Recovery after relief of fetal urinary obstruction: morphological, functional and molecular aspects

DIDIER EDOUGA, BRIGITTE HUGUENY, BERNARD GASSER, LAURENCE BUSSIÈRES, AND KATHLEEN LABORDE
Department of Physiology, Necker-Enfants Malades Hospital, Institut National de la Santé et la Recherche Médicale Unité 356, Institut Fédératif de Recherche 58, Paris, France

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Edouga, Didier, Brigitte Hugueny, Bernard Gasser, Laurence Bussières, and Kathleen Laborde. Recovery after relief of fetal urinary obstruction: morphological, functional and molecular aspects. Am J Physiol Renal Physiol 281: F26–F37, 2001.—The effects of obstruction [urinary tract obstruction (UTO)] and relief on renal development were examined in an experimental model in the fetal lamb. Bladder outlet obstruction was performed at 60 days of gestation; relief was performed by vesicoamniotic shunting at 90 days of gestation. Studies were carried out in obstructed (OF60; n = 11), shunted (SF; n = 5), and control fetuses (CF; n = 11) at 120 days of gestation. Fetal UTO produced either hydronephrosis (64%) or dysplasia (36%); dysplasia was always associated with a reduction in the number of glomeruli (950 ± 99 dysplasia vs. 1,852 ± 249 (CF) glomeruli/section). Obstructed fetuses had lower creatinine clearance 0.76 ± 0.41 (OF60) vs. 0.96 ± 0.21 (CF) ml·min⁻¹·kg⁻¹, higher sodium fractional excretion 17.2 ± 20.3 (OF60) vs. 2.4 ± 3.7% (CF)], and higher urinary concentration 80 ± 30 (OF60) vs. 43 ± 22 (CF) μmol/l than controls. In SF, the number of glomeruli was increased at 120 days of gestation (1,643 ± 106 glomeruli/section) compared with nondiverted fetuses (1,379 ± 502 glomeruli/section), and the temporal pattern of PAX2, disrupted after obstruction, was restored. In conclusion, early fetal UTO leads to either renal hydronephrosis with normal glomerular development or dysplasia with a decreased number of glomeruli; in utero urine diversion performed before the end of nephrogenesis may allow a reversal of the glomerulogenesis arrest observed.

Renal development; dysplasia; PAX2; urinary hyperpression

CONGENITAL URINARY TRACT OBSTRUCTION (UTO) represents a significant cause of end-stage renal dialysis in children (22) and, in boys under the age of 4 yr, has been considered to underlie the condition in >90% of patients (24). The advent of fetal ultrasonography has permitted the obstruction to be identified in utero, providing a new window through which a condition, previously seen only in its latter stages, may be viewed. Prenatal observations suggest that many of our assumptions regarding developmental renal abnormalities and obstruction previously described from a postnatal perspective may not be completely valid when seen from a perinatal viewpoint. On one hand, the condition appears more severe than expected, as, in a series of postnatally diagnosed patients, fetuses that were terminated, stillborn, or dead perinatally were excluded; on the other hand, a considerable proportion of the antenatally diagnosed cases demonstrates a benign postnatal clinical course that might have been ignored postnatally (17, 19, 33). An unexplained continuous spectrum of situations can be observed at any fetal stage, and its natural history remains unknown. Prenatal diagnosis has also encouraged efforts to de-compress the urinary tract before the child is born (16, 18). However, it is still unclear whether antenatal drainage leads to better preservation of renal function; many factors preclude accurate assessment, including variability of patient selection, treatment, outcome measurement, and the lack of a valid natural history for comparison (6, 11, 21). Both the difficulties of the therapeutic management of fetal congenital obstructive uropathies and the controversies about the subject underline the gap between clinical practice and the understanding of the pathophysiology of the observed kidney changes. Clearly, optimal management of the fetus with congenital UTO depends on a thorough understanding of the pathophysiology of renal injury and its progression.

Experimental models of fetal UTO may provide a better understanding of the mechanisms of progression of fetal renal injury as a result of UTO and be the basis for developing methods to modulate progression and perhaps prevent further injury. Available experimental data (14, 29) suggest that fetal UTO may produce renal lesions and that the renal response to injury may differ at different points in renal development. However, differences in experimental models (neonatal or fetal), the small number of fetuses studied, and the heterogeneity in the timing and type of obstruction performed (29) still make interpretation of data ambiguous. In the present study, we systematically examined the effects of bilateral urinary obstruction and its relief on renal morphological, functional, and molecular de-
velopment in an experimental model in the fetal lamb.

**MATERIALS AND METHODS**

Three groups of fetal lambs were studied: 1) normal fetuses of different gestational ages; 2) fetuses undergoing urethral obstruction at 60 days of gestation; and 3) fetuses undergoing urethral obstruction at 60 days of gestation and subsequent drainage at 90 days of gestation.

Time-dated pregnant ewes (Pre´alpes) obtained by the induced ovulation technique underwent general endotracheal anesthesia (halothane-O2) after intravenous thiopental sodium (1 g) induction. Jugular vein and arterial catheters were placed for blood sampling, intravenous infusions, and maternal monitoring. Maternal respiratory rate and volume were adjusted to maintain normal arterial pH, Pco2, and Po2 values.

