Caspase-3 and apoptosis in experimental chronic renal scarring

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Caspase-3 and apoptosis in experimental chronic renal scarring. Background. Caspase-3 is a member of the caspase enzyme family, having a central role in the execution of apoptosis. However, the significance of Caspase-3 in the inappropriate and excessive apoptosis that contributes to the progression of non-immune–mediated renal scarring has not been established.

Methods. Kidneys from sham-operated and subtotal nephrectomized (SNx) rats were harvested on days 7, 15, 30, 60, and 120 post-surgery. These were analyzed for apoptosis (in situ end labeling of DNA, light and electron microscopy), Caspase-3 activity (fluorometric substrate cleavage assay), protein and mRNA (Western and Northern blotting), as well as distribution (immunohistochemistry), inflammation (ED-1 immunohistochemistry) and fibrosis (Masson’s Trichrome staining).

Results. Apoptosis, inflammation and fibrosis gradually increased in glomeruli, tubules and interstitium of SNx rats. Caspase-3 was mainly located in damaged tubules, but also was found in some glomerular and interstitial cells. Little or no staining was noted in sham-operated kidneys. In SNx kidneys, Caspase-3 activity was significantly increased from day 30 and peaked on day 120 (2.5-fold). This resulted from increases in the 17 and 24 kD active protein subunits. The 32 kD precursor was increased at all time points (1861% on day 120, \( P < 0.01 \)). Caspase-3 changes were transcription-dependent with the 2.7 kb caspase-3 mRNA significantly increased at all time points (287% on day 120). Caspase-3 activity was a better predictor of apoptosis (Std \( \beta \) coefficient = 0.347, \( P < 0.05 \)) than Caspase-3 proteins or mRNA; however, Caspase-3 at all levels correlated with apoptosis, inflammation and fibrosis (all \( P < 0.01 \)).

Conclusions. Up-regulation of apoptosis in remnant kidneys is likely to be Caspase-3-dependent as it is associated with increases in Caspase-3 at the activity, protein and mRNA levels. Therefore, Caspase-3 is a potential therapeutic target for the modification of renal cell apoptosis and subsequently renal fibrosis.

Apoptosis plays a dual role in the evolution of renal scarring with potential beneficial and harmful influences [1]. The resolution of renal inflammatory changes depends to a large extent on the apoptosis of infiltrating inflammatory cells in addition to their migration out of the kidneys when the initial insult has subsided [2]. On the other hand, inappropriate regulation of apoptosis may lead to an ongoing proliferation of these cells within the kidneys leading to the initiating and progression of renal fibrosis [1]. The harmful side of apoptosis in relation to renal scarring pertains to the deletion by this programmed cell death process of intrinsic renal cells [3, 4]. Such a mechanism has been put forward to explain experimental progressive glomerulosclerosis [3, 4] and tubular atrophy [5] and the ensuing tubulointerstitial fibrosis.

This has been postulated in the remnant kidney model of renal scarring in rats, where ongoing deletion of glomerular cells through apoptosis was instrumental in the progression of glomerulosclerosis [3]. In the same model, we have demonstrated the progressive increase in apoptosis of tubular and interstitial cells, thus contributing to tubular atrophy and the associated renal fibrosis [6].

While it is clear that apoptosis is associated with the progression of chronic experimental and clinical renal diseases [3–6], questions remain as to the causal link and the precise mechanisms and mediators linking programmed cell death with renal fibrosis. The identification of key mediators involved in apoptosis and renal scarring may allow for therapeutic interventions based on their manipulations and aimed at inhibiting renal atrophy, fibrosis and scarring.

An increasing body of evidence suggests that caspases (cysteine proteases) play an essential role in both the regulation and execution phases of apoptotic cell death and act upstream of DNA fragmentation [7, 8]. To date, a family of 14 different caspases has been identified that play a role in both inflammation and apoptosis [8]. They are all produced as inactive precursors (zymogens) that are processed into the large and small active subunits [9]. Caspases are highly specific with an absolute requirement for cleavage after aspartic acid, while individual caspase recognize different tetrapeptide motifs, which might explain their individual substrate specificity [8]. Caspases have diverse functions, with members of this family playing a key role in the execution of apoptosis.
family playing essential roles in both initial signaling events (Caspase-8, Caspase-9) and the downstream proteolytic cleavages (Caspase-3) [7, 8]. Caspase-3 (CPP32, YAMA or apopain) is thought to be key executor of apoptosis and is activated via the mitochondrial (Bcl-2/ Bax, Caspase-9), death receptor (Fas/FasL, Caspase-8), or endoplasmic reticulum (Caspase-12) routes [7, 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 [8, 11].

