Induction of Renal Tubular Cell Apoptosis in Focal Segmental Glomerulosclerosis: Roles of Proteinuria and Fas-Dependent Pathways

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The hypothesis that apoptosis represents a proximate mechanism by which tubule cells are damaged in FSGS was tested. Thirty kidney biopsy specimens from children with idiopathic early FSGS were studied retrospectively. Unexpected, apoptosis was evident in both proximal and distal tubule cells. There was a significant correlation between the degree of proteinuria and the number of apoptotic cells. Fas protein was detected predominantly in the tubule cells that underwent apoptosis. When compared with patients with other chronic proteinuric states, those with FSGS displayed a proliferation/apoptosis ratio in favor of proliferation in the glomerulus but dramatically in favor of apoptosis in the tubules. When both proteinuria and apoptosis were included in a stepwise logistic regression procedure, only apoptosis was found to predict independently the development of ESRD. Prolonged incubation of cultured Madin-Darby canine kidney (distal/collecting) cells with albumin also resulted in a dose- and duration-dependent induction of apoptosis and activation of the Fas pathway, lending support to the novel finding of distal tubule cell apoptosis in patients with FSGS. The results indicate that an elevated tubule cell apoptosis rate at the time of initial biopsy represents an independent predictor of progression to ESRD in patients with early FSGS.


Proteinuria is the hallmark of glomerular diseases, and the magnitude of proteinuria is an established adverse prognostic factor in a variety of progressive nephropathies (1–5). Ongoing proteinuria has been proposed to lead to tubular injury primarily via excessive tubular cell uptake of filtered proteins and/or protein-bound substances such as fatty acids (3–6). Albumin, the most abundant protein in the glomerular filtrate, is endocytosed by proximal tubule cells (7) and can cause direct tubule cell injury in animals (8–11) and cell culture models (12). Several mechanisms remain under investigation in these experimental settings, including activation of Fas-mediated (12) and peroxisome proliferator–activated receptor–γ-dependent (6) apoptosis, and induction of proinflammatory molecules such as monococyte chemoattractant protein-1 (13,14), osteopontin (13), regulated on activation normal T cell expressed and secreted (15), endothelin-1 (16), IL-8 (17), NF-κB (18), and fractalkine (10). There is, however, a paucity of mechanistic information in human diseases characterized by chronic proteinuria.

One such common clinical condition is primary focal segmental glomerulosclerosis (FSGS). This is an enigmatic heterogeneous disease that is present in approximately 25% of patients with idiopathic nephrotic syndrome. Approximately two thirds of these patients progress inexorably toward ESRD (19–21). The disease is more prevalent, more severe, and more likely to progress to ESRD among black and Hispanic individuals, for reasons that are unclear (19). It is, however, well known that the degree of proteinuria is predictive of the course and prognosis in patients with FSGS. More than half of the patients with nephrotic-range proteinuria were shown in clinical studies to reach ESRD within 8 yr of the diagnosis, in marked contrast to a 10-yr renal survival rate of >80% in those with nonnephrotic proteinuria (19–21). In addition to the pathognomonic segmental glomerular sclerosing lesions, the renal histopathology in progressive FSGS is characterized by widespread tubular atrophy and interstitial fibrosis, and the extent of tubulointerstitial disease correlates well with the degree of functional renal impairment (22). The biogenesis of these lesions remains puzzling, but the well-documented correlation between tubulointerstitial damage, proteinuria, and renal functional impairment has led to the protein overload hypothesis to explain the tubule cell injury (2,23). However, the proximate mechanisms by which
proteinuria may damage tubular cells in humans with FSGS remain to be explored.

In this study, we tested the hypothesis that induction of apoptosis may represent a mechanism by which kidney tubule cells are damaged in children with FSGS. The rationale for this line of investigation was derived from findings in animal studies that progressive proteinuria is associated with tubule cell apoptosis (9,11). In biopsy samples from patients with idiopathic FSGS, apoptosis was correlated with cell proliferation and expression of proapoptotic molecules. Associations between extent of apoptosis, degree of proteinuria, and other clinical outcomes were determined. Because apoptosis in FSGS was surprisingly evident in both proximal and distal tubule cells, the roles of protein overload and Fas-dependent apoptosis were also examined in vitro in cultured Madin-Darby canine kidney (MDCK) (distal/collecting) cells. Our results show that the extent of Fas-associated tubule cell apoptosis at initial presentation correlates with the degree of proteinuria and progression to ESRD. Albumin-induced apoptosis in cultured distal tubular cells also proceeds at least in part via Fas-mediated pathways.

