Urinary Outflow Obstruction Increases Apoptosis and Deregulates Bcl-2 and Bax Expression in the Fetal Ovine Bladder

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During organogenesis, net growth of tissues is determined by a balance between proliferation, hypertrophy, and apoptotic death. Human fetal bladder outflow obstruction is a major cause of end-stage renal failure in children and is associated with complex pathology in the kidney and lower urinary tract. Experimental manipulation of the fetal sheep urinary tract has proved informative in understanding the pathobiology of congenital obstructive uropathy. In this study we used an ovine model of fetal bladder outflow obstruction to examine effects on apoptotic cell death in the developing urinary bladder. While 30 days of obstruction *in utero* between 75 and 105 days gestation resulted in overall growth of the fetal bladder as assessed by weight, protein, and DNA measurements, we found that apoptosis, as assessed by *in situ* end-labeling, was up-regulated in fetal bladder detrusor muscle and lamina propria cells and that this was accompanied by a down-regulation of the anti-death protein Bcl-2 and an up-regulation of the pro-death protein Bax. Moreover, activated caspase-3, an effector of apoptotic death, was increased in obstructed bladders. This is the first study to define altered death in an experimental fetal model of bladder dysmorphogenesis. We speculate that enhanced apoptosis in detrusor smooth muscle cells is part of a remodeling response during compensatory hyperplasia and hypertrophy. Conversely, in the lamina propria, an imbalance between death and proliferation leads to a relative depletion of cells. (*Am J Pathol* 2003; 162:1271–1282)

In utero bladder outflow obstruction (BOO) is a common cause of end-stage renal failure in young children.¹ The specific diseases associated with BOO include posterior urethral valves (PUV)² and prune belly syndrome (PBS)³ which is associated with a mechanically or functionally

obstructed fetal bladder. The renal histological lesion which accompanies severe BOO comprises congenital dysplasia and subcortical cysts.⁴ However, fetal BOO is also associated with bladder dysmorphogenesis and poor bladder emptying, frequency of urination, incontinence, and a tendency to urinary tract infection.⁵ Furthermore, this bladder dysfunction is implicated in postnatal loss of renal excretory function, a process which summates with congenital kidney damage.^{2,6}

Experimental models of fetal urinary tract obstruction have exploited the sheep as a model because ovine fetuses can be manipulated relatively early in gestation without major mortality^{7–10} and by mid-gestation, ovine bladders receive 5 to 6 ml of urine each hour from the metanephroi.¹¹ Investigation of human fetal bladder development illustrates how the bladder thickens and has increased elastic fibers and decreased collagen deposition during maturation of the fetus.¹² The triggering events for this maturational process remain unknown; one possible mechanism is the triggering of growth by mechanical distension of the developing fetal bladder by the increasing volume of urine.¹³

While insights into the effects of fetal urinary tract obstruction on kidney development have been made using studies of ureteric obstruction,^{14–16} such experiments cannot perturb bladder development; this is an important deficiency because human congenital uropathy generally affects morphogenesis of both the upper tracts and the bladder. In contrast to humans, in which the urachus obliterates at 4 to 5 months gestation,¹⁷ the sheep urachus remains patent until late gestation.⁷ This structure constitutes another outflow tract from the developing bladder into the uterine cavity and must be ligated in sheep, in addition to obstructing the urethra, to generate severe BOO. Therefore, investigators have combined fetal sheep urethral and urachal ligation to model bladder pathology which might reflect human diseases with congenital BOO.^{8–10} Peters et al⁸ produced ovine BOO at 60

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days gestation and sacrificed fetuses after 35 or 85 days; a marked growth response was found in obstructed bladders as assessed by increased bladder weight, DNA and protein content, and detrusor smooth muscle cell (SMC) size. Using a similar methodology, we reported that severe BOO¹⁰ between 75 and 105 days gestation reduced bladder muscle contractility, compliance, and innervation; this model uses severe BOO to produce an experimental phenotype which resembles dysmorphic, massively dilated bladders reported in autopsy cases of severe human fetal PUV and PBS.³

In recent years, developmental biologists have emphasized that normal fetal organ growth is not simply mediated by the increase in number and size of cells, but is the net result between these positive processes and programmed cell death by apoptosis.^{18–20} Apoptotic death is implicated in sculpting (eg, loss of webs between digits), deleting unwanted formed structures (eg, involution of the mesonephros), and adjusting cell numbers (eg, deleting excess precursors in the metanephric kidney). Furthermore, advances have been made in determining molecular death pathways, with Bcl-2 being the archetypal survival molecule and Bax and caspase-3 as examples of molecules associated with death.^{19,21} We hypothesized that fetal BOO might be associated with altered apoptosis in the developing bladder and that this might be accompanied by a deregulation of expression of pro- and anti-apoptotic molecules.

