Apoptosis and Caspase-3 in Experimental Anti-Glomerular Basement Membrane Nephritis

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Abstract. The caspase family is central to the proteolytic events of apoptosis. In particular, caspase-3 plays a key role in the execution of apoptosis. However, the importance of caspase-3 in renal cell apoptosis during kidney scarring has not been established. Here, nephrotoxic nephritis (NTN) was induced in Wistar Kyoto rats by a single intravenous injection of rabbit anti-rat glomerular basement membrane serum, with analysis at days 7, 15, 30, and 45 after injection. Cell apoptosis (in situ end labeling of DNA, light and electron microscopy), proliferation (proliferating cell nuclear antigen–positive cells), and inflammation (ED1-positive cells) all increased in NTN kidneys, peaking early (day 7) in the glomeruli and later (days 30 to 45) in the tubules and interstitium. The expression of caspase-3 mRNA (Northern blotting) was increased in NTN kidneys on days 7, 30, and 45 (173.3%, 228%, and 241.7%, respectively; P < 0.05). Western blotting showed that a 24-kD protein band (caspase-3 active subunit) increased with time in NTN kidneys (P < 0.01) and reached a maximum on day 45 (6.08-fold increase). A 32 kD band (caspase-3 precursor) was also increased on day 45 (3.92-fold; P < 0.01). Elevated caspase-3 activity (two- to threefold) was observed in NTN kidneys at all time points (P < 0.01). Upregulated expression of caspase-3 at all levels positively correlated with apoptosis, whereas both correlated closely with inflammation, proliferation, and subsequent fibrosis in glomeruli, tubules, and interstitium (P < 0.05). Inhibition of caspase-3 during the course of experimental nephritis may offer a new therapeutic approach for the prevention of renal apoptosis and the associated renal tubular atrophy and fibrosis.

After acute renal injury, the loss of glomerular and tubular cells with the concomitant proliferation of renal fibroblasts and accumulation of extracellular matrix leads to progressive renal scarring (1). Apoptosis, a morphologic form of programmed cell death required for the control of cell populations, has been shown to have a role in the cell deletion associated with renal scarring (2–5). Normally, the deletion by apoptosis of excessive, damaged, or nonfunctioning renal cells and infiltrating inflammatory cells is beneficial for the resolution of glomerulonephritis such as in the anti-thy1.1 model (6). However, continuous and inappropriate renal cell loss by apoptosis may be detrimental as demonstrated in various models of progressive chronic renal failure (CRF), such as the nephrotoxic nephritis (NTN) and subtotal nephrectomy (SNx) models (2,5). These studies indicate that apoptosis may have a dual role in experimental renal injury, being involved in the resolution of the acute inflammatory changes but also in the progression of tubular atrophy (TA) and fibrotic changes. Therefore, the control of apoptosis and its mediators could be a potential target for therapeutic interventions aimed at manipulating the early inflammation as well as the late atrophic and fibrotic changes.

Although many different signals initiate apoptosis, the phenotype of apoptosis (a series of distinct morphologic and biochemical changes) is surprisingly similar even in different cell types, suggesting that the final stages of apoptotic death are highly conserved (7). A recently identified class of cysteine proteases termed caspases coordinates the execution of the death program. A family of 14 different caspases that play a role in inflammation and apoptosis have been identified (8). They all are produced as inactive precursors (zymogens) that contain an N-terminal prodomain and a large (approximately 20 kD) and a small (approximately 10 kD) subunit. The N-terminal domain is highly variable between the different members and contains important structural domains for the regulation of the activation. Activation requires processing of the precursor into the large and small subunits, and in some cases the prodomain is removed as well. The individual subunits dimerize and associate to form a α2β2 heterotetramer with two independent active sites (9). Caspases are highly specific with an absolute requirement for cleavage after aspartic acid, whereas individual caspases recognize different tetrapeptide motifs, which might explain their individual substrate specificity (10). Caspases have diverse functions; members of this family play essential roles in both initial signaling events (caspase-8, caspase-9) and the downstream proteolytic cleavages (caspase-3) (8,10). Protease inhibitors, including macromolecular and peptide-based inhibitors of caspases, are highly
effective in preventing apoptotic cell death in both in vitro and in vivo models of apoptosis (11,12).

Among identified caspases, the best functionally correlated with the phenotype of apoptosis is caspase-3 (CPP32, YAMA, apopain) (12), which is mainly activated via both the death receptor and mitochondrial routes (8). However, there are few studies on the regulators of apoptosis in CRF models, with little data on the contribution of the caspases, except in an acute ischemic renal injury model (13) and experimental cyclosporin A–induced nephrototoxicity (14). The investigation of caspase-3–deficient mice suggested that this enzyme, in particular, might be an appropriate target for therapeutic intervention in diseases that result from inappropriate apoptosis (11,15). Such an approach, based on the inhibition of caspase-3, has already been shown to be protective in a rat model of neonatal hypoxic-ischemic brain injury and in a murine model of renal ischemia (16,17).