**Materials and Methods**

**Patient Population**

The study protocols were approved by the Institutional Review Boards. We examined the kidney biopsy specimens of all patients with the diagnosis of biopsy-proven primary FSGS made during the period of 1995 to 2000. Patients with an estimated GFR <80 ml/min per 1.73 m² and those with kidney biopsies showing chronic changes such as significant tubulointerstitial fibrosis and tubular atrophy were excluded from the study. For this study, we thus chose a homogeneous cohort of 30 biopsy samples from patients with the clinical and histologic diagnosis of idiopathic early FSGS, who at the time of their biopsy had near-normal GFR and no chronic changes on their biopsy. Proteinuria was calculated on the basis of 24-h urine collections, and patients with >2.5 g/d were designated to have nephrotic-range proteinuria. All children were followed for a minimum of 2 yr. For comparison purposes, we also examined the kidney biopsy specimens of patients with minimal-change disease (MCD; n = 4) and membranoproliferative glomerulonephritis type 2 (MPGN; n = 8). These samples were chosen from age- and gender-matched children who, at the time of their biopsy, also had near-normal GFR and a degree of proteinuria that was comparable to that of our FSGS cohort. In all three patient populations, renal biopsies had been performed because the clinical presentations were atypical for MCD, primarily an older age of onset and/or resistance to corticosteroid treatment. As an additional comparison, sections of normal kidney (controls, n = 4) were obtained from histologically normal tissue flanking surgically excised Wilms’ tumors.

**Apoptosis Assay in Kidney Biopsy Samples**

All studies involving human kidney biopsy tissues were carried out in a double-blinded manner. Internucleosomal DNA fragmentation was detected in situ using the TdT-mediated dUTP nick end-labeling (TUNEL) assay (ApoAlert DNA Fragmentation Assay Kit; Clontech, La Jolla, CA), as described previously (24–26). Briefly, 4-µm sections of formalin-fixed, paraffin-embedded renal tissue were deparaffinized through xylene and descending grades of ethanol, fixed with 4% formaldehyde/PBS for 30 min at 4°C, permeabilized with proteinase K at room temperature for 15 min and 0.2% Triton X-100/PBS for 15 min at 4°C, and incubated with a mixture of nucleotides and TdT enzyme for 60 min at 37°C. The reaction was terminated with 2× SSC, and the sections were washed with PBS and mounted with Crystal/mount (Biomedia, Foster City, CA). Fluorescence nuclei were detected by visualization with a microscope equipped with fluorescein filters (IX70; Olympus, Melville, NY). As a negative control, sections were incubated in the absence of TdT enzyme. Serial sections were stained with propidium iodide to identify TUNEL-positive images as nuclei and to highlight the morphologic changes typical of apoptotic nuclei. The degree of apoptosis was estimated by counting the number of TUNEL-positive cells per 100 cells examined. Only cells that displayed the characteristic morphology of apoptosis, including nuclear fragmentation and nuclear condensation by propidium iodide staining and TUNEL staining, and intensely fluorescence nuclei by TUNEL assay were counted as apoptotic. Merely TUNEL-positive cells, in the absence of morphologic criteria, were not considered apoptotic. Proximal and distal tubular cells were identified primarily by morphologic criteria, including the presence of a brush border, the cell size, and nuclear-cytoplasmic ratio. Lectin histochemistry with Phaseolus vulgaris erythroagglutinin (Sigma, St. Louis, MO) and Arachis hypogaea (AH; Sigma) was used as a confirmatory assay to identify proximal and distal tubules, respectively, as described previously (26,27). Briefly, serial sections were deparaffinized, treated with 1.25% H₂O₂ in methanol for 30 min, incubated with either Phaseolus vulgaris erythroagglutinin (1 µg/ml) or AH (10 µg/ml) for 60 min, and developed with diaminobenzidine.

**Proliferation Assay in Kidney Biopsy Samples**

For detection of proliferating cells, sections were incubated with a monoclonal antibody to proliferating cell nuclear antigen (PCNA; 1:500 dilution; Upstate Biotechnology, Lake Placid, NY) and detection was accomplished by immunoperoxidase staining as recommended by the manufacturer (Immunocruz Staining System; Santa Cruz Biotechnology, Santa Cruz, CA). Slides were examined in a blinded manner, and proliferation was quantified by counting the number of PCNA-positive cells per 100 cells counted in an average of five high-power fields (×40) in each section.