Materials and Methods

Experimental Strategy

All work was conducted in accordance with the United Kingdom Home Office Animals Act (Scientific Procedures) of 1986. All chemicals were from Sigma (Poole, Dorset, UK) unless otherwise stated. *In utero* BOO was produced in fetal sheep as previously described.¹⁰ In brief, male fetuses at 75 days gestation, from Romney Marsh ewes (Royal Veterinary College, Potters Bar, UK), were allocated to a sham control group or an obstructed group. The obstructed group ($n = 11$) underwent experimental partial urethral obstruction by placement of an ω -shaped silver ring around the fetal urethra and complete ligation of the fetal urachus. The sham group ($n = 11$) underwent urethral and urachal exposure only. In addition, five female fetuses, discovered at operation, underwent the sham procedure. After weekly ultrasound examination, to confirm fetal viability and the extent of any urinary tract dilation, pregnant ewes were sacrificed at 105 days gestation (ie, 30 days after surgery) and urinary bladders collected. At autopsy of the obstructed group, amniotic fluid was present within the uterus; furthermore, a urine leak was evident at the urethral meatus, indicating that the bladder outflow obstruction was unlikely to be functionally complete *in utero*. Bladder samples, taken from the bladder body (defined as the superior portion of the bladder excluding the bladder trigone), were placed in 10% paraformaldehyde (BDH, Poole, UK) or snap-frozen in liquid nitrogen. To study the effects of *in utero*

Figure 1. Experimental design. Scheme of fetal sheep gestation, from conception to birth, with timing of sham and obstructed group operations and analyses of urinary bladders. To place these procedures in relation to upper renal tract development, the percent glomerular complement of the developing sheep metanephric kidney is also shown (adapted from results in Edouga et al⁴⁶).

BOO, obstructed and sham male fetuses were used because human fetal BOO occurs almost exclusively in males.¹ As depicted in Figure 1, five sham male and five obstructed male fetuses were used for measurement of bladder weight, protein, DNA, and capsase-3 activity, and samples from the remaining six fetuses in each group were analyzed by histology by Masson's trichrome stain, proliferating cell nuclear antigen (PCNA) immunohistochemistry, Bcl-2 and Bax immunohistochemistry and Western blot, and TUNEL *in situ* end-labeling. In addition, a limited "maturation" study was also performed, in which we additionally analyzed bladders from two 75-day gestation fetuses (one male and one female), five sham (105-day gestation) female fetuses, six 145-day gestation fetuses (three male and three female) and four adults (age 4 to 8 years, two male and two female).

Bladder Weight, Protein, and DNA Measurement

Before placement in liquid nitrogen for snap-freezing, samples from the bladder body (of approximately 100 mg) were blotted dry and weighed. For dry weight measurement, samples were slowly dehydrated by warming at 65°C and were repeatedly weighed until a base-line weight was reached; in this way, a ratio of dry weight to wet weight was established for each sample and then extrapolated to total bladder weight. Further sections of bladder domes were homogenized at 4°C in radioimmunoprecipitation assay buffer containing protease inhibitors; 30 μ l/ml aprotinin (2.2 mg/ml), 10 μ l/ml phenylmethylsulfonyl fluoride (10 mg/ml), and 10 μ l/ml sodium orthovanadate (100 mmol/L in 95% ethanol). After centrifugation at 13,000 rpm at 4°C, protein concentration in supernatants was measured using a BCA protein assay (Pierce, Rockford, IL, USA). For DNA measurement, sections of bladder dome were left overnight at 50°C in lysis buffer (20 mmol/L Tris (pH 7.4), 100 mmol/L EDTA (pH 8.0), and 1 mg/ml proteinase K). After the addition of 0.2

mol/L NaCl, an equal volume of phenol:chloroform:isomyl alcohol (25:24:1) was added to each sample, and after centrifugation at 13,000 rpm at 4°C for 15 minutes, the supernatant was added to equal volume of isopropanol. After precipitation at -70°C and further centrifugation, the remaining DNA pellet was resuspended in Tris-EDTA. DNA concentrations were calculated from the emission wavelength of 260 nm using a luminescence spectrometer and purity was assessed by measuring the emission wavelength of 280 nm and by ethidium bromide-incorporated 0.2% agarose gel electrophoresis and immunofluorescent detection: all DNA samples had 260:280 nm absorbancy ratio of >1.7 and had appropriate ethidium bromide staining patterns on agarose gel electrophoresis (data not shown).

Histology and Morphometric Analyses

Fixed bladders were dehydrated in alcohol, wax-embedded, and sectioned at 4 μ m. Sections were dewaxed, rehydrated, and stained with Masson's trichrome. Using image analysis software (KS 300, Carl Zeiss, Oberkochen, Germany), mean detrusor SMC area (μ m²) was calculated by measuring the area of detrusor muscle bundles factored for the number of nuclei in each bundle. Muscle bundles were examined from four randomly placed fields in the muscle layers of bladders from each group ($n = 6$). Cell density in the lamina propria was measured by counting the number of nuclei in a fixed square area of known size, using a 20 $\times$ objective, and then correcting cell density to 1 mm² area; these areas were from four randomly placed fields in the lamina propria of bladders from each group ($n = 6$).