In this study using the NTN model of experimental progressive glomerulonephritis in Wistar Kyoto rats, we measured caspase-3 at the mRNA, protein, and activity levels in both the early inflammatory and the late fibrotic stages of disease. Furthermore, we addressed the association between the observed changes of caspase-3 and apoptosis with the morphologic acute and chronic changes during the course of NTN.

Materials and Methods

Nephrotoxic Serum Nephritis

Male Wistar Kyoto rats weighing 200 to 300 g were subjected to nephrotoxic serum nephritis. Rats were housed at constant temperature (20°C) and humidity (45%) on a 12-h light/dark cycle. They were fed ad libitum on standard laboratory rat chow (Lab Sure Ltd., March, Cambridge, UK) and had free access to tap water. All of the experiments were carried out according to the rules and regulations laid down by the Home Office (Animal Scientific Procedure Act 1986, UK).

Nephrotoxic serum nephritis was induced in 28 rats by a single intravenous injection of 0.5 ml of rabbit anti-rat glomerular basement membrane (GBM) serum (nephrotoxic serum) into the tail vein. Intravenous injection of 0.5 ml of rabbit anti-rat glomerular basement membrane (GBM) serum (nephrotoxic serum) into the tail vein. Among identified caspases, the best functionally correlated with the phenotype of apoptosis is caspase-3 (CPP32, YAMA, apopain) (12), which is mainly activated via both the death receptor and mitochondrial routes (8). However, there are few studies on the regulators of apoptosis in CRF models, with little data on the contribution of the caspases, except in an acute ischemic renal injury model (13) and experimental cyclosporin A–induced nephrototoxicity (14). The investigation of caspase-3–deficient mice suggested that this enzyme, in particular, might be an appropriate target for therapeutic intervention in diseases that result from inappropriate apoptosis (11,15). Such an approach, based on the inhibition of caspase-3, has already been shown to be protective in a rat model of neonatal hypoxic-ischemic brain injury and in a murine model of renal ischemia (16,17).

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Nephrotoxic serum nephritis was induced in 28 rats by a single intravenous injection of 0.5 ml of rabbit anti-rat glomerular basement membrane (GBM) serum (nephrotoxic serum) into the tail vein. Sixteen rats were used as controls and were injected with the same amount of normal rabbit serum (Vector Laboratories, Peterborough, UK) (18). Rats were killed in groups (n = 4 to 7) at days 7, 15, 30, and 45 after serum injection. Removed kidney tissues were fixed in 10% (wt/vol) neutral buffered formalin and paraffin embedded for histologic and immunohistochemical examination. For electron microscopy, tissues were fixed in 2.5% (vol/vol) glutaraldehyde solution in phosphate buffer (pH 7.4). Tissues were snap-frozen and stored in liquid nitrogen for mRNA, protein, and activity analyses. Serum creatinine (Scr) concentration (standard autoanalyzer techniques) and 24-h urinary protein excretion (Biuret method) were determined respectively. ISEL of DNA while associated with apoptosis can also be seen in necrotic (nonspecific DNA degradation) and mitotic (transient DNA strand break) cells. To substantiate the specificity of our results, we confirmed apoptosis by light microscopic evaluation of the characteristic morphologic features; only strongly positive ISEL cells with observable morphologic features of apoptosis, such as shrunken cells with condensed nuclei surrounded by a narrow cytoplasmic halo, were counted (2,23,24). Fewer than 5% of ISEL-labeled cells in both NTN and control kidneys failed to demonstrate morphologic features of apoptosis.

Evaluation of Cellular Proliferation and Inflammation by Proliferating Cell Nuclear Antigen and ED1 Immunostaining

Cellular proliferation and inflammation were evaluated by proliferating cell nuclear antigen (PCNA) and ED1 (a specific monocye/macrophage marker) immunostaining. Localization of PCNA and ED1 was performed in 10% (wt/vol) neutral buffered formalin-fixed, paraffin-embedded kidney tissues by immunohistochemistry using a standard avidin-biotin peroxidase complex technique as described previously (19). Primary antibodies (monoclonal mouse anti-human PCNA [clone PC10; Dako, Glostrup, Denmark]; monoclonal mouse anti-rat ED1 antibody [Serotec Ltd., Oxford, UK]) were both diluent 1:50 and applied overnight at 4°C in a humid atmosphere. Thereafter, antibody binding was revealed using the ABC Elite Kit (Vector Laboratories). 3′-amino-9-ethylcarbazole (AEC) was used as the substrate. Sections were counterstained with hematoxylin and mounted in Glycyergel (Dako). Negative control sections were incubated with normal mouse IgG at the same protein concentration as the primary antibody. The immunohistochemical staining pattern of PCNA and ED1

