Downregulation of Bcl-2 by Podocytes Is Associated with Progressive Glomerular Injury and Clinical Indices of Poor Renal Prognosis in Human IgA Nephropathy

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Abstract. Bcl-2 defines a new class of proto-oncogenes that block cell death without promoting cell proliferation. To elucidate the role of Bcl-2 in the development of glomerular lesions in human IgA nephropathy (IgAN), we applied immunohistochemistry coupled with in situ hybridization to detect the expression of Bcl-2 products and their association with Bax, p27kip1, and p57kip2 in modulating the apoptotic, proliferative, and sclerotic events in progressive glomerular injury. Glomerular cell apoptosis was examined by TdT-mediated dUTP-biotin nick-end labeling (TUNEL) staining. A total of 51 IgAN cases were categorized into four subgroups (A to D) according to the severity of their histopathological lesions. Creatinine levels, creatinine clearance, and magnitude of proteinuria based on 24-h urine collections at the time of diagnostic renal biopsy were available for the majority of subjects. Bcl-2 expression was observed predominantly in podocytes in IgAN. Podocyte expression of Bcl-2 was found to be upregulated in early-stage disease and downregulated in late-stage disease. Bcl-2 downregulation in progressive IgAN was associated with an increased Bax/Bcl-2 ratio in glomerular epithelial cells and correlated with the downregulation of high endogenous podocyte p27kip1 and p57kip2 expression. Bax/Bcl-2 ratios positively correlated with glomerular cell apoptosis and the degree of glomerulosclerosis, whereas p27kip1 and p57kip2 expression levels were inversely correlated with mesangial hypercellularity and glomerulosclerosis. Clinicopathologic correlations demonstrated that downregulation of Bcl-2 protein expression was associated with indices of poor renal prognosis in human IgAN. The results suggest that Bcl-2 expression by podocytes may exert modulatory effects on cellular processes that contribute to progressive glomerular injury and play an important role in determining renal outcome in human IgA nephropathy.

Bcl-2 defines a new class of proto-oncogenes that block cell death without promoting cell proliferation (1). Studies also support an additional role for Bcl-2 in the regulation of cell cycle progression, which is independent of its function in prosurvival (2). Bcl-2 family members appear to play crucial roles in regulating the balance between entry into apoptosis and survival capacity during early hematopoiesis (3), as well as in the embryonic development of normal and abnormal kidney (4).

To date, only limited data on the role of Bcl-2 in mediating renal injury have been reported. In human glomerulonephritis, Bcl-2 glomerular expression was modest (5) and observed only in a few capsular epithelial cells, infiltrating leukocytes (5), or mesangial cells (6,7). Bcl-2 in podocytes was restricted to those podocytes near intraglomerular fibrotic lesions and to epithelial cells of early adhesions and cellular crescents (5). No difference in Bcl-2 expression was found between cases of proliferative and nonproliferative glomerulonephritis (5). In contrast, increased glomerular expression of Bcl-2 protein was reported in lupus nephritis, IgA glomerulonephritis, and focal glomerulosclerosis, which was shown to correlate with the number of proliferating cell nuclear antigen–positive or Ki-67–positive intraglomerular cells, glomerular α-smooth muscle actin expression, the grade of mesangial cell increase, and the magnitude of proteinuria (6,7). However, Bcl-2 positivity was limited to less than two cells per glomerulus, which could represent expression by infiltrating leukocytes (7). Thus, two seemingly opposed hypotheses have emerged from previous studies regarding the role or roles of Bcl-2 in glomerular injury: (1) increased glomerular expression of Bcl-2 plays an antiapoptotic and proproliferative role in proliferative forms of glomerulonephritis, in which hypercellularity may be at least partly the result of the increased expression of survival factors such as Bcl-2 (8); and (2) near absence or minimal expression of Bcl-2 in both normal and diseased glomeruli reflects unopposed active apoptotic events in injured glomeruli (5).

To further elucidate the role of Bcl-2 in glomerular injury, we examined the expression patterns of Bcl-2 molecules and their association with Bax and the cyclin-dependent kinase
inhibitors (CKIs) p27kip1 and p57kip2 in representative renal biopsy specimens exhibiting mild to severe histopathological features characteristic of progressive human IgA nephropathy (IgAN). The modulatory roles of TNF-α and inducible nitric oxide synthase (iNOS) on Bcl-2 products were also investigated. We now report the novel observation of abundant Bcl-2 podocyte expression in progressive IgAN. Bcl-2 expression was upregulated in early-stage disease and was downregulated in advanced- and end-stage disease. Bcl-2 downregulation was closely associated with the development of progressive glomerular injury as well as clinical prognostic indicators of poor renal outcome in human IgAN.

Materials and Methods

Patients

Tissue samples were obtained from 51 patients with IgAN (24 men and 27 women; mean age, 38.3 yr; range, 15 to 70 yr) during the period 1997 to 2000, either by percutaneous renal biopsy at the National University Hospital of Singapore (NUH) or through referral from local nephrologists to the NUH pathology department for definitive diagnosis. Cases were selected on the basis of at least five glomeruli in light microscopic analysis of renal tissue, and the availability of adequate samples for immunopathology and electron microscopy. Creatinine levels, creatinine clearance rate, and magnitude of proteinuria based on 24-h urine collections at the time of diagnostic renal biopsy were available for the majority of subjects. Biopsy specimens were processed for routine microscopic analyses for the diagnosis of IgAN, including hematoxylin and eosin, periodic acid–Schiff, and Masson trichrome-silver impregnation staining, as well as electron microscopic analysis and immunofluorescence analysis (IgA, IgG, IgM, C3, C4, C1q, and fibrinogen) (9,10).

