Effects of caspase inhibition on the progression of experimental glomerulonephritis

BIN YANG, TIMOTHY S. JOHNSON, JOHN L. HAYLOR, BART WAGNER, PHIL F. WATSON, MOHSEN M.H. EL KOSSI, PETER N. FURNESS, and A. MEGUID EL NAHAS

Sheffield Kidney Institute, Department of Histopathology and Division of Clinical Sciences, Sheffield Teaching Hospitals, Sheffield University, Sheffield; and Department of Pathology, Leicester General Hospital Trust, Leicester University, Leicester, United Kingdom

Effects of caspase inhibition on the progression of experimental glomerulonephritis.

Background. Caspase-3 has a central role in the execution of apoptosis. In a nephrototoxic nephritis (NTN) model, we previously demonstrated an up-regulation of caspase-3 that was associated with inappropriate renal apoptosis, inflammation, tubular atrophy, and renal scarring.

Methods. We applied a pan caspase inhibitor, Boc-Asp (OMe)-fluoro-methyl-ketone (B-D-FMK), directly to rat NTN kidney using an intrarenal cannula fed from an osmotic pump. Animals were treated either for the first 7 days (acutely) to determine the effects on renal inflammation (ED-1 staining) and apoptosis (in situ end labeling of fragmented DNA), or for 28 days commencing 15 days after NTN (chronically) to observe the effects on cell death and renal fibrosis. Changes of caspase-3 and caspase-1 activity were detected by fluorometric substrate cleavage assay. Changes in caspase-3 and caspase-1, interleukin-1β (IL-1β), and collagen I, III, and IV proteins and mRNA were detected by Western blotting and Northern blotting, respectively.

Results. In both treated groups, caspase-3 activity was inhibited, and 17 and 24 kD active caspase-3 proteins were reduced significantly. A compensatory increase of caspase-3 mRNA occurred in the acutely treated group, but decreased in the chronically treated group (P < 0.05). Although there were no significant changes in caspase-1 activity and its active protein, the observed decrease in its precursor in the chronic group was increased by treatment (P < 0.05). Further, IL-1β precursor and its mRNA were significantly reduced by treatment only in the chronically treated group. Apoptosis was decreased in the glomeruli of acutely treated rats, and in the tubules and interstitium of chronically treated animals (P < 0.05). Glomerular inflammation was decreased only in the acutely treated group, whereas tubulointerstitial inflammation was lowered in both treated groups (P < 0.05). Glomerulosclerosis was reduced in both inhibitor groups, with a reduction in tubulointerstitial fibrosis and collagen I, III, and IV mRNA restricted to chronically treated animals (P < 0.05). Proteinuria was significantly decreased with caspase inhibition in both treated groups, but not serum creatinine level.

Conclusion. This study clearly indicates that caspase inhibition reduces renal apoptosis, ameliorates inflammation and fibrosis, and improves proteinuria in experimental glomerulonephritis, which may mainly be related to changes in the caspase enzymatic system.

Chronic glomerulonephritis remains one of the most common causes of chronic renal failure (CRF) worldwide. Progressive glomerulonephritis is associated with initial inflammatory changes followed by glomerulosclerosis, tubular atrophy, and interstitial fibrosis [1]. Apoptosis, a morphologic form of programmed cell death, has a dual role in renal injury. On one hand, it may serve as a healing mechanism related to the resolution of inflammation [2, 3], while on the other hand, inappropriate apoptosis causes cell deletion that affects renal function and remodeling [4–7]. Therefore, the control of apoptosis and its mediators could be a potential target for therapeutic interventions manipulating both the early inflammation as well as the late atrophic and fibrotic changes in progressive glomerulonephritis.

Although different signals initiate apoptosis, the phenotype of apoptosis is surprisingly similar even in different cell types, suggesting that the final stage of apoptotic death is highly conserved [8]. A class of cysteine proteases (caspase family, 14 different members) coordinates the execution of the death program. In particular, caspase-3 is a major execution enzyme acting upstream of DNA fragmentation [9–11]. All proforms of caspase contain both recognition and cleavage sites implying that activation of caspase occurs either autocatalytically or by other caspase. Two major pathways of caspase-3 activation exist, either mediated by death receptors (through caspase-8) or by mitochondria (through caspase-9) [10]. Recently, it was also reported that caspase could be activated via an endoplasmic reticulum (ER) pathway (through caspase-12) [12].
Apart from the promotion of apoptotic cell death, the caspase family of enzymes also participates in the processing of proinflammatory cytokines [13, 14]. Caspase-1 (interleukin (IL)-1β-converting enzyme (ICE)) is the pro-IL-1β processing enzyme [15]. IL-1β, a cytokine with a wide range of proinflammatory actions, is increased in the kidneys of rat antiglomerular basement membrane (GBM)–mediated glomerulonephritis (nephrotoxic nephritis (NTN)) and plays an important role in crescent formation and tubulointerstitial injury [16]. Early and late treatment with an IL-1 receptor antagonist prevents and suppresses the progression of crescentic glomerulonephritis in this model [17, 18]. Caspase-1 knockout mice treated with lipopolysaccharide have a blunted IL-1β response [15] and demonstrate reduced ischemic brain injury [19].