Estimation of Renal Scarring

The extent of renal scarring after NTN was determined by observers who were blinded to the experimental code according to a previously published arbitrary scale (19–21). At ×200 magnification, Masson’s trichrome–stained sections were examined and scored by two authors (B.Y. and G.L.T.). Glomerulosclerosis (GS): a normal glomerulus scored 0, mild segmental GS affecting up to 25% of the glomerular tuft scored 1, moderate GS affecting between 25 and 50% of the tuft scored 2, and severe GS affecting in excess of 50% of the tuft scored 3. Tubulointerstitial scarring: normal tubules (tubule cell number approximately 1000/×200 magnification field) and interstitium scored 0, mild tubular atrophy (TA; tubule cell number approximately 800) and interstitial edema or fibrosis (IF) affecting up to 25% scored 1, moderate TA (tubule cell number approximately 600) and IF affecting 25 to 50% scored 2, and severe TA (tubule cell number approximately 400) and IF scored 3. To determine the level of TA, tubule cells per ×200 magnification field were counted. The data were collected from a minimum series of 12 randomly selected fields in the cortex or such number of fields until 30 glomeruli had been counted.

In Situ End Labeling for the Detection of Apoptotic Cells

Using 10% neutral buffered formalin-fixed, paraffin-embedded sections, fragmented nuclear DNA associated with apoptosis was labeled in situ with digoxigenin-deoxyuridine (dUTP) by terminal deoxynucleotidyl transferase (TdT), using the ApopTag Plus peroxidase kit (Appligene Oncor, Illkirch, France) according to the manufacturer’s instructions (22). For negative controls, slides were incubated in TdT buffer without TdT. For biochemically induced positive controls, slides were pretreated with 10 μg/ml DNase I (Sigma, Poole, UK) in DNA buffer.

For each experimental animal, more than 30 glomerular cross sections and 20 high-power (×400 magnification) fields of tubulointerstitium were examined by two authors who were blinded to the experimental code (B.Y. and G.L.T.). The number of in situ end labeling (ISEL) positive-staining nuclei per glomerulus (Gapo), per 100 tubule cells (%) (Tapo), or per interstitial field (Iapo) was determined respectively. ISEL of DNA while associated with apoptosis can also be seen in necrotic (nonspecific DNA degradation) and mitotic (transient DNA strand break) cells. To substantiate the specificity of our results, we confirmed apoptosis by light microscopic evaluation of the characteristic morphologic features; only strongly positive ISEL cells with observable morphologic features of apoptosis, such as shrunken cells with condensed nuclei surrounded by a narrow cytoplasmic halo, were counted (2,23,24). Fewer than 5% of ISEL-labeled cells in both NTN and control kidneys failed to demonstrate morphologic features of apoptosis.
was semiquantitatively assessed using the same counting system used with ISEL staining.

**Double Staining for Apoptosis with ED1 and α-Smooth Muscle Actin**

Double immunohistochemical staining was undertaken on paraffin sections. ISEL was carried out as described above. Before application of the antidigoxigenin antibody, sections were preincubated with blocking serum for 30 min, labeled with the anti-ED1 or α-smooth muscle actin (α-SMA; monoclonal mouse anti-human α-SMA, diluted 1:250; Dako) antibody at 4°C overnight. Sections were labeled with biotinylated secondary anti-mouse IgG at 37°C for 30 min and with alkaline phosphatase streptavidin for another 30 min and were developed with Fast Red TR/Naphthol AS-MX solution to produce a bright pink color. Subsequently, anti-digoxigenin-peroxidase antibody was applied on the sections and the development was achieved by the solution of diaminobenzidine to provide positive staining as yellow brown. Control sections were incubated with nonimmune normal mouse IgG in place of primary antibody and with the omission of TdT enzyme as ISEL controls.

**Northern Blot Analysis of Caspase-3 mRNA**

Northern blot analysis was carried on the snap-frozen kidney tissues. Total RNA was extracted using the TRizol reagent (Life Technologies BRL, Paisley, UK) and quantified by scanning spectrophotometer at 260 nm. Fifteen μg of total RNA were electrophoresed on a 1% (wt/vol) agarose TAE gel, and RNA was then transferred to a nylon membrane (Hybond-N, Amersham Life Science, Little Chalfont, UK) and cross linked to the nylon membrane. After bacterial amplification and plasmid purification using Prep-A-Gene DNA Purification Systems (Bio-Rad Laboratories Ltd., Hertfordshire, UK). Product confirmation was by molecular Analyst version 4 analysis software (Bio-Rad Laboratories, Inc.).