After excluding cases of Henoch-Schönlein purpura and known causes of secondary IgA glomerulonephritis (systemic lupus erythematosus, liver cirrhosis, or other systemic disease), we selected 51 patients whose biopsy specimens fulfilled the immunohistologic criteria for primary or idiopathic IgAN (predominant or codominant mesangial staining for IgA). To study mechanisms underlying the progression of proliferative and sclerosing glomerular lesions, we categorized the patient biopsy samples into four major descriptive subgroups according to the presence of predominant pathologic features: subgroup A, minor changes with mild thickening of the mesangium and no superimposed lesions; subgroup B, focal segmental proliferative/sclerosis with the majority of the uninvolved glomeruli showing minor changes; subgroup C, diffuse mesangial hypercellularity and hypertrophy, with focal superimposed lesions of sclerosis, adhesions, or crescents in less than 40% of the glomeruli; and subgroup D, diffuse mesangial hypercellularity and hypertrophy, with more than 40% of the glomeruli showing superimposed lesions as in group C. Ten nephrectomy samples without glomerular lesions were used as normal controls.

We use the term “progressive IgAN” in general throughout this study to denote the IgAN subgroups with progressively severe glomerular lesions. Our use of this term is not intended to imply that the disease process always runs a natural history of progression from “minimal” and “focal segmental” lesions (subgroups A and B) to “diffuse proliferative disease with varying degrees of sclerosis” (subgroups C and D). Indeed, IgAN appears to be a heterogeneous disease characterized by diverse clinical manifestations and varied glomerular morphology at presentation, associated with different clinical outcomes (10–14).

Antibodies and Probes

Monoclonal mouse anti-human p27kip1 (clone DCS-72.F6) (15), p57kip2 (57P06) (16), Bax (2D2) (17), and Bcl-2 (100/D5) (18,19) were purchased from Neomarker (Fremont, CA). Polyclonal rabbit anti-human iNOS (20,21) and TNF-α were obtained from Biomol (Plymouth, PA) and Rockland (Gilbertsville, PA), respectively. The anti-Bcl-2 (clone 100) used recognizes epitopes encoded by amino acids 41 to 54 of the human Bcl-2 sequence, the same epitopes recognized by anti-Bcl-2 clone 124 used in previous studies (5,7,22). A similar pattern of reactivity was reported with clones 100 and 124 when applied to either immunostaining or Western blotting (18,23).

The specificity of other antibodies was confirmed by their reactivity with specific human targets in immunoprecipitation and/or Western blotting, as documented in company data sheets and/or the above-cited references.

Digoxigenin (DIG)-labeled oligonucleotide probe cocktails against human Bcl-2 (GenBank accession number M14745), Bax (L22473), iNOS (L09210), and TNF-α (M10988) mRNA were commercially available from R&D systems (Minneapolis, MN). The use of DIG-labeled oligonucleotide probe cocktails in nonradioactive in situ hybridization method have been reported to have a detection sensitivity akin to methods that use radioisotope-labeled antisense RNA probes (24,25). This detection sensitivity can be boosted by incorporating the signal amplification systems (26,27). Every probe cocktail consisted of an equimolar mixture of three to four individual single-stranded DNA antisense oligonucleotides, each 27 to 31 bp long, with DIG labeling at both ends. Their specificity was validated by dot blot analysis against their corresponding specific cDNAs, and by the absence of homology with any other known nucleic acid sequences in the human genome, as determined by the BLAST software. The specificity of TNF-α antibodies was further confirmed by the absence of cross-reactivity with mRNA sequences encoding other known cytokines, as per the manufacturer’s data sheet.

Immunohistochemistry

Four-micron-thick paraformaldehyde-fixed tissue sections cut from paraffin-embedded renal biopsy specimens were used in the study, and a two-step EnVision+ System peroxidase kit (Dako, Carpinteria, CA) was used for immunohistochemistry. Slides were subjected to microwave antigen retrieval with wet heat at 98°C for 10 min under 150 W (Milestone microwave system, Italy) before the addition of antibodies against p27kip1 (1:200 dilution) and p57kip2 (1:400 dilution) overnight at 4°C (16,28). Colorimetric detection was performed with 3,3′-diaminobenzidine tetrahydrochloride, followed by mild counterstaining with periodic acid–Schiff reagents and hematoxylin. The specificity of labeling was confirmed by the absence of staining upon substitution of PBS or equal concentration of irrelevant nonimmune mouse serum for the primary antibody, or upon omission of secondary antibody. Tonsil and breast carcinoma tissues served as positive controls.

In Situ Hybridization Coupled with Immunohistochemistry

In situ hybridization was performed as described elsewhere with minor modifications (29). After deparaffinization and rehydration, sections were permeabilized by sequential treatment with Triton X-100, hydrogen chloride, and proteinase K digestion accordingly. Slides were then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine buffer, pH 8.0, for 10 min, followed by dehydration in graded alcohol, and allowed to air dry. Prehybridization and hybridization proceeded in hybridization buffer containing 5× SSC, 60%
formamide, hybridization accelerator, RNase inhibitor, and blocking reagents. After prehybridization at 42°C for 30 min, the DIG-labeled oligonucleotide probe cocktail for Bax, Bcl-2, TNF-α, or iNOS mRNA (30,31) was added at a concentration of 1 to 5 ng/ml. Sections were covered with parafilm and hybridized at 42°C overnight in a moist chamber. Posthybridization washes were performed in 2× SSC, 1× SSC, and 0.5× SSC at 37°C sequentially on the next day, 15 min each with two changes. Immunohistological detection was performed with a commercially available peroxidase–alkaline phosphatase system (APAAP, Dako). Briefly, horseradish peroxidase–conjugated mouse anti-DIG (Dako, 1: 50) and unconjugated mouse anti-DIG (Roche Diagnostics, 1:200) were applied before the addition of biotinylated Tyramide working solution (1:50), followed by alkaline phosphatase-conjugated streptavidin (SA-AP, Roche Diagnostics, 1:10,000), or mouse APAAP (Dako, 1:50) after rabbit anti-mouse (Dako, 1:50), respectively. Colorimetric detection was developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate at 37°C for 24 h. Negative controls consisted of sections that were not incubated with probes and those preincubated with RNase (20 μg/ml at 37°C for 30 min) before hybridization. There were no positive signals from any of the negative controls. Positive controls were normal renal sections hybridized with an oligo-dT probe cocktail (R&D Systems).