Immunohistochemistry

To assess proliferation, sections were probed with an antibody to PCNA which functions as an auxiliary protein for DNA polymerase- δ .²² To assess cell death by apoptosis, the TUNEL method (see below) was used and for a study of apoptotic regulatory proteins, Bcl-2 and Bax immunoreactive proteins were examined. Sections were prepared as previously described.¹⁶ In brief, primary antibodies were biotin-conjugated mouse anti-human PCNA antibody (1:50; Pharmingen, San Diego, CA, USA), monoclonal anti-mouse Bcl-2 antibody (1:50) or rabbit anti-human Bax antibody (1:50; Chemicon, Temecula, CA, USA) and secondary antibodies were biotinylated sheep anti-mouse antibody (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) for anti-Bcl-2 sections or donkey anti-rabbit (Amersham) for anti-Bax sections at 1:200 dilution. This step was omitted for the biotin-conjugated anti-PCNA antibody. Omission of primary antibodies gave no signal (data not shown). Sections were counterstained in hematoxylin to detect cell nuclei, dehydrated, and mounted in dextropropoxyphene (BDH). Slides were examined and photographed on a Zeiss Axiophot II microscope (Carl Zeiss). To quantify proliferation, nuclear counts were made of PCNA positively stained nuclei within each of the three layers of the

fetal bladder: the detrusor muscle layer, the lamina propria, and the urothelium. For the detrusor layer, by random field placement using a 20 $\times$ objective, 100 cells were counted within muscle bundles and the number of PCNA positively stained cells (cells with brown stained nuclei of any shade) were counted; this was performed 10 times in different fields, so that 1000 nuclei for each fetal sample were analyzed. The Proliferative Index for each sample was the percentage of cells positive for PCNA. For cells from lamina propria and urothelial layers, 100 nuclei were counted 10 times in randomly placed fields within these layers to generate the Proliferative Indices. In addition, a Proliferative Index, as described for urothelial and lamina propria layers, was determined for the prominent cell population which was found to surround detrusor muscle bundles in the obstructed group (see Results).

TUNEL In Situ End-Labeling

The TUNEL method was used to assess cell death; it employs a fluorescein-labeled nucleotide that attaches onto strand breaks in digested DNA found in apoptotic nuclei by *in situ* end-labeling (terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) In Situ Cell Death Detection kit, Boehringer Mannheim, Mannheim, Germany). After dewaxing and rehydration in water, sections were incubated with trypsin for 30 minutes at 37°C and then exposed to terminal deoxytransferase and fluorescein-isothiocyanate (FITC)-conjugated UTP. Control sections were exposed to FITC-conjugated UTP only. Sections were then stained with 4 mg/L propidium iodide containing 100 mg/L RNase A. Sections were mounted in Citifluor (Chemical Labs, Canterbury, UK) and viewed with either a Zeiss Axiophot II microscope (Carl Zeiss) or a confocal microscope (Leica TCS SP2, Leica, Heidelberg, Germany). Using appropriate wavelengths, TUNEL-labeled nuclei were detected as a green color (515 to 565 nm) and could be confirmed as nuclear in origin by staining with propidium iodide (617 nm), a dye which intercalates with DNA. To quantify apoptosis, apoptotic nuclei were counted as described for PCNA-positive cells (see above) and expressed as the Apoptotic Index in each of the three layers in the sham and obstructed groups. Our preliminary data (not shown) indicated that, in all samples, about two thirds of TUNEL-labeled nuclei were also pyknotic (small, irregular, and brightly-staining) as assessed by propidium iodide staining; this is explained because the molecular *in situ* end-labeling methodology is likely to be more sensitive than the morphological method at detecting apoptotic cells.

Western Blotting

Sham and obstructed fetal Bcl-2 and Bax were measured using Western blotting. In addition, PCNA was measured in bladder samples from the four groups of developing fetal bladders. Samples were prepared as previously described.¹⁶ Briefly, the primary antibodies were mouse

anti-human PCNA antibody (1:1000; Amersham Pharmacia Biotech), monoclonal anti-mouse Bcl-2 antibody (1:1000), rabbit anti-human Bax antibody (1:1000; Chemicon) or monoclonal anti-mouse β -actin antibody (1:10000), a "house-keeping" protein. Secondary antibodies were HRP-linked donkey anti-rabbit antibody (1:1000), for Bax detection, or HRP-linked sheep anti-mouse antibody, 1:500 for PCNA detection, 1:1000 for Bcl-2 detection or 1:5000 for β -actin detection. Bands were detected by chemiluminescence (Amersham Pharmacia Biotech), quantified using the Phoretix ID program (Phoretix International, Newcastle on Tyne, UK) and expressed as the ratio of β -actin chemiluminescence. Omission of primary antibodies gave no signal (data not shown).

Assay of Activated Caspase-3 Activity

The Caspase-3 Colorimetric Activity Assay kit (Chemicon) is based on the spectrophotometric detection of the chromophore *p*-nitroaniline (*p*NA) after cleavage from the labeled substrate DEVD-*p*NA; the DEVD amino acid sequence is derived from the caspase-3 cleavage site in enzyme poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme. Bladder samples were incubated at room temperature in Cell Lysis Buffer (proprietary buffer in kit) and subsequent supernatant was incubated with 1 mmol/L ATP at 37°C for 30 minutes. Samples were incubated with caspase-3 substrate (Ac-DEVD-*p*NA) at 37°C for 1 hour. Peptide cleavage was measured at an emission wavelength of 405 nm using a luminescence spectrometer. Absorbency readings were also determined for a *p*NA standard curve and for quantitative purposes, decreasing dilutions of recombinant active caspase-3 (Chemicon) was also tested by the Assay Kit. Caspase activity was expressed in units where one unit was the amount of enzyme that will cleave 1.0 nmol of the colorimetric substrate Ac-DEVD-*p*NA per hour at 37°C under saturated substrate concentrations. In addition, for control purposes, two samples (one each from the sham and obstructed groups) were pre-incubated with caspase-3 inhibitor (Ac-DEVD-CHO) for 10 minutes at room temperature before adding caspase-3 substrate solution. Addition of inhibitor produced enzyme inhibition in the sham fetal bladder sample (2.3 versus 0.1 enzyme units, $n = 1$) and obstructed fetal bladder sample (3.3 versus 0.3 enzyme units, $n = 1$).