All experimental animal procedures performed in the laboratory were conducted in conformity with the Guide for the Care and Use of Laboratory Animals (Washington, DC: Natl. Academy Press, 1996).

**Control Fetal Lambs**

Open hysterotomy was performed via a midline laparotomy in 37 pregnant ewes (44 fetuses) at 50 (n = 8), 60 (n = 5), 80 (n = 5), 90 (n = 3), 100 (n = 2), 120 (n = 15), or 140 (n = 6) days of gestation. When fetus size allowed it, a renal clearance study was performed before nephrectomy. In all fetuses, both kidneys were removed before death and immediately processed for morphological study.

**Bladder Outlet Obstruction**

UTO was performed at 60 days of gestation in 11 fetuses. The ewe's abdomen was shaved and opened under aseptic conditions, and a laparotomy was performed by median infraumbilical incision; the uterine horn was exposed, allowing withdrawal of the greatest part of amniotic fluid, which was preserved in a sterile pocket stored in the abdominal cavity maintained at a constant physiological temperature. The fetal lamb hindquarters were then exteriorized. In male fetuses, a small clip was placed on the abdominal urethra dissected through a small bulbar incision; in female fetuses, the clip was placed on the bladder neck through a median suprapubic incision. The fetal abdominal wall was closed with an ethibon (4/0) ligature. In both sexes, the urachus was repositioned, and the amniotic fluid was turned back into the uterine cavity. Antibiotics were injected into the ewe: 0.5 M piperacillin + 0.5 g dihydrostreptomycin (iu and im; Rhone Mérieux). The fetal membrane and uterine cavity were closed with a double continuous suture (Vicryl 2/0), the abdominal wall in separate layers (Vicryl 2).

**Vesicoamniotic Shunt Therapy**

Five fetuses obstructed at 60 days of gestation underwent vesicoamniotic shunting at 90 days of gestation. The fetal hind end was exposed, the abdomen was opened, and a Rodeck double pig-tailed shunt (Rocket of London, Watford, UK) was placed near the midline between the pubis and umbilical cord insertion. All shunts were documented to be functioning before the fetus was turned back into the uterine cavity. To evaluate the effect of the shunting procedure, an additional group of four fetuses, obstructed at 60 days of gestation and not subjected to shunting, was studied at day 90.

In the postoperative course, all ewes with fetuses that underwent surgery were kept in a restricted area and fed a standard diet; after 4 days of observation, the ewes were returned to the farm (Clayes-Souilly, France), and the pregnancies were allowed to proceed until the date of the renal clearance study, at 120 days of gestation.

**Renal Function Study**

Renal function was measured by half-hourly split urine and blood collections for determination of creatinine clearance and fractional sodium excretion during seven periods. Creatinine clearance was calculated by the conventional formula: Ucr - VPcr, meaned for the seven periods, and expressed as milliliters per minute per kilogram body weight, where Ucr and Pcr are urinary and plasma creatinine concentration, respectively, and V is urine output (ml/min). Fractional sodium excretion was calculated as UNa - VPNa, Ccr, where Ccr is creatinine clearance, and UNa and PNa are urinary and plasma sodium concentration, respectively. Creatinine was measured in blood and urine samples by using the modified Jaffe kinetic technique. Sodium and potassium were measured in blood and urine samples by flame photometry. Other parameters were measured on an IL 919 analyzer.

**Histology**

At the end of the functional study, a caesarian was performed to allow fetal urinary tract dissection and bilateral nephrectomy; kidneys were hemisection and fixed in a formaldehyde solution. Longitudinal frontal renal cross sections were taken through the hilus and embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and phloxin.

**Number of glomeruli**

The total number of glomeruli was determined on whole longitudinal sections taken through the hilus, assuming the result would be representative of the number of nephrons within the entire kidney (12), using a modification of the method of McVary and Maizels (23). Cortical and nephrogenic zones were not separated, but the initial stages of glomerular development were excluded from this count; glomeruli were considered as primitive or mature when they clearly demonstrated both distinct Bowman's spaces and vascularized floculi, regardless of the glomerular width. The mean number of glomeruli counted on seven microscopic fields was determined by using a Reichert-Jung Microstar 10 microscope fitted with ×10 W.F. oculars and a plane 10/0.25 lens (microscopic field surface: 3.14116 mm2), and the average number of glomeruli per square millimeter was calculated. After determination of cortical surface, the glomerular count was given by the product of the mean number of glomeruli per square millimeter × cortical surface. This procedure was used for kidneys of fetal lambs older than 80 days but was not relevant to those of younger ones, because of the lack of clearly delineated medullocortical differentiation. In those cases, glomeruli were counted one by one, by scanning the entire kidney sections.

**Morphological analysis.** In normal fetuses, the state of development of the blastema was carefully analyzed; in obstructed fetuses, the morphological classification of kidney changes was done according to the diagnostic criteria of Osathanondh and Potter (27): i.e., hydrenephrosis: renal pelvic distention, conservation of renal architecture, normal glomeruli, dilated tubules with flat epithelium, and normal nondilated tubules; type IV dysplasia: renal structure conserved as a whole, renal pelvic distention and subcapsular glomerular or tubular cysts, frequent abnormal glomeruli, normal or abnormal tubular epithelium with high columnar

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cells and mesenchymal peritubular collars; and type II dysplasia: total disturbance of renal architecture with cysts of varying sizes, scattered within connective tissue, and only a few nephrons and primitive tubules.

