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CLINICAL INVESTIGATION

Expression of Fas antigen and Bcl-2 in human glomerulonephritis

TSUKASA TAKEMURA, KATSUMI MURAKAMI, HIROFUMI MIYAZATO, KAZURO YAGI, and KAZUO YOSHIOKA

Department of Pediatrics, Kinki University School of Medicine, Osaka-sayama 589, Japan

Expression of Fas antigen and Bcl-2 in human glomerulonephritis. To understand the regulatory mechanism of apoptosis in human glomerulonephritis, we examined the expression of Fas antigen (CD95) and Bcl-2 in five normal human kidney specimens and 80 tissues from patients with several types of glomerular diseases. These proteins were detected in glomeruli by immunofluorescence. The number of intraglomerular cells positive for Fas antigen was high in Henoch-Schönlein purpura nephritis and lupus nephritis, and that of Bcl-2-positive intraglomerular cells was high in lupus nephritis, focal glomerular sclerosis, and IgA nephritis. Dual-labeling and staining on serial sections indicated that mesangial cells and occasionally infiltrating leukocytes expressed Fas antigen and Bcl-2. *In situ* hybridization detected Bcl-2 mRNA in glomerular cells. Electron microscopy revealed apoptotic cells and apoptotic bodies in proliferated mesangial areas and within the glomerular capillaries. Fragmented DNA was detected in glomeruli by *in situ* nick end labeling, the data of which paralleled the number of Fas antigen-positive intraglomerular cells. In mesangial proliferative types of glomerulonephritis, the population of Bcl-2-positive intraglomerular cells, but not that of Fas antigen-positive cells, was significantly correlated with the number of proliferating cell nuclear antigen-positive glomerular cells, the grade of mesangial cell increase, and the magnitude of proteinuria. This study showed that Fas antigen and Bcl-2 are up-regulated in the glomeruli of several types of human renal diseases. Bcl-2 overexpression might play a role in the prolonged proliferation of mesangial cells and glomerular hypercellularity in glomerulonephritis.

Apoptosis or programmed cell death plays a crucial role in embryology, immunology and neoplasia [1, 2]. In the kidney, apoptotic cell death has been observed in glomeruli of proliferative types of human glomerulonephritis [3], and in mesangial cells of anti-Thy 1 antibody-induced glomerulonephritis [4, 5]. It is likely that apoptosis of infiltrating leukocytes and intrinsic cells in the inflamed glomeruli is a mechanism for the clearance of such cells, thus promoting resolution from glomerular injury. Recovery from proliferative glomerulonephritis occurs in several human renal diseases, such as post-streptococcal acute glomerulonephritis and Henoch-Schönlein purpura nephritis [6]. On the other hand, in most chronic forms of human glomerulonephritis, intrinsic glomerular cells, particularly mesangial cells, persistently proliferate. It is postulated that the mechanism for inhibiting apoptotic cell death plays a role in the progression of glomerular injury in those types of glomerulonephritis.

The protein encoded by the Bcl-2 (B-cell lymphoma/leukemia-2) protooncogene is a critical regulator of apoptosis and can block apoptotic cell death and prolong the lifespan of lymphoid cells [7–9]. The expression of Bcl-2 is not specific to neoplastic lymphoid nodes, and it has been identified in a number of nonhematolymphoid tissues [10–12].

There are no precise descriptions of the expression of Fas antigen (APO-1/CD95) and Bcl-2 protein in human glomerulonephritis. Here, we studied the expression of Fas antigen and Bcl-2 mRNA and protein in patients with various glomerular diseases.

Methods

Tissues

Kidney tissue specimens were obtained by percutaneous needle or surgical biopsy from 80 patients with glomerular diseases: 12 had minimal change nephrotic syndrome, 7 had focal glomerular sclerosis, 38 had IgA nephritis, 10 had mesangial proliferative (IgA-negative) glomerulonephritis, 7 with Henoch-Schönlein purpura nephritis and 6 with lupus nephritis (WHO Class IV) [13]. Laboratory data including urinalysis, 24-hour protein excretion, and creatinine clearance were collected from each patient. Most of the patients, excluding three with focal glomerular sclerosis,