Statistics

Group results were expressed as the mean $\pm$ SD. Independent samples Student's *t*-test were used to examine differences in values between sham and obstructed groups. The null hypothesis was rejected at $P < 0.05$. One-way analysis of variance (analysis of variance) was performed to compare difference in means $\pm$ SD of the Proliferative Indices and Apoptotic Indices within the three layers of the fetal bladder, with a Bonferroni correction to allow for multiple comparisons. For the developing

Figure 2. Autopsy specimens. Sham urinary tract (**left**) and obstructed specimen (**right**) at 105 days gestation. In the latter, note the increased size of the bladder, the dilatation of the ureters and the increased size of the kidneys that were hydronephrotic on sectioning (**inset**). These appearances were representative of 11 fetuses in each experimental group. Black thread was used for orientation following dissection. K, metanephric kidney; Ur, ureter; Ura, urachus; B, bladder; U, urethra.

groups, statistics were not performed as there were insufficient samples ($n = 2$) in the 75-day gestation group.

Results

Gross Changes of the Fetal Bladder after in Utero BOO

As assessed by weekly ultrasound examination over 30 days of BOO, the obstructed fetal urinary tracts showed progressive dilatation with hydroureteronephrosis and a large, dilated bladder (data not shown); a similar appearance was found at autopsy at 105 days gestation (Figure 2). Previous histological examination of kidneys from sham fetuses that had undergone the same protocol¹⁰ had revealed a prominent nephrogenic zone, with normal caliber tubules and developing glomeruli; by contrast, in obstructed kidneys, a perturbation of nephrogenesis had been noted, with morphological disruption of the nephrogenic cortex and subcortical cysts.¹⁰ See Table 1 for bladder weights, protein, and DNA content in sham and obstructed groups. To summarize, after BOO, the aver-

Table 1. Bladder Weight, DNA, and Protein Content

	Sham bladders	Obstructed bladders
Weight, g	1.14 $\pm$ 0.25	7.28 $\pm$ 4.41*
Dry weight, g	0.16 $\pm$ 0.06	1.07 $\pm$ 0.57*
Dry/wet weight ratio, %	14.2 $\pm$ 7.0	15.8 $\pm$ 2.7 [†]
Total protein content, mg	64.0 $\pm$ 6.0	303.4 $\pm$ 114.9 [†]
Total DNA content, mg	1.49 $\pm$ 0.47	5.60 $\pm$ 3.08*
Protein:DNA ratio (arbitrary units)	0.05 $\pm$ 0.03	0.06 $\pm$ 0.03 [‡]

Bladder weight, protein and DNA content in fetal bladders from sham and obstructed groups, * $P, < 0.05$, [†] $P, < 0.005$, [‡] not significant versus sham males.

Figure 3. Histology of experimental fetal bladders. Masson's trichrome staining of sham (**A** and **B**) and obstructed (**C** and **D**) fetal bladders at 105 days gestation, representative of 6 bladders from each group. In the detrusor layer (**A** and **C**), distinct muscle bundles stain **red** and collagenous tissues between bundles stain **blue**; note the apparent increase in SMC size and increased collagenous material (indicated by an asterisk) in the obstructed group. **B** and **D** show the lamina propria (LP) and the urothelium; note the apparent decrease in cell density in the lamina propria and also the flattening of the urothelium in the obstructed group. Bars, 50 μ m.

age significant increase in fetal bladder wet weight was 539%, in dry weight was 569%, in protein content was 374%, and in DNA content was 276%.

Histology

Sham fetal bladders at 105 days gestation showed a detrusor muscle layer containing discrete muscle bundles with few cells between bundles, a lamina propria containing cells surrounded by collagen and a urothelium which was three to four cell layers thick (Figure 3, A and B). In contrast, obstructed bladders (Figure 3, C and D) had a detrusor layer with muscle bundles separated by prominent spaces containing collagen and cells, a lamina propria layer that appeared to a lower density of cells, and an attenuated, predominantly single cell-layered, urothelium. High power images suggested that myocytes in obstructed bladders were larger than cells found in the sham group; this was confirmed by measuring a significant increase ($P < 0.001$) in the average area of detrusor SMC by morphometric analysis ($132 \pm 59 \mu\text{m}^2$ versus $64 \pm 15 \mu\text{m}^2$, $n = 5$ each group). The impression of a relatively acellular lamina propria was confirmed by morphometric analysis, with a significantly lower ($P < 0.001$) cell density in this layer ($3144 \pm 1101/\text{mm}^2$ versus $4718 \pm 946/\text{mm}^2$, $n = 5$ each group).