After washing, sections were subjected to microwave antigen retrieval in Dako target retrieval solution (anti-Bcl-2 and Bax) (33–35), or in 10 mM citrate buffer (anti-iNOS and TNF-α). The Dako EnVision+ peroxidase system was applied accordingly (36). Sections were then mildly counterstained with periodic acid–Schiff reagents before being dehydrated, cleared, and mounted.

**TdT-Mediated dUTP-Biotin Nick-End Labeling**

TdT-mediated dUTP-biotin nick-end labeling (TUNEL) staining was performed as per the manufacturer’s instructions. This method utilizes the In Situ Cell Death Detection Kit, Peroxidase (Boehringer Mannheim, Germany). Positive and negative controls were established by DNase I digestion before the addition of TdT and by omitting TdT, respectively. To avoid overestimation of apoptotic activity in TUNEL staining, only cells with observable morphologic features of apoptosis were counted.

**Semiquantitative Histologic Analysis**

Histologic evaluation was performed under light microscopy by two independent pathologists who were blinded to the clinical and demographic characteristics of the patients. The agreed-upon score was adopted for the final data set. In cases of disagreement in the scoring, the process was repeated and the final score was determined by consensus of both observers. The glomerular positivity was expressed as the mean size by each subgroup. The averaged glomerular size was 1.7 ± 0.3 mm² when disease subgroups and controls were compared; glomeruli were slightly larger in subgroup C than those in subgroups B and D (P < 0.05). A staining score was used for analyzing the TNF-α immunoreactivity in glomeruli (TNF-α glomerular immunohistochemistry score), ranging from 0 (negative staining) to 7 (maximum staining intensity with ≥75% of stained glomerular tufts) (37).

The proliferation index (PI) was assessed as a measure of the degree of cellular proliferation in the mesangium, and expressed on an arbitrary scale (0 to 3) based on the number of affected lobes: absent (0); mild, one lobe (1); moderate, two to three lobes (2); severe, more than four lobes (3). The mean glomerulosclerosis score (GS score) was measured on a semiquantitative scale of 0 to 4 for the degree of glomerulosclerosis (0, no glomerulosclerosis; 1, <25%; 2, 26% to 50%; 3, ≥51% to 75%; and 4, ≥76% to 100% glomerulosclerosis) per glomerulus and averaged as mean GS score per tissue section (38,39).

The index of glomerular lesion (IGL) took into account both proliferative and sclerotic changes as described previously (40,41). In short, the degree of glomerular damage was graded 0 to 4 according to the percentage of injured lobules as the result of mesangial proliferation and glomerular sclerosis. The average of degrees for all glomeruli in one tissue section was calculated and registered as the IGL: [(0 × N₀) + (1 × N₁) + (2 × N₂) + (3 × N₃) + (4 × N₄)]/N, where N₀ – N₄ = number of glomeruli showing changes of grades 0 to 4, respectively, and N is the total glomeruli per tissue section.

**Statistical Analyses**

Results were expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA, followed by Fisher’s protected least significant difference post hoc analysis for multiple comparisons among four diseased subgroups and normal tissue. For nonparametric data, the Mann-Whitney U–Wilcoxon rank sum W test was used for multiple comparisons. Correlation was determined with Spearman correlation coefficients. P < 0.05 was considered to be significant.

**Results**

**Categorization of IgAN Subgroups**

To confirm the validity of our descriptive subgroup definitions, we applied additional semiquantitative histologic indices (PI, IGL, and mean GS score) that address the outcome variables of glomerular proliferative and sclerotic changes. Table 1 shows the degree of glomerular proliferation and sclerosis characteristic of each subgroup. The validity of our original “descriptive” IgAN subgroup definitions was confirmed by the combined ability of the PI, IGL, and GS scores to distinguish statistically significant differences among these subgroups. Subgroups A and B differed from subgroups C and D by both

**Table 1. IgAN subgroups are distinguished by semiquantitative indices of glomerular proliferation and sclerosis**

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>n</th>
<th>PI</th>
<th>IGL</th>
<th>Mean GS Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgAN A</td>
<td>5</td>
<td>0.85 ± 0.22</td>
<td>0.67 ± 0.11</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>IgAN B</td>
<td>21</td>
<td>1.05 ± 0.83</td>
<td>1.27 ± 0.10</td>
<td>0.44 ± 0.08</td>
</tr>
<tr>
<td>IgAN C</td>
<td>15</td>
<td>1.84 ± 0.15</td>
<td>2.37 ± 0.14</td>
<td>1.50 ± 0.18</td>
</tr>
<tr>
<td>IgAN D</td>
<td>10</td>
<td>1.64 ± 0.15</td>
<td>3.42 ± 0.07</td>
<td>2.97 ± 0.12</td>
</tr>
</tbody>
</table>

a PI, proliferation index; IGL, index of glomerular lesion; Mean GS Score, mean glomerulosclerosis score. Results expressed as mean ± SEM.

b P < 0.01 versus IgAN A.

c P < 0.01 versus IgAN B.

d P < 0.001 versus IgAN A and B.

e P < 0.001 versus IgAN A, B, and C.
the degree of proliferation and sclerosis; subgroup C was distinguished from subgroup D by the extent of sclerotic lesions.