**PAX2 In Situ Hybridization**

A PAX2 probe was used by cloning the human PAX2 gene, exon 5 (37), into the pCR-Script Amp SKI (+) plasmid vector. We first checked the homology between PAX2 exon 5 in humans and sheep by comparing their sequences. The two exon 5 primers used to amplify human and sheep genomic DNA were 5’-GGTCAGCACGAGGCTTCACC-3’ (sense), located from bp 65 to 84, and 5’-TCAGTCTCTCTCTCACC-3’ (antisense), from bp 161 to 180, giving a 115-bp fragment. Each 50-μl PCR reaction contained 200 ng of DNA fraction, 5 μl 10× PCR buffer (Boehringer), 5 μl of 2 mM dNTPs, 20 μM of each primer, and 1 U of Taq DNA polymerase (GIBCO). Amplification was performed for 30 cycles (40 s of denaturation at 94°C, 40 s of annealing at 60°C, and 40 s of elongation at 72°C). The 115-bp fragments were sequenced on both strands by using the fluorometric method (DyeDeoxy Terminator Cycle Sequencing Kit, Applied Bio Systems) and showed 95% of homology, predicting a specific hybridization of the human probe on lamb kidney sections. Sense and antisense riboprobes were generated by using either T7 or T3 RNA polymerase in the presence of [α-35S]UTP (1,200 Ci/mmol; NEN). Labeled probes were purified on Sephadex G50 columns.

Tissue sections were deparaffinized in toluene and rehydrated in descending ethanol series (100–30%). Then, they were treated for 20 min by proteinase K (20 μg/ml), postfixed for 20 min in 4% paraformaldehyde, rinsed for 5 min in 0.85% standard sodium citrate (SSC) at room temperature. The specific signal was detected by using the enhanced chemiluminescence Western blotting detection system (Amersham). No specific signal was detected in absence of the primary antibody (data not shown).

**PAX2 Immunohistochemistry**

Tissue sections were deparaffinized and rehydrated as indicated in PAX2 In Situ Hybridization. They were washed for 10 min in PBS (10 mM, pH 7.4) at room temperature. Then, to optimize antigen retrieval, tissue sections were heated in a microwave oven for 10 min up to 100°C in citrate buffer (0.01 M, pH 8) (38). Endogenous peroxidase activity was quenched by incubating tissue sections in 0.3% hydrogen peroxide (Merck) for 30 min. Tissue sections were then incubated at room temperature for 2 h with PAX2 antibody (1:100; Zymed Labs, South San Francisco, CA). In control experiments, the primary antibody was omitted. Sections were rinsed in PBS (10 mM, pH 7.4) with 1% dry milk for 30 min before incubating with the Envision system for 30 min at room temperature. Sections were rinsed in PBS (10 mM, pH 7.4) with 1% dry milk for 30 min, and peroxidase activity was identified with 3,3′-diaminobenzidine (DAB) tetrahydrochloride as chromogen substrate (DAKO). After a last wash in 50 mM Tris buffer, pH 7.6, sections were dehydrated, mounted with Eukitt, and photographed using a Dialux 20 microscope surrounded by a camera.

Western blot analysis was performed to confirm the specificity of the PAX2 antibody. Total protein was extracted from a 60-day-old half-frozen normal fetal sheep kidney by homogenization in PBS lysis buffer (0.5 mM DTT, 1 mM polymethlysulfonyl fluoride, and 0.5 mM EDTA; protease inhibitors aprotinin (1 μg/ml), benzamidin (1 mM), and pepstatin A (1 μg/ml); and 1% Triton X-100, pH 7.4) on ice. The homogenate was centrifuged at 600 g for 15 min at 4°C. Protein concentration of the supernatant was determined by the method of Lowry et al. (20) with BSA as a standard. Fractions of 100 and 150 μg of total protein extracted were separated on 12% running polyacrylamide and 5% stacking gels. Molecular weight standards were prestained broad-range molecular standards (Bio-Rad). Electrophoresis was carried out at 20 mA and constant voltage in duplicate. The proteins were transferred to nitrocellulose membrane (Hybond-C super, Amersham). The membrane was saturated at room temperature in 10 mM PBS, pH 7.4, with 6% dry milk for 1 h and then split into two smaller membranes, leaving one lane from each total protein fraction on each membrane. These two identical membranes were then processed strictly in parallel: one was incubated with the PAX2 antibody (1:100), the other in PBS with 1% dry milk instead of the antibody, and both were incubated overnight at 4°C. Then, the blots were washed and incubated with the secondary antibody (1:1,000) for 1 h at room temperature. The blots were then washed twice, once for 15 min in PBS with 1% dry milk, and then for 30 min in PBS with 0.1% Tween at room temperature. The specific signal was detected by using the enhanced chemiluminescence Western blotting detection system (Amersham). No specific signal was detected in absence of the primary antibody (data not shown).

