. J Am Soc Nephrol 1997, 8:653–663
5. Wintour EM, Alcorn D, Butkus A, Congiu, Earnest L, Pompolo S, Potocnik SJ: Ontogeny of hormonal and excretory function of the meso- and metanephros in the ovine fetus. Kidney Int 1996, 50:1624–1633
6. Beck AD: The effect of intrauterine urinary obstruction upon the development of the fetal kidney. J Urol 1971, 105:784–789
7. Tanagho EA: Surgically induced partial urinary obstruction in the fetal lamb. III. Ureteral obstruction. Invest Urol 1972, 10:35–52
8. Peters CA, Carr MC, Lais A, Retik AB, Mandell J: The response of the fetal kidney to obstruction. J Urol 1992, 148:503–509
9. Carr MC, Schlussel RN, Peters CA, Uchida T, Mandell J, Freeman MR: Expression of cell growth related genes in the fetal kidney: relevance to in utero obstruction. J Urol 1995, 154:242–246
10. Woolf AS: Multiple causes of human kidney malformations. Arch Dis Child 1997, 77:471–473
11. Dressler GR, Deutsch U, Chowdhury K, Nornes HO, Gruss P: Pax2, a new murine paired-box-containing gene, and its expression in the developing excretory system. Development 1990, 109:787–795
12. Winyard PJD, Risdon RA, Sams VR, Dressler GR, Woolf AS: The PAX2 transcription factor is expressed in cystic and hyperproliferative dysplastic epithelia in human kidney malformations. J Clin Invest 1996, 98:451–459
13. Rothenpieler UW, Dressler GR: Pax-2 is required for mesenchyme-to-epithelium conversion during kidney development. Development 1993, 119:711–720
14. Torres M, Gomez-Pardo E, Dressler GR, Gruss P: Pax-2 controls multiple steps of urogenital development Development 1995, 121: 4057–4065
15. Dressler GR, Wilkinson JE, Rothenpieler UW, Patterson LT, Williams-Simons L, Westphal H: Deregulation of Pax-2 expression in transgenic mice generates severe kidney abnormalities. Nature 1993, 362:65–67
16. Sanyanusin P, Schimmentl LA, McNoe LA, Ward TA, Pierpoint MEM, Sullivan MJ, Dobyns WB, Eccles MR: Mutations of the PAX2 gene in a family with optic nerve colobomas, renal anomalies and vesicoureteral reflux. Nature Genet 1995, 9:358–364
17. Koseki C, Herzlinger D, Al-Awqati Q: Apoptosis in metanephric development. J Cell Biol 1992, 119:1322–1333
18. Coles HSR, Burne JF, Raff MC: Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. Development 1993, 118:777–784
19. Winyard PJD, Nauta J, Lirenman DS, Hardman P, Sams VR, Risdon AR, Woolf AS: Deregulation of cell survival in cystic and dysplastic renal development. Kidney Int 1996, 49:135–146
20. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ: Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys and hypopigmented hair. Cell 1994, 75:229–240
21. Sorenson CM, Rogers SA, Korsmeyer SJ, Hammerman MR: Fulminant metanephric apoptosis and abnormal kidney development in bcl-2-deficient mice. Am J Physiol 1995, 268:73–81
22. Bravo R, Frank R, Blundell PA, MacDonald-Bravo H. 1987. Cyclin/PCNA is the auxillary protein of DNA polymerase-delta. Nature 1987, 326:515–517
23. Gavrelli Y, Sheman Y, Ben-Sasson SA: Identification of programmed cell death in situ via specific labeling of DNA fragmentation. J Cell Biol 1992, 119:493–501
24. Mandell J, Peters CA, Estroff JA, Allred EN, Benacerraf BR: Human fetal compensatory renal growth. J Urol 1993, 150:790–792
25. Glazebrook KN, McGrath FP, Steele BT: Prenatal compensatory renal growth: documentation with US. Radiology 1994, 189:733–735
26. Peters CA, Gaertner RC, Carr MC, Mandell J: Fetal compensatory renal growth due to unilateral urethral obstruction. J Urol 1993, 150: 597–600
27. Dressler GR, Douglas EC: Pax-2 is a DNA-binding protein expressed in embryonic kidney and Wilms tumor. Proc Natl Acad Sci USA 1992, 89:1179–1183
28. Phelps DA, Dressler GR: Identification of novel Pax-2 binding sites by chromatin precipitation. J Biol Chem 1996, 271:7978–7985
29. Winyard PJD, Bao Q, Hughes RC, Woolf AS: Epithelial galectin-3 during human nephrogenesis and childhood cystic diseases. J Am Soc Nephrol 1997, 8:1647–1657
30. Loughna S, Hardman P, Landels E, Jussila L, Alitalo K, Woolf AS: A molecular and genetic analysis of renal glomerular capillary development. Angiogenesis 1997, 1:84–101
31. Bernstein J: Glomerulocystic kidney disease: nosological considerations. Pediatr Nephrol 1993, 7:464–470
32. Matsell DG, Bennett T, Armstrong RA, Goodyer P, Goodyer C, Han VKM: Insulin-like growth factor and IGF binding protein gene expression in multicystic renal dysplasia. J Am Soc Nephrol 1997, 8:85–94