**Bcl-2 Oncoprotein Was Overexpressed Predominantly by Podocytes in Glomerular Lesions of IgAN**

Bcl-2 immunoreactivity was readily detectable in glomerular lesions of IgAN, which was predominantly localized to visceral epithelial cells (podocytes), and to a lesser extent, to a few parietal epithelial cells, in both normal and diseased glomeruli (Figures 1 and 2). A low endogenous level of Bcl-2 oncoprotein by podocytes (an average of 1.4 cells per glomerular cross section) was detected in normal tissue, while a robust increase in Bcl-2 immunoreactivity was observed in podocytes of subgroups A and B (P < 0.001 versus normal control), followed by a gradual decrease in the number of Bcl-2-expressing podocytes in subgroups C and D (IgAN A versus IgAN C and D, P < 0.001; IgAN B and C versus IgAN D, P < 0.01, Figure 1). This pattern was inversely correlated with the PI (r = −0.367, P = 0.011), IGL (r = −0.634, P < 0.001), and mean GS score (r = −0.575, P < 0.001). Immunoreactive Bcl-2 was also detectable in cellular lesions (cellular adhesions or crescents) (Figure 2, D and E). In contrast, Bcl-2 mRNA expression in glomerular lesions of IgAN was minimal (Figure 2).

**Increased Ratio of Bax/Bcl-2 by Glomerular Epithelial Cells Was Identified in Progressive Glomerular Lesions of IgAN**

Unlike Bcl-2, a modest expression of Bax mRNA was observed in glomerular epithelium, endothelium, and mesangium in all four IgAN subgroups (Figure 3). Upregulation of Bax mRNA in podocytes was observed in subgroup B; expression was less pronounced in subgroups C and D (data not shown). Immunoreactive Bax was detected in fewer podocytes in comparison with Bcl-2. However, although a pattern of progressive decrease in podocyte Bcl-2 expression was observed, Bax overexpression persisted in all IgAN subgroups (subgroups A to D versus normal control, P < 0.01, Figure 1), despite the increased percentage of severe glomerulosclerosis and extensive collapse of glomerular tufts characteristic of late-stage disease. Bax protein immunoreactivity was also observed in glomerular mesangium and endothelium (Figure 3).

Notably, an associated stepwise increase in the Bax/Bcl-2 ratio by glomerular epithelial cells (GECs) with increasing severity of histopathological lesions was observed, reflecting concurrent Bax upregulation (r = 0.729, P < 0.001) and Bcl-2 downregulation (r = −0.511, P = 0.001, Table 2). This increased Bax/Bcl-2 ratio correlated with glomerular cell apoptosis as assessed by TUNEL staining (r = 0.363, P = 0.018). TUNEL-positive cells were identified in all cell types of the glomerular compartment, including mesangial, endothelial, and visceral epithelial cells, as well as cells present in capsular adhesions and cellular crescents. A significant increase in the number of apoptotic cells was observed in advanced-stage disease (subgroup C), as shown in Table 2. The observed increased Bax/Bcl-2 ratio in GECs was further related to the PI (r = 0.361, P = 0.041), IGL (r = 0.465, P = 0.002), and mean GS score (r = 0.464, P = 0.002), indicating a role for Bax/Bcl-2 signaling in the development of progressive glomerular injury during the chronic course of IgAN.

**Bcl-2 Overexpression in Progressive IgAN Was Associated with the Expression Levels of p27kip1 and p57kip2 by Podocytes**

High endogenous podocyte expression of p27kip1 (p27) and p57kip2 (p57) persisted in early-stage disease (subgroups A and B, Figure 4A), in the presence of high levels of Bcl-2 oncoprotein. This robust expression of p27 and p57 by podocytes was notably downregulated in advanced- and end-stage disease (Figure 4A, subgroups C and/or D), in association with decreased levels of Bcl-2 oncoprotein by glomerular epithelium. Podocyte expression of both p27 and p57 in progressive glomerular injury was highly correlated with the overexpression of Bcl-2 oncoprotein in progressive IgAN (Figure 4, B and C), and their downregulation in late-stage disease was inversely correlated with the grade of mesangial hypercellularity and glomerulosclerosis (Table 3).

**Glomerular Induction of TNF-α and iNOS Products Paralleled Bcl-2 Overexpression in Progressive IgAN**

To explore the mechanism or mechanisms underlying the variable expression patterns of Bcl-2 oncoprotein in progressive IgAN, we examined the association between Bcl-2 levels and the upregulation of cytokine and iNOS products. We observed dramatic upregulation of TNF-α mRNA in podocytes of early-stage disease (subgroups A and B), together with the induction of iNOS protein by podocytes in intermediate-stage disease (subgroups B and C), in contrast to their decreased levels in advanced- and/or end-stage disease (Figure 5, subgroups C and/or D). Intense staining for TNF-α protein was similarly observed in all IgAN subgroups, with peak levels in subgroup C, and was primarily localized to glomerular endothelium, mesangium, and epithelium in addition to a few infiltrating leukocytes (Figure 6). The induction of TNF-α mRNA in podocytes and its increased staining score for im-
munoreactive TNF-α in glomerular lesions were well correlated with the overexpression of iNOS protein by podocytes (Figure 7), while the TNF-α glomerular staining score was additionally related to the increased number of apoptotic cells in advanced-stage disease ($r = 0.357$, $P = 0.009$), suggesting its role in mediating proapoptotic activity. The increased expression of iNOS and TNF-α at levels of mRNA and/or protein was strongly correlated with the overexpression of Bcl-2 protein, and to a lesser extent with Bax protein, by podocytes in both normal kidneys and diseased glomeruli in progressive IgAN (Table 4).