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<table>
<thead>
<tr>
<th>Days</th>
<th>UP (mg/24 h)</th>
<th>SCr (µmol/L)</th>
<th>GS</th>
<th>TA</th>
<th>IF</th>
<th>Gpca (±/G)</th>
<th>Tpca (%)</th>
<th>Ipcna (±/400)</th>
<th>Ged1 (±/G)</th>
<th>Ied1 (±/400)</th>
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<tbody>
<tr>
<td>7</td>
<td>12.0 ± 0.6</td>
<td>33.0 ± 2.1</td>
<td>3.2 ± 0.2</td>
<td>21</td>
<td>0.22 ± 0.02</td>
<td>980.0 ± 10.0</td>
<td>0.05 ± 0.05</td>
<td>3.2 ± 0.2</td>
<td>925.0 ± 12.7</td>
<td>0.02 ± 0.02</td>
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<tr>
<td>15</td>
<td>15.0 ± 2.1</td>
<td>39.3 ± 1.1</td>
<td>0.32 ± 0.02</td>
<td>21</td>
<td>0.17 ± 0.02</td>
<td>965.9 ± 4.0</td>
<td>0.03 ± 0.03</td>
<td>0.32 ± 0.02</td>
<td>925.0 ± 12.7</td>
<td>0.02 ± 0.02</td>
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<tr>
<td>30</td>
<td>19.7 ± 1.8</td>
<td>34.8 ± 2.1</td>
<td>0.32 ± 0.02</td>
<td>21</td>
<td>0.17 ± 0.02</td>
<td>925.0 ± 12.7</td>
<td>0.02 ± 0.02</td>
<td>0.32 ± 0.02</td>
<td>925.0 ± 12.7</td>
<td>0.02 ± 0.02</td>
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<tr>
<td>45</td>
<td>7.8 ± 1.0</td>
<td>34.1 ± 0.6</td>
<td>0.20 ± 0.06</td>
<td>21</td>
<td>0.20 ± 0.06</td>
<td>954.0 ± 16.5</td>
<td>0.03 ± 0.03</td>
<td>0.20 ± 0.06</td>
<td>954.0 ± 16.5</td>
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<td>NTN</td>
<td>34.0 ± 11</td>
<td>177 ± 0.12</td>
<td>1.77 ± 0.12</td>
<td>0.17</td>
<td>1.77 ± 0.12</td>
<td>845.3 ± 3.36</td>
<td>0.77 ± 0.07</td>
<td>1.77 ± 0.12</td>
<td>1088 ± 1.58</td>
<td>0.07 ± 0.07</td>
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<tr>
<td>15</td>
<td>222 ± 18.5</td>
<td>53.0 ± 3.2</td>
<td>0.33 ± 0.03</td>
<td>128</td>
<td>0.20 ± 0.03</td>
<td>695.0 ± 1.08</td>
<td>0.18 ± 0.01</td>
<td>0.33 ± 0.03</td>
<td>695.0 ± 1.08</td>
<td>0.18 ± 0.01</td>
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<tr>
<td>30</td>
<td>319 ± 16.5</td>
<td>62.4 ± 1.2</td>
<td>0.38 ± 0.04</td>
<td>128</td>
<td>0.20 ± 0.04</td>
<td>790.3 ± 1.35</td>
<td>0.20 ± 0.04</td>
<td>0.38 ± 0.04</td>
<td>790.3 ± 1.35</td>
<td>0.20 ± 0.04</td>
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</table>

**Table 1.** Changes with time in renal function, fibrosis, cell proliferation, and inflammation in the kidneys of rats submitted to NTN. a NTN, nephrotoxic nephritis; UP, proteinuria; SCr, serum creatinine; GS, glomerulosclerosis; TA, tubular atrophy; IF, interstitial edema or fibrosis; Gpcna, proliferating cell nuclear antigen (PCNA) positive-staining glomerular nuclei; Tpcna, PCNA positive-staining tubular nuclei; Ipcna, PCNA positive-staining interstitial nuclei; Ged1, ED1 positive-staining glomerular cells; Ied1, ED1 positive-staining interstitial cells. Controls (n = 4) and NTN (n = 6 to 8).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NTN</th>
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<tbody>
<tr>
<td>SCr</td>
<td>126.0 ± 0.2</td>
<td>364.2 ± 0.0</td>
<td></td>
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<tr>
<td>Gpca</td>
<td>7.8 ± 1.0</td>
<td>34.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Tpca</td>
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<td>954.0 ± 16.5</td>
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</table>

A value for NTN is significantly different from control (P < 0.05).
using the housekeeping gene cyclophilin (27). Results were expressed as percentage of control sample mRNA densities. Transcript size was determined by comparison to RNA molecular weight markers (Promega) using same analysis package and by visual comparison to the ribosomal RNA subunits.

