Altered Signaling and Regulatory Mechanisms of Apoptosis in Focal and Segmental Glomerulosclerosis

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Abstract. The purpose of this study was to investigate signaling and regulatory mechanisms of apoptosis in a model of focal and segmental glomerulosclerosis. Sprague-Dawley rats received two doses of puromycin aminonucleoside (PAN) (day 0 and week 3) and a uninephrectomy (PAN model). Apoptosis was detected with the use of the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling technique. Bax, Bcl-2, Fas, and Fas ligand expression was analyzed by competitive reverse transcription-PCR. Bax, Bcl-2, and Fas mRNA were localized by in situ hybridization. Renal function was transiently impaired after the first PAN dose. After the second PAN dose, further progressive renal impairment, tubular atrophy, interstitial fibrosis, and glomerulosclerosis were evident. Eighteen percent of PAN samples demonstrated up to 4 apoptotic cells/50 glomeruli, compared with 7% of sham controls (not significant). No consistent significant change in glomerular Bax, Bcl-2, Fas, and Fas ligand mRNA was evident by reverse transcription-PCR, although focal increases in glomerular Bcl-2 mRNA were demonstrated by in situ hybridization. In the tubulointerstitium, apoptosis was increased from weeks 1 to 12 (P < 0.01 PAN versus sham), correlated to renal function and tubulointerstitial injury (P < 0.01). Total renal Bax, Fas, and Fas ligand mRNA were upregulated in the PAN model, peaking at week 17 (P < 0.01 versus sham), whereas Bcl-2 mRNA was not significantly different in PAN versus sham controls. In situ hybridization in the PAN model demonstrated prominent Bax mRNA in dilated tubules and infiltrating leukocytes. Fas mRNA signal was localized to tubular epithelial cells and leukocytes. The results suggest that altered apoptotic signaling and regulatory mechanisms contribute to the tubulointerstitial injury in this model.

Clinically, focal and segmental glomerulosclerosis (FSGS) is characterized by proteinuria, poor response to steroid therapy, and progressive renal dysfunction. GS, tubular atrophy, and interstitial fibrosis are the main histopathologic characteristics (1). Because of the lack of an effective treatment, most patients with FSGS eventually develop end-stage renal failure that requires dialysis and/or renal transplantation. Recent studies have shown that apoptosis is involved in the pathophysiologic changes seen in various renal diseases, such as IgA nephropathy, lupus nephritis, and crescentic nephritis (2). This study evaluates the incidence of apoptosis and describes changes in apoptosis signaling and regulatory pathways in a model of FSGS induced by puromycin aminonucleoside (PAN).

Apoptosis, a process of intrinsically programmed cell suicide, is characterized morphologically and biochemically by nuclear shrinkage, chromatin condensation, condensation of cytoplasmatic matrix and organelles, cytoplasmatic blebbing, and internucleosomal DNA fragmentation. Apoptosis can be triggered by a variety of stimuli, including physiologic and pathologic factors, that activate one of several signaling pathways (3). The Fas ligand/Fas pathway is one of best-characterized apoptotic signaling pathways. Fas (CD95, Apo-1) is a member of the tumor necrosis factor receptor family (4,5). Fas has a death domain in its cytoplasmic tail, which can associate via various combinations of adaptors with the death effector domain present in the N-terminal prodomains of caspases 8 and 10. Once activated, these caspases translocate to the cytoplasm, where they can activate downstream caspases, e.g., caspase 3 (6).

The Bcl-2 family is defined as proteins that share one or more of the conserved domains, BH1, BH2, BH3, and BH4 (7). The Bcl-2 family includes survival (e.g., Bcl-2, Bcl-xL) and proapoptotic factors (e.g., Bax, Bad, and BID). Cytosolic Bax can translocate to the mitochondrial membrane, and its dimerization is associated with mitochondrial dysfunction and cell death. Bcl-2 is tethered to the outer mitochondrial membrane and may prevent the release of cytochrome c from mitochondria (8). Cytochrome c in conjunction with ATP induces a conformational change in apoptotic protease activating factor 1, leading to activation of caspase-9, which in turn activates downstream caspases (9).

Renal lesions that are similar histologically to human FSGS can be induced in rats through administration of PAN, a nephrotoxic antibiotic. The PAN model used in this study is based on a dual dose of PAN combined with uninephrectomy (10). The model closely resembles human FSGS disease and is characterized clinically by progressive renal dysfunction and...
proteinuric, and pathologically by progressive FSGS, tubular dilation followed by tubular atrophy, and interstitial fibrosis. The present study was designed to determine whether apoptosis is involved in the pathophysiologic changes seen within this model and the roles of the signaling and regulatory molecules Fas, Fas ligand, Bax, and Bcl-2.