**Downregulation of Bcl-2 Oncoprotein Was Associated with Clinical Indices of Poor Renal Outcome**

Notably, both the downregulation of Bcl-2 oncoprotein and the decreased expression levels of p27 and p57 by podocytes in late stage of IgAN were strongly and inversely correlated with the elevated serum creatinine, with the decline in creatinine clearance rate and with the magnitude of proteinuria at the time of diagnostic renal biopsy (Table 5). Weak and inverse correlations were also observed between the impairment of renal function and the expression levels of TNF-α mRNA or iNOS protein by podocytes in progressive IgAN. In contrast, the Bax/Bcl-2 ratio of GECs was positively correlated with impairment of renal function as well as the magnitude of proteinuria (Table 5).

*Figure 2. Detection of Bcl-2 mRNA and protein expression using in situ hybridization (purplish blue) coupled with immunohistochemistry (brown). Bcl-2 mRNA was barely detectable in both normal and diseased glomeruli. In contrast, upregulation of Bcl-2 protein by podocytes in early-stage disease (IgA nephropathy [IgAN] A and B) and downregulation in late-stage disease (IgAN C and D) was evident. Immunoreactive Bcl-2 and mRNA overexpression were observed in cellular adhesions and crescents (D and E); representative immunoreactive cells and mRNA expressing cells indicated by arrows and arrowheads, respectively. Counterstained with periodic acid–Schiff. Original magnification, ×400.*

*Figure 3. Detection of glomerular Bax mRNA (purplish blue) and protein (brown) in progressive IgA nephropathy (IgAN). (A) Occasional Bax mRNA, but little protein was detected in normal kidney. (B to D), modest overexpression of Bax mRNA (representative cells indicated by arrowheads) was observed in glomerular endothelium, mesangium, and epithelium, whereas upregulation of Bax protein was notable in glomerular epithelium, endothelium, and to a lesser degree in mesangium in disease subgroups. There was coexpression of Bax mRNA and protein in glomerular cells (B, C) as well as in an infiltrating leukocyte in the lumen of an afferent arteriole (D, arrows). Counterstained with periodic acid–Schiff. Original magnification, ×250 (D); ×400 (A to C).*
Table 2. Increased ratio of Bax/Bcl-2 expression in glomerular epithelial cells associated with glomerular cell apoptosis in the course of IgAN

<table>
<thead>
<tr>
<th>Grouping</th>
<th>Bax (cells/GCS)</th>
<th>Bcl-2 (cells/GCS)</th>
<th>Bax/Bcl-2</th>
<th>TUNEL in Glomeruli (cells/GCS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>1.081 ± 0.738</td>
<td>1.439 ± 0.565</td>
<td>0.002 ± 0.002</td>
<td>0.006 ± 0.056</td>
</tr>
<tr>
<td>IgAN A</td>
<td>6.167 ± 2.912</td>
<td>15.122 ± 3.172b</td>
<td>0.342 ± 0.117b</td>
<td>0.184 ± 0.079</td>
</tr>
<tr>
<td>IgAN B</td>
<td>5.928 ± 0.907b</td>
<td>10.961 ± 0.775b</td>
<td>0.566 ± 0.098b</td>
<td>1.298 ± 0.378b</td>
</tr>
<tr>
<td>IgAN C</td>
<td>7.402 ± 1.178b</td>
<td>10.174 ± 1.255b</td>
<td>1.160 ± 0.363b</td>
<td>2.068 ± 0.375e</td>
</tr>
<tr>
<td>IgAN D</td>
<td>7.230 ± 1.342b</td>
<td>6.076 ± 1.155bcd</td>
<td>2.115 ± 1.096bcd</td>
<td>0.643 ± 0.181</td>
</tr>
</tbody>
</table>

* The ratio was compared at the level of protein inclusive of both parietal epithelial cells and visceral epithelial cells as a whole.

b *P < 0.05 versus NC.

c *P < 0.05 versus IgAN A.
d *P < 0.05 versus IgAN B.
e *P < 0.05 versus all other groups.

Figure 4. Podocyte expression of p27kip1 and p57kip2 protein levels was highly correlated with the expression of Bcl-2 in progressive IgA nephropathy (IgAN). (A) High endogenous expression of p27kip1 and p57kip2 by podocytes decreased with progressive glomerular disease (*P < 0.05 versus NC; *P < 0.05 versus IgAN A; *P < 0.05 versus IgAN B; *P < 0.05 versus all other groups). (B and C) Downregulation of podocyte p27kip1 and p57kip2 expression was well correlated with the decreased level of Bcl-2 protein in podocytes in the progression of glomerular injury.

Discussion

Previous studies that reported an increased expression of Bcl-2 in human proliferative glomerulonephritis proposed that the increased expression of glomerular Bcl-2 may be a mechanism for the maintenance of glomerular hypercellularity in human glomerular disease by preventing cell death and by counteracting the functions of Bax (5,7,8). However, the results of our study question this hypothesis by observing a variable expression pattern of Bcl-2 oncoprotein in progressive IgAN, characterized by upregulation at the initial and early stage of IgA glomerulonephritis, followed by downregulation in advanced- and end-stage disease. The podocyte overexpression of Bcl-2 oncoprotein was inversely related to the severity of glomerular damage as defined by indices of glomerular hypercellularity and glomerulosclerosis, as well as by clinical indices such as impairment of renal function and the magnitude of proteinuria. These findings suggest that the glomerular expression of Bcl-2 protein may play an antiproliferative role in progressive glomerular injury, rather than mediating a pro-proliferative state as previously proposed (8). Moreover, the downregulation of Bcl-2 expression was statistically significant between subgroups C and D (Figure 1), which differ only by the degree of sclerotic, but not proliferative, changes. These data suggest a specific role of Bcl-2 expression in the development of sclerotic lesions in late-stage disease. Consistent with this hypothesis, the Bcl-2 expression level correlated better with indices of sclerosis (mean GS score) than proliferation (PD) (Table 3).