Caspase inhibitors, including peptide-based inhibitors of caspase, are highly effective in preventing apoptotic cell death in in vitro models of apoptosis [20, 21]. The investigation of caspase-3–deficient mice has suggested that this enzyme may be an appropriate target for therapeutic interventions in diseases resulting from inappropriate apoptosis [22]. For example, the elevated apoptosis in hepatic parenchymal cells induced by tumor necrosis factor-α (TNF-α)–mediated endotoxicemia was prevented by benzoyloxycarbonyl-Val-Ala-Asp (OMe) (ZVAD), providing a caspase-3 inhibition, which reduced the severity of the disease [23]. In addition, the administration of Boc-Asp (OMe)-fluoro-methyl-ketone (B-D-FMK) after cerebral hypoxia/ischemia, inhibited caspase-3 activity and was neuroprotective [24]. More recently, it has been reported that ZVAD-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/reperfusion [25].

Previous studies have demonstrated the increase of caspase-3 in various experimental models of renal disease, including ischemic renal injury [26] and cyclosporin A–induced nephrotoxicity [27]. Our work has highlighted the importance of apoptosis in the development of glomerulosclerosis and tubular atrophy [28], where up-regulation of caspase-3 activity, protein, and synthesis has been described in both the subtotal nephrectomy (SNx) [7] and NTN [29] models of experimental renal scarring. Given these findings, a similar blockade of caspase-3 inhibition on the inflammatory reaction while the chronically treated group was intended to allow the evaluation of chronic scarring phase. Rats were injected with normal rabbit serum and received DMSO as controls for both time points (each experimental group consisted of six to eight rats). Rats were killed at the end of the experiment. Kidneys were removed and quartered, processed for histologic, immunohistochemical, and electron microscopic examination. The remaining tissue was snap-frozen and stored in liquid nitrogen for activity, protein, and mRNA analyses. Serum creatinine (standard autoanalyser techniques) and 24-hour urinary protein (Lowry method) were measured at sacrifice. Rats were housed at constant temperature and humidity on a 12-hour light/dark cycle and fed ad libitum on standard rat chow. All procedures were carried out according to the regulations laid down by the Home Office (Animal Scientific Procedure Act 1986, UK).

**Detection of tissue caspase-3 and caspase-1 activity**

Caspase-3 or caspase-1 activity was detected by the modified Fluorometric CaspACETM Assay System (Promega, Southampton, UK) using 100 µg of protein ex-
tracted from caspase-3 or 1 to cleave the fluorogenic substrate of either Ac-DEVD-amino-4-methyl coumarin (AMC) or Ac-YVAD-AMC. The specificity of the assay was determined using the caspase-3 or 1 inhibitor Ac-DEVD-CHO or Ac-YVAD-CHO. The proteolytic cleavage of substrates was monitored in a fluorescence microplate reader (SOFTmax PRO, Molecular Devices Corp., Sunnyvale, CA). The fluorescence intensity was calibrated with standard concentrations of AMC and caspase-3 or caspase-1 activity was expressed as pmol s−1.

Measurement of tissue caspase-3, caspase-1, and IL-1β protein by Western blot

Twenty μg of protein [20% (wt/vol) homogenate] was separated on a 15% (wt/vol) polyacrylamide denaturing gel and electro-blotted onto Hybond-C membrane (Amersham Life Science, Buckinghamshire, UK). This was blocked with 5% (wt/vol) bovine serum albumin (BSA) and probed with a polyclonal rabbit antirat full-length caspase-3 (Santa Cruz Biochemicals, Santa Cruz, CA, USA), caspase-1 (Upstate Biotechnology, Milton Keynes, UK), and IL-1β (NIBSC, Potters Bar, UK) antibodies at 1:1,000, 1:500, and 1:1000 dilution, respectively. The antibody binding was revealed using an antirabbit peroxidase conjugate (Dako, Glostrup, Denmark) or the ABC Elite Kit (Vector Laboratories, Peterborough, UK) diluted at 1:1000 and the ECL chemiluminescent detection system (Amersham Life Science). Developed films were semiquantitatively analyzed by scanning volume density using a Bio-Rad GS-690 densitometer and Molecular Analyst version 4 software (Bio-Rad Laboratories, Ltd., Herts, UK). Results were expressed as the percentage of the average controls’ mRNA volume densities. Transcript size was determined by comparison to ribosomal RNA subunits.

Northern blot analysis of caspase-3, IL-1β, collagen I, II, and IV mRNA

Fifteen μg of total RNA extracted using TRIzol® reagent (Life Technologies BRL, Paisley, UK) were electrophoresed on a 1% (wt/vol) agarose/3-(N-Morpholino)propanesulfonic acid (MOPS)/formaldehyde gel, capillary blotted onto Hybond-N membrane (Amersham Life Science) and crosslinked with 70 mJ/cm² radiation (UV Crosslinker, Amersham Life Science) [31]. This was then probed with 32P-labeled deoxyctydilimine triphosphate (dCTP) random primed DNA probes. Specific random primed DNA probes were constructed from the following sequences: rat caspase-3 [26], IL-1β [32], human collagen α1 (I) [33], rat collagen α1 (III) [34] and human collagen α1 (IV) [35]. Prehybridization and hybridization were performed using the Church buffer system (0.5 mol/L sodium phosphate and 7% (wt/vol) sodium dodecyl sulfate (SDS)) at 65°C [7, 36]. The filter was exposed to Kodak Biomax MS film (Sigma) for 24 to 48 hours. Autoradiographs were semiquantitatively analyzed by scanning volume density using a Bio-Rad GS-690 densitometer and Molecular Analyst version 4 software. Volume density was corrected for loading using the housekeeping gene cyclophilin [7], which remained stable during the experiment. Results were expressed as the percentage of the average controls’ mRNA volume densities. Transcript size was determined by comparison to RNA molecular weight markers (Promega) using the same analysis package and by visual comparison to the ribosomal RNA subunits.

