Cellular apoptosis and proliferation in experimental renal fibrosis

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Abstract

Background. The progression of chronic renal failure (CRF) is associated with the progressive deletion of renal cells along with the fibrosis of the kidney. We have studied the role of programmed cell death (apoptosis) in the progression of experimental CRF and renal scarring.

Methods. The sub-total (5/6th) nephrectomy (SNx) model of CRF was studied in adult male Wistar rats, with renal tissue collected from experimental and control animals on days 7, 15, 30, 60, 90, and 120 post SNx (n = 6 per group). These were examined for morphological signs of apoptosis by light and electron microscopy. Further, we stained the nuclear chromatin by the acridine orange fluorescent method and detected signs of DNA cleavage by endonucleases via the principal of TUNEL staining (ApopTag™). Rates of cellular proliferation were measured simultaneously by immunohistochemical staining for the proliferating cell nuclear antigen (PCNA). In addition, cell division was monitored by counting of morphologically mitotic motifs detectable by light microscopy.

Results. Progressive renal insufficiency associated with glomerulosclerosis and tubulointerstitial fibrosis took place in the majority of SNx rats. In these animals, we noted a marked and progressive increase in the number of apoptotic glomerular, tubular as well as interstitial cells. The most significant apoptotic changes were seen in the tubules of remnant kidneys peaking at day 120 post-SNx. At this stage, the increase in apoptosis compared to controls was 10.33 ± 2.67 (M ± SEM) fold for glomerular cells (P ≤ 0.006), 26.20 ± 4.56 fold for tubular cells (P < 0.0001) and 4.66 ± 0.81 fold for interstitial cells (P ≤ 0.001). Parallel changes in the number of PCNA positive renal cells were observed. Maximal PCNA staining was seen at day 120 when the increase with respect to controls was 14.00 ± 4.93 fold (P ≤ 0.05) for glomerular cells, 60.01 ± 12.20 fold (P ≤ 0.05) for tubular cells and 28.59 ± 4.45 fold (P ≤ 0.05) for interstitial cells. As expected, the number of cells undergoing division and detectable by conventional light microscopy was lower at any time point to those expressing PCNA. We also observed a close correlation between the severity of tubular atrophy and tubulointerstitial fibrosis with the rate of tubular apoptosis (r = 0.970, R² = 0.941, P ≤ 0.001).

Conclusions. We have shown a time-dependent increase in apoptosis and PCNA antigen positive staining in the sub-total nephrectomy model of chronic renal failure correlating with the progression of renal fibrosis. PCNA staining did not match analysis for mitosis and was considered to overestimate the number with renal tissue collected from experimental and control animals on days 7, 15, 30, 60, 90, and 120 post SNx (n = 6 per group). These were examined for morphological signs of apoptosis by light and electron microscopy. Further, we stained the nuclear chromatin by the acridine orange fluorescent method and detected signs of DNA cleavage by endonucleases via the principal of TUNEL staining (ApopTag™). Rates of cellular proliferation were measured simultaneously by immunohistochemical staining for the proliferating cell nuclear antigen (PCNA). In addition, cell division was monitored by counting of morphologically mitotic motifs detectable by light microscopy.

Key words: apoptosis; subtotal nephrectomy; cellular proliferation; glomerulosclerosis; tubulointerstitial fibrosis; chronic renal failure

Introduction

The relentless progression of many renal diseases results in end-stage renal failure, independently of the initial pathogenic mechanism. The progression of chronic renal failure (CRF) and the underlying renal scarring process, involving glomerulosclerosis and tubulointerstitial fibrosis, continue to baffle both nephrologists and scientists alike [1]. Typically, the early stages of renal injury involve compensatory renal growth associated with cellular hypertrophy and hyperplasia [2]. However, the infiltration of the glomeruli and interstitium by platelets, lymphocytes and monocytes also appears to contribute to the progression of renal scarring [3, 4]. Later on, as the scarring process evolves it is characterized by a progressive deletion of glomerular and tubular cells. This leads ultimately to...
glomerular obsolescence and tubular atrophy. With end-stage renal disease, renal cells are replaced by fibrous tissue contributing to the sclerotic changes observed in the glomeruli and the interstitium [3,4]. The transition from renal growth and hypercellularity to cell deletion and atrophy raises the question as to which process is responsible for cell loss.