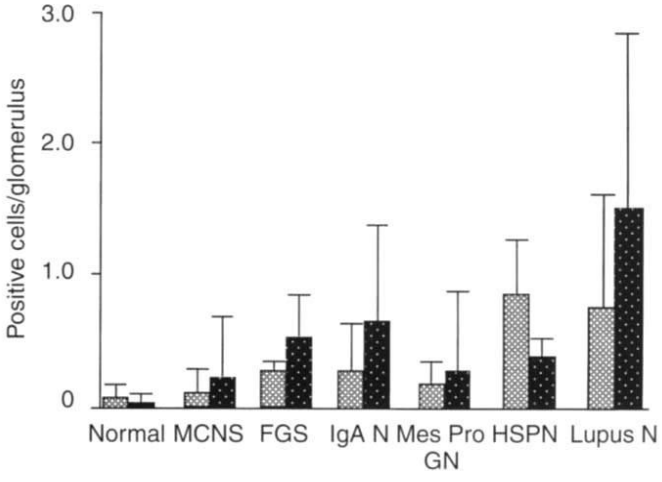

Fig. 1. *The proportion of intraglomerular cells positive for (▨) Fas antigen and Bcl-2 (▩) protein in various glomerular diseases.* Positive cells per glomerular cross section are expressed as means ± SD. Abbreviations are: MCNS, minimal change nephrotic syndrome; FGS, focal glomerular sclerosis; IgA N, IgA nephritis; Mes Pro GN, mesangial proliferative (IgA-negative) glomerulonephritis; HSPN, Henoch-Schönlein purpura nephritis; Lupus N, lupus nephritis.

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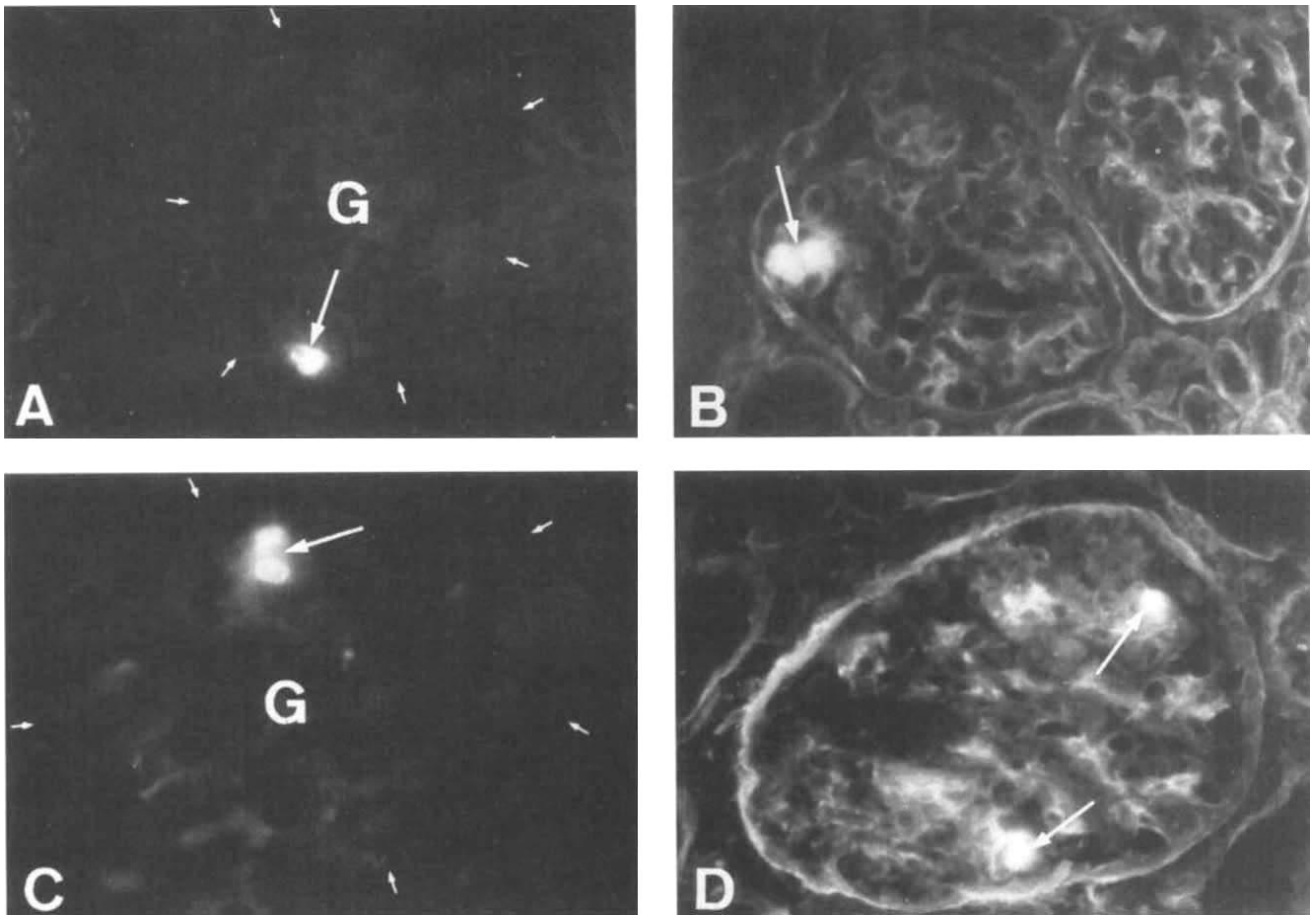