In situ end labeling (ISEL) of apoptotic cells

Using paraffin 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) [28, 37]. For each experimental animal, more than 30 glomerular cross-sections and 20 high-power (×400 magnification) fields of tubulointerstitium were examined blinded to the experimental code by two of the authors (B. Yang and A.M. El Nahas). In situ end labeling (ISEL) + nuclei per glomerulus, per 400 tubular cells, and per interstitial field were determined, respectively. To substantiate the specificity of our results, we confirmed apoptosis by light and electron microscopy to evaluate the morphologic features; only strongly positive ISEL cells with observable morphologic features of apoptosis such as shrunken cells with condensed nuclei were counted [38, 39].

Evaluation of inflammation by ED-1 immunostaining

Cellular inflammation was evaluated by ED-1 (a specific rat monocyte/macrophage marker) immunostaining on paraffin sections using a standard avidin-biotin peroxidase complex technique [40]. The sections were pretreated with 0.125% (wt/vol) trypsin and incubated with the primary antibody (monoclonal mouse antirat ED-1 antibody, Serotec Ltd., Oxford, UK) at 1:50 dilution or normal mouse immunoglobulin G (IgG) at the same protein concentration of primary antibody as negative control. The antibody binding was revealed using the ABC Elite Kit (Vector Laboratories, Peterborough, UK) and 3’-amino-9-ethylcarbazole substrate. ED-1+ cells in glomeruli and interstitium were semiquantitatively assessed by the same counting system as described above for ISEL.

Estimation of renal scarring

The extent of renal scarring was semi-quantitatively analyzed on Masson’s Trichrome (MT)-stained sections at ×200 magnification. Two authors (B. Yang and A.M. El Nahas) assessed by the same counting system as described above for ISEL.
El Nahas) blinded to the experimental code scored the severity of glomerular injury/sclerosis, tubular atrophy, and tubulointerstitial fibrosis, according to a previously published arbitrary scale (from 0 to 3) [41]. Scores were attributed as following: a normal glomerulus scored 0; mild glomerular sclerosis affecting up to 25% of glomerular tuft scored 1; moderate glomerular sclerosis affecting between 25% and 50% of tuft scored 2; and severe glomerular sclerosis affecting in excess of 50% of tuft scored 3. Tubulointerstitial scarring was defined and scored as normal tubules with tubular cell number (TCN) approximately 1000 and no expansion of interstitium scored 0; mild tubular atrophy with TCN approximately 800 and interstitial edema or fibrosis (IF) affecting up to 25% of the field scored 1; moderate tubular atrophy with TCN approximately 600 and IF affecting 25% to 50% of the field scored 2; and severe tubular atrophy with TCN approximately 400, IF exceeding 50% of the field scored 3. The data were collected from a series of adjacent fields extending perpendicularly from the cortex toward the outer medulla. Three such randomly selected linear series of minimum 12 fields were measured in the cortex or such number of fields until 30 glomeruli had been counted per section.

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 Student t test. P < 0.05 was considered to be significant.

RESULTS

Effect of B-D-FMK on caspase-3 activity, proteins, and mRNA

Caspase-3 activity (pmol AMC liberated per minute per μg protein at 30°C) was increased in the early (0.059 ± 0.005) and late (0.084 ± 0.009) untreated (DMSO/vehicle-infused) NTN kidneys compared to their controls (0.021 ± 0.004 and 0.027 ± 0.005, respectively, P < 0.01). This elevated caspase-3 activity was reduced in the NTN kidneys treated with B-D-FMK in both acute (−40%, P < 0.01) and chronic (−29%, P < 0.05, Figure 1A) treatment groups. The specific and competitive tetrapeptide inhibitor of caspase-3, Ac-DEVD-CHO, almost fully inhibited the caspase-3 activity in the assays, demonstrating the specificity of the assay. In the noncannulated contralateral NTN kidneys, caspase-3 activity was also significantly reduced to a similar level of the treated kidneys suggesting a systemic effect (data not shown).

Caspase-3 proteins in kidney tissues, determined by Western blotting analysis, showed that 17 kD and 24 kD bands, representing caspase-3 active subunits, were increased in the acutely (+292% and +296%) and chronically (+361% and +265%) untreated NTN kidneys (P < 0.05). These caspase-3 subunits were diminished in the NTN kidneys treated with B-D-FMK in acute (−61% for both, P < 0.05) and chronic (−46% for 17 kD and −37% for 24 kD, P < 0.05) experiments. The 32 kD band, precursor of caspase-3, increased by +392% (P < 0.05) only in the chronically untreated NTN kidneys, which was reduced by −38% (P < 0.01) after B-D-FMK treatment. The 29 kD processing intermediate was also present in the kidneys of all groups (Fig. 1B).