Materials and Methods

**Dual-Dose PAN Animal Model**

Male outbred Sprague-Dawley rats, weighing 200 to 250 g, were obtained from Monash Animal Services. At day 0, the rats received a left unilateral nephrectomy, followed by administration of PAN (Sigma Chemicals, St. Louis, MO) 5 mg/100 g body wt intravenously (IV). At week 3, they received a second dose of PAN, 2.5 mg/100 g IV. Groups of five to eight rats were killed at day 2 and weeks 1, 3, 6, 9, 12, and 17. Their kidneys were retrieved and processed for histopathology, immunohistochemistry, and RNA extraction. Sham control rats underwent laparotomy and removal of the perinephric fat and peritoneum from around the hilum of the left kidney. Tail-vein blood samples and 24-h urine were collected for analysis of serum creatinine concentration (Scr) and urinary protein excretion, which were performed on a Cobas Bio analyzer (Hoffman-La Roche, Basel, Switzerland). All experimental protocols conformed to the National Health & Medical Research Council code of practice and were approved by the Alfred Animal Experimentation Ethics Committee.

**Isolation of Glomeruli**

A sample of cortex was cut into 1- to 2-mm³ blocks and passed sequentially through 80-, 100-, and 200-mesh wires with the use of diethylpyrocarbonate-treated phosphate-buffered saline (PBS). Glomeruli were collected from the surface of the 200-mesh wire. Glomerular samples were centrifuged at 750 × g for 10 min, and the pellet was resuspended in either 1.5 ml of 5 M GTC or 700 ml of RNase lysis buffer (Qiagen Inc., Chatsworth, CA) before being transferred into a 2-ml freezing vial and stored at −80°C until RNA was extracted.

**Histologic Analysis**

Sections of the formalin-fixed, paraffin-embedded tissues were stained with periodic acid-Schiff and silver Masson trichrome to assess glomerular and tubulointerstitial damage by light microscopy. Glomerular tuft size was determined by measurement of the area of 20 to 30 glomeruli sectioned through the vascular pole, with the use of an image analysis system with Image Pro Plus (Media Cybernetics, Silver Spring, MD). Glomerular sclerotic changes were graded in 50 to 100 randomly selected glomeruli per tissue section, with the use of a severity score: grade 1, increased mesangial material involving up to one fourth of the glomerular tuft; grade 2, involving one fourth to one half the glomerular tuft; grade 3, involving one half to three fourths of the glomerular tuft; or grade 4, involving three fourths to a whole of the glomerular tuft. A GS score for each tissue was given as the product of the percentage of glomeruli affected by the severity score, the maximum GS score being 400. Glomerular cells in each sample were counted in 15 glomeruli sectioned through the vascular pole. For assessment of tubular damage, 20 random fields of renal cortex (equivalent to an area of 3 mm²) were examined at a final magnification of 400×. Tubular dilation, tubular atrophy, and fibrosis were graded in each field as follows: grade 0, well-preserved renal architecture; grade 1, less than 25% of the field involved; grade 2, 25 to 50% of the field involved; grade 3, 50 to 75% of the field involved; grade 4, 75 to 100% of the field involved. Scores were recorded for each tissue, with the maximum score being 4.

**Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling**

Five-µm sections of formalin-fixed, paraffin-embedded renal tissue were dewaxed, rehydrated, washed in PBS, and incubated in terminal deoxynucleotidyl transferase (TdT) buffer for 10 min at room temperature before incubation with TdT enzyme (Promega, Madison, WI) and digoxigenin-labeled dUTP (Boehringer Mannheim, Mannheim, Germany) for 1 h at 37°C. After the sections were washed in PBS, a sheep anti-digoxigenin F(ab)₂ was applied for 1 h at room temperature. Endogenous peroxidase was blocked, and a peroxidase-conjugated rabbit anti-sheep antibody was applied, followed by peroxidase-conjugated swine anti-rabbit antibody. The reaction was developed with the use of Pierce metal-enhanced diaminobenzidine substrate (Rockford, IL). On the basis of terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining (11) and morphologic changes, the apoptotic cells were counted under light microscope in 50 glomerular cross sections and 10 random areas of 0.15 mm² in the interstitium, with the use of a grid at 400× magnification. Negative controls without TdT enzyme and positive controls with DNase treatment were included for each tissue. No paraffin-embedded tissues of the PAN model were available to assess apoptosis by TUNEL technique at week 17.

**Immunohistochemistry**

A four-layer immunoperoxidase technique was applied to detect the infiltration of monocyte/macrophages, T cells, and interleukin-2 (IL-2) receptor–positive cells in 4-µm frozen sections, as described previously (12). Antibodies used included ED1 (anti-rat monocyte/macrophages, kindly provided by Dr. Dijkstra, Free University, Amsterdam, The Netherlands) (13), R73 (anti-rat α/β T-cell receptor), and NDS61 (anti-CD25) (both from Serotec, Oxford, England). Proliferating cell nuclear antigen (PCNA) was detected by four-layer immunohistochemistry on formalin-fixed, paraffin-embedded tissues. T cells, monocytes/macrophages, and IL-2 receptor–positive cells were counted in 10 glomerular cross sections and 10 random areas of 0.15 mm² in interstitial areas, respectively, with the use of a grid at 400× magnification. PCNA-positive cells were counted in 10 to 43 glomerular cross sections, and results are expressed as cells per glomerulus.