Proliferation

As assessed by PCNA immunostaining (Figure 4, A-D), all layers in sham and obstructed bladders at 105 days

gestation contained proliferating cells. The quantification is shown in Figure 4E. In the sham controls ($n = 6$), there were no significant differences in Proliferative Indices between the cells in the three main layers of the bladder wall ($44 \pm 17\%$ in detrusor SMC, $45 \pm 4\%$ in lamina propria layer, and $46 \pm 8\%$ in urothelium; $P > 0.05$). In contrast, in the obstructed group ($n = 6$), there was a significantly lower Proliferative Index in lamina propria cells ($40 \pm 5\%$) versus detrusor SMC ($68 \pm 2\%$; $P < 0.001$) and versus urothelial cells ($57 \pm 1\%$; $P < 0.05$). Further analysis showed a significant increase ($P < 0.05$) in the Proliferative Index in detrusor SMC of the obstructed versus the sham group but there was no significant differences of Proliferative Indices of lamina propria or urothelial cells between these two groups. Lastly, a prominent proliferating cell population was observed around obstructed muscle cell bundles (Figure 4F) which were not evident in sham bladders; the Proliferative Index of these cells was $44 \pm 10\%$ ($n = 6$) (Figure 4G).

Apoptosis

TUNEL *in situ* end-labeling (Figure 5, A-C and data not shown) revealed that some cells were undergoing death by apoptosis in all layers in both sham and obstructed bladders at 105 days gestation. The quantification is shown in Figure 5D. There were no significant differences between Apoptotic Indices in cells of the three layers from either the sham ($n = 6$) ($0.14 \pm 0.09\%$ in detrusor SMC, $0.11 \pm 0.06\%$ in lamina propria layer and $0.04 \pm 0.05\%$ in urothelium; $P > 0.05$) or obstructed ($n = 6$)

Figure 4. Cell proliferation in experimental bladders. **A–D**, Immunohistochemistry for PCNA in sham (**A** and **B**) and obstructed bladders (**C** and **D**) in the detrusor layer (**A** and **C**) and lamina propria and urothelial layers (**B** and **D**) at 105 days gestation. Sections were counterstained with hematoxylin. Note PCNA-positive nuclei in all three main layers of the bladders in both experimental groups. Note also, in **C**, the increased size of obstructed detrusor SMC. **E**: Proliferative Index in sham (closed bars, $n = 6$) and obstructed (open bars, $n = 6$) detrusor SMC, lamina propria cells, and urothelial cells. There was a significant increase in detrusor SMC Proliferative Index in the obstructed *versus* the sham group, and there was a significantly lower Proliferative Index in lamina propria cells *versus* both detrusor SMC and also urothelial cells in the obstructed group. **F**: In the obstructed group, a proliferating population of cells was noted between muscle bundles (to the right of the dotted line, indicated by an asterisk); no proliferation was found in the sham group in this location (data not shown). **G**: The Proliferative Index of the cell population described in **F** was about 44%. Bars, **A–D** and **F** are 30 μ m.

Figure 5. Apoptosis in experimental fetal bladders. **A–C** is the same field from an obstructed muscle bundle visualized in appropriate wavelengths for propidium iodide (**A**) and TUNEL-FITC (**B**), with **C** representing the merged image. Five TUNEL-labeled nuclei are indicated (arrows). In this field, the numbers of apoptotic nuclei were higher than average; these images were simply used to illustrate the TUNEL technique. Bars, 10 μ m. **D:** The Apoptotic Index in sham (closed bars, $n = 6$) and obstructed (open bars, $n = 6$) bladders in detrusor SMC, lamina propria cells, and urothelial cells. Note the significant increase of death in obstructed *versus* sham detrusor SMC and lamina propria cells. **E:** Increased caspase-3 enzyme activity in full thickness samples from obstructed bladders (closed bars, $n = 5$) *versus* sham bladders (open bars, $n = 5$).

($0.31 \pm 0.07\%$ in detrusor SMC, $0.29 \pm 0.07\%$ in lamina propria layer and $0.09 \pm 0.09\%$ in urothelium; $P > 0.05$) groups. As depicted in Figure 5D, the Apoptotic Index was significantly increased in the obstructed *versus* sham bladders in detrusor SMC ($P < 0.001$) and also lamina propria cells ($P < 0.001$); there was no significant difference in apoptosis between obstructed and sham urothelial cells. Apoptosis was not observed in the population of proliferating cells located between muscle bundles in the obstructed bladders. By a substrate cleavage assay, activated caspase-3 was detected in the sham bladder at 105 days gestation (2.0 ± 0.4 enzyme units, $n = 5$) and was significantly increased ($P < 0.005$) in time-matched obstructed bladders (4.2 ± 0.5 , enzyme units, $n = 5$) (Figure 5E).