**Kidney Immunohistochemistry for Proapoptotic Pathways**

Because Fas-dependent extrinsic apoptotic mechanisms have been implicated in albumin-induced proximal tubule cell injury in vitro (12), serial kidney sections were examined with Fas antibody and compared with propidium iodide or TUNEL labeling. Paraffin-embedded sections were deparaffinized and rehydrated through two changes of xylene and graded alcohols, fixed with 4% formaldehyde/PBS, blocked with goat serum, and incubated with primary antibody (Fas monoclonal, 1:2500; Transduction Laboratories, Lexington, KY) for 1 h at room temperature, as described previously (24–26). Slides then were exposed for 30 min at room temperature in the dark to secondary antibodies conjugated with Cy5 (Amersham, Piscataway, NJ) and visualized using rhodamine filters. Serial sections were also examined for activation of the intrinsic or mitochondrial apoptotic pathways by staining with antibodies to activated Bax-NT and activated Bak-NT (both at 1:1000 dilution; Upstate Biotechnology), as described previously (26).

**Cell Culture and Albumin Overload Protocol**

Because apoptosis was surprisingly evident in both proximal and distal tubule cells and albumin uptake has previously been shown to occur only in proximal tubule cells, the roles of protein overload and Fas-dependent apoptosis were also examined in vitro in cultured MDCK (distal/collecting) cells. MDCK cells obtained from American
Type Culture Collection (Manassas, VA) were grown on cover slips in six-well tissue culture plates (Costar, Cambridge, MA) in DMEM with 5% FBS (Life Technologies, Gaithersburg, MD). Upon reaching confluence, cells were washed and incubated in medium that contained endotoxin-free BSA (Sigma) at concentrations of 0, 5, 10, or 20 mg/ml, for up to 3 d. The concentrations of BSA were chosen on the basis of our previous studies with cultured proximal tubular cells (12). The maximum duration of 3 d was chosen because incubation for longer periods resulted in morphologic features of cellular necrosis. For some experiments, delipidated endotoxin-free BSA (Sigma) was used instead, at the same concentrations, because albumin-bound lipids and fatty acids may contribute to apoptosis of tubule cells (3–6). For additional experiments, MDCK cells were cultured on collagen-coated inserts (BD Biosciences, San Jose, CA) to rule out any effects on survival conferred by the resultant integrin-mediated cell–matrix interactions. For yet additional experiments, MDCK cells were cultured on Transwell filters (Costar, Acton, MA) to ensure complete polarization and incubated with varying concentrations of fatty acid–bearing or delipidated BSA placed on the apical surface to assay for apical albumin uptake.

**Albumin Uptake and Apoptosis Assays in Cultured Cells**

Albumin uptake by MDCK cells was documented by Western blotting and immunofluorescence. Cells that were grown on Transwell filters were incubated with varying quantities of BSA or delipidated BSA for 2 h and subjected to Western analysis with a monoclonal antibody to albumin (1:1000; Sigma). Albumin uptake was also documented by immunofluorescence, as described previously (12). Briefly, cells that were grown on coverslips were incubated with 20 mg/ml BSA for 2 h, fixed in 4% fresh parafomaldehyde, blocked with normal goat serum, incubated with primary antibody (albumin monoclonal, 1:500; Sigma) for 60 min, exposed to secondary antibody conjugated to Cy2, and visualized with fluorescein filters.

For detection of apoptosis, cells were incubated with BSA or delipidated BSA for 72 h, stained with propidium iodide, and visualized with rhodamine filters to identify the characteristic morphology of apoptosis as revealed by propidium iodide staining, including nuclear fragmentation and nuclear condensation. In another set of experiments, cells that were incubated with BSA or delipidated BSA for 72 h were subjected to the TUNEL assay and visualized with fluorescein filters to identify TUNEL-positive, intensely fluorescent, condensed and fragmented apoptotic nuclei. For further confirmation of apoptosis in MDCK cells, we detected the characteristic intercensosomal DNA fragmentation by the DNA laddering assay (28). After 72 h of incubation with various concentrations of BSA, nonadherent cells were pelleted, added to trypsinized and pelleted adherent cells, and incubated in 500 μl of lysis buffer (1% SDS, 20 mM EDTA, 1 mg/ml protease K [pH 8]) overnight at 50°C. Ribonuclease A (10 mg/ml) then was added, for an additional 2-h incubation at 37°C. The chromosomal DNA was extracted with phenol/chloroform, precipitated with ethanol and salt, and analyzed by agarose gel electrophoresis and ethidium bromide staining to reveal the characteristic fragmentation pattern.

To provide a better picture of the albumin-induced cellular homeostasis, we also completed additional cell proliferation assays. The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-based assay kit (Sigma) is designed for the measurement of overall cell viability and proliferation. Briefly, MDCK cells were incubated with varying concentrations of BSA for 72 h. MTT solution (10% of culture volume) was added, and the cells returned to the incubator for an additional 3 h. MTT solvent then was added in an amount equal to the culture volume, and absorbance was measured spectrophotometrically at 570 nm. Background absorbance measured at 690 nm was subtracted, and the values were expressed as percentage of control values.