We performed Northern blot analysis on kidney tissues to address whether B-D-FMK affected caspase-3 transcription. In the acute group, the 2.7 kb caspase-3 mRNA transcript (Fig. 1C) was increased by +250% in the untreated NTN kidneys in comparison to controls, which was further increased by +139% in the NTN kidneys treated with B-D-FMK (P < 0.05). In the chronic study, caspase-3 mRNA was increased by +566% in the untreated NTN kidneys, but decreased by −52% (P < 0.01) in the NTN kidneys treated with B-D-FMK (P < 0.05) (Fig. 1C).

Effect of B-D-FMK on caspase-1 activity and proteins

There was no significant change in renal caspase-1 activity (pmol AMC liberated per minute per μg protein at 30°C) between experimental groups (Fig. 2A). Of note, the competitive tetrapeptide inhibitor, Ac-YVAD-CHO, inhibited the activity of caspase-1 only about 65.5% in the in vitro assay.

The expression of caspase-1 proteins was detected by Western blotting analysis. The active subunit of caspase-1 was a 20 kD band, remained unchanged through both the acute and chronic time courses and was not affected by B-D-FMK (Fig. 2B). However, the precursor of caspase-1, a 45 kD band, was significantly decreased by −40% in chronic NTN kidneys and increased again by +18% after the treatment with B-D-FMK (P < 0.05) (Fig. 2B).

B-D-FMK reduced IL-1β proteins and synthesis in NTN kidneys

Although no significant changes in caspase-1 activity and the active subunit of caspase-1 were seen, the expression of the caspase-1 precursor was reduced and corrected by B-D-FMK. Therefore, we further investigated whether B-D-FMK had an effect on IL-1β at both protein and mRNA levels, as pro-IL-1β may be processed by either caspase-1, caspase-3 (caspase-1 independent), or both pathways [42].

IL-1β proteins in kidney tissues were determined by Western blotting analysis. The 17 kD band representing the IL-1β active subunit was increased in the acutely (+223%) and chronically (+169%) untreated NTN kidneys (P < 0.05). This IL-1β subunit was reduced in the NTN kidneys treated with B-D-FMK of both groups, but did not reach statistical significance (−46% and −42%, P > 0.05). The 35 kD band, precursor of IL-1β, increased by +249% and +181% (P < 0.05) in both acutely and chronically untreated NTN kidneys, which
Fig. 1. Caspase-3 activity, proteins and mRNA in kidney tissues. (A) Caspase-3 activity assayed by the fluorometric substrate cleavage. Data represents mean ± SEM. (B) Expression of caspase-3 proteins detected by Western blot analysis. Sample immunoblots of both acute and chronic studies show 17 kD and 24 kD bands, representing the caspase-3–active subunits; a 32 kD band, representing the precursor of caspase-3; a 29 kD band, representing processing intermediate of caspase-3. Histogram data represent the result of volume density analysis as mean percentage change compared to the average of control values (mean ± SEM). (C) Tissue level of caspase-3 mRNA in rat kidneys assayed by Northern blot analysis. Histogram shows volume density analysis of autoradiographs using cyclophilin corrected for loading. Data are expressed as mean percentage change compared to the average of control values (mean ± SEM). Abbreviations are: Cyc, cyclophilin (N = 6 to 8); NS, normal serum; NTS, nephrotoxic serum; DMSO, dimethyl sulfoxide; B-D-FMK, Boc-Asp (OMe)-fluoromethyl ketone; D7, B-D-FMK administration for 7 days; and D28, B-D-FMK administration for 28 days. Symbols are: (□), NS + DMSO; (■), NTS + DMSO; and (▲), NTS + B-D-FMK. *P < 0.05; **P < 0.01.
remained at a similar level in the acute group and was reduced by −66% \((P < 0.01)\) in the chronic group after B-D-FMK treatment (Fig. 3A).

In the acute study, there was no significant change in the expression of the 1.6 kb IL-1\(\beta\) mRNA (Fig. 3B). In the chronic investigation, the expression of IL-1\(\beta\) mRNA was significantly increased by +270\% in the untreated NTN kidneys, which was subsequently decreased by −59\% in the NTN kidneys treated with B-D-FMK \((P < 0.05, \text{Fig. 3B})\).
Fig. 3. Tissue level of interleukin-1β (IL-1β) proteins and mRNA. (A) Expression of IL-1β proteins detected by Western blot analysis. Sample immunoblots of both acute and chronic studies show a 17 kD band, representing the IL-1β active subunit; a 35 kD band, representing the precursor of IL-1β. Histogram data represents the result of volume density analysis as mean percentage change compared to the average of control values (mean ± SEM). (B) Tissue level of IL-1β mRNA in rat kidneys assayed by Northern blot analysis. Histogram shows volume density analysis of autoradiographs using cyclophilin (Cyc) corrected for loading. Data is expressed as mean percentage change compared to the average of control values (mean ± SEM) (N = 6 to 8). Abbreviations are: NS, normal serum; NTS, nephrotoxic serum; DMSO, dimethyl sulfoxide; B-D-FMK, Boc-Asp (OMe)-fluoromethyl ketone; D7, B-D-FMK administration for 7 days; and D28, B-D-FMK administration for 28 days. Symbols are: ( ), NS + DMSO; ( ), NTS + DMSO; and ( ), NTS + B-D-FMK. *p < 0.05; **p < 0.01.
Apoptotic changes following B-D-FMK administration