**TUNEL/Immunohistochemistry**

Combined TUNEL/immunohistochemistry staining was performed to identify the cells that were undergoing apoptosis in the PAN model. TUNEL was performed as described above, with the omission of the peroxidase-conjugated swine anti-rabbit antibody, to prevent cross reactivity. The TUNEL reaction was developed with diaminobenzidine, resulting in a brown product. Slides were washed in running tap water, followed by Tris-buffered saline. Preincubation in 20% normal rabbit serum in Tris-buffered saline for 10 min at room temperature was followed by overnight incubation with the primary antibody at 4°C. The second layer of rabbit anti-mouse Ig was applied for 30 min followed by mouse alkaline phosphatase anti-alkaline phosphatase. The reaction was developed with New Fuchsir, resulting in a red product. Slides then were counterstained in hematoxylin and mounted.

**Competitive Reverse Transcription-PCR**

**RNA Extraction.** Total renal RNA was extracted from 0.1 to 1 g of renal tissue with the use of guanidine isothiocyanate and caesium
chloride centrifugation (14). Glomerular RNA was extracted and purified with the use of the RNeasy kit (Qiagen Inc., Hilden, Germany). RNA concentrations were assessed spectrophotometrically. Before reverse transcription (RT), the RNA samples were diluted to 0.5 to 1 μg/μl to avoid variability in transcription efficiency.

Reverse Transcription. The RT was carried out with the use of the Perkin-Elmer Reverse Transcription System (Perkin Elmer Inc., Norwalk, CT), as per the manufacturer’s instructions.

Primer Design. On the basis of the published sequences (15–18), four primers were designed for each gene with the use of Oligo 5 Software for Windows (National Biosciences Inc., Plymouth, MN), including one upstream primer, two downstream primers, and a composite primer that combined the two downstream primers. The details of primers are shown in Table 1; their positions in the published sequences are indicated in parentheses.

Competitor Synthesis. Deletion mutants were generated on the basis of the method described by Celi et al. (19). First, native RNA was reverse transcribed into cDNA and amplified with the use of the upstream primer and downstream primer 1 or upstream primer and downstream primer 2 to verify the specificity of the PCR products. When both reactions amplified only one band of the expected product length, the upstream primer and composite primer were used to amplify the native cDNA for generating the competitors, which were purified after electrophoresis (Qiagen DNA gel purification kit), quantified, and stored at −20°C.

Quantitative Analysis of Fas, Fas Ligand, Bax, and Bcl-2 Gene Expression. PCR reactions were set up in a 25-μl volume with the use of a PCR amplification kit with AmpliTaq Gold (Perkin-Elmer). It contained 10 mmol/L Tris, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L each deoxyribonucleotide triphosphate, 0.5 μmol/L each primer pair (fluorescein-labeled upstream primer and unlabeled downstream primer 2), 1.25 U AmpliTaq Gold, 2.5 μl of cDNA sample, and 2.5 μl of varying dilutions of the competitor. Thirty μl of mineral oil was added to avoid evaporation. Annealing temperatures were 59°C (Bax and Fas), 60°C (Bcl-2), and 61°C (Fas ligand), and the number of cycles were 40 for Fas and Bax and 50 for Fas ligand and Bcl-2. PCR products were run on a 3% agarose gel and scanned with the use of a fluorimager (FluorImager 575, Molecular Dynamics, Sunnyvale, CA). The log of the competitor copy number was plotted against the log of the ratio cDNA/competitor bands. At least five dilutions of competitor were used per sample. Linear regression analysis was performed to assess the copy number of each gene in the test samples, and the result was expressed as copy number per nanogram of RNA.

In Situ Hybridization for Bax, Bcl-2, and Fas mRNA
For probe generation, primers were designed to incorporate T7 phage RNA polymerase and SP6 phage RNA polymerase promoters in the 5’ ends of the upper and lower primers respectively. Bax: upper primer, TAATACGACTCACTATAGGGGAATTCAGTGAAGATTG; lower primer, CAGTGAAGACAGGAAATTCGCTACTG.
PCR products of 199 bp, 368 bp, and 236 bp for reverse transcribed and amplified by PCR as described above, generating for the generation of linear DNA templates for probes, rat total RNA was lower primer, ATTTAGGTGACACTATAGACGGGATCTTGTGCT.

TAATACGACTCACTATAGGGGTTTGGCAATTCTATTTGT; product purification kit (Qiagen). Fluorescein labeled sense and antisense respectively. The PCR products were purified with the use of a PCR

and quantity of probes were verified by fluorimager scanning.