**Caspase Assays**

Caspase 8 and caspase 9 activity assays were performed using the ApoAlert Caspase 8 Colorimetric Assay and the Caspase 9 Fluorescence assay kits (both from Clontech), respectively, as described previously (12,28,29). After 72 h of incubation with various concentrations of BSA, cells were incubated in cell lysis buffer for 10 min and centrifuged. The supernatants were incubated in reaction buffer that contained IETD-AFC (specific substrate for caspase 8) or LEHD-AMC (caspase 9 substrate) at 37°C for 1 h. The activity was assayed using a spectrophotometer for caspase 8 and a fluorometer for caspase 9. Because even control cells displayed a minimal degree of caspase 8 and 9 activity, perhaps reflective of a low degree of "physiologic" apoptosis in cultured cells (12,28,29), changes in caspase activity were expressed as percentage change from control, which was set at 100%.

**Western Blots**

Protein concentrations of cell lysates were determined by the Bradford assay (Bio-Rad, Hercules, CA), and 30 μg of total protein was loaded in each lane. Samples were subjected to SDS-PAGE and transferred to nitrocellulose paper. After blocking with 5% milk for 60 min at room temperature, primary antibodies were applied for 60 min at room temperature as recommended by the manufacturers. The antibodies used were monoclonals to Fas (1:2500; Transduction Laboratories) and Fas-associating protein with death domain (FADD; 1:200; Transduction Laboratories) and a polyclonal to caspase 8 that detects only the inactive pro-caspase form (1:200; Upstate Biotechnology). A monoclonal antibody to tubulin (1:10,000; Sigma) was used to confirm equal sample loading. After incubation with horseradish peroxidase–conjugated secondary antibodies, immunodetection of transferred proteins was performed using enhanced chemiluminescence (Amersham).

**Statistical Analyses**

All values are means ± SD. The SAS 8.0 statistical software was used for the analysis. A two-sample t test or Mann-Whitney rank sum test was used to compare continuous variables, and the χ² test was used to compare categorical variables. The correlation between degree of proteinuria and apoptosis was evaluated by Spearman test. Stepwise multiple logistic regression analysis was performed to assess whether proteinuria and apoptosis were independent predictors of ESRD. Potential independent predictor variables included age, gender, race, serum creatinine, proteinuria, and the number of apoptotic tubular cells, all at the time of initial biopsy. P < 0.05 was considered significant.

**Results**

**Detection and Quantification of Apoptosis and Proliferation in FSGS**

We tested the hypothesis that apoptosis may represent a mechanism by which tubule cells are damaged in chronic proteinuric states. We examined the kidney biopsies of 30 children who had the clinical and histologic diagnosis of idiopathic FSGS of short duration and had near-normal GFR and no chronic changes at the time of biopsy. For comparison, we also examined kidney biopsies of patients with MCD and MPGN and normal kidney tissue flanking surgically excised Wilms’ tumors. Apoptosis was documented by morphology and TUNEL staining. In patients with FSGS, apoptosis was observed by TUNEL staining predominantly in tubular epithelial cells. Both proximal and distal/collection tubule cells displayed condensed and fragmented nuclei that were intensely TUNEL positive.
The identity of TUNEL-positive nuclei as apoptotic was confirmed by propidium iodide staining in serial sections, which revealed the characteristic morphology, including nuclear condensation and fragmentation in TUNEL-positive tubule epithelial cells (Figure 2). Necrotic tubular cells were only rarely detected in this homogeneous cohort of patients with early-onset FSGS. Occasional apoptotic cells were located within the glomerulus.

Because of the unexpected finding of apoptosis in distal tubule cells, lectin histochemistry with AH was used as a confirmatory assay to identify distal nephron segments. In serial sections, tubules that were shown to be distal contained condensed intensely staining nuclei that were also TUNEL positive (Figure 2). In patients with MPGN, tubule cell apoptosis was less pronounced, but apoptotic nuclei were clearly detected in the glomerulus (Figure 1). In contrast, the kidneys

Figure 1. Apoptosis and proliferation in selected glomerular diseases. Serial sections of kidneys with biopsy-proven minimal-change disease (MCD), primary FSGS, and membranoproliferative glomerulonephritis type 2 (MPGN) were stained for apoptosis using the TdT-mediated dUTP nick end-labeling assay and for proliferation using proliferating cell nuclear antigen (PCNA). White arrows point to cells that display the nuclear fragmentation and condensation characteristic of apoptosis by TUNEL staining, and black arrows identify proliferating cells with PCNA-positive nuclei. Figure is representative of results obtained from patients with nephrotic-range proteinuria.
of children with MCD (Figure 1) and normal kidney tissues (data not shown) were essentially devoid of glomerular or tubular cell apoptosis.