We have recently observed changes of Caspase-3 in an immune-mediated glomerulonephritis model of renal scarring [12]. This suggested that Caspase-3 is associated with apoptosis, inflammation and fibrosis, and highlighted it as a potential therapeutic target for preventing renal scarring. However, there are no data concerning changes of Caspase-3 during the progression of renal scarring in non-immune–mediated chronic renal scarring. With that in mind, we have measured Caspase-3 activity, protein and mRNA levels throughout the 120-day course of renal scarring in rats submitted to extensive renal ablation, and correlated the observed changes with those of apoptosis, inflammation and fibrosis during the progression of the disease.

METHODS

Experimental animals and protocol

Male Wistar rats (Sheffield University strain) weighing 250 to 300 g were subjected to subtotal (5/6) nephrectomy (SNx). Rats were housed at constant temperature (20°C) and humidity (45%) on a 12-hour 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. Subtotal nephrectomy was undertaken in 32 rats as a one-step procedure: left 2/3 nephrectomy through the ligation and ablation of the kidney upper and lower poles as well as a right uninephrectomy [13]. Rats were sacrificed in groups (N = 4 to 6) at days 7, 15, 30, 60, 90 and 120 after SNx. Sham-operated rats (N = 29) were used as controls, being sacrificed at the same time points as those with SNx. Rats were housed in metabolic cages for 24 hours prior to sacrifice to facilitate urine collection. All the experiments were carried out according to the rules and regulations laid down by the Home Office (Animal Scientific Procedure Act 1986, UK).

Removed kidney tissue was fixed in formal calcium [4% (wt/vol) paraformaldehyde and 2% (wt/vol) calcium chloride, pH 7.4] and paraffin-embedded for histological and immunohistochemical examination. For electron microscopy, small tissue blocks were fixed in 2.5% (vol/vol) glutaraldehyde solution in phosphate buffer (pH 7.4). Snap-frozen tissues were stored in liquid nitrogen for Caspase-3 activity, protein and mRNA analyses. Serum creatinine concentration (standard autoanalyzer techniques) and 24-hour urinary protein excretion (Biuret method) were determined in each group at all time points.

Estimation of renal scarring

The extent of renal scarring following SNx was determined by two authors who were blinded to the experimental code according to a previously published arbitrary scale [14–16]. Using a ×200 magnification, sections stained with Masson’s Trichrome were scored as follows. For glomerulosclerosis, a normal glomerulus scored 0; mild glomerulosclerosis (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 was defined and scored as: normal tubules with approximately 1000 tubule cells per ×200 magnification field and no expansion of the interstitium scored 0; mild tubular atrophy (TA), with approximately 800 tubular cells per field and interstitial edema or fibrosis (IF) affecting up to 25% of the section scored 1; moderate TA with tubular cell number approximately 600 per field and IF affecting 25% to 50% of the section scored 2; and severe TA with tubular cell number approximately 400 per field, IF exceeding 50% of the section scored 3. To determine the level of tubular atrophy, tubular 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

In formal calcium-fixed and paraffin-embedded 4 μm sections, fragmented nuclear DNA 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 [6, 17]. Briefly, after deparaffinization and hydration, sections were digested by incubation with 15 μg/mL proteinase K for 15 minutes at 37°C. Endogenous peroxidase was inactivated by 2% (vol/vol) H₂O₂ in phosphate-buffered saline (PBS). The sections were then immersed in TdT reaction buffer, and incubated with TdT and digoxigenin-dUTP for 60 minutes at 37°C. The slides were transferred to stop buffer at 37°C for 30 minutes to terminate the reaction. The sections were incubated with the antidigoxigenin-peroxidase complex for 30 minutes at 37°C and developed by using the 3’-amino-9-ethylcarbazole (AEC) substrate kit (Vector Laboratories, Peterborough, UK) and counterstained with hematoxylin. For negative controls, slides were incubated in TdT buffer without TdT. For biochemically induced positive controls, slides were pre-
treated with 10 µg/mL of DNAsc I (Sigma, Poole, UK) in DNA buffer.