**Statistical analysis.** Results are expressed as means ± SD. Control and obstructed groups were compared by ANOVA followed by an unpaired Dunnett’s t-test in the case of a significant global test. Correlation coefficients were calculated by linear regression analysis and assessed for significance by using Fisher’s t-test. Probabilities of <5% were considered as significant.

**RESULTS**

**Renal Morphological, Functional, and Molecular Development in Normal Fetal Lambs**

Renal morphology and number of glomeruli. Metanephros development was studied in control fetuses from day 50 after conception to the end of gestation, 140 days after conception. Fifty days after gestation, ureteral bud divisions were clearly visible, and, as in other mammals, nephrogenesis proceeded in a centrifugal pattern. Nephrogenic blastema appeared as a continuous, highly cellular band running along the whole subcapsular part of the cortex, showing the initial stages of glomerular differentiation: vesicles, comma-shaped, and S-shaped bodies surrounded by undifferentiated mesenchymal tissue. In the inner cortex, only a few glomeruli showed more developed structures. At this stage, the medulla did not appear organized. From days 60–80 (Fig. 1a), the renal cortex became organized into three layers: the subcapsular
blastemic layer, the midcortical layer of primitive nephrons, and the inner juxtamedullary layer, composed of differentiated nephrons. Progressively, the nephrogenic blastema decreased; it was still present up to day 100 of gestation but had disappeared on day 120 (Fig. 1b). At the end of gestation, most glomeruli were fully developed, and even ones not fully developed showed the different structures (epithelium, mesangium, and endothelium). The boundary between the inner and the outer medulla was well defined.

The number of primitive glomeruli present in a sagittal section was used as a time-dependent marker of renal development. The evolution of glomerular count during gestation showed an S-shaped pattern (Fig. 1c), as a consequence of variable glomerular growth depending on gestational age. Two periods could be distinguished: from days 50–60 of gestation, the number of glomeruli increased with a high rate of proliferation to reach an upper limit at ~ 100; from day 120, no significant increase in number of glomeruli could be further demonstrated (Table 1). The number of glomeruli was correlated

![Fig. 1. Histology of the fetal sheep kidney sections used in this study (a and b) and evolution of the glomerular count during gestation (c). a: 60-Day-old normal fetal sheep kidney section. Note well-defined and dense superficial nephrogenic zone. b: 120-Day-old normal fetal sheep kidney section. Cortical region is well organized; nephrogenic zone has disappeared. c: Evolution of the glomerular count during gestation. At 120 days of gestation, no significant increase in number of glomeruli could be found. u, Ureteric bud branch tips; mm, metanephrogenic mesenchyme cells; ns, nephron differentiating structure. Magnification: ×80 (a and b).

<table>
<thead>
<tr>
<th>Gestational Age, days</th>
<th>Kidney Wt, g</th>
<th>Glomerular No., (no./section)</th>
<th>Glomerular Density, (no./mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.22 ± 0.05</td>
<td>99 ± 12</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0.39 ± 0.03*</td>
<td>194 ± 18†</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>3.39 ± 1.08*</td>
<td>555 ± 130*</td>
<td>11.02 ± 1.90</td>
</tr>
<tr>
<td>90</td>
<td>5.85 ± 0.80*</td>
<td>967 ± 110*</td>
<td>13.32 ± 0.33*</td>
</tr>
<tr>
<td>100</td>
<td>6.31 ± 1.66†</td>
<td>1,556 ± 596*</td>
<td>13.68 ± 2.36†</td>
</tr>
<tr>
<td>120</td>
<td>9.69 ± 1.09*</td>
<td>1,852 ± 249†</td>
<td>13.28 ± 1.48†</td>
</tr>
<tr>
<td>140</td>
<td>12.58 ± 2.9*</td>
<td>1,974 ± 402†</td>
<td>13.25 ± 0.96†</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.05 vs. previous gestational age values. †Not significant.
with kidney weight \((r = 0.93; P < 0.001)\); glomerular density did not increase significantly from 90–140 days after conception.

**Renal function.** There was no significant change in fetal plasma sodium, potassium, or urea concentration during gestation (Table 2). Fetal plasma creatinine increased significantly during gestation (from \(59 \pm 8 \mu\text{mol/l} \) at 90 days of gestation to \(149 \pm 18 \mu\text{mol/l} \) at 140 days of gestation, \(P < 0.05\)) and was significantly correlated with fetal weight \((r = 0.81; P < 0.001)\). Creatinine clearance corrected for body weight increased progressively during gestation (from \(0.27 \pm 0.13 \text{ ml/min} \cdot \text{kg}^{-1} \) at 90 days of gestation to \(1.01 \pm 0.13 \text{ ml/min} \cdot \text{kg}^{-1} \) at 140 days of gestation, \(P < 0.05\)) and was correlated with glomerular count \((r = 0.58; P < 0.01)\). Urinary sodium concentration and fractional excretion, high at 90 days of gestation, decreased progressively from 90 to 120 days but increased again at the end of gestation (day 140).