Apoptotic cells were detected by ISEL of fragmented DNA. Positively stained cells were counted and confirmed by distinct morphologic motifs using light and electron microscopy. Very few apoptotic cells were noted in the glomeruli (0.012 ± 0.009/glomerulus), tubules (0.026 ± 0.008/400 tubular cells) and interstitium (0.057 ± 0.014/400 field) of control kidneys (Fig. 6A). In the acute study, apoptotic cells in glomeruli (0.467 ± 0.073/glomerulus) were increased in untreated NTN kidneys (Fig. 4a and a’) compared with controls (P < 0.01). This was decreased by −55% (P < 0.01) in the NTN kidneys treated with B-D-FMK (Figs. 4b and 6A). In the chronic group, apoptotic cells in glomeruli were also higher in untreated NTN kidneys (P < 0.05), and were not affected by B-D-FMK treatment. Apoptotic cells in tubules (1.112 ± 0.238/400 tubular cells) and interstitium (0.680 ± 0.072/400 field) were also increased in the untreated NTN kidneys (P < 0.01, Fig. 4c); however, this was significantly decreased by −64% (P < 0.05) and −57% (P < 0.01), respectively, in the B-D-FMK–treated NTN kidneys (Fig. 4d and 6A). In both groups, the areas mostly contained apoptotic cells were the inflamed or sclerotic glomeruli (Fig. 4e) as well as the dilated or atrophied tubules (Fig. 4f) and expanded interstitium (Fig. 4g). Electron microscopy confirmed apoptotic cells with distinct morphologic motifs in some glomeruli (not shown), tubules (Fig. 4h) and interstitium (Fig. 4i).

Inflammatory (ED-1+) changes following B-D-FMK administration in NTN kidneys

Inflammatory mononuclear infiltration of the kidneys was evaluated by immunostaining of ED-1. A small number of ED-1+ cells were seen in the glomeruli (0.22 ± 0.01/glomerulus) and interstitium (1.02 ± 0.12/400 field) of control kidneys (Fig. 6B). Over the first 7 days, ED-1+ cells were increased in the glomeruli (20.61 ± 1.71/glomerulus) and interstitium (18.91 ± 1.20/400 field) of untreated NTN kidneys (P < 0.01). This was decreased by −27% (P < 0.01) in the glomeruli and by −22% (P < 0.05) in the interstitium following B-D-FMK treatment. In the chronic experiment, ED-1+ cells in the glomeruli (7.51 ± 1.39/glomerulus) were less than in the acutely untreated NTN kidneys but still significantly higher than controls. No significant change was noted after B-D-FMK treatment. In the interstitium of the late untreated NTN kidneys, ED-1+ cells (34.57 ± 3.15/400 field) were almost double that on the normal, but reduced by −53% (P < 0.01) in those receiving B-D-FMK treatment (Fig. 6B). Positive ED-1 cells were distributed in the inflamed glomeruli (Fig. 5 a, b, c, e, and f), interstitium and tubular lumen (Fig. 5 c, d, g, and h).

Fibrotic and functional changes after B-D-FMK treatment

The extent of glomerular sclerosis, tubular atrophy, and tubulointerstitial fibrosis were semiquantitatively analyzed on the MT-stained sections. Glomerular sclerosis was severe and progressive in the untreated NTN kidneys (Fig. 7 a and c, acute; c and g, chronic). This was reduced by −27% (Fig. 7 b and f, acute) and −31% (Fig. 7 d and h, chronic) in NTN kidneys treated with B-D-FMK for both time periods (Fig. 7i, P < 0.05). No significant difference in tubular atrophy or tubulointerstitial fibrosis was noted in the early (acute) untreated and treated NTN kidneys (Fig. 7 a, b, and i) compared to controls (TCN, 960.0 ± 8.2 tubular cell/×200 field). In the chronic study, tubular atrophy (TCN, 570.0 ± 31.3 tubular cell/×200 field, P < 0.01) and tubulointerstitial fibrosis (Fig. 7 c, g, and i) were increased in the untreated NTN kidneys compared to controls (TCN, 952.5 ± 8.54 tubular cell/×200 field). Caspase inhibition significantly improved tubular atrophy (TCN, 714.00 ± 39.32 tubular cell/×200 field, P < 0.05) and reduced tubulointerstitial fibrosis by −34% (Fig. 7 d, h, and i).

These results were supported by the assay of tissue collagen I, III, and IV mRNA using Northern blot analysis. In the acute study, the 4.8 kb/5.8 kb of collagen I mRNA was increased by +220% in the untreated NTN kidneys, and further increased to +480% (P < 0.05) in the NTN kidneys treated with B-D-FMK. There was no significant change in the expression of 5.4 kb collagen III and 6.5 kb/4.5 kb collagen IV mRNA transcripts in the acute study. In the chronic study, the expression of collagen I, III and IV mRNAs increased by +1487%, +522% and +442% in the untreated NTN kidneys and were decreased by −63%, −58% and −72% in the treated kidneys (P < 0.05, Fig. 7j to l).