For each experimental animal, more than 30 glomerular cross-sections and 20 high power (×400) fields of tubulointerstitium were examined blinded to the experimental code by two authors. The number of in situ end labeling (ISEL) positive-staining nuclei per glomerulus (Gapo), per 400 tubular cells (Tapo), or per interstitial field (Iapo) was determined, respectively. ISEL of DNA, while associated with apoptosis, also can be seen in necrotic (nonspecific DNA degradation) and mitotic (transient DNA strand break) cells. To substantiate the specificity of our results, apoptosis was confirmed by light microscopic evaluation of the characteristic morphological features; only strongly positive ISEL cells with observable morphological features of apoptosis such as shrunken cells with condensed nuclei surrounded by a narrow cytoplasmic halo were counted [4, 6, 18, 19].

**Evaluation of distribution of Caspase-3 and cellular inflammation (ED-1) by immunostaining**

Localization of Caspase-3 and ED-1 (a specific monocye/macrophage marker) was performed in paraffin-embedded kidney tissues by immunohistochemistry using a standard avidin-biotin peroxidase complex technique as described previously [15]. ED-1 immunostaining was analyzed to evaluate the cellular inflammation. Sections were pretreated with 0.25% or 0.125% (wt/vol) trypsin at 37°C for 10 minutes. A polyclonal rabbit anti-human Caspase-3 antibody (PharMingen, San Diego, CA, USA) recognizing the 32 and 17 kD Caspase-3 subunits with no cross reactivity against other caspase family members (manufacturer’s specification) or a monoclonal mouse anti-rat ED1 antibody (Serotec Ltd., Oxford, UK) were diluted 1:100 or 1:50 and then applied overnight at 4°C. The extract was centrifuged at 12,000 × g for 10 minutes and supernatant was collected. A volume of supernatant equivalent to 100 µg 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 minutes before the substrate. Proteolytic cleavage of the substrates was monitored in a fluorescence microplate reader (SOFTmax PRO; Molecular Devices Corp., Sunnyvale, CA, USA) 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 calculated from the slope of the recorder trace and expressed in picomols per minute per µg of protein at 30°C.

**Double staining for both apoptosis and Caspase-3, ED-1 or α-smooth muscle actin**

Double immunohistochemical staining was undertaken on paraffin sections. ISEL was carried out as described above. Before application of the anti-digoxigenin antibody, sections were pre-incubated with blocking serum for 30 minutes, labeled with the anti Caspase-3, anti-ED-1 or anti-α-smooth muscle actin (α-SMA; monoclonal mouse anti-human α-SMA, diluted 1:250; Dako) antibodies at 4°C overnight. The α-SMA antibody was used to detect myofibroblasts typically expressing a high immunostaining for this cytoskeletal protein [20]. Sections were labeled with biotinylated secondary anti-mouse or -rabbit IgG at 37°C for 30 minutes, with alkaline phosphatase streptavidin for another 30 minutes and developed with Fast Red TR/Naphthol AS-MX solution (Sigma) to produce a bright pink color. Subsequently, anti-digoxigenin peroxidase antibody was applied to the sections and revealed by the addition of dianinobenzidine to provide positive staining as a yellow/brown color. Control sections were incubated with non-immune normal mouse IgG or normal rabbit serum in place of primary antibody and with the omission of TdT enzyme as ISEL controls.

**Detection of Caspase-3 activity in renal tissue**

The modified Fluorometric CaspACE™ Assay System (Promega, Cambridge, UK) was used to detect the activity of Caspase-3 in tissue. Kidney tissue (20 to 50 mg) from control and SNx 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 [21]. The extract was centrifuged at 12,000 × g for 10 minutes and supernatant was collected. A volume of supernatant equivalent to 100 µg 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 minutes before the substrate. Proteolytic cleavage of the substrates was monitored in a fluorescence microplate reader (SOFTmax PRO; Molecular Devices Corp., Sunnyvale, CA, USA) 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 calculated from the slope of the recorder trace and expressed in picomols per minute per µg of protein at 30°C.

**Measurement of tissue Caspase-3 protein level**

Tissue level of Caspase-3 protein was determined by immunoprobing of Western blots. Ten percent (wt/vol) tissue homogenate was prepared in the STE buffer [0.32 mol/L sucrose, 5 mmol/L Tris, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 5 mmol/L benzamidine and 20 µg/mL leupeptin], then centrifuged at 4°C, 14,000 × g for 10 minutes. Twenty micrograms of protein from the supernatant was separated on a 15% (wt/vol) poly acrylamide denaturing gel and then electro-blotted onto Hy bond-C nitrocellulose membranes (Amersham Life Science, Little Chalfont, UK). Membranes were blocked by the addition of 3% (wt/vol) bovine serum albumin.
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 micrograms of total RNA were electrophoresed on a 1% (wt/vol) agarose (N-Morpholino) propane sulfonic acid (MOPS)/formaldehyde gel. RNA was then transferred to a nylon membrane (Hybond-N, Amersham Life Science) by capillary blotting using 20 × SSC and cross-linked to the nylon filter using a UV crosslinker (Amersham Life Science) at 70 mJ/cm² energy [22, 23].