33. Eccles MR, Wallis LJ, Fidler AE, Spurr NK, Goodfellow PJ, Reeve AE: Expression of the PAX2 gene in human fetal kidney and Wilms' tumor. Cell Growth Differ 1992, 3:279–289
34. Gnarra JR, Dressler GR: Expression of Pax-2 in human renal cell carcinoma and growth inhibition by antisense oligonucleotides. Cancer Res 1995, 55:4092–4098
35. Maulbecker CC, Gruss P: The oncogenic potential of Pax genes. EMBO J 1993, 12:2361–2367
36. Stuart ET, Haffner R, Oren M, Gruss P: Loss of p53 function through PAX-mediated transcriptional repression. EMBO J 1995, 14:5638–5645
37. Granata C, Wang Y, Puri P, Tanaka K, O'Briain DS: Decreased bcl-2 expression in segmental renal dysplasia suggests a role in its morphogenesis. Br J Urol 1997, 80:140–144
38. Mesrobian H-GJ, Rushton HG, Bulas D: Unilateral renal agenesis may result from in utero regression of multicystic dysplasia. J Urol 1993, 150:793–794
39. Yamada T, Horiuchi M, Dzau VJ: Angiotensin II type 2 receptor mediates programmed cell death. Proc Natl Acad Sci USA 1996, 93:156–160
40. Yerkes E, Nishimura H, Hohenfellner K, Hunley TE, Yoshida H, Kon V, Philips JA III, Brock JW III, Ichikawa I: Association between the angiotensin type 2 receptor (AT2) genotype and congenital urinary

tract anomalies in two independent cohorts. J Am Soc Nephrol 1997, 8:397A
41. Feather SA, Winyard PJD, Dodd S, Woolf AS: Oral-facial-digital syndrome type 1 is another dominant polycystic kidney disease: clinical, radiological and histopathological features of a new kindred. Nephrol Dial Transplant 1997, 12:1354–1361
42. Feather S, Woolf AS, Malcolm S, Donnai D, Winter R: The oral-facial-digital syndrome type 1, a cause of polycystic kidney disease and associated malformations, maps to Xp22.2–22.3. Hum Mol Genet 1997, 7:1163–1167
43. Gage JC, Sulik KK: Pathogenesis of ethanol-induced hydronephrosis and hydroureter as demonstrated following in vivo exposure of mouse embryos. Teratology 1991, 44:299–312
44. Chevalier RL, Thornhill BA, Gomez RA: EDRF modulates renal hemodynamics during unilateral obstruction in the rat. Kidney Int 1992, 42:400–406
45. Peters C, Belman AB: Urinary tract obstruction in children. J Urol 1995, 154:1874–1884
46. Truong LD, Petrusevska G, Yang G, Gurpinar T, Shappell S: Cell apoptosis and proliferation in experimental chronic obstructive uropathy. Kidney Int 1996, 50:200–207
47. Kaneto H, Morrisey J, Klahr S: Increased expression of TGF-β1 mRNA in the obstructed kidney of rats with unilateral ligation. Kidney Int 1993, 44:313–321
48. Gilbert T, Lelievre-Pegorier M, Malienou R, Meulemans A, Merlet-Benichou C: Effects of prenatal and postnatal exposure to gentamycin on renal differentiation in the rat. Toxicology 1987, 43:301–313
49. Chevalier RL: Growth factors and apoptosis in neonatal ureteral obstruction. J Am Soc Nephrol 1996, 7:1098–1105
50. Berman DJ, Maizels M: The role of urinary obstruction in the genesis of renal dysplasia: a model in the chick embryo. J Urol 1982, 128: 1091–1096
51. Tanner GA, McQuillan PF, Maxwell MR, Keck JK, McAteer JA: In vitro test of the stretch-proliferation hypothesis of renal cyst enlargement. J Am Soc Nephrol 1995, 6:1230–1241
52. Nadasdy T, Laszik Z, Lajoie G, Blick KE, Wheeler DE, Silva FG: Proliferative activity of cyst epithelium in human renal cystic diseases. J Am Soc Nephrol 1995, 5:1462–1468
53. Woo DDL, Miao S, Pelayo J, Woolf AS: Taxol inhibits progression of congenital polycystic kidney disease. Nature 1994, 368:750–753
54. Grantham JJ: Fluid secretion, cellular proliferation, and the pathogenesis of renal epithelial cysts. J Am Soc Nephrol 1992, 3:1841–1857
55. Foxall PJD, Price RG, Jones NK, Nield GH, Thompson FD, Nicholson JK: High resolution proton magnetic resonance spectroscopy of cyst fluid from patients with polycystic kidney disease. Biochim Biophys Acta 1992, 1138:305–314
56. Qian F, Watnick TJ, Onuchic LF, Germino GG: The molecular basis of focal cyst formation in human autosomal dominant polycystic kidney disease type 1. Cell 1996, 87:979–987
57. Woolf AS, Cale CM: Roles of growth factors in renal development. Curr Opin Nephrol Hypertens 1997, 6:10–14
58. Rogers SA, Ryan G, Purchio AF, Hammerman MR: Metanephric transforming growth factor β1 regulates nephrogenesis in vitro. Am J Physiol 1993, 264:F996–F1002
59. Woo DDL: Apoptosis and loss of renal tissue in polycystic kidney diseases. N Engl J Med 1995, 333:18–25
60. Steinhardt GF, Liapis H, Philips B, Vogler G, Nag M, Yoon K-W: Insulin-like growth factor improves renal architecture of fetal kidneys with complete ureteral obstruction. J Urol 1995, 154:690–693