There was no detectable change in the body and kidney weight or serum creatinine among groups in the acute study (Table 1). In the chronic study, kidney weight and serum creatinine, but not body weight, were significantly increased in the untreated rats. However, neither was affected by B-D-FMK treatment (Table 1). Proteinuria was significantly increased in both early and late untreated NTN rats (113.45 ± 13.18 and 399 ± 33.33 mg/24 hours) compared to controls (P < 0.01). Of note, NTN kidneys treated with B-D-FMK both acutely (−38%) and chronically (−29%) had a significant reduction of proteinuria (P < 0.05, Table 1).

DISCUSSION

Apoptosis has a critical role in the resolution of inflammatory response, such as that occurring in glomerulonephritis [2, 3]. However, if the induction of apoptosis becomes uncontrolled, it leads to glomerular and tubular cell deletion, and glomerular sclerosis as well as tubular...
atrophy and interstitial fibrosis subsequently ensued [5, 7]. In previous experiments on rats with NTN and SNx, we have observed the progressive glomerular and tubular cell apoptosis and postulated that renal cellular apoptosis contributed to glomerulosclerosis and tubular atrophy [7, 28]. In these studies, a strong association was noted between the up-regulation of caspase-3, the occurrence of apoptosis, and the progression of scarring/fibrosis [7, 29]. Recent evidence linking caspase-3 and inflammation indicates that caspase-3 may also play a role in the inflammatory changes that characterize early glomerulonephritis, as well as the late interstitial inflammation, which precedes renal fibrosis in progressive glomerulonephritis [7, 25]. The pivotal role of caspase-3 in the machinery of apoptosis and inflammation makes it as an attractive target to regulate the progression of glomerulonephritis and renal fibrosis.

In this study, we have used the intrarenal administration of a cell-permeable pan caspase inhibitor, B-D-FMK, to test the effect of caspase inhibition on both apoptosis and inflammation, and the ensuing renal scarring in the in vivo rat NTN model. The administration of B-D-FMK in the acute phase (starting 1 day before NTN and lasting 7 days) of NTN prevented the early onset of glomerular apoptosis and inflammation. In addition, the early inhibition of caspase also blunted tubulointerstitial inflammation. These effects may have contributed to the attenuation of glomerular injury and proteinuria. Conversely, when B-D-FMK was administered chronically starting on day 15 after the induction of NTN and continuing for 28 days, it predominantly ameliorated the tubular apoptosis, interstitial inflammation, and fibrosis. It also reduced glomerulosclerosis and proteinuria. These results indicate that this pan caspase inhibitor has a potential not only to prevent, but also to halt, the progression of glomerulosclerosis and interstitial fibrosis [43].

In order to evaluate the protective mechanisms of B-D-FMK in renal injury, we detected the expression of caspase-3 at activity, protein and mRNA levels in renal tissues. Caspase-3 is a key enzyme in the apoptotic process and is also involved in the progression of inflammation [11, 25]. Application of B-D-FMK led to a decrease in caspase-3 activity in the infused and contralateral kidneys of both acute and chronic experimental groups. This indicated that the local delivery of B-D-FMK for 7 or 28 days had a systemic effect. It also led to a decrease of caspase-3 protein levels in renal tissues. More interestingly, this decrease was despite an early up-regulation of caspase-3 transcription as a compensatory reaction to the inhibition of this enzyme, which declined later in the experimental time course. These changes may reflect the effect of autoadjustment to the caspase enzyme inhibitor at mRNA level in this in vivo model. All these results indicate that the caspase-3 activity, proteins, and mRNA were affected by B-D-FMK. In other words, the action of B-D-FMK on apoptosis and inflammation during the course of NTN may be predominantly through the inhibition of caspase-3.