Apoptosis is a likely mechanism involved in the progressive loss of renal cells during the course of renal fibrosis. Apoptosis is a particular type of cell death, which has several distinguishing features from necrosis, and is often referred to as physiological or programmed cell death [5]. In apoptotic cells, chromatin is digested by a non-lysosomal Ca$^{2+}$ and Mg$^{2+}$-dependent endonuclease into oligonucleosome length fragments [5]. Typical morphological changes are observed during this process, including condensation and fragmentation of the nucleus and condensation of the cytoplasm. Apoptotic cells are rapidly eliminated via phagocytosis by neighbouring cells and macrophages in the absence of a prolonged inflammatory response [5,6]. Therefore, it is regarded as a cell clearance mechanism of beneficial value in the resolution of inflammation but potentially harmful when it involves viable tissues.

Apoptosis is thought to play an important role in the regulation of renal cell number in both healthy and diseased kidneys [7]. Efficient deletion by apoptosis of excessive, damaged or non-functioning renal cells and infiltrating inflammatory cells is beneficial for the resolution of glomerulonephritis [7]. This is the case in the anti-thy 1.1 model of glomerulonephritis where the resolution of mesangial proliferation relies on the apoptotic deletion of mesangial cells [8], being the major cell clearance mechanism counter-balancing cell division [8]. During the progression of experimental crescentic glomerulonephritis to end-stage renal failure, apoptosis plays an essential role in the resolution of intra- and extraglomerular inflammation by the elimination of cell membrane compromised cells [9]. However, this model also indicates that cell loss by apoptosis may be detrimental. Furthermore, apoptosis has been implicated in the progressive renal atrophy that follows experimental obstruction [10]. Indeed, an increased number of apoptotic glomerular cells has been described during the development of glomerulosclerosis in rats submitted to extensive renal ablation [11]. Nevertheless this study was short in duration and did not fully explore the consequences of measured apoptosis for tissue kinetics in remnant kidneys. In humans, apoptosis has been described in a wide range of glomerulonephritides [7] as well as in patients with polycystic kidney disease [12] and glomerulosclerosis [11], but the significance of these observations remains uncertain.

In this study, we intended to define the progressive changes in cellular apoptosis and proliferation during the different stages of experimental renal scarring in rats submitted to subtotal nephrectomy. We attempted to correlate these changes with the functional and morphological changes that characterize this model of progressive renal insufficiency; namely glomerulosclerosis and tubulointerstitial fibrosis. Finally, we speculated as to the relevance of apoptosis to the progression of experimental renal scarring.

Methods

Experimental design

Male Wistar rats (University of Sheffield strain) weighing 300–400 g were studied. They were housed at constant temperature and humidity on a 12 h light/dark cycle. These rats were fed ad libitum on standard laboratory rat chow (Lab Sure Ltd, March, Cambridge, UK) and had free access to tap water. All the experiments were carried out according to the rules and regulations laid down by the Home Office (Animal Scientific Procedure Act 1986, UK). Subtotal nephrectomy (SNx) was undertaken in 32 rats as a one-step procedure: left 2/3 nephrectomy through the ligation and ablation of kidney upper and lower poles as well as a right uninephrectomy [13]. Sham-operated rats (n=29) were used as controls. Rats were sacrificed in groups (n=4 controls, 6 experimental) 7, 15, 30, 60, 90, and 120 days after SNx. Sham animals were sacrificed on the same time points with SNx.

Serum and urine analysis for renal function

Serum creatinine concentration (standard autoanalyser techniques) and 24-h urinary protein excretion (Biuret method) were determined in each group, 24 h prior to sacrifice at all time points.

Histological and microscopical preparation of tissues

At sacrifice, excised kidney tissue was fixed in formol calcium (4% (w/v) paraformaldehyde and 2% (w/v) calcium chloride, pH 7.4) and paraffin-embedded for histological and immunohistochemical examination. For electron microscopic study, small blocks of tissue 1 mm$^2$ were fixed in 2.5% (w/v) electron microscopy grade glutaraldehyde solution in phosphate-buffered saline (PBS, pH 7.4). Acridine orange staining to confirm apoptotic chromatin cleavage. Tissue sections were dewaxed and deparaffinized. This was followed by the pre-treatment of sections with RNase A (100 µg/ml) for 15 min at 37°C to degrade any contaminating RNA which may cause non-specific staining. The sections were washed twice with PBS then stained with acridine orange (10 µg/ml) for 5 min at room temperature. After staining, the sections were washed twice with PBS then rehydrated and cleared through graded alcohol and xylene. Sections were mounted with DPX and examined microscopically under ultraviolet epifluorescence. Normal cells have brightly stained spherical nuclei while apoptotic cells stained with an irregular pattern of acridine orange uptake which characterizes cleaved DNA and the condensed chromatin normally attributed to apoptosis [8]. This method was used as a second qualitative proof of apoptosis (results not shown) in conjunction with electron microscopy.