**PAX2 distribution.** PAX2 mRNA expression was confined in the nephrogenic area of the 60-day-old fetal sheep kidney; no PAX2 mRNA expression was detected

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### Table 2. Body weight and biochemical parameters in urine and plasma of control fetuses from 90 to 140 days of gestation

<table>
<thead>
<tr>
<th>Gestational Age, days</th>
<th>Body Wt, g</th>
<th>(P_{cr}), (\mu\text{mol/l})</th>
<th>(P_{Na}), mmol/l</th>
<th>(P_{K}), mmol/l</th>
<th>(P_{U}), mmol/l</th>
<th>(U_{Na}), mmol/l</th>
<th>FE(_{Na}), %</th>
<th>(C_{Cr}) ml/min</th>
<th>(C_{Cr}) ml/min⁻¹·kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>510 ± 14</td>
<td>59 ± 8</td>
<td>134 ± 1</td>
<td>3.2 ± 0.4</td>
<td>8.2 ± 0.2</td>
<td>84 ± 5</td>
<td>13.5 ± 5.6</td>
<td>0.14 ± 0.07</td>
<td>0.27 ± 0.13</td>
</tr>
<tr>
<td>100</td>
<td>1,350 ± 537†‡</td>
<td>78 ± 18‡</td>
<td>134 ± 1‡</td>
<td>3.3 ± 0.7‡</td>
<td>5.4 ± 0.8‡</td>
<td>35 ± 8‡</td>
<td>1.2 ± 0.7‡</td>
<td>0.94 ± 0.58‡</td>
<td>0.66 ± 0.17‡</td>
</tr>
<tr>
<td>120</td>
<td>2,308 ± 566*</td>
<td>122 ± 31‡</td>
<td>133 ± 2‡</td>
<td>3.8 ± 0.5‡</td>
<td>7.7 ± 1.9‡</td>
<td>43 ± 22‡</td>
<td>2.4 ± 3.7‡</td>
<td>2.25 ± 0.81*</td>
<td>0.96 ± 0.21‡</td>
</tr>
<tr>
<td>140</td>
<td>2,915 ± 355±</td>
<td>149 ± 18‡</td>
<td>134 ± 3‡</td>
<td>3.8 ± 0.4‡</td>
<td>5.7 ± 0.7‡</td>
<td>91 ± 45*</td>
<td>13.7 ± 9.4†</td>
<td>3.1 ± 0.45‡</td>
<td>1.01 ± 0.13‡</td>
</tr>
</tbody>
</table>

\(P_{cr}\), \(P_{Na}\), \(P_{K}\), \(P_{U}\), plasma creatinine, Na, K, and urea concentration, respectively; \(U_{Na}\), urine Na concentration; FE\(_{Na}\), fractional Na excretion; \(C_{Cr}\), creatinine clearance. Significant vs. previous gestational age value: *\(P < 0.05\); †\(P < 0.01\). ‡Not significant.

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**Fig. 2.** Darkfield observations of the PAX2 gene expression in 60-day-old normal fetal sheep kidney (A). Note descending gradient expression of PAX2 gene in this kidney from the superficial nephrogenic zone to deeper, more mature medullary region. No hybridization signal was detected with the labeled sense probe (B), confirming the specificity of the in situ hybridization pattern obtained with the labeled antisense probe. Each figure is representative of the results in 3 different experiments. Magnification: \(×12.5\).
in the deeper, more mature regions (Fig. 2A). No hybridization signal was detected with the $\alpha^{35}$S-labeled sense probe, confirming the specificity of the in situ hybridization patterns obtained with the $\alpha^{35}$S-labeled antisense probe (Fig. 2B).

As described above for PAX2 mRNA, expression of PAX2 protein was highly prominent in the nephrogenic zone of the 60-day-old fetal sheep kidney (Fig. 3a). As expected for a transcription factor, staining was predominantly nuclear. Ureteric bud branch tips and condensing mesenchyme cells showed strong PAX2 expression. The expression of PAX2 was also high in the nuclei of the growing collecting duct in the deeper area of the nephrogenic zone (Fig. 3a). At 80 days of gestation, PAX2 protein was still clearly present in the nephrogenic zone, predominantly in S-shaped bodies and ureteric buds. At 100 days of gestation, the nephrogenic zone had decreased and the staining was restricted to collecting ducts. At 120 days of gestation, PAX2 immunoreactivity was significantly reduced in the cortex (Fig. 4); only faint staining could be detected in the medulla. Moreover, PAX2 antibody specificity was confirmed in Western blot analysis. Two isoforms of PAX2 protein were found: a major band at 46 kDa and a minor band at 48 kDa. Signal intensity of these bands increased in the presence of high protein concentration on blot (data not shown).

**Effects Of Bladder Outlet Obstruction on Renal Development**

**Effects on renal morphology and number of glomeruli.** The effects of fetal outlet obstruction on renal morphology are presented in Table 3. Outlet obstruction produced variable but grossly symmetrical kidney changes, ranging from mild hydronephrosis to severe dysplasia; renal pelvic distension was constant. Spontaneous renal decompression under the form of ascites...
was present in one case only; no urinoma was found. Hydronephrosis, pure or with a few tubular and glomerular microcysts scattered among the cortex, was present in seven cases (64%) (Fig. 5a). More severe disturbances, representative of renal dysplasia, were seen in four cases (36%): irregular areas of subcapsular cysts with distortion of renal architecture between the cysts in three cases (Fig. 5b) and, in a single case, type II dysplasia; in no specimen was there any evidence of heterotopic tissue as cartilage. The persistence of S-shaped bodies (without nephrogenic blastema) more numerous than expected at 120 days of gestation was noted in five cases (45%).