Cell proliferation was assayed by PCNA staining. In patients with FSGS, proliferating cells were evident most strikingly in the glomeruli but also to a lesser degree in proximal tubule cells (Figure 1). In patients with MPGN, the situation was reversed, with greater numbers of proliferating cells in the tubules than in the glomeruli (Figure 1). In contrast, the kidneys of children with MCD (Figure 1) and normal kidney tissues (data not shown) displayed only rare glomerular or tubular cell proliferation.

The quantification of these data are depicted in Figure 3. In patients with FSGS, apoptosis was more pronounced in tubule cells (8.5 ± 3.4%) than in the glomeruli (0.82 ± 0.4%), whereas proliferation was more marked in the glomeruli (9.1 ± 2.2%) than in tubules (2.6 ± 0.5%). Patients with MPGN displayed decreased tubule cell apoptosis (3.4 ± 1.5%) but increased glomerular apoptosis (3.9 ± 1.5%), in comparison with the FSGS cohort. The panels to the extreme right illustrate a mathematical ratio of proliferation over apoptosis per 100 cells counted. In patients with FSGS, the ratio was significantly increased in favor of proliferation in the glomerulus (11.1:1) but markedly in favor of apoptosis in the tubules (0.3:1).

Tubular Cell Apoptosis in FSGS Correlates with the Degree of Proteinuria

The clinical characteristics of patients included in this study are shown in Table 1. There were no significant differences between the patient groups with respect to age, race, initial serum creatinine, or degree of proteinuria. The correlation between degree of proteinuria and apoptosis at the time of initial biopsy with various clinical characteristics in patients with FSGS is illustrated in Table 2. Patients in the nonnephrotic group (n = 10) displayed 1.0 ± 0.4 g/d proteinuria, whereas those in the nephrotic group (n = 20) excreted 7.9 ± 5.1 g/d protein (P < 0.001). The nephrotic group displayed a significantly greater degree of apoptotic tubular cells (11.1 ± 3.2 versus 4.7 ± 1.1 g/d; P < 0.001). Overall, there was a very strong positive correlation between the degree of proteinuria and incidence of apoptosis at the time of initial biopsy in this cohort of FSGS patients, as illustrated in Figure 4 (r = 0.92, P < 0.001).
Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>MCD (n = 4)</th>
<th>MPGN (n = 8)</th>
<th>FSGS (n = 30)</th>
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<tbody>
<tr>
<td>Age (yr)</td>
<td>6.8 ± 4.5</td>
<td>10.9 ± 4.2</td>
<td>9.6 ± 5.2</td>
</tr>
<tr>
<td>Male:female</td>
<td>1:1</td>
<td>1:7</td>
<td>1:1</td>
</tr>
<tr>
<td>White:minority</td>
<td>1:1</td>
<td>1:1</td>
<td>0.8:1</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.71 ± 0.3</td>
<td>0.76 ± 0.4</td>
<td>0.68 ± 0.3</td>
</tr>
<tr>
<td>Proteinuria (g/d)</td>
<td>7.5 ± 2.1</td>
<td>8.1 ± 3.1</td>
<td>5.8 ± 5.2</td>
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*MCD, minimal-change disease; MPGN, membranoproliferative glomerulonephritis type 2. There were no significant differences in each patient group, with the exception of a female preponderance in MPGN.

Tubule Cell Apoptosis in FSGS Is an Early Marker for Progression

All 30 FSGS patients were followed up for a minimum of 2 yr (range, 2 to 5 yr) for progression. During this period, 12 developed ESRD, defined as calculated GFR <10 ml/min per 1.73 m². Patients who progressed to ESRD displayed a significantly greater degree of proteinuria at the time of their initial biopsy than those who did not progress (10.4 ± 5.8 versus 2.5 ± 1.6 g/d; P < 0.001; Table 2). Importantly, patients who progressed to ESRD were found to exhibit a significantly greater number of apoptotic tubular cells at the time of their initial biopsy than those who did not progress (12.8 ± 3.6 versus 6.4 ± 2.2/100 cells counted; P < 0.001; Table 2). It is important to reiterate that this subset of patients was chosen for study because they had early-onset FSGS, with near-normal GFR and no chronic changes at the time of biopsy. Thus, the degree of apoptosis in FSGS may represent an early marker for progression.