Experimental studies have established a role for Bcl-2 in slowing the progression of cell cycle (42,43). This antiproliferative effect of Bcl-2 may occur through modulation of the expression of p27 and p57kip2, two members of the cip/kip family of CKIs. In cultured fibroblasts, Bcl-2 was shown to retard cell cycle entry by increasing the levels of p27 and p130, a member of the pRb family (42). p27 has been reported to be required for the cell cycle function of Bcl-2 (44). Bcl-2 may delay cell cycle entry by inhibition of c-Myc activity through the elevation of p27 (44). Upregulation of Bcl-2 results in both an increase in the level of p27 and inhibition of cell proliferation (45). A previous report has implicated Bcl-2 in CKI regulation of the podocyte lineage (46). Consistent with these observations, we observed persistence of high levels of p27 and p57 expression by podocytes in IgAN subgroups A and B, which may be because of the upregulation of Bcl-2 in early-stage disease. Conversely, downregulation of Bcl-2 in late-stage disease may be responsible for the downregulation of p27 and p57 expression in glomeruli characterized by severe forms of glomerular damage.

Notably, the onset and grade of mesangial hypercellularity was observed to be strongly associated with the expression...
levels of p27 and p57 by podocytes in our study of human IgAN, a similar finding in experimental anti-Thy1.1 nephritis (47,48). Furthermore, our observation provides evidence for the association of CKIs, particularly p57 downregulation, with the development of sclerotic injury in glomeruli of IgAN patients. These data support a process in which the elaboration of regulatory factors by podocytes may play a direct role in the activation of mesangial cell proliferation. Another potential role for podocyte expression of Bcl-2 in mediating glomerular lesions may relate to the ability of Bcl-2 to delay E2F1 expression, as shown in cultured fibroblasts (42). Consistent with this report, we have observed an association between overexpression of mesangial E2F1 and downregulation of p27 and p57 in glomeruli exhibiting mesangial hypercellularity (Qiu et al., unpublished data).

Downregulation of Bcl-2 in late-stage disease was found to be correlated with an increased Bax/Bcl-2 ratio in GECs, which was related to increased glomerular cell apoptosis as well as severe glomerular damage in progressive IgAN. Pro-apoptotic Bax and antiapoptotic Bcl-2 are two important regulators of apoptosis whose heterodimerization can nullify the functions of each (49,50). The outcome of a cell that receives an apoptotic stimulus is thought to depend partly on the ratio of the death promoter (Bax) to the death suppressor (Bcl-2) (51,52). Thus, an overexpression of Bcl-2 protein observed in early stages of IgAN may confer protection against apoptotic injury to glomerular cells by counteracting the opposing activities of Bax protein. It was shown in activated T cells that Bcl-2 overexpression delayed the degradation of p27, whereas Bax accelerated the degradation of p27 (53,54). Bax expression may be associated with matrix expansion in progressive glomerular injury. Increased Bax mRNA has been reported to

**Table 3.** Correlations of the severity of glomerular damage with podocyte expression levels of different markers in progressive glomerular injurya

<table>
<thead>
<tr>
<th></th>
<th>PI</th>
<th></th>
<th>IGL</th>
<th></th>
<th>Mean GS Score</th>
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<tbody>
<tr>
<td></td>
<td>CC</td>
<td>P</td>
<td>CC</td>
<td>P</td>
<td>CC</td>
<td>P</td>
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<td>0.002</td>
<td>0.464c</td>
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<td>iNOS</td>
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<td>TNFα Glomerular Score IHC</td>
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<td>0.313</td>
<td>0.400c</td>
<td>0.005</td>
<td>-0.398c</td>
<td>0.005</td>
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a Protein level of various markers expressed by podocytes was used in the analysis unless otherwise specified. PI, proliferation index; IGL, index of glomerular lesion; Mean GS Score, mean glomerulosclerosis score; CC, Spearman Rho correlation coefficient; GEC, glomerular epithelial cells; ISH, *in situ* hybridization for the comparison at level of mRNA; IHC, immunostaining for the analysis at protein level.

b Correlation was significant at the 0.05 level (two-tailed).

c Correlation was significant at the 0.01 level (two-tailed).

**Figure 5.** Induction of TNF-α and inducible nitric oxide synthase (iNOS) products in podocytes (A) and glomerular lesion (B) in progressive IgA nephropathy (IgAN). Results were expressed as mean ± SEM (A) or mean (bar) with clouds for individual values (B). *P < 0.05 versus NC; aP < 0.05 versus IgAN A; cP < 0.05 versus IgAN C; dP < 0.05 versus IgAN D.
correlate positively with matrix expansion or type IV collagen accumulation in the glomerulus of proliferative forms of glomerulonephritis including IgAN (8). In anti-Thy1.1 nephritis, a hydramate-based matrix metalloproteinase inhibitor was demonstrated to attenuate excess mesangial cell proliferation and extracellular matrix accumulation in a Bax-dependent manner, through the induction of cell cycle arrest followed by apoptosis (55). Indeed, we observed that the downregulation of Bcl-2, associated with an increased ratio of Bax/Bcl-2 by GECs in progressive IgAN, was strongly and positively correlated with the severity of glomerulosclerosis.