Estimation of renal scarring

The extent of renal scarring following SNx was determined blindly according to a previously published arbitrary scale
normal glomerulus (score=0); mild segmental glomerulosclerosis affecting ≤25% of the glomerular tuft (score=1); moderate glomerulosclerosis affecting 25–50% of the tuft (score=2) and severe glomerulosclerosis affecting >50% of the tuft (score=3). Similarly, damaged tubulointerstitium were scored separately from normal ones. Normal tubulointerstitium (score=0); mild tubular atrophy and interstitial oedema or fibrosis affecting up to 25% of an objective field at 200 magnification (score=1); moderate tubulointerstitial fibrosis affecting 25–50% of a given field (score=2); severe tubulointerstitial fibrosis >50% of a field (score=3).

For this, sections stained with H&E, periodic acid-Schiff (PAS) or Masson’s trichrome (MT) stain were examined. These were scored (AM EN) blinded to status of each sample, using an Olympus BH-2 (Olympus, UK) light microscope with a ×20 flat field objective. The data was collected from a minimum series of 12 randomly selected fields in the cortex, or such number of fields until 20 glomeruli had been counted. The mean of the glomerular and tubulointerstitial scores constituted the global score for individual kidneys/ remnant kidneys.

In situ end-labeling for the detection of apoptotic cells

In histological sections, fragmented nuclear DNA associated with apoptosis were labeled in situ with digoxigenin-deoxyuridine (dUTP), introduced by terminal deoxynucleotidyl transferase (TdT), according to standard methods [16] using ApopTag™ Plus, peroxidase kit (Appligene, Oncor, France). After deparaffinization and dehydration, tissue sections (4 μm thick) were digested by incubation with 15 μg/ml proteinase K for 15 min at 37°C to enable the enzymatic permeabilization of the section for even incorporation of nucleotides, and then washed in distilled water four times for 2 min each wash. Endogenous peroxidase was inactivated by 2% (v/v) H2O2 in PBS for 5 min at room temperature. The sections were rinsed with PBS, immersed in TdT labeling buffer at 37°C for 15 min, and then incubated with TdT and digoxigenin-dUTP at 37°C for 60 min. The reaction was terminated by transferring the slides to ‘stop’ buffer at 37°C for 30 min. The sections were washed in PBS three times for 5 min each wash. The slides were then incubated with the anti-digoxigenin-peroxidase complex for 30 min at 37°C and then rinsed in PBS three times for 5 min each wash. The slides were developed by using 3-amin-9-ethylcarbazole (AEC) substrate kit (Vector Laboratories). For negative controls, some slides were incubated in TdT buffer without TdT. For biochemically induced positive controls, some slides were pre-treated with 10 μg/ml of DNAse I (Sigma, UK) in DNA buffer. For physiological positive controls, sections of rat mammary gland or pig small intestine were studied. These controls were subsequently subjected to the rest of the same procedure.

Immunolocalization of proliferating cells, expressing PCNA

Proliferating cells were identified in formal calcium-fixed, paraffin-embedded, kidney tissue by the detection of proliferating cell nuclear antigen (PCNA) cyclin polypeptide through immunohistochemistry, using a standard avidin-biotin immunoperoxidase complex technique. Briefly, 4 μm sections were dewaxed, dehydrated and stained as described previously [13]. After the quenching of the endogenous peroxidase activity, sections were incubated with the primary antibody (monoclonal mouse anti-human PCNA [clone PC 10] 1:50, Dako, UK) overnight at 4°C in a humid atmosphere. Thereafter, the sections were stained immunohistochemically with a commercial avidin-biotin-peroxidase procedure using a commercially available kit (ABC Elite, Vector Laboratories, Peterborough, UK). AEC was used as the substrate. Finally, sections were counter stained with dilute haematoxylin and mounted in Glycergel (Dako Patts, High Wycombe, UK). Control sections were incubated with non-immune normal mouse γ-globulin at the comparative protein concentration.

Double staining for both apoptosis and PCNA

The hypothesized link between those cells which express PCNA but exit the cell cycle prior to completion, in favor of entering apoptosis has been investigated by double staining of fixed tissue sections for both TUNEL and PCNA positive cells. Briefly the method was as follows. Tissue sections were dewaxed and dehydrated according to standard protocols, endogenous peroxidase was blocked with hydrogen peroxide in methanol (3% [v/v]) followed by the blocking of endogenous alkaline phosphatase with levamisole in PBS (2.5 mg/10 ml). Sections were then digested with proteinase K, as in the ApopTag method. A biotinylated anti-PCNA monoclonal antibody (clone PC10, Pharmingen) was applied overnight at 4°C at a concentration of 1:200. After removal of unbound anti-PCNA antibody, the sections were taken through the ApopTag protocol form equilibration buffer to anti-digoxigenin-peroxidase antibody. Development of the anti-PCNA antibody was achieved by application of streptavidin alkaline phosphatase (Vector Laboratories) followed by the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BICIP/NBT, substrate tablets Boehringer Mannheim). Development of the anti-digoxigenin antibody used the AEC method as described earlier. Control sections were incubated with non-immune normal mouse γ-globulin in place of anti-PCNA antibody (at a comparative protein concentration) and with the omission of TdT enzyme as apoptosis controls.