The average number of glomeruli and density were significantly decreased ($P < 0.05$) in obstructed kidneys compared with controls (Table 3). A second analysis of obstructed kidneys was made on the basis of the morphological lesions observed (Table 3). In obstructed groups, the number of glomeruli and density were more decreased in kidneys with dysplasia than in kidneys with hydronephrosis. As changes in either kidney volume or glomerular diameter may interfere with glomerular count, these parameters were studied in the different groups by using ANOVA. There was no significant difference between the groups in glomerular diameter and indirect estimates of kidney volume: kidney weight, volume after fixation (length $\times$ width $\times$ thickness), cortex perimeter, and surface (data not shown). The only significant difference observed was a decrease in cortex perimeter in kidneys from the group with dysplasia, which would lead to a possible overestimation and therefore a further decrease in the number of glomeruli in this group.

**Renal function after bladder outlet obstruction.** Plasma creatinine, sodium, potassium, and urea concentration did not differ in the obstructed group compared with control. Creatinine clearance (ml/min) was significantly lower, and urinary sodium concentration and fractional excretion were significantly higher than in controls. Among the different morphological groups, the group with renal dysplasia exhibited lower creatinine clearances and significantly higher plasma creatinine and higher sodium fractional excretion than the group with hydronephrosis (Table 4).

**Effects of obstruction on PAX2 distribution.** PAX2 immunostaining was still highly present in the 60-day-old obstructed sheep kidney sections, in cortical (Fig. 3c) and medullary regions, compared with normal 120-day-old fetal sheep kidney sections (Fig. 3b). Staining was especially intense in the abnormal persistent

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**Table 3. Effects of bladder outlet obstruction on kidney morphology**

<table>
<thead>
<tr>
<th>Group</th>
<th>$n$</th>
<th>Kidney Wt. g</th>
<th>Glomerular No., no./section</th>
<th>Glomerular Density, no./mm$^2$</th>
<th>Morphology</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N</td>
</tr>
<tr>
<td>CF</td>
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<td>1,852 ± 249</td>
<td>13.3 ± 1.5</td>
<td>11</td>
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<tr>
<td>OF60</td>
<td>11</td>
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<td>1,379 ± 503$^*$</td>
<td>9.6 ± 2.9$^*$</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>7</td>
<td>8.5 ± 1.3</td>
<td>1,706 ± 189</td>
<td>11.3 ± 2.0$^*$</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>7.6 ± 2.6$^*$</td>
<td>808 ± 295$^*$</td>
<td>6.5 ± 1.0$^*$</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. $n$, No. of fetuses; N, normal renal morphology; H, hydronephrosis; D, type IV (n = 3) and type II (n = 1) renal dysplasia. Kidney weight, morphology, glomeruli number/section and density at 120 days of gestation in control fetuses (CF), and in those with vesical outlet obstruction performed at 60 days (OF60) are shown. Obstructed fetuses are divided into groups according to kidney morphology. $^*$ $P < 0.05$ vs. CF. $^\dagger$ $P < 0.05$ vs. H.
nephrogenic zone (Fig. 3c). In the dysplastic zone, staining was present in dilated structures; in the atrophic cortical area, only faint expression of PAX2 was found. No significantly PAX2 mRNA expression could be detected in control or obstructed fetal sheep kidneys sections at day 120 (data not shown).

**Vesicoamniotic Shunting**

*Renal morphology and number of glomeruli.* Renal morphology in shunted fetuses was nearly normal; only pure residual hydronephrosis without visible cysts was present. The number of glomeruli and density were higher in the group obstructed at 60 days and subjected to vesicoamniotic shunt than in the nonderived obstructed kidney group; as to the difference from the 60-day obstructed kidney group, their values did not differ significantly from those of the control group (Table 5). Creation of the obstruction at 60 days of gestation led to a 20% reduction in the number of glomeruli at day 90 and 25% at day 120 compared with age-matched fetuses; the shunting procedure at day 90 allowed limitation of the reduction in the number of glomeruli to 5% (at day 120). A representation of the effect of obstruction and relief on the evolution of the number of glomeruli is provided in Fig. 6.

**Renal function.** Plasma creatinine, sodium, potassium, and urea concentration did not differ significantly among the different groups; urinary sodium concentration, sodium fractional excretion, and creatinine clearance did not differ significantly from values in nondiverted fetuses (Table 6).

**PAX2 expression.** Persistence of PAX2 protein expression observed in 60-day obstructed sheep kidneys was reversed by the shunting procedure, so that in contrast to what was observed in obstructed fetuses, PAX2 immunostaining intensity and pattern were similar in shunted fetuses and in age-matched controls (Fig. 7).