To produce a Caspase-3 cDNA probe, Caspase-3 exonic DNA was amplified from rat cDNA by the polymerase chain reaction (PCR) using the following previously published primers: 5'-sense ATGGACAACAACGAAACCTCCGTG, 3'-antisense CCACTCCCAGTCATTCCTTTAGTG [24]. Amplification reactions were performed with 100 μmol/L of each dNTP in amplification buffer (containing 1.5 mmol/L MgCl₂) and 1 unit Taq polymerase at 85°C for five minutes, before 20 picomoles of primers were added. Thirty-nine cycles of amplification were completed using the following conditions: 94°C for one minute, 48°C for one minute, 72°C for two minutes. The 850 bp PCR product was cloned into the pCR®2.1 vector (Invitrogen, UK). Following bacterial amplification and plasmid purification, the Caspase-3 insert was excised with BstXI and EcoRV, separated by electrophoresis on a 1.5% (wt/vol) agarose TAE gel and purified using Prep-A-Gene DNA Purification Systems (Bio-Rad Laboratories Ltd., Hertfordshire, UK). Product confirmation was by restriction mapping using EcoRI and KpnI [12]. Purified cDNA was then random primed with 32P-labeled dCTP (NEN, USA) using the Prime-a-Gene® Labeling System (Promega). Unincorporated label was removed using a Sephadex® G-50 NICK™ column (Pharmacia Biotech, UK).

Prehybridization and hybridization were performed using the Church buffer system (0.5 mol/L sodium phosphate and 7% SDS) at 65°C [25]. The filter was washed three times in church wash buffer (40 mmol/L sodium phosphate, 1% SDS) at 65°C for 20 minutes and then exposed to Kodak Biomax MS film for 24 hours. Autoradiographs were quantitatively analyzed by scanning volume density using a Bio-Rad GS-690 densitometer and Molecular Analyst version 4 software (Bio-Rad Laboratories Ltd.). Optical density values for Caspase-3 were corrected for loading using the housekeeping gene cyclophilin [12, 26]. Results were expressed as percentage of control sample mRNA densities. Transcript size was determined by comparison to RNA molecular weight markers (Bio-Rad Laboratories Ltd.) using the same analysis package.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). The statistical difference was assessed by a single factor variance (ANOVA) or the Student t test. Linear correlation analysis using GraphPad InStat (GraphPad Software Inc., San Diego, CA, USA) and multiple linear regression analysis using SSPS (SPSS Inc., Chicago, IL, USA) 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 SNx rats was significantly raised from day 30 (SNx 40.4 ± 8.0 mg/24 h vs. Sham 9.3 ± 0.7 mg/24 h, P < 0.05) while serum creatinine was significantly raised from day 7 (SNx 68.5 ± 5.8 μmol/L vs. Sham 33.8 ± 3.1 μmol/L). Both reached maximum levels on day 120 (proteinuria, SNx 256.8 ± 56.8 mg/24 h vs. Sham 7.4 ± 0.8 mg/24 h; serum creatinine, SNx 140.6 ± 36.5 μmol/L vs. Sham 40.0 ± 1.4 μmol/L; Table 1). Significant evidence of GS (0.22 ± 0.10), TA (936 ± 26 tubular cells per ×200 field) and IF (0.32 ± 0.13 at ×200 field) following SNx was noted from day 7 and progressively increased thereafter. Maximum changes were recorded on day 120 after SNx with GS reaching 1.86 ± 0.15, TA at 613.62 ± 47.54 tubular cells per ×200 field and an IF score of 1.93 ± 0.24 at ×200 field (Table 1). This indicated the progressive renal insufficiency and a moderate degree of renal scarring as previously documented in our earlier studies using this model [6].