The probes were run on a denaturing agarose gel, and the size, integrity, for nonradioactive RNA polymerase and T7 RNA polymerase, respectively (RNA color kit GTGACACTATAGAGAAGGGCGTCAGGTGC; Fas TATAGGCGGGAGATCGTGATGAAGTA; lower primer, ATTTAG-

6 min, fixed with 4% paraformaldehyde for 15 min at room temper-

with 0.2 M HCl followed by 0.25% protease VIII (Sigma) at 37°C for 6 min, fixed with 4% paraformaldehyde for 15 min at room temperature, and further treated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. Sections then were incubated in prehybridization buffer (2 × SSC, 20% deionized formamide, 10 × Denhardt’s, 20% Dextran Sulfate, 0.25 mg/ml yeast t-RNA, and 0.5 mg/ml freshly denatured herring sperm DNA) at 50°C for 1.5 to 2 h. Hybridization was performed with the use of a probe concentration of 0.8 µg/ml (Bax) or 0.6 µg/ml (Bcl-2 and Fas) in hybridization buffer (in situ hybridization grade buffer containing 50% deionized formamide; Amersham) at 50°C overnight. After hybridization, sections were washed twice with 1 × SSC/0.1% sodium dodecyl sulfate, followed by two high stringency washes with 0.2 × SSC/0.1% sodium dodecyl sulfate at 50°C. Sections were treated with blocking solution (Amersham) before addition of alkaline-phosphatase–conjugated sheep anti-fluorescein antibody for 1 h. Color was developed with Nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphoric acid; Amersham) at 50°C overnight. After hybridization, sections

Statistical Analyses

All statistical analyses were performed with the use of SPSS for Windows (SPSS, Inc., Chicago, IL). Competitive PCR results were log transformed to attain a normal distribution. After demonstration of significant difference by ANOVA, group pairs were compared by independent t test. Nonparametric data were analyzed by Kruskal-Wallis ANOVA by ranks followed by Mann-Whitney U test for pairwise comparisons.

Results

Renal Function and Histopathology

Sham control animals had normal Scr concentration, urine protein excretion, and renal histology throughout the 17-wk period. In contrast, PAN-treated animals demonstrated transiently impaired renal function after the first dose of PAN; Scr peaked at week 1 and returned to normal range at week 3 (Figure 1). After the second dose of PAN, a progressive increase in Scr was demonstrated. Heavy proteinuria also was evident after the first dose of PAN (Figure 1). Proteinuria subsided at week 3, although it remained elevated compared

<table>
<thead>
<tr>
<th>Postoperative Time</th>
<th>Glomerular Tuft Size (µm)</th>
<th>Glomerular Proliferation</th>
<th>Glomerulosclerosisb</th>
<th>Tubular Damageb</th>
<th>Interstitial Fibrosisb</th>
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<tbody>
<tr>
<td>PAN</td>
<td>SHAM</td>
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<tr>
<td>Day 2</td>
<td>8730 ± 216</td>
<td>10336 ± 272</td>
<td>7.43 ± 0.7c</td>
<td>2.88 ± 0.03</td>
<td>0.8 ± 0.4d</td>
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<td>Week 1</td>
<td>10950 ± 308</td>
<td>9568 ± 333</td>
<td>5.03 ± 0.45</td>
<td>5.35 ± 0.64</td>
<td>1.6 ± 1.1d</td>
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<tr>
<td>Week 3</td>
<td>12647 ± 506d</td>
<td>10112 ± 127</td>
<td>7.97 ± 0.77c</td>
<td>3.60 ± 0.73</td>
<td>13.7 ± 1.7d</td>
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<tr>
<td>Week 6</td>
<td>19832 ± 459d</td>
<td>9691 ± 208</td>
<td>8.01 ± 0.80d</td>
<td>5.31 ± 0.92</td>
<td>165.1 ± 37.2d</td>
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<tr>
<td>Week 9</td>
<td>20560 ± 661d</td>
<td>10008 ± 236</td>
<td>10.07 ± 1.69g</td>
<td>4.31 ± 0.38</td>
<td>189.0 ± 24.5d</td>
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<tr>
<td>Week 12</td>
<td>20436 ± 578d</td>
<td>11297 ± 167</td>
<td>6.73 ± 0.26d</td>
<td>4.46 ± 0.44</td>
<td>170 ± 33.9d</td>
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<tr>
<td>Week 17</td>
<td>19979 ± 893d</td>
<td>10506 ± 140</td>
<td>5.86 ± 0.61c</td>
<td>4.12 ± 0.48</td>
<td>209 ± 13.4d</td>
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*Results are expressed as mean ± SEM.  
*b Scores were calculated as described in the Materials and Methods section.  
*c P < 0.05, PAN versus sham controls.  
*d P < 0.01, PAN versus sham controls.

lower primer, ATTTAGGTGACACTATAGACAGAAAGGGAACC; Bcl-2: upper primer, TAATACGACTCAC-TATAGGGCGGAGATCGTGATGAAGTA; lower primer, ATTAGGTGACACTATAGACAGGAAGGGGTCAATGGTGT; lower primer, ATTTAGGTGACACTATAGACGGGCATCTTGCT. For the generation of linear DNA templates for probes, rat total RNA was reverse transcribed and amplified by PCR as described above, generating PCR products of 199 bp, 368 bp, and 236 bp for Bax, Bcl-2, and Fas, respectively. The PCR products were purified with the use of a PCR product purification kit (Qiagen). Fluorescein labeled sense and antisense cRNA probes for Bax and Bcl-2 were transcribed with the use of SP6 RNA polymerase and T7 RNA polymerase, respectively (RNA color kit for nonradioactive in situ hybridization; Amersham, Uppsala, Sweden). The probes were run on a denaturing agarose gel, and the size, integrity, and quantity of probes were verified by fluorimager scanning.