**Measurement of Tissue Caspase-3 Protein Level**

Tissue levels of caspase-3 protein were determined by immunoblotting of Western blots. Twenty μg of protein was separated on a 15% (wt/vol) polyacrylamide denaturing gel and then electroblotted onto Hybond ECL nitrocellulose membranes (Amersham Life Science). Membranes were blocked by the addition of 3% (wt/vol) bovine serum albumin in 0.1% (vol/vol) Tween 20 TBS (TTBS) at 4°C overnight before being probed with a polyclonal rabbit anti-rat full-length caspase-3 at 1:2000 dilution in TTBS buffer at room temperature for 2 h. Primary antibody binding was revealed using an anti-rabbit peroxidase conjugate (Dako) diluted at 1:2000 in TTBS buffer for 1 h and the ECL chemiluminescence detection system (Amersham Life Science). Recombinant caspase-3 (17 kD and 12 kD subunits; Sigma) was used to verify antibody efficacy under experimental conditions. Developed films were semiquantitatively analyzed by volume density using a Bio-Rad GS-690 scanning densitometer and Molecular Analyst version 4 software. Translation size was determined by comparison to protein molecular weight markers (Bio-Rad Laboratories Ltd.) using the same analysis package.

**Detection of Caspase-3 Activity in Renal Tissue**

The activity of caspase-3 in tissue was detected by the modified Fluorometric CaspACE Assay System (Promega). Kidney tissue (20 to 50 mg) from control and NTN rats was ground in liquid nitrogen using a pestle and mortar. A 1:9 (wt/vol) tissue:buffer extract was prepared in Tris/acetate buffer (pH 7.5) at 30°C (28). The extract was centrifuged at 12,000 × g for 10 min, and the supernatant was collected. A volume of supernatant equivalent to 100 μg of protein was assayed for caspase-3 activity by the ability to cleave the fluorogenic substrate Ac-DEVD-AMC. The specificity of the assay was determined using the caspase-3 inhibitor Ac-DEVD-CHO by adding to the sample 30 min before the substrate. Proteolytic cleavage of the substrates was monitored in a fluorescence microplate reader (SOFTmax PRO, Molecular Devices Corp., Sunnyvale, CA) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The fluorescence intensity was calibrated with standard concentrations of AMC, and the caspase-3 activity was calculated from the slope of the recorder trace and expressed in picomoles per minute per μg of protein.

**Statistical Analyses**

Results are expressed as mean ± SEM. The statistical difference was assessed by a single factor variance (ANOVA) or t test. Linear correlation analysis (GraphPad InStat, GraphPad Software, Inc., San Diego, CA) and multiple linear regression analysis (SSPS; SPS Inc., Chicago, IL) were applied to determine the correlation and association between parameters. P < 0.05 was considered to be significant.

**Results**

**Renal Function and Histology Studies**

Proteinuria in NTN rats was significantly raised from day 7 onward and peaked at 364.2 ± 62.3 mg/24 h by day 45 (Table 1), whereas SCr was significantly raised from day 15 and peaked at day 30 (77.0 ± 5.2 μmol/L; Table 1). These measurements are consistent with chronic and progressive renal insufficiency. Significant evidence of GS, TA, and IF after NTN was noted from day 7 and day 15 and was progressively severe thereafter. At day 45, the scores of GS, TA, and IF were 2.73 ± 0.12 (severe GS), 500.0 ± 15.3 (tubule cells/×200 magnification field), and 2.50 ± 0.03 (moderate-severe TA and IF), respectively (Table 1).
Detection of Apoptotic Cells

Apoptotic cells with distinct morphologic motifs were counted from ISEL-stained sections and confirmed by morphology at light and electron microscopy levels. Very few apoptotic cells were noted in glomeruli (0.01 ± 0.01/glomerulus), tubules (0.01 ± 0.01%), and interstitium (0.04 ± 0.04/×400 magnification field) of normal rabbit serum-injected rats. In NTN kidneys, glomerular apoptosis was significantly