Detection of apoptotic cells

Using ISEL, very few apoptotic cells were noted in the glomeruli, tubules and interstitium of sham-operated
rats. Remnant kidneys demonstrated a significant and gradual increase in positively stained nuclei in the glomeruli from day 15 (SNx 0.07 ± 0.02 vs. Sham 0.01 ± 0.01 per glomerulus), in the tubules from day 7 (SNx 1.08 ± 0.24 vs. Sham 0.09 ± 0.01 per 400 tubular cells) and in the interstitium from day 7 (SNx 0.43 ± 0.05 vs. Sham 0.17 ± 0.02 per interstitial field at ×400) until the end of the time course. Maximum changes were seen at day 120 for apoptosis in glomeruli (SNx 0.25 ± 0.04 vs. Sham 0.03 ± 0.02), tubules (SNx 2.77 ± 0.44 vs. Sham 0.06 ± 0.02 cell per 400 tubular cells) and interstitium (SNx 1.04 ± 0.25 vs. Sham 0.11 ± 0.03 cell per interstitial field at ×400; Table 1). The highest rates of apoptosis were in the sclerotic glomeruli (Fig. 1A), dilated or atrophied tubules (Fig. 1B) and expanded interstitium (Fig. 1C). In positive control sections treated with DNase I before the TdT reaction, nearly all of the cells stained, but most of positive nuclei showed normal shape, and no cytoplasmic condensation. No staining was present in the negative control sections using buffer lacking TdT (data not shown). Electron microscopy confirmed apoptotic cells with distinct morphological motifs (Fig. 2).

**Distribution of Caspase-3 and detection of cellular inflammatory ED-1**

There was no or very faint Caspase-3 immunostaining in sham-operated kidneys (Fig. 1D). In contrast, Caspase-3 staining was seen in dilated tubules (Fig. 1E), damaged glomerular capsule and a few glomerular cells (Fig. 1E), the loop of Henle (Fig. 1F) and interstitial cells (Fig. 1G) of remnant kidneys. Some Caspase-3-positive cells had the typical morphological features of apoptosis (Fig. 1G).

In kidneys from sham-operated rats, a small number of ED-1 positive cells were seen in the glomeruli (0.28 ± 0.12 per glomerulus) and interstitium (2.61 ± 0.49 per ×400 field; Fig. 1J). In contrast, ED-1 staining cells were gradually and significantly increased throughout the experimental time course in SNx kidneys with a peak on day 120 in the glomeruli (15.90 ± 3.64 per glomerulus) and interstitium (27.40 ± 4.78 per ×400 field; Table 1). ED-1+ cells were distributed in inflamed or sclerotic glomeruli (Fig. 1K), expanded interstitium (Fig. 1L) and dilated tubular lumens (Fig. 1M).

**Double staining for ED-1, α-SMA and Caspase-3 with apoptosis**

Double staining cells of Caspase-3 and ISEL were noted in glomerular cells (Fig. 1H) and atrophied tubular cells (Fig. 1I). In remnant kidneys ED-1 and ISEL double-staining, positive cells were found in inflamed interstitium (Fig. 1J) and glomeruli (not shown). Some cells stained positively for both apoptosis (ISEL) and α-SMA in the interstitium (Fig. 1O).

**Caspase-3 activity**

There was a gradual increase in Caspase-3 protease activity in SNx rat kidneys at all time points compared with the controls reaching significance by day 30 and reaching a peak on day 120 (2.5-fold of control; Fig. 3). 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 (data not shown).

**Tissue level of Caspase-3 protein**

Western blot analysis showed a considerable variation in Caspase-3 levels in animals from the same experimental groups in this study (Fig. 4). However, the magnitude of the observed changes was statistically significant. The 17 kD Caspase-3 active subunit was significantly increased from day 30 onwards in SNx kidneys with maxi-
mal expression on day 120 (1942% of control). A 24 kD band also representing a Caspase-3 active subunit was gradually and significantly increased at all time points, reaching a peak on day 120 (921% of control). The 29 kD Caspase-3 processing intermediate was present in all kidneys throughout the time course. The 32 kD precursor of Caspase-3 was significantly increased as early as day 7 (747%), peaked on day 60 (2704%), and then dropped with time until day 120 (1861% of control). This was in contrast with the continuous increase of both 17 kD and 24 kD active subunits (Fig. 4).

To validate antibody reactivity, Western blots were performed utilizing recombinant active Caspase-3 protein. The full length Caspase-3 antibody strongly bound with 12 and 17 kD recombinant Caspase-3 proteins (Fig. 4).

**Expression of Caspase-3 mRNA**

Northern blot analysis revealed the expression of a Caspase-3 mRNA transcript at 2.7 kb (Fig. 5). In comparison with the control rat kidneys, the level of Caspase-3 mRNA was significantly increased at all time points, and reaching a peak at day 120 (287% of control; Fig. 5).