Five-µm paraffin-embedded sections were dewaxed and pretreated with 0.2 M HCl followed by 0.25% protease VIII (Sigma) at 37°C for 6 min, fixed with 4% paraformaldehyde for 15 min at room temperature, and further treated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. Sections then were incubated in prehybridization buffer (2 × SSC, 20% deionized formamide, 10 × Denhardt’s, 20% Dextran Sulfate, 0.25 mg/ml yeast t-RNA, and 0.5 mg/ml freshly denatured herring sperm DNA) at 50°C for 1.5 to 2 h. Hybridization was performed with the use of a probe concentration of 0.8 µg/ml (Bax) or 0.6 µg/ml (Bcl-2 and Fas) in hybridization buffer (in situ hybridization grade buffer containing 50% deionized formamide; Amersham) at 50°C overnight. After hybridization, sections were washed twice with 1 × SSC/0.1% sodium dodecyl sulfate, followed by two high stringency washes with 0.2 × SSC/0.1% sodium dodecyl sulfate at 50°C. Sections were treated with blocking solution (Amersham) before addition of alkaline-phosphatase–conjugated sheep anti-fluorescein antibody for 1 h. Color was developed with Nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate substrate. Sections were counterstained with nuclear fast red and coverslipped with aqueous mount. A sense probe control was included for each sample.

Figure 2. Histopathologic changes in the PAN model. Silver Masson trichrome stain of a kidney from a PAN model animal at week 17. Note focal glomerulosclerosis, tubular atrophy, interstitial fibrosis, and inflammatory cell infiltrate. Magnification, ×200.
A

![Graph A](image1)

**Figure 3.** Glomerular and tubulointerstitial apoptosis in the PAN model. The terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique was performed as described in the Materials and Methods section. Apoptotic cells were counted in 50 glomeruli (A) and in the interstitium (B) with the use of a grid at 400× magnification. ◆, PAN; ○, sham; *, P < 0.05; **, P < 0.01, PAN versus sham.

with baseline. After the second dose of PAN, there was a further increase in urinary protein concentrations.

Histologically, the PAN-treated animals demonstrated glomerular hypertrophy and mild degrees of GS as early as week 3 (Table 2). As disease progressed, the glomerular hypertrophy reached steady-state levels by week 6; the GS became progressively more severe, although it remained focal. Figure 2 depicts a PAN-treated animal at week 17. Two glomeruli are seen, with damage scores of 1 and 4, respectively. PCNA staining demonstrated significantly increased glomerular cell proliferation in the PAN model at day 2 and from weeks 3 to 17 (Table 2), compared with sham rats.

Progressive tubulointerstitial damage was evident throughout the cortical and medullary regions of the kidney (Table 2). Tubular dilation was seen at day 2, and tubular atrophy and interstitial fibrosis were evident from week 3, their severity increasing with time. Figure 2 demonstrates tubular atrophy and interstitial fibrosis, as well as a focal area of interstitial inflammatory cell infiltrate in the PAN model at week 17.

**Apoptosis**

Glomerular apoptosis was not a prominent feature in the PAN model; only 18% of PAN animals demonstrated any glomerular cell apoptosis (between 1 and 4 cells/50 glomeruli). This did not differ significantly from results obtained in shams, in which apoptotic glomerular cells (1 to 2 cells/50 glomeruli) were detected in 7% of tissues analyzed (Figure 3A). Glomerular apoptosis was not associated with GS score.

Glomerular apoptotic cells can be cleared rapidly by phagocytosis or by detachment into Bowman’s capsule and thus may not be detected by the TUNEL method. To investigate further glomerular cell changes in the PAN model, glomerular cellularity was assessed in relation to glomerular damage (Figure 4). Glomerular hypercellularity was evident from week 6 to week 17 in glomeruli with up to 50% sclerosis (grades 0, 1, and 2; P < 0.001). In the PAN model, glomeruli with more than 50% sclerosis (grades 3 and 4) had significantly decreased cellularity compared with grades 0 to 2 (P < 0.0001). Nevertheless, glomerular cellularity at weeks 12 and 17 was increased in glomeruli with more than 50% sclerosis as compared with sham controls (P < 0.01)

In the tubulointerstitium, significantly increased apoptosis was evident in the PAN model from week 1, persisting until week 12, whereas very few apoptotic cells were seen in the sham controls (Figure 3B). Apoptotic cell numbers were correlated significantly with the tubulointerstitial damage scores (Spearman coefficient, 0.62; n = 33; P < 0.01). Apoptotic cells were identified morphologically and by two-color TUNEL/immunohistochemistry. Figure 5A depicts a TUNEL-positive cell also staining for ED1 monocyte/macrophage marker (brown and red staining, arrow), whereas Figure 5B demonstrates an apoptotic tubular epithelial cell (brown staining), identified morphologically. Figure 5, C and D, shows examples of double staining with α-smooth muscle actin, a marker for interstitial myofibroblasts and mesangial cell activation, as well as for vascular smooth muscle cells. No TUNEL-positive/α-smooth muscle actin–positive cells were identified in the samples examined by two-color staining. This result was consistent with morphologic assessment of TUNEL-positive cells in other tissues, which tended to be clearly tubular epithelial cells or seemed to be within foci of infiltrating leukocytes rather than in myofibroblast areas. Figure 5C depicts a glomerular apoptotic cell (brown); the red staining demonstrates mesangial cell activation and periglomerular myofibroblasts. Figure 5D exhibits an apoptotic tubular epithelial cell and staining of peritubular myofibroblasts and vascular smooth muscle cells.