Notably, a progressive increase in the ratio of Bax/Bcl-2 was associated with a decreased number of apoptotic cells in glomeruli of end-stage disease. The relatively low level of apoptotic activity may be because of the predominant presence of glomerulosclerosis in end-stage glomeruli, and/or may reflect a possible “apoptotic defect” characteristic of end-stage injury. Both hypercellularity (unchecked mesangial cell growth due to an apoptotic defect) and hypocellularity (a consequence of excess apoptosis preceding advanced-stage disease) may contribute to glomerulosclerosis, as hypothesized by Haas et al. in 1999 (56).

Abundant Bcl-2 expression has been reported in cellular and fibrocellular crescents in human lupus nephritis, rapidly progressive glomerulonephritis, and transplant rejection (5,57). In line with these observations, we provide evidence for Bcl-2 overexpression in cellular lesions in progressive IgAN. It has been reported that the major component of the cellular crescents is parietal epithelial cells, and that apparent overexpression of cyclins and Bcl-2 coupled with restrained expression of p27 may be synergistically associated with the development of cellular crescents in human crescentic glomerulonephritis (58). The report of Bcl-2 overexpression without detectable p27 and p57 in cellular adhesions/crescents may be associated with the deregulation of apoptosis and the possible role of apoptosis in the progression of glomerular scarring (5), or alternatively,

Figure 6. Detection of inducible nitric oxide synthase (iNOS) and TNF-α mRNA (purplish blue) and protein (brown) levels in progressive IgA nephropathy (IgAN). (A and B) Negative and positive controls for in situ hybridization, counterstained with nuclear fast red. (C and D) iNOS and TNF-α expression in normal tissue (original magnification, ×200). Induction of iNOS protein (E and F) and TNF-α mRNA in podocytes as well as TNF-α immunoreactivity in glomerular lesions (G and H) was evident in early and midstage disease, but was decreased in end-stage disease. Induction of iNOS and TNF-α products was also notable in glomerular endothelium, mesangium, and cellular crescents (representative mRNA expressing cells indicated by arrowheads). Original magnification, ×400. C to H, counterstained with periodic acid–Schiff.
human IgAN, we speculate that the early induction of TNF-α products in early-stage disease (subgroup A) and their strong in-
dependent cell death processes such as mitochondria-dependent cell killing through alterations in the membrane permeability of mitochondria (60,61). Therefore, TNF-α products may be involved in the elevation of Bcl-2 protein levels in podocytes. This could be partly achieved through the activation of NF-κB, which in turn may upregulate antiapoptotic factors such as Bcl-2/Bcl-xl.

On the basis of extensive evidence that reactive oxygen intermediates mediate TNF-α–induced signaling (60), we determined whether TNF-α–induced Bcl-2 overexpression may be mediated via the induction of iNOS in podocytes. Our results demonstrate a close association between iNOS, TNF-α, and Bcl-2 overexpression. Thus, elevated levels of iNOS products in response to TNF-α stimulation may contribute to the upregulation of Bcl-2 protein in early-stage disease, while low levels of NO and its metabolites may underlie the downregulated Bcl-2 and p27 proteins in late-stage disease. A dual role for iNOS and NO in regulating antiapoptotic and proapoptotic effects through the modulation of Bcl-2 protein levels has been previously reported (62,63). iNOS may serve as a “biosensor” of disease exacerbation in inflammatory conditions such as IgAN. An association between TNF-α and iNOS products in mediating glomerular injury has also been observed in an experimental model of diabetic nephropathy (64). However, a differential effect of iNOS and TNF-α products on the patterns of pathologic injury has been noted in this study. iNOS was shown to correlate more with glomerulosclerosis, probably as a result of its capacity in modulating Bax expression as well, whereas TNF-α products were related to both proliferative and sclerotic changes as mentioned above. These descriptive observations raise the possibility of heterogeneous roles for TNF-α and iNOS regulation and signaling in the pathogenesis of glomerulonephritis. Although our descriptive finding that TNF-α and iNOS expression correlates with Bcl-2 and Bax levels does not prove a cause-and-effect relationship, it is nonetheless a compelling and hypothesis-generating observation that deserves further study.

In summary, our results show that the overexpression of Bcl-2 protein by podocytes may exert a protective effect on the overall development of glomerular lesions in progressive IgAN through its affect on the Bax/Bcl-2 ratio, thus limiting glomerular cell apoptosis, as well as by maintaining high endogenous protein levels of p27 and p57. The latter may limit the devel-

| Table 4. Correlations among the induction of tumor necrosis factor α (TNF-α), inducible nitric oxide synthase (iNOS), and the protein levels of Bcl-2/Bax by podocytes in glomerular injury of IgANa |
|-----------------|-----------------|-----------------|
|                | Bcl-2            | Bax             |
|                | CC, P            | CC, P           |
| TNF-α ISH      | 0.558b <0.001    | 0.285c 0.041    |
| TNF-α Glomeruli Score IHC | 0.495b <0.001    | 0.350b 0.009    |
| iNOS IHC       | 0.669b <0.001    | 0.465b <0.001   |

a Analysis was made in podocytes at level of protein unless specified. ISH, in situ hybridization for mRNA level; IHC, immunostaining for the protein expression.

b Correlation was significant at the 0.01 level (two-tailed).

c Correlation was significant at the 0.05 level (two-tailed).

represent a compensatory response to the proliferative activity in these lesions.