**Correlation between apoptosis, inflammation, fibrosis and Caspase-3**

Cellular apoptosis in glomeruli, tubules and interstitium closely correlated with inflammation (r = 0.403, 0.820 and 0.732, P < 0.01), as well as with GS, TA and IF (r = 0.871, -0.873 and 0.773, P < 0.01 [28]). Cellular inflammation also positively correlated with GS, TA and IF (r = 0.679, -0.698 and 0.698, P < 0.01). Multiple regression analysis showed that apoptosis was more closely associated with GS and TA (Std β coefficients = 0.531 and -0.723, respectively, P < 0.01) than inflammation or proliferation and interstitial inflammation was more closely related with IF (Std β coefficients = 0.458, P < 0.01) than apoptosis and proliferation (refer previous data [6]).

There were close associations between the expression of Caspase-3 activity and proteins (17 kD, r = 0.512 and 24 kD, r = 0.440, P < 0.01; and 32 kD, r = 0.302, P < 0.05); proteins and mRNA (17 kD, r = 0.634; 24 kD, r = 0.637; and 32 kD, r = 0.583, all P < 0.01); and activity and mRNA (r = 0.698, P < 0.01). The Caspase-3 at different levels positively correlated with overall apoptosis (activity, r = 0.589, 17 kD, r = 0.530; 24 kD, r = 0.573; and 32 kD, r = 0.577; and mRNA, r = 0.642, all P < 0.01), inflammation (activity, r = 0.722; 17 kD, r = 0.642; 24 kD, r = 0.621; and 32 kD, r = 0.544; and mRNA, r = 0.805, all P < 0.01) and fibrosis (activity, r = 0.728; 17 kD, r = 0.679; 24 kD, r = 0.711; and 32 kD, r = 0.589; and mRNA, r = 0.818, all P < 0.01). Among Caspase-3 activity, protein and mRNA, multiple regression analysis showed that Caspase-3 activity was the best predictor of apoptosis (Std β coefficient = 0.347, P < 0.05) and Caspase-3 mRNA was a better predictor of inflammation and fibrosis (Std β coefficient = 0.435 and 0.394 respectively, P < 0.01).

**DISCUSSION**

Subtotal nephrectomy in rats is a non-immune mediated experimental model of chronic renal scarring [27]. Previously we described a progressive and sustained increase in the number of apoptotic cells in the glomeruli, tubules and interstitium in this model, with maximal apoptosis detected in sclerotic glomeruli, atrophied tubules and expanded interstitium [6]. Further studies in this model have demonstrated that apoptosis is likely to be influenced by the interplay between Bax (pro-apoptotic antigen) and Bcl-2 (anti-apoptotic antigen) with changes at both the mRNA and protein levels [28]. An increase in Bax coupled with a decreased level of Bcl-2 was shown to have strong associations with the changes in apoptosis and the progression of renal scarring [28]. In this study, we extended our observations to encompass Caspase-3, which is thought to be a key enzyme for the execution of the apoptotic program [7, 8]. There is a clear up-regulation of Caspase-3 activity that is dependent on changes not only at the translation and transcription levels, but also by post-translational modification of the latent precursor. Multiple regression analysis demonstrated that Caspase-3 activity was the best predictor of apoptosis and strongly correlated with its protein and mRNA levels. Furthermore, we have highlighted changes in Caspase-3 that are consistent with variations in apoptosis, inflammation and fibrosis over the time course.

The caspase enzyme family, and in particular Caspase-3, has a central role in the execution of apoptosis that results in the phenotype of apoptosis. Caspase-3 is translated as an inactive 32 kD precursor that is proteolytically processed to become a functionally active enzyme [29–32].

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_Fig. 1. (A–C) Positive apoptotic cells (indicated by arrows) in glomeruli (A, ×400), tubules (B, ×200) and interstitium (C, ×400) of subtotally nephrectomized (SNx) kidneys by ApopTag staining. (D–I) Caspase-3 immunostaining in sham-operated (D, ×200) and remnant kidneys (E–G) within the dilated tubules (E, ×200; G, ×400), glomerulus (E), loops of Henle (F, ×200) and interstitial cells (G). (H and I) Double staining of Caspase-3 (bright pink) with ISEL (brown) in the glomerulus (H, ×800) and atrophied tubule (I, ×800) in the remnant kidney with dark brown indicating composite staining. (J–M) ED-1 positive cells in the glomerulus and interstitium of sham-operated rat kidney (J, ×200), and in glomeruli, interstitium (K and L, ×100) and tubular lumens of remnant kidneys (M, ×200). (N and O) Double staining of ISEL (brown) with ED-1 (bright pink) (N, ×400) and α-smooth muscle actin (α-SMA; bright pink; O, ×1000) in the interstitium of SNx kidneys._
Activation of Caspase-3 requires two proteolytic cleavage events. Removal of the NH$_2$ terminal pro-domain generating a 29 kD processing intermediate that is subsequently cleaved into 17 kD and 11 kD or 12 kD subfragments [30–32]. However, other active fragments, such as 24, 20 and 18 kD, have also been reported [12, 24, 33]. These subfragments then heterodimerize to form the activated protease [31, 32].