**Bax, Bcl-2, Fas, and Fas Ligand Expression**

Competitive reverse transcription-PCR methods were established for the quantification of Bax, Bcl-2, Fas, and Fas ligand mRNA. Figure 6 shows a representative image. The expression of Bax, Bcl-2, Fas, and Fas ligand in isolated glomerular RNA is shown in Figure 7. There were no consistent significant trends in the expression of the genes examined, despite occasional significant differences being observed between the PAN and sham groups at isolated time points. In contrast, there was
increased expression of Bax, Fas, and Fas ligand in total renal RNA, consistent with the increased numbers of apoptotic cells in the tubulointerstitium (Figure 8). In the PAN model, Bax was upregulated at day 2 and week 1 \( (P < 0.001 \) versus sham controls) and returned to normal by week 3. After the second dose of PAN, Bax was upregulated from week 6 and persisted until week 17 \( (P < 0.01 \) versus sham controls; Figure 8A). Bax mRNA was correlated significantly with renal dysfunction and with the incidence of apoptosis in the tubulointerstitium (Spearman coefficients, 0.74 \( [n = 45] \) and 0.71 \( [n = 38] \), respectively; \( P < 0.01 \)). No significant difference was detected in total renal Bcl-2 mRNA between the PAN model and sham controls (Figure 8B). Significantly increased expression of Fas was demonstrated at day 2 and from week 6 to week 17 in the PAN model \( (P < 0.05 \) versus sham controls; Figure 8C). Significantly increased expression of Fas ligand was evident from week 1 in the PAN model \( (P < 0.05 \) versus sham controls), peaking at week 17 \( (P < 0.01 \) versus sham controls; Figure 8D). The mRNA concentrations of Fas and Fas ligand were correlated significantly with the incidence of apoptosis in the tubulointerstitium (Spearman coefficients, 0.49 \( [n = 30] \) and 0.84 \( [n = 34] \), respectively; \( P < 0.001 \)).

In Situ Hybridization

The pattern of expression of Bax detected by in situ hybridization was consistent with the results of competitive reverse transcription-PCR. Figure 9A demonstrates low levels of Bax mRNA on distal tubular epithelial cells and on few glomerular epithelial cells in a sham animal at week 17. Bax also was expressed on collecting ducts in sham animals. Bax mRNA was upregulated in PAN animals at week 17. Bax was expressed by distal tubular epithelial cells and collecting ducts, being particularly prominent in dilated tubules (Figure 9B). Few cells that were identified morphologically as infiltrating leukocytes had a strong Bax mRNA signal (Figure 9C). Bax also was expressed by glomerular visceral and parietal epithelial cells. In sham animals, Bcl-2 mRNA was localized by in situ hybridization to visceral and parietal epithelial cells in glomeruli and to tubular epithelial cells (Figure 9D). Although no overall upregulation of Bcl-2 was demonstrated, increased expression was evident segmentally in glomeruli in some PAN animals (Figure 9E), presumably expressed by mesangial cells. Few cells within foci of infiltrating leukocytes also expressed Bcl-2 (Figure 9F). Fas was not detected by in situ hybridization in the sham animals. In the PAN model, it was detected weakly from week 3, and a progressively stronger signal was evident from week 6, expressed on tubular epithelial cells and infiltrating leukocytes. There was marked variability in expression between tubules and between different cells within one tubule (Figure 9, G and H). No signal was detected when sense probes were used (Figure 9I).

Analysis of Infiltrating Leukocyte Subpopulations

Sham controls had normal numbers of T cells and monocytes/macrophages in both the glomerular and the tubulointerstitial compartments. In contrast, analysis of infiltrating leukocytes in the PAN model showed progressively increased numbers of T cells, IL-2 receptor–positive cells, and monocytes/macrophages in tubulointerstitial areas (Figure 10). T cells and IL-2 receptor–positive cells in the tubulointerstitium were correlated significantly with the total renal mRNA levels of Fas ligand (Spearman coefficient, 0.70 \( [n = 38; P < 0.01] \).
and 0.73 \( [n = 38; P < 0.001] \). Most glomeruli had no T cells or IL-2 receptor–positive cells. Most PAN animals had normal ED1-positive cells in glomeruli (1 to 3/glomerulus), but an increase up to 6 cells/glomerulus was seen at weeks 6 and 17.