Bcl-2 and Bax Immunohistochemistry and Western Blot

Bcl-2 immunohistochemistry revealed this anti-apoptotic protein was present within the cytoplasm of most cells within each layer of the sham (Figure 6, A and B) and obstructed (Figure 6, C and B) bladders at 105 days gestation. Western blot of full thickness bladder samples showed a band at 26 kd (Figure 6I), as previously reported,²³ and quantification, using Bcl-2/ β actin

ratios, showed a significant down-regulation (approximately 50%) of Bcl-2 protein in obstructed *versus* sham bladders ($n = 6$ each group, $P < 0.05$). Bax immunohistochemistry revealed that this pro-death protein was also present within the cytoplasm of cells within all layers of the sham (Figure 6, D and F) and obstructed (Figure 6, G and H) fetal bladders at 105 days gestation; moreover, the staining intensity was subjectively increased in the obstructed *versus* sham detrusor SMC and lamina propria (eg, compare Figure 6G with 6D, and compare Figure 6H with 6F) and also appeared prominent within the luminal layers of the urothelium in bladders from both groups. Western blot of full thickness bladder samples showed a band at 22 kd (Figure 6I), as previously reported,²³ and quantification, using Bax/ β actin ratios, showed a significant up-regulation (approximately 200%) of Bax protein in obstructed *versus* sham bladders ($n = 6$ each group, $P < 0.001$).

The Developing Fetal Bladder

Staining with Masson's trichrome (Figure 7, A–C) revealed progressive thickening of the fetal bladder wall from 75 days gestation through 105 and 145 days. Detrusor muscle bundles were present at all three time points, but appear to become increasingly organized with increasing gestation. During this period, the bladder grows, as assessed by the total content of protein (57.0 mg, $n = 2$; 68.7 ± 22.4 mg, $n = 10$; 126.9 ± 48.6 mg, $n = 6$, at 75, 105 and 145 days gestation, respectively) and DNA (1.36 mg, $n = 2$; 1.44 ± 0.37 mg, $n = 10$; 2.97 ± 2.13 mg, $n = 6$, at the same time points). PCNA Western blot of full thickness bladder samples harvested from fetuses at these time points, and also from adult sheep, showed a band at 36 kd (Figure 7D), as previously reported;¹⁵ as assessed by PCNA/ β -actin ratios (Figure 7E), there was a progressive fall in levels of this protein which is a surrogate marker for proliferation. Furthermore, using activated caspase-3 activity as a surrogate marker of apoptotic death (Figure 7F), a similar downward trend was seen found between 75 and 145 days gestation.

Discussion

Known Effects of Experimental BOO on the Fetal Bladder

Three decades ago, Tanagho⁷ established a model of fetal BOO in which male ovine fetuses underwent partial urethral obstruction at 70 to 75 days gestation; the urethral obstruction became complete as fetuses grew. However, probably because the urachus was not ligated, neither the bladder not the upper tracts were severely damaged. Peters and colleagues,^{8,14,24} refined the model to include experimental urachal obstruction, a protocol which resulted in hydronephrosis and renal cystic changes. After BOO from 60 days through to term, bladder weight increased approximately fivefold *versus* sham controls.⁸ As assessed by various morphometric analyses performed at term, detrusor muscle average cell

volume was found to have increased, as had "total smooth muscle mass;" total bladder DNA increased approximately twofold but there was a significantly greater increase in protein. The authors concluded that the response to fetal BOO was mainly accounted for by hypertrophy, with a lesser contribution from hyperplasia; in a limited analysis after only 35 days obstruction, obstructed bladders were also heavier than controls. Concomitant with this growth response, a deregulation of extracellular matrix proteinases was found to correlate with bladder fibrosis.²⁴ More recently, investigators have examined the effects of BOO on bladder contractility. Levin et al⁹ caused BOO at 90 days gestation by urachal and urethral obstruction; after 3 to 5 days, obstructed bladders were hypocontractile, as assessed by electrical field stimulation of bladder strips. More prolonged BOO, from 75 to 105 days gestation, using an identical protocol to the current study, produced hypocontractility with evidence of denervation;¹⁰ such obstructed fetal bladders had large capacities, were flaccid but retained stress-relaxation.

BOO Deregulates Proliferation and Apoptosis in the Developing Bladder (Figure 8)

In the current study we used an ovine model of fetal BOO to examine effects on proliferation and apoptotic cell death in the developing urinary bladder. Thirty days of BOO resulted in enhanced growth of the fetal bladder as assessed by whole bladder weight, protein, and DNA measurements, somewhat similar to the results of Peters and colleagues,⁸ discussed above. Assessing PCNA protein expression, a surrogate marker of proliferation, we noted that experimental fetal BOO was associated with a significantly increased percentage of proliferating detrusor SMC *versus* sham controls, with no significant difference in the lamina propria or urothelial cells between the two groups. In addition, the average area of detrusor SMC was increased after 30 days of BOO. Furthermore, in obstructed bladders only, a prominent population of proliferating cells was noted between muscle bundles; we speculate that these cells represent fibroblast-like cells which may be implicated in the increased collagen in this area, as assessed by Masson's trichrome staining (compare Figure 3, A and C). If we make the assumption that these alterations in proliferation and hypertrophy have occurred for a significant period during BOO, our results are consistent with the conclusion that both proliferation and hypertrophy of detrusor SMC, and also proliferation of interstitial cells between muscle bundles make an important contribution to bladder growth after BOO. Furthermore, an outer serosal layer exists that may also undergo cellular proliferation;²⁵ this was not