In this SNx model, Western blot analysis of remnant kidneys showed significant increases in both the 17 kD and 24 kD subunits representing active Caspase-3. The appearance of the active Caspase-3 fragments correlated well with the changes in enzyme activity. However, while elevated, Caspase-3 mRNA did not increase in line with the Caspase-3 activity. Thus, the changes in activity due to the increases in 17 kD and 24 kD proteins would appear to be a consequence of changes in level of activation of the 32 kD Caspase-3 precursor, rather than solely down to an increased transcriptional rate. This also explains the decrease in 32 kD protein at the latter time points in spite of a continuing up-regulation of the mRNA levels, suggesting this de novo Caspase-3 precursor is immediately processed to the smaller active forms when apoptosis is at its highest. Furthermore, the levels of 32 kD Caspase-3 protein are not wholly consistent with the changes of Caspase-3 mRNA level at earlier time points. The steadily increasing levels of the 32 kD protein up to day 60 in comparison to the static elevation in mRNA level indicates that changes in mRNA stability or alteration to the rate of translation also may be important in Caspase-3 production.

Immunolocalization of Caspase-3 in rat kidneys has previously proved difficult with a few studies restricted to analysis of human tissues [34, 35]. Here for the first time, we have been able to localize Caspase-3 in paraffin-embedded rat kidney by pretreating sections with a high concentration of trypsin and applying an anti-human full-length Caspase-3 antibody. This revealed no or very faint Caspase-3 positive immunostaining in sham-operated kidneys. Given the substantial levels of Caspase-3 protein and mRNA detectable in normal kidneys (assumption made on band development time in Western and Northern blotting analysis) this would be consistent with a low expression of Caspase-3 in many cells falling below the threshold for immunohistochemical detection. In contrast within remnant kidneys, there was a strong Caspase-3 staining especially in damaged tubules, but also within some glomerular and interstitial cells. This staining pattern is consistent with that of ISEL, which in combination with double staining provides direct evidence for the involvement of Caspase-3 in apoptosis in this model of renal scarring. While the location allows identification of tubular cells as producers of Caspase-3, it is not directly evident from our study that cell types in the glomeruli and interstitium have elevated Caspase-3. These may be
Fig. 3. Activity of Caspase-3 in kidney tissues assayed by the fluorometric measurement of AMC cleaved from a specific Caspase-3 substrate. Symbols are: (□) control; (■) SNx. Data represent mean ± SEM. N = 4 for control and N = 6 for SNx. **P < 0.01; ***P < 0.001 compared with the control kidneys.

Fig. 4. Western blot analysis for Caspase-3 protein in SNx kidneys on day 7, 15, 30, 60, 90 and 120. A 17 kD band and a 24 kD band, representing the Caspase-3 active subunit; a 29 kD, representing processing intermediate of Caspase-3; a 32 kD band, representing the precursor of Caspase-3. Rec3: 5 ng recombinant Caspase-3. Symbols are: (□) control; (■) 17 kD; (□) control; (■) 24 kD; (□) control; (■) 32 kD. Data represent the mean percentage change in volume density compared to the average control value (mean ± SEM). N = 4 for control, N = 5 for SNx. *P<0.05; **P<0.01 compared with the control kidneys.
either resident renal cells or inflammatory cells. Double staining for cell markers appears to be a solution, although markers such as ED-1 and α-SMA are rapidly lost once the apoptotic pathway has commenced. Given that most Caspase-3 positive cells are likely to be at some stage in the apoptotic program and requirement of strong trypsinization for Caspase-3 immunostaining, then the co-localization of a specific cell marker with Caspase-3 has obvious problems. However, we have had some success double-staining ISEL-positive cells with ED-1 or α-SMA, which by inference suggests that most cells expressing Caspase-3 would be monocytes or myofibroblasts in the glomeruli and interstitium.