**Discussion**

The PAN model used in this study, which combined administration of a dual dose of PAN and a left uninephrectomy, was characterized by progressive renal dysfunction, GS, and tubulointerstitial damage, pathologically similar to human FSGS disease. Previous studies of animals that had received a single dose of PAN and a uninephrectomy did not result in progressive renal disease, and there was significantly lower glomerular hypertrophy, GS, and tubulointerstitial damage than that seen in the dual-dose PAN model \( (10) \). Sham control rats received vehicle and underwent laparotomy and isolation of the hilum of

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**Figure 5.** Identification of apoptotic cells. Double TUNEL/immunohistochemical staining was performed in a PAN animal at week 6 as described in the Materials and Methods section. (A) TUNEL/ED1 staining. The arrow indicates an apoptotic monocyte/macrophage exhibiting brown (TUNEL) and red (ED1) staining; note other infiltrating ED1-positive monocyte/macrophages. (B) TUNEL/ED1 staining. The arrow indicates an apoptotic tubular epithelial cell, with positive TUNEL staining and typical morphologic changes; scattered infiltrating ED1-positive cells are evident. (C) TUNEL/\( \alpha \)-smooth muscle actin (\( \alpha \)SMA) staining. The arrow shows an apoptotic glomerular cell; note \( \alpha \)SMA staining on activated mesangial cells and periglomerular myofibroblasts. (D) TUNEL/\( \alpha \)SMA staining. The arrow points to a TUNEL-positive tubular epithelial cell; \( \alpha \)SMA staining is evident on peritubular myofibroblasts and vascular smooth muscle cells. Magnification, \( \times 630 \).

**Figure 6.** Representative image of the quantification of Bax mRNA by competitive reverse transcription-PCR (RT-PCR). Total cellular RNA was extracted from a sham sample and reverse transcribed. The cDNA was co-amplified with different copy numbers of Bax competitor (from \( 10^5 \) to \( 10^7 \)) with the use of the fluorescein-labeled Bax upstream primer and downstream primer 2 as described in the Materials and Methods section. The fluorescein-labeled PCR products were run on a 3\% agarose gel and scanned with the use of a fluorimager.
the left kidney. No nephrectomy was performed in the sham animals, because the uninephrectomy was an integral part of the model and its effects contributed to the development of injury. This study describes the incidence of apoptosis and the expression of \textit{Bax}, \textit{Bcl-2}, \textit{Fas}, and \textit{Fas ligand} genes in the dual-dose PAN model of FSGS.

The involvement of apoptosis in the evolution of glomerular pathologic changes in various renal diseases has been demonstrated in a variety of clinical and experimental studies (20–23). In the present study, glomerular apoptosis was not a prominent feature, and no consistent significant changes in expression of the genes analyzed were observed. All PAN and sham control animals demonstrated \textit{Bax} and \textit{Bcl-2} mRNA on glomerular visceral and parietal epithelial cells, as previously observed by others (24). Segmental increased \textit{Bcl-2} expression was evident in some glomeruli. \textit{Fas} was not detected in glomeruli by \textit{in situ} hybridization, suggesting a relatively lower level of expression. Shiiki \textit{et al.} (25) described similar numbers of glomerular apoptotic cells (4/100 glomeruli) and increased \textit{Bcl-2} in an FSGS model induced by administration of 10 subcutaneous injections of PAN. However, other studies associated glomerular cell apoptosis with progression of GS in the later stages of the remnant kidney model (22), and glomerular apoptotic cells in IgA nephropathy and lupus nephritis have been shown to correlate with glomerular sclerosis index (22).

Analysis of glomerular cell numbers revealed increased cellularity in the PAN model from week 6, consistent with increased cell proliferation and low glomerular cell apoptosis. However, comparison of cell numbers in glomeruli with more or less than 50% sclerosis revealed that a degree of cell deletion occurred, associated with extracellular matrix (ECM) accumulation. Recent studies demonstrated an important role for the ECM in the regulation of survival or death of mesangial cells. Normal glomerular ECM components, such as type IV collagen and laminin, protect mesangial cells from apoptosis induced by serum deprivation or by DNA damage induced by etoposide.

Figure 7. Quantification of \textit{Bax}, \textit{Bcl-2}, \textit{Fas}, and \textit{Fas ligand} mRNA in glomeruli. Competitive RT-PCR was performed as described in the Materials and Methods section with the use of isolated glomerular RNA extracted from the PAN and sham animals at different times after disease induction. (A) \textit{Bax} mRNA; (B) \textit{Bcl-2} mRNA; (C) \textit{Fas} mRNA; (D) \textit{Fas ligand} mRNA. Results are expressed as geometric mean and 95% confidence interval on a logarithmic scale. ■, PAN; □, sham; *, \( P < 0.05 \), PAN versus sham.
treatment (26,27). In contrast, collagen type I, fibronectin, and osteopontin/SPARC, ECM components that are overexpressed in diseased glomeruli, failed to promote mesangial cell survival. It therefore is likely that the glomerular cell loss detected in glomeruli with >50% sclerosis is a response to the pathologic changes in ECM composition. The TUNEL method detects apoptosis at a particular point in time, and rapid clearance of apoptotic cells by phagocytosis or detachment into the Bowman’s space may result in an underestimation of the importance of this mechanism.