Figure 7. Histology and cellular turnover in the developing fetal bladder. **A–C** depict Masson's trichrome-stained sections at 75 (**A**), 105 (**B**) and 145 (**C**) days gestation, representative of 2, 11 and 6 fetal bladders, respectively. Note increasing thickness of the fetal bladder through gestation. **Bars**, 400 μ m. **D:** Western blot for PCNA (36 kD) and β -actin (42 kD) of full thickness bladders; note the trend to down-regulation of PCNA expression through gestation into adulthood. This impression was confirmed by PCNA/ β -actin ratios. **F:** A similar decrease in activated caspase-3 activity was noted between 75 and 145 days gestation. In **E** and **F**, each dot represents a single bladder sample.

ascertained in the current study. We speculate the generation of new, larger SMC may be an adaptation which attempts to overcome the experimental urinary flow impairment; however, at the same time, another cell population, between muscle bundles, increases and this could lead to fibrosis which is detrimental to net detrusor contractility, as reported by Nyirady et al.¹⁰

However, the response to BOO in terms of cell turnover is seen to be more complex, when programmed cell death is also considered. Within detrusor muscle bundles, we found that BOO was associated with a significant increase in the percentage of apoptotic SMC; if we make the assumption that the clearance of cells undergoing programmed cell death is equal in the sham and obstructed groups, the extent of apoptosis is three times higher after BOO. This up-regulation may have biological significance as follows. First, it may represent a deletion of "excess" cells in a rapidly proliferating population, as occurs in the metanephric kidney to control cell numbers incorporated into developing nephrons;^{18,20} in this context, some detrusor SMC may also need to be deleted as their neighbors hypertrophy and fill a limited space. On the other hand, the apoptosis we documented may represent a pathological loss of potentially functional muscle cells that might help to overcome BOO. Overall, we believe the balance of growth is mediated by cell prolifera-

Figure 6. Bcl-2 and Bax proteins in experimental fetal bladders. **A–D** show Bcl-2 and **D–H** show Bax immunohistochemistry with hematoxylin counterstaining, representative from 6 samples in each group. **A, B, E,** and **F** are sham bladders at 105 days gestation, while **C, D, G,** and **H** are time-matched obstructed bladders. Note cytoplasmic staining of these two apoptotic-regulatory proteins in all bladder layers in both experimental groups; Bax immunostaining appeared more prominent in the detrusor and lamina propria layers of the obstructed bladders and appeared prominent within the luminal layers of the urothelium from sham and obstructed bladders. **I:** Western blot of Bcl-2 (26 kD), Bax (22 kD), and β -actin (42 kD) from representative sham and obstructed bladders (representative of six full thickness bladder samples in each group). **J:** Bcl-2/ β -actin and Bax/ β -actin ratios; **closed bars** from sham ($n = 6$) and **open bars** from obstructed bladders ($n = 6$). Note significant down-regulation in Bcl-2 expression and significant up-regulation in Bax expression in obstructed samples.

Figure 8. Cell turnover of sham and obstructed bladders. **A:** Sham bladder, with proliferating (red) and apoptotic (black) cells within the detrusor and lamina propria layers. Yellow cells are non-proliferative and non-apoptotic. **B:** Obstructed bladder, with increase in proportions of proliferating and apoptotic cells in the detrusor layer and increase in apoptotic cells only in the lamina propria layer. Note also the larger SMC in the muscle bundles which are separated by widened spaces containing proliferating cells, the lower cell density in the lamina propria, and the single-layered urothelium in the obstructed bladder.

tion and hypertrophy, with this process modified by programmed cell death. Further studies are necessary to determine what factors are involved in controlling the fine balance of proliferation and apoptosis; investigations of the proto-oncogene, c-myc,²⁶ show this protein can stimulate cell proliferation in the presence of appropriate survival factors and trigger apoptosis in their absence. Such dual capacity may have an important impact on cell turnover in the obstructed fetal bladder. In future, it would be interesting to assess c-myc in our model.

In the lamina propria layer, we documented a significant, approximately threefold increase in apoptosis in obstructed bladders, whereas, unlike the detrusor layer, the percentage of proliferating cells was not different from sham controls. Furthermore, the number of cells per unit area significantly decreased in this layer. Collectively, these observations suggest that enhanced death may have led to a decrease in cell density in the lamina propria. In the early stages of murine bladder development, the organ is relatively simple, with a prominent mesenchymal layer enveloping a simple epithelial layer.^{27,28} Through a poorly understood process of mutual inductive events,²⁷ murine bladder mesenchyme differentiates into the detrusor muscle layer and the lamina propria, while the epithelium acquires a complex, multi-layered phenotype. While it is not established whether ovine lamina propria or adjacent epithelium may have similar inductive roles in the experimental period of current study, it is interesting to note that attenuation of the lamina propria layer, described above, was accompanied by "simplification" of the urothelium, which had only one or two layers of cells in obstructed bladders versus three to four layers in sham controls. It is also possible that this simplification of the urothelium may result directly from the mechanical effects of increased urine volume within the obstructed bladder. As the bladder matures, neurovascular bundles traverse the lamina propria;²⁹ while we did not formally localize apoptotic nuclei in our study, our impression was that most dying cells were not

in vessel walls and they were therefore likely to be interstitial cells.