Figure 2. Positive apoptotic cells (arrows) in glomeruli (A and D), tubules (B and E), and interstitium (C and F) of NTN kidneys by ApopTag staining (A through C) and electron microscopy. Magnifications: ×200 in A through C; ×11,124 in D and E; ×8829 in F.
increased on days 7, 15, and 30 and peaked on day 7 (0.69 ± 0.15/glomerulus; Figure 1A). In the tubules and interstitium of NTN kidneys, apoptosis was significantly increased from day 15 onward, maximally on day 45 (0.83 ± 0.04% and 0.82 ± 0.10/×400 magnification field, respectively; Figure 1, B and C). Maximal areas of apoptotic cells were detected in the inflamed or sclerotic glomeruli (Figure 2A) as well as in the dilated or atrophied tubules (Figure 2B) and expanded interstitium (C) of NTN kidneys. ED1 staining positive cells in glomerulus and interstitium of nonimmune rabbit serum-injected rat kidney (D) and NTN kidneys (E and F). Magnifications: ×200 in A, B, and D; ×400 in C; ×100 in E and F.
stitium (Figure 2C). In positive control sections that were treated with DNAse I before the TdT reaction, nearly all of the cells stained, but most of the positive nuclei showed normal shape and no cytoplasmic condensation (not shown). No staining was present in the negative control sections using buffer that lacked TdT (not shown). Electron microscopy confirmed apoptotic cells with distinct morphologic motifs in some glomeruli, tubules, and interstitium (Figure 2, D through F).

Detection of Proliferation (PCNA +) and Inflammation (ED1 +)

In kidneys from nonimmune serum-injected rats, a few PCNA-positive cells were noted in glomeruli (0.03 ± 0.02/ glomerulus), tubules (0.37 ± 0.10%), and interstitium (0.09 ± 0.03/×400 magnification field; Table 1). In contrast, in NTN kidneys, PCNA staining was dramatically and significantly increased in the glomeruli throughout the experimental time...
course and peaked at day 7 (10.88 ± 1.53/glomerulus). In the tubules and interstitium, PCNA staining was elevated by day 15, peaked on day 30 (3.09 ± 0.43% and 4.40 ± 0.93/×400 magnification field), and remained raised until the end of the experiment (Table 1). Positive PCNA nuclei were localized in inflamed or sclerotic glomeruli, dilated tubules, and expanded interstitium (Figure 3, A through C).

A small number of ED1-positive cells were seen in the nonimmune serum-injected rat kidneys in glomeruli (1.04 ± 0.32/glomerulus) and interstitium (3.76 ± 0.50/×400 magnification field; Table 1, Figure 3D). ED1 staining in NTN kidneys was significantly increased in glomeruli between day 6 and Caspase-3 protein in the samples of different species. 1, Wistar Kyoto rat normal kidney; 2, Wistar Kyoto rat NTN kidney day 45; 3, Wistar rat normal kidney; 4, Wistar rat 120-d subtotal nephrectomized kidney; 5, rat proximal tubule cells; 6, rat proximal tubule cells treated with 100 μM cisplatin for 24 h; 7, normal human kidney; 8, 5 ng of recombinant caspase-3.

Figure 7. Western blot analysis for caspase-3 protein in the samples of different species. 1, Wistar Kyoto rat normal kidney; 2, Wistar Kyoto rat NTN kidney day 45; 3, Wistar rat normal kidney; 4, Wistar rat 120-d subtotal nephrectomized kidney; 5, rat proximal tubule cells; 6, rat proximal tubule cells treated with 100 μM cisplatin for 24 h; 7, normal human kidney; 8, 5 ng of recombinant caspase-3.

Figure 8. The activity of caspase-3 in kidney tissues assayed by the fluorometric measurement of AMC cleaved from a specific caspase-3 substrate. Data represent mean ± SEM. n = 4 for control and n = 7 for NTN. *, P < 0.05; **, P < 0.01 compared with the control kidney.

Double Staining for ED1 and α-SMA with Apoptosis

ED1 and ISEL double staining positive cells were found in inflamed glomeruli (Figure 4A) and tubulointerstitium (not shown). Some cells stained positively for both apoptosis (ISEL) and α-SMA in the interstitium (Figure 4B).

Expression of Caspase-3 mRNA

Northern blot analysis revealed a caspase-3 mRNA transcript at 2.7 kb transcript (Figure 5). In comparison with the control rat kidneys, the level of caspase-3 mRNA was increased at all time points, significantly so on days 7, 30, and 45 (173.3%, 228.0%, and 241.7%, respectively; P < 0.05), reaching a peak on day 45 (Figure 5).

Tissue Levels of Caspase-3 Protein

A 24 kD band possibly representing a caspase-3 active subunit was gradually and significantly increased with time in NTN kidneys compared with the controls, with maximal expression on day 45 (6.08-fold). A 32 kD band, representing the precursor of caspase-3, was found to be increased on day 45 in NTN kidneys (3.92-fold of controls; P < 0.01). The 29 kD processing intermediate was also present in all kidneys (Figure 6).