Tubule Cell Apoptosis in FSGS Is an Independent Predictor of Progression

Because our results indicated that both the degree of proteinuria and the extent of apoptosis correlated with progression, we next performed regression analysis to determine whether these were independent risk factors. Both proteinuria and apoptosis independently predicted the development of ESRD (R² = 0.45, P < 0.001 for proteinuria; R² = 0.54, P < 0.001 for apoptosis). However, when both proteinuria and apoptosis were included in a stepwise multiple logistic regression analysis, only apoptosis was found to predict independently the development of ESRD (R² = 0.54, P < 0.001). Thus, an elevated apoptosis rate emerged as an accurate and independent predictor of ESRD in FSGS.

Tubule Cell Apoptosis in FSGS Is Associated with Induction of Fas Expression

Because the proapoptotic molecule Fas has previously been implicated in albumin-induced apoptosis in cultured proximal tubule cells (12), it next was of interest to determine the status of Fas expression in FSGS kidneys. We noted that, just like apoptosis, Fas expression was observed in a patchy distribution in both proximal and distal/collection tubular cells. Indeed, in serial sections, it was evident that Fas expression was detected primarily in the cells that underwent apoptosis; conversely, the majority of cells that were TUNEL positive also exhibited Fas staining (Figure 2). In contrast, immunostaining for activated Bax or Bak was weak and in serial sections did not correlate with TUNEL staining (data not shown). Thus, apoptosis in FSGS is associated primarily with activation of the extrinsic (Fas-dependent) mechanisms and not the intrinsic mitochondrial pathways.

Table 2. Proteinuria, apoptosis, and selected clinical characteristics at the time of initial biopsy in patients with FSGS

<table>
<thead>
<tr>
<th></th>
<th>Proteinuria (g/d)</th>
<th>Apoptosis (% Cells)</th>
</tr>
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<tbody>
<tr>
<td>Nonnephrotic (n = 10)</td>
<td>1.0 ± 0.4</td>
<td>4.7 ± 1.1</td>
</tr>
<tr>
<td>Nephrotic (n = 20)</td>
<td>7.9 ± 5.1b</td>
<td>11.1 ± 3.2b</td>
</tr>
<tr>
<td>Non-ESRD (n = 18)</td>
<td>2.5 ± 1.6</td>
<td>6.4 ± 2.2</td>
</tr>
<tr>
<td>ESRD (n = 12)</td>
<td>10.4 ± 5.8c</td>
<td>12.8 ± 3.6c</td>
</tr>
<tr>
<td>White (n = 13)</td>
<td>6.1 ± 5.9</td>
<td>8.2 ± 4.4</td>
</tr>
<tr>
<td>Minority (n = 17)</td>
<td>5.4 ± 4.0</td>
<td>9.5 ± 4.2</td>
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*Those with nephrotic-range proteinuria displayed significantly greater apoptosis (P < 0.001). Patients who subsequently developed ESRD displayed a significantly greater degree of proteinuria and apoptosis at the time of initial biopsy than those who did not (P < 0.001). There was no significant difference between white and minority patients with regard to proteinuria (P = 0.27) or apoptosis (P = 0.43).

Cultured Distal Tubular Cells Uptake Albumin and Undergo Apoptosis

The novel finding of Fas-associated apoptosis in distal tubular cells of patients with FSGS prompted us to examine an in vitro model of protein overload in cultured distal tubular cells. Because albumin is known to be the predominant protein excreted in the urine of patients with glomerular disease, confluent MDCK cells were subjected to varying concentrations of BSA for different periods of time. In preliminary studies, we established that MDCK cells that were incubated in albumin began to display morphologic changes suggestive of apoptosis, including appearance of gaps in the monolayer and nuclear condensation, after 72 h (data not shown). For first documenting the ability of distal tubular cells to take up albumin, MDCK cells were incubated in varying concentrations of BSA or delipidated BSA for 2 h and subjected to Western analysis with a monoclonal antibody to albumin. A significant dose-dependent increase in intracellular albumin was documented after exposure to both BSA and delipidated BSA, as shown in Figure 5 (top). For further confirming albumin uptake, MDCK cells that were incubated as above were immunostained with a monoclonal antibody to albumin. Albumin in these cells was easily detected in a punctate cytoplasmic distribution (Figure 5, bottom), similar to our previous findings in cultured proximal tubule cells (12).