Counts of mitotic (H&E), apoptotic (TUNEL) and PCNA positive cells

In rat kidney tissue, more than 30 glomerular cross-sections and 20 high power (×400 stage objective) fields of tubulointerstitium were examined from all animals in each time point (GLT and BY independently counted all sections, both blinded to the status of each sample). Those cells with distinct mitotic motifs, showing signs of chromosome separation and chiasma formation were counted in 20 random fields of tubulointerstitium at × 400 magnification.

The number of TUNEL positive-staining nuclei (red) per glomerulus was determined to be the glomerular apoptotic cell score. In tubules, the number of positive-staining nuclei per 400 tubular cells was regarded as the tubular apoptotic cell score. As to the interstitium, the number of positive nuclei per field was evaluated as the interstitial apoptotic cell score. Only cells with observable morphological features of apoptosis were counted as apoptotic cells. Typically condensed nuclei with irregular cell shape and intact cell membranes. Discrete apoptotic bodies comprising a large dense nuclear fragment surrounded by a narrow cytoplasmic halo, if found isolated, were given a single count; clusters of small apoptotic bodies were also given a single count. This retrospective counting method could not establish whether two small apoptotic bodies located near each other were in...
fact from two discrete apoptotic cells or fragments from a single apoptotic cell, thus it is feasible that our results may have been an underestimate of total apoptotic cell numbers. The corresponding score of positive PCNA cells were calculated in the same manner. Double stained sections were used for qualitative evidence of those cells which were both PCNA positive and TUNEL positive; actual counts were made on the single stained sections.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). The statistical difference was assessed by a single factor variance (ANOVA). Linear regression analysis was applied to determine both the correlation values (r) and goodness of fit (R^2) between parameters. A P value ≤ 0.05 was considered to be significant. Multiple regression analysis (RGRESS) and a test of individual predictor significance within any group of predictors (BREG), was carried out using Minitab version 8.2, this allowed individual parameters with high significance to be identified for progression of glomerulosclerosis and tubulointerstitial fibrosis.

Results

Renal function studies

Serum creatinine (Cr) concentrations were significantly raised from day 7 onward and gradually increased until the end of the experiment, which is consistent with chronic and progressive renal failure (Table 1). Some SNx rats showed signs of proteinuria on day 7, and all rats showed significant proteinuria after day 30 (Table 1). In these animals, urinary protein levels continued to increase until the end of the experimental period of 120 days. All rats survived until the end of the experimental period (day 120).

Development of renal fibrosis in SNx rats

Significant evidence of glomerulosclerosis and tubulointerstitial fibrosis after subtotal nephrectomy were noted from day 15 onward, and progressively increased thereafter (Table 1). The morphological appearance of glomerulosclerosis can be seen displayed on Figure 7a absissa and tubulointerstitial fibrosis on Figure 7b absissa. By day 120, the score of glomerulosclerosis and the score of tubulointerstitial fibrosis were 1.86 ± 0.15 and 1.93 ± 0.24, respectively. This suggests that by the end of the experiment the renal scarring was moderate in severity. This is supported by the degree of renal functional impairment (Table 1). We also noted a progressive tubular atrophy affecting the remnant kidneys throughout the course of the experiment.

Light and electron microscopy detection of apoptosis in the remnant kidney

The apoptotic cells containing condensed chromatin in glomeruli, tubules and interstitium were identified by light and electron microscopy (Figure 1a, b) thus confirming the true nature of these changes. Counts of cells with distinct morphological motifs of both apoptosis and the final stages of mitosis were made from the H&E stained sections. Figure 2 shows two inset plates of the typical apoptotic and mitotic morphology seen on H&E stained sections, combined with graphical representation of the results. It can be seen from these data that the numbers of apoptotic cells rise steadily from day 30 post SNx, whereas the numbers of late stage mitotic cells remains more constant. Evidently, not all those cells expressing PCNA are traversing the cell cycle to completion. This was confirmed later by double staining for both apoptosis and PCNA.