To validate antibody efficacy further, we performed Western blots using recombinant active caspase-3 protein, kidney tissue from Wistar rats with apoptosis induced by SNx (5), rat proximal tubule cells with cisplatin-induced apoptosis, and normal human renal tissue (Figure 7). The full-length caspase-3 antibody strongly bound with 12 and 17 kD recombinant caspase-3 protein. In SNx Wistar rats, the antibody reacted with both 24 kD and 17 kD bands at a greater intensity than in a control animal. In a rat proximal tubule cell line treated with cisplatin, the antibody revealed a similar upregulation of a 24 kD but not a 17 kD band. In normal human renal tissue, a 20 kD band was detectable.

Activity of Caspase-3

There was a significant increase of caspase-3 protease activity in NTN rat kidneys at all time points compared with the controls (Figure 8). On day 7, this increase was 2.12-fold in controls and dropped to 1.85-fold on day 15 before rising again on day 30 (2.29-fold) and reaching a peak on day 45 (2.38-fold). The specific and competitive tetrapeptide inhibitor of caspase-3, Ac-DEVD-CHO, almost fully inhibited the caspase-3 activity in the assays, demonstrating assay specificity.

Correlation among Renal Histology, Apoptosis, and Caspase-3

Cellular apoptosis correlated closely with inflammation and proliferation in glomeruli (r = 0.676 and 0.656), tubules (r = 0.658, no inflammation), and interstitium (r = 0.653 and 0.624) (all P < 0.01). Apoptosis also correlated with GS (r = 0.412; P < 0.05), TA, and IF (r = −0.917 and 0.917; P < 0.01) in NTN kidneys. Multiple regression analysis showed that apoptosis was more closely associated with TA and IF (Std β coefficients = −0.674 and 0.612; P < 0.01) than inflammation or proliferation and that tubular apoptosis was a better predictor of changes in SCr and proteinuria (Std β coefficients
Discussion

Nephrotic serum nephritis is a model of anti-GBM glomerulonephritis that evolves into end-stage kidney disease and eventually to CRF (2,29). The early stage is characterized by a severe necrotizing and crescentic glomerulonephritis followed by a progressive sclerosis of the glomeruli associated with TA and interstitial fibrosis (30,31). This type of nephritis is particularly severe in Wistar Kyoto rats, which are more susceptible to anti-GBM antibody than other strains (32,33). In NTN, the decrease in glomerular cellularity during the resolution of glomerular inflammation and proliferation, and progression to sclerotic scarring has previously been associated with apoptosis (2). In this study, an initial increase in glomerular cell number occurred as a result of inflammatory cell infiltration (ED1-positive cells) as well inflammatory and glomerular cell proliferation (PCNA-positive cells). This is consistent with intraglomerular proliferation of infiltrating monocytes reported in this model (34). The initial proliferative glomerulonephritis was rapidly followed by an increase in glomerular cell apoptosis. With disease progression, interstitial cell number increased, partially as a result of inflammatory cell infiltration but also of interstitial cell proliferation. Interstitial proliferation was partially paralleled by apoptosis; however, tubule cell number decreased with time, showing a strong negative correlation with tubular apoptosis. This progressive increase in glomerular and tubulointerstitial apoptosis is in contrast to cellular apoptosis in the anti-thy1.1 model of glomerulonephritis, which returns to normal level after approximately 2 wk (6). This may explain the difference in outcome between the two experimental models of glomerulonephritis with resolution of injury and healing in the anti-thy1.1 model compared with progressive TA and scarring in NTN. The comparison between these two models therefore provides strong evidence that apoptosis is an important mechanism for progressive loss of glomerular and tubule cells from a kidney destined for progressive scarring (35,36).

However, elevated apoptosis within a diseased kidney should not always be viewed as harmful. The apoptotic deletion of infiltrating neutrophils (myeloperoxidase positive) in glomerulonephritis limits neutrophil-mediated glomerular injury and may play a role in the resolution of glomerular inflammation. Failure of these mechanisms might lead to disintegration of neutrophils within the inflamed glomerulus and the development of persistent inflammation leading to scarring (37). Furthermore, the role of macrophage apoptosis may be a central regulator of the progression and resolution of macrophage-mediated tissue injury (34).

In this study, the peak in glomerular apoptosis (day 7) is likely to result from the appropriate clearance of the inflammatory cells recruited into the glomeruli during the initial immune response, as demonstrated by double staining of ISEL and ED1. The later peak of apoptosis occurring from days 30 to 45 coincides with the severe TA and interstitial injury that characterizes the late stages of this experimental model. However, determining the cellular origin of apoptotic cells is complicated by changes in cell surface proteins that occur early in apoptosis and complicating identification by double staining for interstitial inflammatory cells (ISEL and ED1 positive) or myofibroblasts (ISEL and α-SMA positive), thus using location for general identification is essential.