It has been suggested that Bcl-2 transcription may be up-regulated by prosurvival cytokines (59). In this study, we demonstrated an association between the induction of TNF-α and Bcl-2 upregulation in the development of glomerular lesions. TNF-α and its downstream effectors, including nuclear factor kappa B (NF-κB) and c-Jun N-terminal kinase, have been recognized as inducers of both apoptosis and proliferation (60). TNF-α–triggered intracellular transduction pathways involve not only death receptor signaling, leading directly to the activation of caspase cascades, but also various caspase-independent cell death processes such as mitochondria-dependent cell killing through alterations in the membrane permeability of mitochondria (60,61). Therefore, TNF-α signaling in glomerular cells could serve as a link between two distinct apoptotic pathways, and may play a role in determining the apoptotic response to glomerular injury. As evidenced by the concomitant upregulation of TNF-α transcripts and Bcl-2 protein products in early-stage disease (subgroup A) and their strong inverse correlation with the development of glomerular lesions in human IgAN, we speculate that the early induction of TNF-α products may be involved in the elevation of Bcl-2 protein levels in podocytes. This could be partly achieved through the activation of NF-κB, which in turn may upregulate antiapoptotic factors such as Bcl-2/Bcl-xl.

On the basis of extensive evidence that reactive oxygen intermediates mediate TNF-α–induced signaling (60), we determined whether TNF-α–induced Bcl-2 overexpression may be mediated via the induction of iNOS in podocytes. Our results demonstrate a close association between iNOS, TNF-α, and Bcl-2 overexpression. Thus, elevated levels of iNOS products in response to TNF-α stimulation may contribute to the upregulation of Bcl-2 protein in early-stage disease, while low levels of NO and its metabolites may underlie the downregulated Bcl-2 and p27 proteins in late-stage disease. A dual role for iNOS and NO in regulating antiapoptotic and proapoptotic effects through the modulation of Bcl-2 protein levels has been previously reported (62,63). iNOS may serve as a “biosensor” of disease exacerbation in inflammatory conditions such as IgAN. An association between TNF-α and iNOS products in mediating glomerular injury has also been observed in an experimental model of diabetic nephropathy (64). However, a differential effect of iNOS and TNF-α products on the patterns of pathologic injury has been noted in this study. iNOS was shown to correlate more with glomerulosclerosis, probably as a result of its capacity in modulating Bax expression as well, whereas TNF-α products were related to both proliferative and sclerotic changes as mentioned above. These descriptive observations raise the possibility of heterogeneous roles for TNF-α and iNOS regulation and signaling in the pathogenesis of glomerulonephritis. Although our descriptive finding that TNF-α and iNOS expression correlates with Bcl-2 and Bax levels does not prove a cause-and-effect relationship, it is nonetheless a compelling and hypothesis-generating observation that deserves further study.

In summary, our results show that the overexpression of Bcl-2 protein by podocytes may exert a protective effect on the overall development of glomerular lesions in progressive IgAN through its affect on the Bax/Bcl-2 ratio, thus limiting glomerular cell apoptosis, as well as by maintaining high endogenous protein levels of p27 and p57. The latter may limit the devel-

Figure 7. Induction of inducible nitric oxide synthase (iNOS) protein by podocytes was associated with TNF-α products in podocytes (A) and glomerular lesions (B) in progressive IgA nephropathy (IgAN).

Table 4. Correlations among the induction of tumor necrosis factor α (TNF-α), inducible nitric oxide synthase (iNOS), and the protein levels of Bcl-2/Bax by podocytes in glomerular injury of IgANa
Table 5. Clinicopathological correlation between the expression levels of different markers by podocytes and the severity of renal dysfunction in progressive IgAN

<table>
<thead>
<tr>
<th>Markers</th>
<th>Creatinine (µmol/L)</th>
<th>Creatinine Clearance Rate (ml/min)</th>
<th>24h Urine Protein (g/d)</th>
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<tr>
<td></td>
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<td>n</td>
</tr>
<tr>
<td>p27</td>
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</table>

* Protein levels of different markers expressed by podocytes were analyzed unless otherwise specified. CC, Spearman correlation coefficients; GEC, glomerular epithelial cells.

Correlation was significant at the 0.05 level (two-tailed).

Correlation was significant at the 0.01 level (two-tailed).

opment of progressive glomerular proliferative and sclerotic lesions. A schematic model for the modulation of mesangial cell proliferation, glomerular cell apoptosis, and glomerulosclerosis in progressive IgAN is outlined in Figure 8. We propose that the variable expression levels of Bcl-2 during the course of progressive IgAN may be mediated by the induction of TNF-α and iNOS products in response to glomerular injury and thus play a role in the pathogenesis of human glomerulonephritis through the coordination of cell-proliferation and cell-death pathways. The observed associations between podocyte Bcl-2 expression and the degree of glomerular cell proliferation, apoptosis, and glomerulosclerosis were reflected in clinical indices that are well established predictors of poor renal outcome in patients with IgAN.

**Figure 8.** Schematic model of the variable expression of Bcl-2 protein levels by podocytes in the regulation of glomerular response to injury in progressive IgA nephropathy (IgAN).

**Table 5.** Clinicopathological correlation between the expression levels of different markers by podocytes and the severity of renal dysfunction in progressive IgAN

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**Acknowledgments**

We thank Jon Choon Evan Lee (senior consultant nephrologist at National University Hospital, Singapore), Drs. Gordon Ku, Akira Wu, Wai Choong Lye, Serh Sheng Wei (Mount Elizabeth Hospital, Singapore), See Odd Leong (Gleneagles Hospital, Singapore), Simon Ching King Wong (Timberland Medical Center, Malaysia), and Thian Chai Lee (Johor Specialist Center, Malaysia) for their assistance in providing the clinical data on the biopsy cases included in the study presented here.

**References**