To determine the effects of prolonged albumin exposure on overall distal tubule cellular homeostasis, we performed cell
viability assays. A dose-dependent decrease in cell viability was demonstrated after 72 h of exposure to albumin (Figure 6) and delipidated albumin (data not shown). This loss of cell viability was documented to be primarily due to induction of apoptosis, using three complementary assays. The DNA laddering assay revealed the characteristic internucleosomal nuclear fragmentation induced by prolonged exposure to BSA (Figure 7) or delipidated BSA (data not shown) in a dose-dependent manner. The characteristic nuclear changes of apoptosis were further confirmed in these cells by staining with propidium iodide or TUNEL (Figure 7). In additional experiments, MDCK cells that were cultured on collagen-coated inserts also underwent albumin-induced apoptosis (data not shown), despite any confounding effects on survival conferred by the additional integrin-mediated cell–matrix interactions.

**Albumin-Induced Apoptosis in Distal Tubular Cells Is Associated with Induction of the Fas-FADD-Caspase 8 Pathway**

It next was of interest to identify the intracellular apoptotic pathways induced by albumin in distal tubular cells, and we sought first to determine the type of pro-caspase that is activated. Significant caspase 8 activation was evident in a dose-dependent manner, whereas the activity of caspase 9 was only minimally above control levels (Figure 8). Cleavage of caspase 8 in a dose-dependent manner was also documented by Western analysis with an antibody that recognizes only the inactive pro-caspase form (Figure 9). These results suggest that caspase 8–dependent pathways predominate in albumin-induced apoptosis. This was confirmed by Western analysis showing a marked dose-dependent induction of Fas and FADD expression after albumin incubation (Figure 9). Densitometric analysis revealed a 3.5-fold increase in Fas expression after 72 h of 5 mg/ml albumin and a 6-fold increase with 10 or 20 mg/ml albumin. The expression of FADD was increased by approximately threefold in all albumin concentrations tested.

**Discussion**

In this study, we examined the hypothesis that induction of apoptosis may represent a proximate mechanism by which kidney tubule cells are injured in FSGS, as a result of the ongoing proteinuria. The rationale for undertaking this study was that (1) there is a well-documented correlation between tubular damage and proteinuria in patients with FSGS (19–23), and (2) ongoing proteinuria has been associated with tubular cell apoptosis both in vitro (12) and in animal models (9,11). Apoptosis, characterized by cell shrinkage, nuclear condensation, and internucleosomal DNA fragmentation, has recently been documented in an increasing array of renal disorders, and renal tubule cell apoptosis is emerging as a possible common pathway in response to a variety of insults that are applied at an intensity below the threshold for necrotic cell death (30,31). Significant attention therefore has been directed toward identification of the intracellular pathways involved in the stimulus recognition, signal transduction, and execution phases of tubular cell apoptosis in various pathophysiologic states.

For this study, we chose to examine a homogeneous cohort of patients who had the clinical and histologic diagnosis of primary early-onset FSGS and had near-normal GFR and no chronic changes at the time of their initial biopsy. This choice was based on previous observations that tubular epithelial cell apoptosis is a well-known feature of chronic renal failure itself, which could add a confounding variable to our analysis (32). We used stringent morphologic criteria and three complementary assays (propidium iodide staining, TUNEL assay, and Fas immunolocalization) for the detection of apoptosis in the biopsy samples in a double-blind manner. Perhaps predictably, we found a strong positive correlation between the degree of proteinuria and incidence of tubule cell apoptosis. This finding, however, does provide a possible mechanistic explanation for the observation that the extent of proteinuria is predictive of the degree of tubulointerstitial disease. In addition, we have made the novel observation that patients who progressed to ESRD
exhibited a significantly greater number of apoptotic tubular cells than those who did not progress. Thus, the degree of tubule cell apoptosis in FSGS at the time of initial biopsy may represent an adverse prognostic factor (similar to proteinuria) and a novel marker for progression. Indeed, when both proteinuria and apoptosis were included in a stepwise multiple logistic regression analysis, only apoptosis was found to predict independently the development of ESRD. Thus, an elevated tubule cell apoptosis rate at the time of initial biopsy has emerged as a novel, accurate, and independent predictor of ESRD in our cohort of patients with early FSGS lesions.

Apoptosis in early FSGS was evident in both proximal and distal nephron segments. Although proximal tubule cells have been shown to respond to albumin overload by programmed cell death both in vitro (12) and in animal models (9,11), our surprising findings necessitated confirmation of albumin-induced apoptosis in a cell culture model of the distal tubule. MDCK cells are known to possess mechanisms for endocytosis (32), and our results indicate for the first time that they do take up albumin. We used stringent morphologic criteria and three complementary assays (propidium iodide staining, TUNEL assay, and DNA laddering) to demonstrate that these distal/collecting cells undergo a dose-dependent apoptotic cell death.
similar to that previously reported for proximal tubule cells (12). Both lipid-laden BSA and delipidated BSA were equally effective in inducing apoptosis, indicating that this effect on cultured distal tubule cells was independent of protein-bound substances such as fatty acids (3–6). It is intriguing to speculate that in patients with FSGS, the ongoing heavy proteinuria may overwhelm the transport mechanisms of the proximal tubule, leading to delivery of plasma proteins to the distal segments. Alternatively, it is possible that apoptotic loss of proximal tubule cells may lead to a diminished reabsorptive capacity, with resultant exposure of distal tubule cells to protein.