Given these two peaks of apoptosis, we demonstrated for the first time at the mRNA, protein, and activity levels significant increases in caspase-3 that coincide with elevated apoptosis. During the NTN time course, caspase-3 mRNA transcription was increased, particularly in the early inflammatory phase and late fibrotic phases, indicating the requirement for de novo synthesis of caspase-3 in both phases. This increase resulted in both elevated precursor and active caspase-3 protein, which were associated with a parallel change in the enzyme activity within NTN rat kidneys.

Caspase-3 is translated as an inactive 32 kD precursor that is proteolytically processed to become a functionally active enzyme (7,38–40). Activation of caspase-3 requires two proteolytic cleavage events: removal of the NH2 terminal prodomain for interstitial inflammatory cells (ISEL and ED1 positive) or myofibroblasts (ISEL and α-SMA positive), thus using location for general identification is essential.

Western blot analysis of caspase-3 in this study revealed a 24 kD band that we believe is also an active caspase-3 protein. Interestingly, this band is approximately 4 kD bigger than the recognized largest active caspase-3 (13,41) and larger still than other reported active caspase-3 proteins (7,39,40). However, other active fragments, such as 20 kD and 18 kD, have also been reported (13,41). These subfragments then heterodimerize to form the activated protease (39,40). Western blot analysis of caspase-3 in this study revealed a 24 kD band that we believe is also an active caspase-3 protein.
Furthermore, the 24 kD band was the only nonprecursor/intermediate band detectable in rat proximal tubule cells (42) when apoptosis was induced by cisplatin (43), which suggests a renal- rather than a strain-specific isof orm. Detection of this unusual caspase-3 subunit may be due to the use of this unique full-length caspase-3 antibody, rather than the more commonly used antibodies to the "active" subunits. In our hands, these antibodies, e.g., polyclonal rabbit anti-caspase-3 antibodies (Pharmingen, San Diego, CA) react with the 17 kD band in remnant kidneys from SNx Wistar rats but fail to show any active band in the NTN Wistar Kyoto rat kidneys even though activity and mRNA levels are comparable. This promotes the 24 kD band as an active caspase-3 subunit. However, without isolation and characterization of this 24 kD protein, its active status cannot be ratified.

Western blot analysis also revealed some interesting findings with regard to the 32 kD pro-caspase-3. Despite early progressive increases in activity and mRNA, there was little change in pro-caspase-3 until day 45. This suggests that all de novo pro-caspase-3 is immediately processed to the active form until this time. The accumulation of pro-caspase-3 may indicate that caspase processing becomes a limiting factor in the level of apoptosis or an attempt to prevent further increases in apoptosis despite increases in caspase-3 mRNA.

Although increased caspase-3 in this model of renal scarring is novel, it is not surprising given the well-documented role of caspases in the execution phase of apoptosis (8,44,45). Caspase-3 is potentially the most important effector enzyme in apoptosis, providing a common pathway to both death receptor- and mitochondria-dependent apoptotic mechanisms (8,46). Caspase-3 has also been linked to the pathogenesis of other models of renal injury associated with apoptosis. For instance, it was found to be upregulated at both mRNA and protein levels during reperfusion in a rat model of acute renal ischemia (13), whereas increased activity was reported after the administration of nephrotoxic doses of cyclosporin A in salt-depleted rats (14).

The pivotal role of caspase-3 in the apoptosis machinery makes it an attractive target to regulate apoptosis-related cell death. In vitro, the induction of apoptosis in mouse proximal tubule cells by cisplatin has been inhibited by Ac-Asp-Glu-Val-Asp-H, a known caspase-3 inhibitor (43). Application of this therapeutic approach in vivo is clearly more problematic, although there has been some success. For example, elevated apoptosis in hepatic parenchymal cells during endotoxemia (tumor necrosis factor-α mediated) was prevented by injection of Z-VAD, providing a caspase-3 inhibition and effective disease treatment (47). It has been also been reported that the administration of B-D-FMK (pan caspase inhibitor) was significantly caspase-3 inhibiting and neuroprotective when given by intracerebral or systemic injection after cerebral hypoxia-ischemia (16). More recent, it has been reported that Z-VAD-FMK reduced the caspase-3 activity and prevented the early onset of not only renal apoptosis but also inflammation and tissue injury in a mouse model of renal ischemia (17). Given these findings, a similar blockade of caspase-3 in progressive renal scarring may provide a novel therapeutic approach to the treatment of renal scarring by controlling inappropriate apoptosis.

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References

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