It is generally accepted that tubulointerstitial damage has a better correlation with renal dysfunction than glomerular lesions. Increased apoptosis has been associated with the progression of tubulointerstitial pathologic changes and tubular atrophy in ureteric obstruction (28), cyclosporine nephropathy (29), and diabetic nephropathy (30). The loss of renal tubules and the accumulation of ECM, including collagens, fibronectin, and laminin, characterize the renal tubulointerstitial damage. In this study, many apoptotic cells in the tubulointerstitial were identified morphologically as tubular epithelial cells, demonstrating a contribution of this process to the development of tubular atrophy. Furthermore, there was a close correlation between apoptosis in the tubulointerstitial areas and the tubulointerstitial damage scores in the PAN model.

Fas and Fas ligand mRNA were increased in total renal tissue but not in glomerular tissue, consistent with increased tubulointerstitial apoptosis. Previous studies demonstrated constitutive expression of Fas by leukocytes, and in the kidney by mesangial cells and tubular cells (31,32). In situ hybridization analysis demonstrated Fas mRNA on infiltrating leukocytes and progressively increasing expression on tubular epithelial cells in the PAN model from week 3, associated with severity of injury. Fas ligand is expressed by activated T cells and can be expressed at low level by the tubular epithelial cells in the kidney (16,31). Fas ligand expression was significantly correlated with activated T-cell numbers, suggesting that these were the primary source of Fas ligand mRNA. The interaction of Fas ligand on the activated T cells with Fas expressed on

Figure 8. Quantification of Bax, Bcl-2, Fas, and Fas ligand mRNA in total renal RNA. Competitive RT-PCR was performed as described in the Materials and Methods section with the use of total renal RNA extracted from the PAN and sham animals at different times after disease induction. (A) Bax mRNA; (B) Bcl-2 mRNA; (C) Fas mRNA; (D) Fas ligand mRNA. Results are expressed as geometric mean and 95% confidence interval, on a logarithmic scale. □, PAN, PAN; ◇, sham; *, P < 0.05; **, P < 0.01; ***, P < 0.001, PAN versus sham.
Figure 9. *In situ* hybridization for *Bax*, *Bcl-2*, and *Fas* mRNA. (A) Sham rat at week 17 demonstrating weak *Bax* mRNA signal in distal tubule epithelial cells and a few glomerular epithelial cells. (B and C) *Bax* mRNA in a PAN rat at week 17. (B) A strong *Bax* mRNA signal was demonstrated in some but not all dilated tubules; (C) strong *Bax* mRNA expression in some leukocytes (an example is indicated by the arrow). (D) *Bcl-2* mRNA in a sham rat at week 1 demonstrating positive signal on tubular epithelial and glomerular epithelial cells. (E) Upregulated *Bcl-2* expression in a glomerulus of a PAN rat at week 6. (F) PAN rat at week 12, demonstrating *Bcl-2* mRNA signal in tubular epithelial cells and some infiltrating leukocytes (an example is indicated by the arrow). (G) *Fas* mRNA expression in a PAN rat at week 6, showing scattered positive tubular cells and infiltrating leukocytes. (H) *Fas* mRNA in a PAN animal at week 12; note the strong signal in tubular epithelial cells. (I) Hybridization with sense probe control for *Fas* mRNA in a PAN rat at week 12. Magnifications: ×200 in A, C, D, F, and G; ×100 in B, H, and I; ×400 in E.
tubular epithelial cells would provide a signal for apoptosis, contributing to the tubular atrophy observed in this model. However, apoptosis was also evident on infiltrating leukocytes, suggesting activation-induced cell death may have contributed to the resolution of the inflammatory injury.

No significant changes in Bcl-2 expression were evident in the tubulointerstitium in the PAN model. Bcl-2 was expressed in PAN and sham animals by proximal and distal tubular cells, the loop of Henle, and papillary collecting ducts, consistent with previous findings (24). Bax and Bak were shown previously to be expressed in tubular cells (33). In sham animals, weak Bax mRNA hybridization signal was apparent in distal tubular epithelial cells and glomerular visceral and parietal epithelial cells. In the PAN model, increased Bax mRNA hybridization signal in the tubulointerstitium was localized to dilated tubules and few infiltrating leukocytes in the PAN model. The close correlation between Bax mRNA levels and the incidence of apoptosis indicated that the upregulation of Bax was associated with the increased incidence of apoptosis. The specific signal for upregulation of Bax in the PAN model is not known. However, increased expression of transforming growth factor β1 is a characteristic of this model (10). This growth factor has been shown to induce apoptosis in a variety of cell types (34–36) and to increase the expression of Bax and downregulate Bcl-2 in a leukemic cell line (37). Thus, the upregulation of transforming growth factor β1 in PAN nephritis may contribute to the increased apoptosis described in this article.

This study demonstrated increased apoptosis in the tubulointerstitial areas of the dual-dose PAN model of FSGS, strongly associated with the severity of pathologic damage in the tubulointerstitium. The balance between survival and apoptotic factors was found to be altered, and the Fas/Fas ligand signaling pathway was shown to be involved in chronic PAN nephropathy. The apoptotic death of tubular epithelial cells is an important link in the complex chain of events, which eventually results in the replacement of functional renal cells by fibrotic tissue, leading to end-stage renal failure.

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