Posttranslation, caspase-3 can be activated through three pathways. First, caspase-3 activation may be mediated by death receptors, such as (Fas/FasL system), which is associated with the activation of caspase-8. Second, caspase-3 activation may also be mediated by the mitochondria (through Bax/Bcl-2) with release of cytochrome c, which is required for caspase-9 activation [10, 44, 45]. Third, caspase-3 can be activated by caspase-12 when the ER is under stress [12]. Auto- or transactivation of initiator caspase-8, caspase-9, and caspase-12 results in the subsequent cleavage and activation of the downstream effector caspase-3 [10]. B-D-FMK is a pan caspase inhibitor and was assumed to have a role on all caspases, including caspase-8, caspase-9, and caspase-12. Therefore, B-D-FMK not only directly inhibited the activity of caspase-3 (demonstrated by the caspase-3 activity assay), but also prevented the formation of active caspase-3 (showed by the expression of caspase-3 proteins) by possibly causing a reduction in the activity of caspase-8, caspase-9, or caspase-12. So the effects of B-D-FMK on apoptosis and inflammation may be attributed to the inhibition of both caspase-3 activity and activation. Further, we cannot rule out a role for B-D-FMK on other caspases, such as caspase-6 and caspase-7 (another two effector caspases), which could be the subject of future studies.
The appearance of apoptosis is closely correlated with the increase of caspase-3 activity, as shown in our previous study in this experimental model of accelerated glomerulonephritis [7]. In the present investigation, B-D-FMK with caspase-3 inhibition decreased the occurrence of apoptosis as revealed by the decreased number of ISEL-positive nuclei. The inhibition of apoptosis occurred early in the glomeruli mainly after an acute administration of B-D-FMK for 7 days and late predominantly in the tubulointerstitium after chronic treatment for 28 days. The inhibition of early apoptosis in the glomeruli may lead to the reduction of secondary inflammation that could happen when the apoptotic inflammatory cells did not be cleared away in time. While initial reports dissociated apoptosis from inflammation, a growing body of experimental evidence links the two, which was clearly demonstrated following renal ischemia/reperfusion injury [25]. On the other hand, we have expected that the inhibition of apoptosis in the acute phase of glomerulonephritis would have led to diminish the resolution of acute glomerulonephritis contributing to further scarring. However, we failed to observe such a deleterious effect in our acute experiment, which may be hidden by the direct effect of B-D-FMK on inflammation. In
Fig. 7. Renal fibrosis analyzed on Masson’s Trichrome–stained sections and by collagen I, III, and IV mRNAs. Glomerular injury and sclerosis and tubulointerstitial fibrosis in Masson’s Trichrome–stained sections at ×200 (A to D) and ×400 (E to H) magnification. Histogram (I) shows semiquantitative analysis of renal fibrosis. Data represents mean ± SEM. Tissue levels of collagen I (J), III (K), and IV (L) mRNAs in rat kidneys assayed by Northern blot analysis. Histogram shows volume density analysis of autoradiographs using cyclophilin (Cyc) corrected for loading. Data is expressed as mean percentage change compared to the average control value (mean ± SEM) (N = 6 to 8). Abbreviations are: NS, normal serum; NTS, nephrotoxic serum; DMSO, dimethyl sulfoxide; B-D-FMK, Boc-Asp (OMe)-fluoromethyl ketone; D7, B-D-FMK administration for 7 days; and D28, B-D-FMK administration for 28 days. Symbols are: (○), NS + DMSO; (●), NTS + DMSO; and (■), NTS + B-D-FMK. *P < 0.05; **P < 0.01.
addition, the resolution of acute glomerulonephritis may also depend on the migration of inflammatory cells out of the glomeruli upon completion of their task [46].

The caspase inhibition by B-D-FMK may have had a direct effect on inflammation. There was a reduction of ED-1+ cells early in the glomeruli and tubulointerstitium, and late in the tubulointerstitium. This may result from the reduction of apoptosis as we postulated above. In addition, the decrease of inflammatory cell infiltration may be linked to the reduction of inflammatory mediators such as IL-1β. B-D-FMK is a broad-spectrum inhibitor of activated caspases, including not only caspase-3 but also caspase-1. Apart from a functional role on apoptosis [47], caspase-1, also referred to as ICE, is associated with the activation of IL-1. However, we did not show a significant change in caspase-1 activity and its active protein in our study, but the precursor of caspase-1 was decreased in chronic NTN kidneys and increased again after treatment with B-D-FMK. These may reveal that there was a continuous activation and consumption of caspase-1 simultaneously during time course, which would have led to a continuous cleavage of the caspase-1 precursor and kept a stable overall level of active caspase-1 in the NTN kidneys. This continuous consumption of active caspase-1 may have been involved in the processing of IL-1β. Furthermore, IL-1β active and precursor subunits were significantly increased in both groups of untreated NTN kidneys and reduced by B-D-FMK treatment, although this only reached significance in the chronic groups. These data found are also consistent with the changes observed in the IL-1β mRNA levels. It suggests that the changes of IL-1β protein (both active and precursor subunits) and mRNA can occur through the activation of caspase-1 [42]. IL-1 could in turn contribute to renal inflammation and scarring. Nevertheless, these processes can be inhibited by a pan caspase inhibitor in vivo. In addition, the processing of other inflammatory mediators, such as IL-18 and macrophage inflammatory protein-2 (MIP-2), may require the activation of caspase-3 [48, 49]. Apoptosis and inflammation could be mutually affected. This was highlighted by the inhibition of apoptosis using by ZVAD-FMK and insulin-like growth factor-1 (IGF-1) in renal ischemia/reperfusion, which prevented the associated inflammation [25]. Inflammation may provide a microenvironment that favors the development of apoptosis. Apoptosis and inflammation may share so far-unknown upstream molecular mediating mechanisms.