Table 1. The time course of changes in renal function and progressive fibrosis in rats submitted to subtotal nephrectomy (SNx) and their sham-operated controls (n = 4 for controls, 6 for experimental, M ± SEM)

<table>
<thead>
<tr>
<th>Time</th>
<th>Serum creatinine (μmol/l)</th>
<th>Urine protein (mg/24 h)</th>
<th>Glomerulo sclerosis (Score 0–3)</th>
<th>Tubulointerstitial fibrosis (Score 0–3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNx animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>68.5 ± 5.8,a</td>
<td>16.9 ± 4.5,a</td>
<td>0.22 ± 0.10,b</td>
<td>0.32 ± 0.13,b</td>
</tr>
<tr>
<td>Day 15</td>
<td>84.2 ± 7.9,a</td>
<td>16.7 ± 2.8</td>
<td>0.18 ± 0.06,b</td>
<td>0.28 ± 0.06,a</td>
</tr>
<tr>
<td>Day 30</td>
<td>78.7 ± 6.4,a</td>
<td>40.4 ± 8.0,a</td>
<td>0.34 ± 0.05,b</td>
<td>0.54 ± 0.06,a</td>
</tr>
<tr>
<td>Day 60</td>
<td>107.4 ± 28.5</td>
<td>131.1 ± 23.0</td>
<td>0.73 ± 0.13,b</td>
<td>1.18 ± 0.25,a</td>
</tr>
<tr>
<td>Day 90</td>
<td>111.2 ± 19.9,a</td>
<td>234.1 ± 45.5,a</td>
<td>1.67 ± 0.16,a</td>
<td>1.73 ± 0.20,a</td>
</tr>
<tr>
<td>Day 120</td>
<td>140.6 ± 36.5,a</td>
<td>256.8 ± 56.8,a</td>
<td>1.86 ± 0.15,a</td>
<td>1.93 ± 0.24,a</td>
</tr>
<tr>
<td>Control animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>33.8 ± 3.1</td>
<td>6.5 ± 0.7</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Day 15</td>
<td>42.6 ± 2.4</td>
<td>9.3 ± 2.7</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Day 30</td>
<td>53.8 ± 4.2</td>
<td>9.3 ± 0.7</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Day 60</td>
<td>42.5 ± 1.8</td>
<td>7.7 ± 2.1</td>
<td>n.d.</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>Day 90</td>
<td>53.4 ± 5.6</td>
<td>9.9 ± 1.1</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Day 120</td>
<td>40.0 ± 1.4</td>
<td>7.4 ± 0.8</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Statistical significance when compared to respective control. (...P ≤ 0.05, ...P ≤ 0.01, ...P ≤ 0.005 and n.d. = not detectable).
Fig. 1. Electron micrographs of apoptotic changes in the interstitium of a remnant kidney on day 90 after SNx; (a) magnification ×3105 single apoptotic endothelial cells (boxed), (b) apoptotic cell from (a) clearly visible is the condensed nuclear chromatin, blebbing on the cell membrane (single arrowhead) and the still intact cell membrane bounding the cell (double arrowhead), magnification ×23679.

but the most of positive nuclei showed normal shape, and no cytoplasmic condensation (data not shown). No staining was present in the negative control using buffer lacking TdT (data not shown). In the sham-operated group, few apoptotic cells were noted in the glomeruli, tubules and interstitium. The remnant kidney samples demonstrated a continued increase in positively stained nuclei. The number of apoptotic cells significantly increased, with respect to age-matched sham samples, in glomeruli on day 90, ($P<0.01$), tubules on day 7 ($P<0.005$) and interstitium on day 30 ($P<0.01$). Thereafter, the number of positive apoptotic cells progressively increased until the end of the experiment (Figure 3a). Maximal areas of positive apoptotic cells were detected in the sclerotic glomeruli, dilated or atrophied tubules and expanded interstitium (Figures 4a–c).

Fig. 2. Scoring of morphological motifs of apoptosis and mitosis, shown by representative plates of H&E sections and graphically. Inset plate (a) apoptosis and inset plate (b) mitosis. Graphically, the numbers as seen in H&E stained sections from the time course of rats submitted to subtotal nephrectomy (M±SEM) in 20 fields at ×400 magnification were examined of tubulointerstitium, only those cells with distinct apoptotic (solid line) and mitotic (broken line) morphology were scored as positive. Values detected in controls have not been shown for clarity, but were consistently below 0.5%.

Detection of apoptotic cells

We used the TUNEL method to detect DNA fragmentation which is the biochemical characteristic of apoptosis. The TUNEL technique as described by [11], stained nearly all of the cells in the positive control sections treated with DNase I before the TdT reaction, but the most of positive nuclei showed normal shape, and no cytoplasmic condensation (data not shown).