**DISCUSSION**

The fetal lamb has been extensively used as both a physiological and pathological experimental model of fetal renal function studies; however, most studies on fetal renal function have been carried out in the latter stages of gestation (34), and the precise and quantitative development of nephrogenesis in the sheep have not been yet fully described. Therefore, to evaluate the renal effects of UTO in the fetal lamb, we first carefully studied the morphological, functional, and molecular development of the kidney in our model. The dispari-
tion of the nephrogenic area and the stability of glomerular count after 120 days of gestation show that no significant nephrogenesis occurs after this time and confirm previous studies that suggested that nephrogenesis was completed before birth in this species (35). Analysis of glomerular count during gestation shows that in sheep, as in humans, nephrogenesis is not a continuous process and is characterized by a period of high proliferation at 70–80 days of gestation, corresponding to the prominent period of PAX2 protein expression (7, 8).

PAX2, an active transcription factor during kidney development, plays a critical role in the inductive transition of mesenchymal cells to the epithelial phenotype, the initial stage of kidney nephrogenesis (8). Previous studies have reported that both PAX2 protein expression and proliferation are rapidly downregulated with normal renal maturation (8, 36). Our immunohistochemical data are in accordance with this PAX2 expression pattern.

Functional studies show that renal fetal function and nephrogenesis are tightly linked; they are in agreement with previous reports and confirm that, after 120 days of gestation, glomerular filtration rate increases in parallel with weight only (34, 35). On the basis of these data, we decided to perform obstruction at 60 days of gestation during the high-proliferation period of nephrogenesis and to evaluate its effects on the fetal kidney after nephrogenesis completion, at day 120.

The question we addressed in the present study is whether performing an obstruction before the end of nephrogenesis is sufficient to produce obstructive dysplasia. The association of renal dysplasia with UTO in the fetus is a long and controversial one (3). The concept that obstruction never causes dysplasia is centered on the idea that dysplasia, when strictly defined, is only the result of an inductive failure and obstruction, a secondary process (2). In this context, there is no indication to develop in utero derivation in congenital UTO: as an embryonic process, dysplasia must then be seen as irreversible. Alternatively, if obstruction produces dysplasia, relief of obstruction may reduce dysplasia (13). Results show that experimental fetal UTO alters renal patterns of both differentiation and growth regulation. As in congenital UTO, the pattern of morphological changes observed is not uniform and displays a wide spectrum of lesions. Two major groups of lesions can be individualized: hydronephrosis, corresponding to normally developed but distended kidneys, and dysplastic kidneys, characterized by abnormal structural and architectural development. The more severe form of renal dysplasia, type II, was not observed except in one case. Although a mechanism other than obstruction may be responsible for these lesions, other hypotheses may explain their low frequency in our model. First, their severity may be incompatible with survival and accounts for a large part of the mortality observed in the model. Second, these lesions may represent later stages of dysplasia, and the duration of observation in the model may be too short to

Table 5. Effects of vesicoamniotic shunting on kidney morphology

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Kidney Wt, g</th>
<th>Glomerular No., no./section</th>
<th>Glomerular Density, no./mm²</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N  H    D</td>
</tr>
<tr>
<td>120 Days of gestation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF</td>
<td>11</td>
<td>9.7 ± 1.1</td>
<td>1,852 ± 249</td>
<td>13.3 ± 1.5</td>
<td>11 0 0</td>
</tr>
<tr>
<td>OF60</td>
<td>11</td>
<td>8.2 ± 1.8</td>
<td>1,379 ± 503*</td>
<td>9.6 ± 2.9*</td>
<td>0 7 4</td>
</tr>
<tr>
<td>SF</td>
<td>5</td>
<td>12.5 ± 7.0</td>
<td>1,784 ± 193*</td>
<td>10.7 ± 2.9</td>
<td>5 0 0</td>
</tr>
<tr>
<td>90 Days of gestation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF*</td>
<td>3</td>
<td>5.85 ± 0.8</td>
<td>967 ± 110</td>
<td>13.3 ± 0.3</td>
<td>3 0 0</td>
</tr>
<tr>
<td>OF60*</td>
<td>4</td>
<td>3.54 ± 1.6</td>
<td>776 ± 308*</td>
<td>8.9 ± 1.5*</td>
<td>0 4 0</td>
</tr>
</tbody>
</table>