As we demonstrated previously, renal apoptosis and inflammation are associated with tubular atrophy and renal fibrosis [7, 29]. It has been revealed that the swift uptake and degradation of apoptotic cells by phagocytes leads to a very short half-life for apoptotic cells [3]. Thus, the changes in the small numbers of detectable apoptotic cells may mask a higher rate of apoptosis and result in a significant loss of renal cell mass. Therefore, the inhibition of renal cell apoptosis and inflammation, in particular, late tubular cell apoptosis and interstitial inflammation, by B-D-FMK could be helpful to preserve renal parenchymal cells and renal function. The reduction in apoptosis and inflammation was associated with the improvement of proteinuria and renal morphology, including glomerular sclerosis, tubular atrophy, and tubulointerstitial fibrosis. The reduction in interstitial fibrosis was supported by the reduction of collagen I, III, and IV mRNA expression in the chronic study group. There was a statistically significant improvement in renal cellular apoptosis, inflammation, and fibrosis with both acute and chronic administration of the pan caspase inhibitor. However, we are aware that there is variation between animals and that this kind of treatment partially halted but did not completely stop the progression of renal scarring. Further, we failed to observe a satisfactory improvement in serum creatinine level. Although we did not notice an obvious toxicity in liver and heart tissues (on hematoxylin and eosin sections, data not shown), the systemic toxicity of B-D-FMK needs further investigation. Adjustment in the dose of B-D-FMK may be required to maximize its protective effects and the delivery method needs to be improved to avoid the damage caused by the intrarenal cannulation, although this was contrasted by the vehicle administration to the control animals.

Apart from caspase, there are another two groups of cysteine proteases, the cathepsin and calpain [50]. Although cathepsins do not appear to play a role in lethal cell injury, calpain plays an injurious role in ischemic injury to liver, heart, brain and renal ischemia/reperfusion [50, 51]. Calpain activation has been associated with the down-

---

### Table 1. Changes of body weight, kidney weight, serum creatinine, and proteinuria in nephrotoxic nephritis (NTN) rats following Boc-Asp (OMe)-fluoro-methyl-ketone (B-D-FMK) treatment.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NS + DMSO</th>
<th>NTS + DMSO</th>
<th>NTS + B-D-FMK</th>
<th>NTN 43 days B-D-FMK 28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight g</td>
<td>306.0 ± 10.0</td>
<td>295.0 ± 1.5</td>
<td>317.4 ± 7.1</td>
<td>324.5 ± 12.1</td>
</tr>
<tr>
<td>Kidney weight g</td>
<td>1.03 ± 0.05</td>
<td>1.08 ± 0.04</td>
<td>1.14 ± 0.03</td>
<td>0.95 ± 0.03</td>
</tr>
<tr>
<td>Serum creatinine μmol/L</td>
<td>37.5 ± 4.1</td>
<td>39.4 ± 2.3</td>
<td>42.6 ± 2.4</td>
<td>43.8 ± 2.0</td>
</tr>
<tr>
<td>Proteinuria mg/24 hours</td>
<td>42.9 ± 4.3</td>
<td>113.5 ± 13.2</td>
<td>69.8 ± 11.1</td>
<td>67.0 ± 20.3</td>
</tr>
</tbody>
</table>

Abbreviations are: NS, normal serum; NTS, nephrotoxic serum; and DMSO, dimethylsulfoxide. *P < 0.01; †P < 0.05
regulation and cleavage of calpastatin, a calpain inhibitor, by caspase-3 [50]. The relationship between caspase-3 and calpain, and the effect of B-D-FMK on calpain in chronic renal scarring rat NTN models warrants further investigation.

Compounds able to modulate the apoptotic pathway are still rare with the exception being the organic small-molecule inhibitors of caspase activity. There is no precedence in humans of drugs that successfully target cysteine proteases, owing to the difficulties in developing electrophiles that are specific enough that they would not attack other biologic nucleophiles. In addition, can apoptosis be selectively modulated in one organ or cell type without adversely affecting other key systems? Furthermore, will cells that are salvaged by inhibiting apoptosis be functional? In all likelihood this will depend on the cell types, the context, and the degree of cellular injury [52]. The side effects of these manipulations need to be strictly investigated, especially the neoplastic potential in long-term therapy. Nevertheless, impressive advances have been made to modulate apoptosis in vitro and in vivo, and some of these as demonstrated in this study show promise in the prevention and attenuation of experimental renal scarring.

CONCLUSION

B-D-FMK was applied in a chronic renal fibrosis NTN model that not only inhibited the caspase-3 activity, expression, and synthesis mostly, but also decreased apoptosis, inflammation, and fibrosis, and reduced proteinuria. These effects may be predominantly mediated by the reduction of caspase-3 activity and activation; even if we cannot rule out the involvement of others such as caspase-1. It is also possible that this pan caspase inhibitor inhibited the production and release of inflammation mediators for instance IL-1β. To our knowledge this is the first report of a novel therapeutic approach to the treatment of experimental renal inflammation and fibrosis by modifying caspases and controlling inappropriate apoptosis that is associated with renal inflammation, tubular atrophy, and fibrosis.

ACKNOWLEDGMENTS

The authors would like to acknowledge the National Kidney Research Fund (grant # R40/1/2000), the Northern General Hospital NHS Trust Research Committee, the Sheffield Kidney Research Foundation, and the Sheffield Kidney Patients Association for their financial support of this study. The authors would also like to thank Enzyme System Products (Livermore, California) for providing us with their caspase inhibitor.

Reprint requests to Bin Yang, M.D., Ph.D., Renal Research Group, Clinical Sciences, Leicester General Hospital Trust, Gwendolen Road, Leicester LE3 4PW, United Kingdom. E-mail: by5@le.ac.uk

REFERENCES

25. Daemen MA, van’t Veer C, Denecker G, et al: Inhibition of...
apoptosis induced by ischemia-reperfusion prevents inflammation. J Clin Invest 104:541–549, 1999