In negative controls, there was no staining at all. In sham operated rats, low grade PCNA positive cells were noted in glomerular, tubular and interstitial cells. The prevalence of staining remaining unchanged throughout the study (Figure 3b). In contrast, in SNx rats, a dramatic, diffuse and significant increase in the number of PCNA positive cells was noted in the glomeruli after day 30 ($P<0.05$), in the tubules from day 7 onward ($P<0.05$) and in the interstitium from day 15 ($P<0.05$). Nuclei positive for PCNA were observed in damaged glomeruli, dilated tubules and expanded interstitium (Figures 5a–c).

Evaluation of cellular proliferation (PCNA staining)

In negative controls, there was no staining at all. In sham operated rats, low grade PCNA positive cells were noted in glomerular, tubular and interstitial cells. The prevalence of staining remaining unchanged throughout the study (Figure 3b). In contrast, in SNx rats, a dramatic, diffuse and significant increase in the number of PCNA positive cells was noted in the glomeruli after day 30 ($P<0.05$), in the tubules from day 7 onward ($P<0.05$) and in the interstitium from day 15 ($P<0.05$). Nuclei positive for PCNA were observed in damaged glomeruli, dilated tubules and expanded interstitium (Figures 5a–c).
Fig. 3. Time course of apoptotic changes (M ± SEM) observed by TUNEL (a) in the glomeruli (blocked bars), interstitial (clear bars) and tubular cells (striped bars) of subtotally nephrectomized rats (SNx) and their sham-operated controls (SNc). Changes in the number of PCNA (b) positive cells (M ± SEM) in the glomeruli (blocked bars), interstitium (clear bars) and tubules (striped bars) of remnant kidneys (SNx) and their sham-operated controls (SNc). (*P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.005, compared to respective controls).
Fig. 4. ApopTag™ stained formol calcium fixed, paraffin embedded 4 μm sections of rat kidney. (a) Positive apoptotic interstitial cell (arrowhead) day 90 post SNx (×400), (b) apoptotic cell of the tubular cell wall (arrowhead) exfoliating into the tubule lumen (×200), (c) apoptotic glomerular cell (arrowhead), day 120 post SNx the cell has fragmented into a number of apoptotic bodies (small arrowhead) (×400).

By contrast, to the significant number of PCNA positive cells noted in remnant kidneys, we observed very few mitotic figures within the tubular cells of normal or remnant kidneys by conventional light microscopy (Figure 2).

Double staining for both apoptosis and PCNA
Having found that the numbers of tubulointerstitial cells with mitotic motifs was lower than the corresponding figures for apoptosis (90 days post SNx, % apoptotic cells 1.54 ± 0.44, % mitotic cells 0.22 ± 0.15). We have hypothesised that those cells which stained solely for PCNA gave a misleading representation of the numbers of cells actually undergoing proliferation. As the biological half-life of PCNA is 20 h, it was reasonable to believe that the antigen may still be present in those cells which had actually exited from the cell cycle and were now in fact undergoing DNA
The number of PCNA positive cells in different renal compartments also showed close correlations the scores of glomerulosclerosis and tubulointerstitial fibrosis (Table 2). Of interest, in the normal kidney and in the absence of fibrotic changes ~0.04–0.06% cells go into apoptosis, a matched percentage to the incidence of cell proliferation. Throughout, the time-course of the study the apparent rate of change of apoptosis seemed to be matched by the rate of changes in cellular proliferation (PCNA positivity).

Discussion

The progression of chronic renal failure (CRF) is associated histologically with glomerulosclerosis and tubulointerstitial fibrosis [3,4]. These are reproduced in the remnant kidney model of CRF in the rat, where the initial compensatory renal growth response to ablation is followed in the long-term by progressive renal scarring [2,17,18]. Typically, the late renal fibrotic changes are associated with the progressive loss of glomerular cells and tubular atrophy and their replacement by fibrous tissue. Simultaneously, we [13] and others [19] have described a proliferative response affecting interstitial renal cells in particular the activated fibroblasts or the so-called myofibroblasts.

In this study, we have attempted to determine whether apoptosis is an important mechanism contributing to the progressive loss of glomerular and tubular cells during the course of experimental CRF. We also investigated changes in the programmed cell death (apoptosis) of interstitial cells associated with progressive renal scarring. As studying apoptosis in isolation without an evaluation of changes in cell proliferation may be misleading, we also attempted to determine in this study the proliferative profile of the glomerular, tubular as well as interstitial cells.