Molecular Correlates of Increased Apoptosis in Fetal BOO

Apoptosis is the end result of a cascade of molecular events.³⁰ Caspases play central roles, and can be activated by via specific cell-surface receptors (eg, Fas ligand stimulation) or a mitochondrial cascade (eg, activated by irradiation and diverse other stimuli). Caspase-3 lies at the convergence of these two pathways, and the activated molecule is able to cleave several cellular substrates, including structural proteins and cellular enzymes, resulting in apoptosis.³¹ Furthermore, members of the Bcl-2 family interact with these pathways, with Bcl-2 favoring survival; Bax heterodimerizes with Bcl-2, and favors cell death.³² In our study, 30-day fetal BOO led to an average twofold increase of caspase-3 activity in fetal bladders. This was associated with an average 50% reduction in Bcl-2 protein level and an average threefold increase in Bax protein level in whole bladders. Using immunohistochemistry, we found that Bcl-2 and Bax were expressed in most bladder cells but, in preliminary studies, we were unable to obtain a convincing signal with antisera to activated caspase-3.

The novel observations we have made regarding the effects of BOO on bladder proliferation and death should be taken in the context of an insult to an actively growing organ. This is emphasized in our limited surveys of whole bladder PCNA protein levels, which were found to show a downward trend between 75 days gestation and adulthood, and activated caspase-3 levels, which were found to show a downward trend from between 75 days gestation and term. These findings are consistent with investigation of cell turnover in the developing murine bladder²⁸ where multiple bladder samples from different time points are more readily available. This study showed that cell

division and programmed death, based on PNCA protein levels and TUNEL labeling, significantly reduced at each time point from the inception of the bladder at embryonic day 14, through gestation and up to maturity at 6 weeks after birth. Therefore, ovine fetal BOO may shift bladder cell turnover to a more embryonic pattern. Further experiments would be necessary to establish whether similar degrees of BOO elicit the same, or different, responses in more mature sheep bladders.

Other studies have examined apoptosis in experimental urinary tract obstruction *in vivo*, although none of these studies addressed fetal bladder apoptosis. Santarosa et al³³ found that BOO caused bladder hypertrophy in adult rabbits, with an increase of apoptosis following relief of obstruction. Chuang et al³⁴ reported that apoptosis and Bax were up-regulated in smooth muscle of obstructed ureters in adult rats. Several studies have, however, examined the effects of experimental urinary tract obstruction on the developing kidney. Attar et al¹⁵ and Matsell et al³⁵ reported that fetal ureteric obstruction, in the sheep and monkey, respectively, was associated with increased apoptosis in the tufts of developing glomeruli. Furthermore, Chevalier et al³⁶ found that ureteric obstruction in neonatal rats caused apoptosis in some tubules which correlated with decreased Bcl-2 protein immunostaining. Finally, Liapis et al³⁷ using ureteric obstruction in the developing opossum, reported a complex picture, with enhanced apoptosis and proliferation in the renal interstitium but a predominantly apoptotic response in tubules; enhanced death was accompanied by increased Bax, and decreased Bcl-2 immunostaining.

What are the possible mechanisms by which BOO leads to altered fetal bladder cell turnover? One possibility is that BOO leads to increased pressure within the fetal urinary tract and that this physical stress, perhaps through increased axial strain, somehow triggers the aberrations of bladder cell biology. There have been several such studies on detrusor and other cells harvested from adults. Orsola et al³⁸ reported that cyclic stretch and relaxation of human detrusor SMC led to increased DNA and protein synthesis but apoptosis was not examined. Galvin et al³⁹ reported similar findings; in addition, apoptosis was decreased with stretch. On the other hand, there are reports that mechanical stretch does lead to increased apoptosis of both vascular smooth muscle⁴⁰ and renal epithelial⁴¹ cells. Furthermore, the variant response observed, of cell proliferation and cell death within different layers of the obstructed bladder in our studies, may be dependent on the different shapes of cells in these layers provoking diverse physical forces induced by the mechanical stretch produced by urinary obstruction. These forces may induce a switch between growth and cell death, as have been shown in other anchorage-dependent cells.⁴² However, we know of no *in vitro* studies which have examined the effects of stretch on isolated populations of fetal bladder cells. Angiotensin II signaling through its type 1 receptor (AT1) has been implicated in the biology (eg, proliferation) of stretched detrusor SMC.⁴³ In the developing embryo, however, another angiotensin II receptor, AT2, is prominently expressed in the urinary tract and is implicated in transduc-

ing a death signal.⁴⁴ We postulate that this signaling pathway might be important in the bladder apoptotic response to BOO described in the current study. Furthermore, the expression of cystein-rich protein 61 (Cyr61), implicated in cell migration, adhesion and proliferation, is up-regulated in cyclically stretched detrusor smooth muscle cells⁴⁵ and suggests a possible role in cell signaling in the obstructed fetal bladder. Further, functional studies should therefore address the effects of stretch on isolated fetal bladder cells, and focus on the possible expression of Cyr61 and the activity of receptors such as AT2.

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