Values are means ± SD. n, No. of fetuses. N, H, and D are as described in Table 3. Kidney weight, morphology, glomerular number/section, and density in control fetuses at 120 (CF) and 90 days of gestation (CF*); in fetuses subjected to vesicoamniotic shunting at 90 days of gestation (SF); and in 120-day-old (OF60) or 90-day-old (OF60*) fetuses with vesical outlet obstruction performed at 60 days of gestation are shown. *P < 0.05 vs. CF. †Not significant.
allow them to develop. In this view, very dysplastic lesions reported as characteristic of type II dysplasia in neonatal or postnatal kidneys, as cartilage metaplasia, are exceptionally observed in fetuses. Regardless, it remains that a form of renal dysplasia can unquestionably be produced by fetal UTO. As in congenital UTO (12), dysplasia is always associated with a decrease in the number of glomeruli, corresponding to an arrest in renal development; similar findings were reported in the fetal lamb (30) and in the fetal rabbit (23). At a molecular level, obstructive dysplasia was associated with the abnormal persistence of PAX2 protein expression, particularly relevant in the cortical zone with persistent immature structures. Persistence of PAX2 protein expression in the developing kidney after UTO was also observed by other authors in the fetal sheep kidney and in human dysplastic kidneys (1, 40). Persistence of PAX2 protein expression in the developing kidney after UTO was also observed by other authors in the fetal sheep kidney and in human dysplastic kidneys (1, 40). Persistence of PAX2 protein expression in the developing kidney attenuates the differentiation potential of renal epithelial cells and generates renal cysts in a transgenic mice model overexpressing PAX2 protein (9); conversely, reduced PAX2 gene dosage slows cyst growth in the mouse model of cystic kidney disease (28). Furthermore, the proliferative state of dysplastic epithelia in vitro correlates with the level of PAX2 expression (41). Previous studies have reported the association of PAX2 persistence expression with renal pathological growth: Wilms' tumor and renal carcinoma (8, 10, 15). Therefore, PAX2 persistence expression may be related to the abnormal differentiation of renal structures in renal obstructive dysplasia. On the other hand, in the atrophic cortical zone, PAX2 expression was completely absent around dysplastic epithelia. Dominant apoptosis activity and low PAX2 expression have been reported in the mesenchyme surrounding dysplastic epithelia in humans (31). Whether the low PAX2 expression in this zone is the cause or the consequence of apoptosis remains unclear. In all cases, it may reflect a definitive loss of differentiation potential.

If the renal damage produced by fetal UTO is reversible after obstruction relief, it appears justifiable to perform in utero diversion. Reversibility of renal damage was suggested in the pioneer experimental study of Glick et al. (13), who evidenced no dysplasia in fetuses diverted before the end of nephrogenesis. Since the above study, diversion has been performed in congenital UTO, but the results are still controversial (11). In the present study we investigated morphological, functional, and molecular effects of diversion performed at 90 days of gestation. The time of diversion was chosen to be before the end of nephrogenesis, to allow renal development recovery but sufficiently late to be comparable to conditions observed in congenital UTO. Results show that even late in the course of nephrogenesis, diversion allows an improvement in kidney condition; although the morphological status of the kidney on the day of diversion was not studied and the number of cases was small, no dysplasia was noted in shunted fetuses; more importantly, there was a clear increase in the number of glomeruli compared with

![Day 120](image1)

![UTO 60 days](image2)

![UTO + Diversion](image3)

**Fig. 7.** PAX2 protein expression at 120 days of gestation in age-matched controls (a), obstructed (b), and shunted fetal sheep kidney (c). Persistence of PAX2 protein expression noted in 60-day obstructed fetal sheep kidney was reversed by the shunting procedure. PAX2 protein expression level in shunted kidneys was similar to that observed in control animals. Magnification: ×250.
that in nondiverted fetuses. This observation suggests that obstruction reduces glomerular formation and that this arrest is reversible with obstruction relief. Persistence of PAX2 protein expression noted in obstructive dysplasia was reversed in shunted kidneys so that the PAX2 protein expression level in shunted kidneys was similar to what was observed in controls. These results, together with the increase in the number of glomeruli, represent, to our knowledge, the first indication that lowering the urinary pressure by the shunting procedure allows the recovery of the normal nephrogenesis process. Recovery of glomerulogenesis may be possible only during a short critical period of development, as recent data in a postnatal model in the rat failed to demonstrate any increase in the number of glomeruli when relief of obstruction was performed 5 days before the end of nephrogenesis (5). The mechanisms by which urinary hyperexpression interfere with the different elements of nephrogenesis remain unknown.

In humans, on the basis of the need to predict renal outcome, different parameters of fetal renal function have been studied (25); among them, fetal urinary sodium concentration is considered to be predictive of renal function, allowing one to distinguish between moderate damage and normal renal function (26, 32). The present study shows, as did previous experimental work (39), that fetal UTO leads to decreased sodium reabsorption and increased urinary sodium concentration. The partial recovery of sodium reabsorption after 30 days of relief suggests that the decrease in urinary pressure improved individual tubule function or the number of tubules reabsorbing sodium. An unexpected finding in this study was the persistence of a decrease in glomerular filtration rate in shunted fetuses despite an increase in the number of glomeruli. Whether this discrepancy between morphological and functional data can be related to the immaturity or low perfusion rate of the new nephrons formed remains to be determined by longer follow-up studies.

In summary, the present study confirms results from previous experimental studies (14, 30) of fetuses subjected to unilateral or bilateral urinary obstruction and shows clearly that fetal UTO during nephrogenesis leads to severe renal morphological and functional abnormalities and disrupts PAX2 pattern expression, all features that are observed in congenital UTO. The severity of the renal damage induced, especially nephronic reduction, is sufficient to reduce postnatal renal function. Urinary diversion before the end of nephrogenesis allows the recovery of this process, confirmed by the normalization of PAX2 pattern expression. More investigations are needed to assess the reversibility of nephrogenesis arrest suggested in the present study.

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REFERENCES


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