We observed a progressive and sustained increase in the number of apoptotic cells in the glomerular, tubular and interstitial cells of remnant kidneys of rats submitted to extensive renal ablation. The nature of apoptotic changes were confirmed by the in situ end-labelling of fragmented DNA as well as the simultaneous observation of condensed nuclear chromatin by light and electron microscopy. This excludes the possibility of detection of random nuclear fragmentation by the TUNEL technique late in the course of cellular necrosis [20]. Our observation of increased renal apoptosis in remnant rat kidneys confirms earlier data made in the same model over a shorter observation time relating to the apoptosis of glomerular cells [11]. We extended these observations to show parallel changes in tubular and interstitial cells. The tubular apoptotic changes are consistent with the observations made in other models of experimental [10] and clinical [12] tubular atrophy [21]. Our observations in the remnant kidney bear strong similarities to those made in rats with obstructive uropathy where tubular followed by interstitial cell apoptosis was described throughout a 90-day period.
Fig. 7. Graphical representation of the severity of glomerulosclerosis and tubulointerstitial fibrosis with the number of apoptotic cells detected by TUNEL. The plates on the abscissa of each graph, show the morphological appearance of sections giving the corresponding score of glomerulosclerosis and tubulointerstitial fibrosis respectively. (a) Correlation between the severity of glomerulosclerosis and the number of apoptotic glomerular cells (b). Correlation between the severity of tubulointerstitial fibrosis and the number of apoptotic tubular cells.
days observation period [10]. Of interest, in this study, active cellular proliferative changes were also described [10]. This was one of the main findings of our study as apoptotic and proliferative changes took place simultaneously in the glomerular, tubular as well as interstitial compartments. Both processes paralleled the progression of glomerulosclerosis and tubulointerstitial fibrosis.

The parallel changes between cellular proliferation, detected by the expression of the proliferating cell nuclear antigen (PCNA) cyclin polypeptide, and those related to the apoptosis of renal cells are intriguing. It may express a compensatory proliferative response to the loss of cells through programmed cell death in an attempt to maintain renal structural integrity. However, the relationship may be more complicated as initial cell cycle events are engaged with the expression of PCNA in both cellular proliferation or apoptosis before cell divergence to its pre-programmed fate [21]. During the course of apoptosis in density-arrested mouse fibroblasts induced by serum deprivation, it was noted that apoptosis was accompanied by the expression of early cell-cycle genes followed by the transition of the cell to the G1/S border when it expressed PCNA [21]. In this case, the expression of PCNA may merely reflect a stage preceding the apoptosis of renal cells and therefore would be expected to shadow and match tissue apoptosis. This would be supported, in our study, by the paucity of mitotic figures detected at any one time within renal tubular cells contrasting with a significant number of apoptotic cells detectable by light microscopy. Although the latter could also underestimate the real number of cells undergoing mitosis. The impression that the expression of PCNA may be a common cellular event in proliferation and apoptosis was supported by the observation that some cells expressing PCNA also staining positively by TUNEL suggesting DNA fragmentation and apoptosis. As a single test, PCNA antigen detection may therefore overestimate cell proliferation by the detection of cells primed for apoptosis. To gain a true perspective of the proliferative state of a tissue it is necessary to look at a cell proliferative marker such as PCNA but also at cell division markers such as mitotic morphology or cyclin D2 simultaneously. With these reservations in mind, and even if we accept that PCNA expression is a rough indication of cell proliferation, the apparent matching of the number of apoptotic and proliferative cells in kidney sections at any time after subtotal nephrectomy may be misrepresentative of the prevalence of the two processes in vivo. In reality, such a numerical match would represent a predominance of the apoptotic changes over the proliferative ones. This could be easily explained by the fact that in vivo a single apoptotic event takes between 1 and 4 h to be completed [22], while the time necessary for a complete proliferative cell cycle is much longer with the detection half-life of PCNA protein being ~20 h [23]. Thus, a 2–8-fold increase in apoptosis over proliferation would be anticipated explaining progressive cell deletion and loss of renal tubular mass.

In the interstitium, the multitude of cell lines including fibroblasts, myofibroblasts and inflammatory cells (lymphocytes and monocytes) makes the interpretation of the apoptotic changes difficult. An increase of fibroblastic cells is anticipated as myofibroblasts can be seen to increase in number within the interstitium of scarred kidneys during the progression of clinical [24] and experimental [13] nephropathies. It has been postulated that wound healing and resolution of tissue injury is associated with the apoptosis of myofibroblasts while tissue scarring and fibrosis is associated with an inappropriate decrease in apoptosis favouring longevity of these cells [25]. This may indeed be the case in our study as the proliferation of interstitial myofibroblasts detected by PCNA may not be matched by their apoptosis. On the other hand, the interstitial apoptotic changes may be taking place in other cell lines such as infiltrating lymphocytes and monocytes.