Other immunohistochemical studies using the same Caspase-3 antibody in normal human kidneys showed strong positive staining in renal tubule epithelium with little or no Caspase-3 immunoreactivity in the glomeruli [35]. The higher tubular staining in normal human kidneys may well represent differences between species; however, it equally could be due to greater sensitivity of the antibody for human than rat Caspase-3. Studies describing changes in Caspase-3 staining in human scarred tissue remain to be performed. However, when this is done it will be interesting to note if the considerable Caspase-3 staining within the glomeruli and interstitium seen in this experimental model also are evident, as they undoubtedly contribute significantly to the Caspase-3 pool.

The predominantly Caspase-3 tubular epithelial staining pattern combined with the positive double staining of Caspase-3 and ISEL clearly implicates Caspase-3 in tubular cell apoptosis and tubular atrophy. Caspase-3 also has been reported to be involved in the pathogenesis of other renal injury models associated with apoptosis. For instance, it was found to be up-regulated at both mRNA and total protein levels during reperfusion in a rat model of acute renal ischaemia [24]. In addition, increased Caspase-3 activity was reported following the administration of nephrotoxic doses of cyclosporine A in salt-depleted rats [36].

The staining profile of Caspase-3 reported here is similar to that reported for Bax in remnant kidneys, especially in dilated tubules, sclerotic glomeruli and fibrotic interstitial areas [28]. This indicates that Bax and Caspase-3 may have a coordinating role in the processing of apoptotic cell death. Caspase-3 is potentially the most important effector enzyme in apoptosis, providing a common pathway for death receptor (Fas/FasL), mitochondria-dependent (Bax/Bcl-2 related) or endoplasmic reticulum-mediated apoptosis [7, 10, 37]. In light of our previous findings of changes in the Bax/Bcl-2 ratio in this model [28], it seems likely that Caspase-3 activation may be linked with the changes of Bax/Bcl-2.

While our results clearly implicate Caspase-3 in apoptosis associated with progressive renal scarring, it is important to note that Caspase-3 also is involved in the inflammation as indicated by the positive correlation between the Caspase-3 activity, proteins, mRNA and cellular inflammation in remnant kidneys. While the remnant kidney model of renal scarring is not initiated by an immune response, the progression of fibrosis has been associated with the severity of the late interstitial inflammatory infiltrate [27, 38]. This was supported in our study by the strong association between the severity of the monocytic interstitial infiltrate and interstitial fibro-

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**Fig. 5.** Tissue level of Caspase-3 mRNA by Northern blot analysis in SNx rat kidneys. Autoradiographs at the top show 2.7 kb Caspase-3 mRNA transcript and the bottom, cyclophilin 1.8 kb transcript. The histogram shows the volume density analysis of autoradiographs corrected for loading using cyclophilin. Data represent the mean percentage change in volume density compared to the average control value (mean ± SEM). N = 4 for control and N = 6 for SNx. *P < 0.05; **P < 0.01 compared with the control kidneys.
sis. In addition, we noted that this inflammatory monocytic (ED-1+) infiltrate paralleled the gradual increase of apoptosis in glomeruli, tubules and interstitium. Such a persistent inflammation may favor a microenvironment for uncontrolled apoptosis of renal cells. This not only may be due to the direct action of various inflammatory cells to instigate apoptosis through cell to cell contact [39], but also due to the inevitable changes in cytokines and growth factors that accompany inflammatory cells. Many of these, such as transforming growth factor-β1 [40–41] and tumor necrosis factor-α (TNF-α) [42], are highly influential on apoptotic rates.

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 it has met with some success despite fears relating to the potential tumorigenic consequences of inhibiting apoptosis. For example, elevated apoptosis of hepatic parenchymal cells during endotoxemia (TNF-α mediated) was prevented by injection of Z-VAD, a strong Caspase-3 inhibitor [44]. It has been also been reported that the administration of B-D-FMK (a pan caspase inhibitor) was neuro-protective when given by intra-cerebral or systemic injection after cerebral hypoxia-ischemia [45]. More recently, it has been reported 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 [46]. 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 of renal cells.

In conclusion, we have demonstrated, to our knowledge for the first time, significant increases in Caspase-3 at the activity, protein and mRNA levels, which coincide with elevated apoptosis in a non-immune mediated chronic renal fibrosis model. During the SNx time course, the increase of Caspase-3 activity was associated with the elevated precursor and active Caspase-3 proteins, which resulted from the increase of Caspase-3 mRNA transcription indicating the requirement for de novo synthesis of Caspase-3. Caspase-3 activity was a good predictor of apoptosis occurrence associated with GS and TA. The manipulation of Caspase-3 could therefore be a therapeutic target for prevention renal cell deletion by uncontrolled apoptosis and the subsequent renal fibrosis.

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