In general, the caspases are the final effectors of apoptosis, and apoptotic pathways may be classified according to the pro-caspase activated (34). Thus, activation of the initiator pro-caspase 8 usually results from signaling via death receptors such as Fas, which are integral membrane proteins that transduce apoptotic signals to downstream molecules such as FADD (35). This Fas-FADD-caspase 8 pathway has been implicated in the renal tubule cell apoptosis after ischemic injury (28), during endotoxemia (36), during transplant rejection (37), and in the tubular atrophy of chronic renal failure (32). However, activation of the initiator pro-caspase 9 is dependent on mitochondrial signaling pathways involving members of the Bcl-2 family such as Bax and Bak (38). In this study, both the human and in vitro data strongly suggest activation of the Fas-FADD-caspase 8 pathway in FSGS. Our results lend support to the concept of using specific cell-permeant inhibitors of caspase 8 such as IETD-fmk (12) as a possible strategy to prevent albumin-induced apoptosis.

One of the limitations of our study is that it is retrospective, and the observed associations between apoptosis, proteinuria, and progression do not necessarily suggest a causal relationship. Another limitation pertains to the possibility that tubule cell apoptosis may not be unique to FSGS and may indeed represent a common feature in several chronic proteinuric states. Indeed, we have found that although apoptosis is not easily evident in patients with MCD, it is readily detected in patients with MPGN type 2. However, our overall analysis of apoptosis and proliferation in these patient populations has revealed important and striking differences. In patients with MPGN, the proliferation:apoptosis ratio was somewhat increased in favor of proliferation in the glomerulus but was near unity in the tubules, suggesting that both proliferation and apoptosis occur at equal rates in the tubules. In contrast, in patients with FSGS, the ratio was markedly increased in favor of proliferation in the glomerulus but dramatically in favor of apoptosis in the tubules. These findings characterize early FSGS as a condition in which the balance of cell fate is slanted toward survival and proliferation in the glomerulus but concomitantly tilted toward apoptosis in the tubules. Whereas tubule proliferation rates were comparable in the two conditions, the degree of tubule cell apoptosis was significantly greater in FSGS than in MPGN, even though there were no differences in the degree of proteinuria between the two patient populations. This analysis raises the possibility that tubule cell apoptosis in FSGS cannot be attributed exclusively to the degree of proteinuria. We speculate that FSGS cannot be considered a disease of the glomerulus alone, and additional hitherto unknown mechanisms may be called into play in the pathogenesis of the widespread tubular atrophy and interstitial fibrosis that are characteristic of FSGS. Our study indicates that one such mechanism may include the induction of Fas-mediated apoptotic pathways, via proteinuria-dependent and -independent mechanisms.

In summary, in FSGS, the extent of Fas-associated tubule cell apoptosis at initial presentation correlates strongly with the degree of proteinuria and progression to ESRD. Albumin-induced apoptosis in cultured distal tubular cells also proceeds at least in part via Fas-dependent pathways. It will be important in future work to confirm and extend these findings in a large, prospective study. It will be of significant interest to determine the utility of identifying apoptosis and apoptotic pathways as novel early biomarkers of disease severity and progression. We speculate that inhibition of specific apoptotic pathways may represent an innovative therapeutic strategy for the amelioration of renal tubule cell damage in FSGS, a common clinical condition that is still associated with unsatisfactory treatment options and an unfavorable prognosis.

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Correction

The authors of this article from the December 2004 issue of *JASN* regret an error in reporting the methods in the Material and Methods section. Under Clinical Chemistry, it was mentioned that total protein was measured by colorimetric assay using molybdate red and albumin with bromocresol green. However, total protein was measured using endpoint measurement with TCA (Nephelometer Analyzer II, Dade Behring, Marburg, Germany), and albumin was not measured at all (and not shown in the paper). This error does not alter any of the published results or conclusions.

Correction

The authors of this article from the February 2005 issue of *JASN* regret an error in figure use. In Figure 1, the section identified as the MCD biopsy (left panel, 3rd row) was erroneous and inadvertently identical to that identified as the MPGN biopsy (right panel, 3rd row). The correct sections from MCD kidneys showed little or no proliferation. This error does not alter any of the published results or conclusions.