The factors triggering apoptosis in the remnant

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**Table 2.** Correlation matrix showing the $R^2$ value derived from the multiple linear regression analysis between clinical and histological variables in rats with remnant kidneys at the time of sacrifice

<table>
<thead>
<tr>
<th>GS</th>
<th>TIF</th>
<th>S Cr</th>
<th>UP</th>
<th>G APO</th>
<th>I APO</th>
<th>T APO</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS</td>
<td>1</td>
<td>0.961a</td>
<td>0.830b</td>
<td>0.981a</td>
<td>0.929b</td>
<td>0.937c</td>
</tr>
<tr>
<td>TIF</td>
<td>0.830b</td>
<td>1</td>
<td>0.877b</td>
<td>0.994a</td>
<td>0.863b</td>
<td>0.941c</td>
</tr>
<tr>
<td>S Cr</td>
<td>0.981a</td>
<td>0.877b</td>
<td>1</td>
<td>0.868c</td>
<td>0.779c</td>
<td>0.911d</td>
</tr>
<tr>
<td>UP</td>
<td>0.929b</td>
<td>0.863b</td>
<td>0.868c</td>
<td>1</td>
<td>0.913d</td>
<td>0.914e</td>
</tr>
<tr>
<td>G APO</td>
<td>0.937c</td>
<td>0.941c</td>
<td>0.911d</td>
<td>0.913e</td>
<td>1</td>
<td>0.977f</td>
</tr>
<tr>
<td>I APO</td>
<td>0.9000</td>
<td>0.859</td>
<td>0.998</td>
<td>0.935f</td>
<td>0.929g</td>
<td>1</td>
</tr>
<tr>
<td>T PCNA</td>
<td>0.9500</td>
<td>0.900</td>
<td>0.930</td>
<td>0.859</td>
<td>0.859</td>
<td>0.981h</td>
</tr>
</tbody>
</table>

**Note:**

1. Glomerular apoptosis though significant proved not to be the best histological predictor for GS and TIF.
2. Urine protein consistently proved to be the best clinical predictor for progression of GS and TIF via multiple regression analysis.
3. Glomerular PCNA staining proved to be the best histological predictor for GS and TIF.
4. Urine protein consistently proved to be the best clinical predictor for progression of GS and TIF via multiple regression analysis.
5. Key: GS = glomerular sclerosis, TIF = tubulo interstitial fibrosis, Scr = Serum creatinine, UP = urine protein, G APO = glomerular apoptosis score, I APO = interstitial apoptosis score, T = tubular apoptosis score, G PCNA = glomerular PCNA score, I PCNA = interstitial PCNA score, T PCNA = tubular PCNA score, NS = value of no significance.
kidney remain unknown. It is well established that apoptosis is associated with the tissue expression of some growth factors including transforming growth factor-β (TGF-β) [26]. This has been clearly demonstrated in the liver cirrhosis where the expression of TGF-β and apoptosis are closely associated [26]. We have previously demonstrated the increased expression of TGF-β in the interstitium of remnant kidneys [14]. Others have shown the active synthesis of this growth factor in the remnant tubules [19]. These observations may establish a link between this growth factor and the apoptosis of tubular and interstitial cells in this experimental model. Of interest, we have also described an upregulation of the expression of immunoreactive insulin-like growth factor-I (IGF-I) in the tubules and interstitium of remnant kidneys [15]. This growth factor is believed to be one of the most potent inhibitors of apoptosis [5,6]. It may be that the balance between these pro- and anti-apoptotic growth factors determine the direction renal cells take during the course of the scarring process.

In conclusion, our observations confirm that apoptosis is a major mechanism of cell loss during the course of progressive experimental renal scarring. Apoptosis in the remnant kidney model clearly exceeds proliferative changes. This results in a loss of homeostasis as the rate of apoptosis outweighed that of proliferation thus favouring cellular deletion with the renal tubular cells being the most severely affected.

More research is warranted to determine the controlling mechanisms that contribute to cellular apoptosis during the course of experimental renal scarring. Conceivably, such an understanding may lead to therapies based on the manipulation of apoptosis and aimed at the preservation of renal integrity.

Acknowledgements. The authors are grateful to the Trent Regional Health Authority for their financial support (grant number: 64358). The support of the research committee of the Northern General Hospital Trust is also acknowledged (grant number: 63025).

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Received for publication: 9.12.97
Accepted in revised form: 8.4.98