Fig. 2. *Immunofluorescent staining for Fas antigen and Bcl-2 protein in tissues from patients with IgA nephritis.* **A.** Cellular staining for Fas antigen. **B.** Fas antigen and type IV collagen were double-stainied with fluorescein- and rhodamine-labeled secondary antibodies, and photographed at the same time. The glomerular and tubular basement membranes, mesangium, and Bowman's capsules are stained for type IV collagen. Fas antigen-positive cells are located in the mesangial area. **C.** Bcl-2 protein-positive cells in a glomerulus. **D.** Double staining of Bcl-2 and type IV collagen, photographed at the same time. Positive cells are localized in the mesangial area and within the capillary walls. Long arrows denote positive cells and short arrows in A and C the Bowman's capsule. Abbreviation is G, glomerulus. Original magnification: A–D, × 400.

three with IgA nephritis and two with lupus nephritis, had normal creatinine clearance. Five other renal specimens with no morphologic abnormalities under light and immunofluorescent microscopy was obtained either by biopsy from two patients with macroscopic hematuria or during the course of surgical management from three patients with renal calculi or renal trauma, were used as normal kidney tissues. For immunofluorescence all the kidney specimens were snap-frozen in isopentane precooled in liquid nitrogen, and stored at −70°C until use.

The tissues were stained with hematoxylin-eosin and periodic acid-Schiff's reagents. The severity of pathologic features in the glomeruli, tubules and interstitium was assessed upon light microscopic observation. Glomerular hypercellularity, mesangial matrix increase, and tubulointerstitial changes were graded semiquantitatively on a 0 to 3+ scale (absent or minimal, mild, moderate, and marked) as described [14]. The percentage of glomeruli involving crescents or global sclerosis/hyalinosis was also determined. Mesangial hypercellularity was mild in patients with mesangial proliferative (IgA-negative) glomerulonephritis, compared with that in patients with IgA nephritis.

For conventional electron microscopy, a portion of the kidney tissue was fixed in 2% glutaraldehyde, washed in PBS, postfixed in 1% osmic acid, and then embedded in Epon 812. Ultrathin sections were cut, and stained with lead citrate and uranyl acetate [15].

Antibodies

The following antibodies were used for immunocytochemistry: mouse monoclonal antibody to Bcl-2 protein [12] (Cambridge Research Biochemicals, London, UK), mouse monoclonal antibody to human Fas antigen (MBL, Nagoya, Japan) [16], mouse monoclonal antibody to proliferating cell nuclear antigen (PCNA; Oncogene Science, Inc., Uniondale, NY, USA), mouse monoclonal antibody to human leukocyte common antigen (LCA) (CD45; DAKO, Grostrup, Denmark), and rabbit antibody to human type IV collagen (Advance, Tokyo, Japan). Fluorescein-isothiocyanate (FITC)-labeled goat anti-mouse IgG, peroxidase-labeled goat anti-mouse IgG, rhodamine-conjugated goat anti-rabbit IgG were purchased from Cappel (Durham, NC, USA).

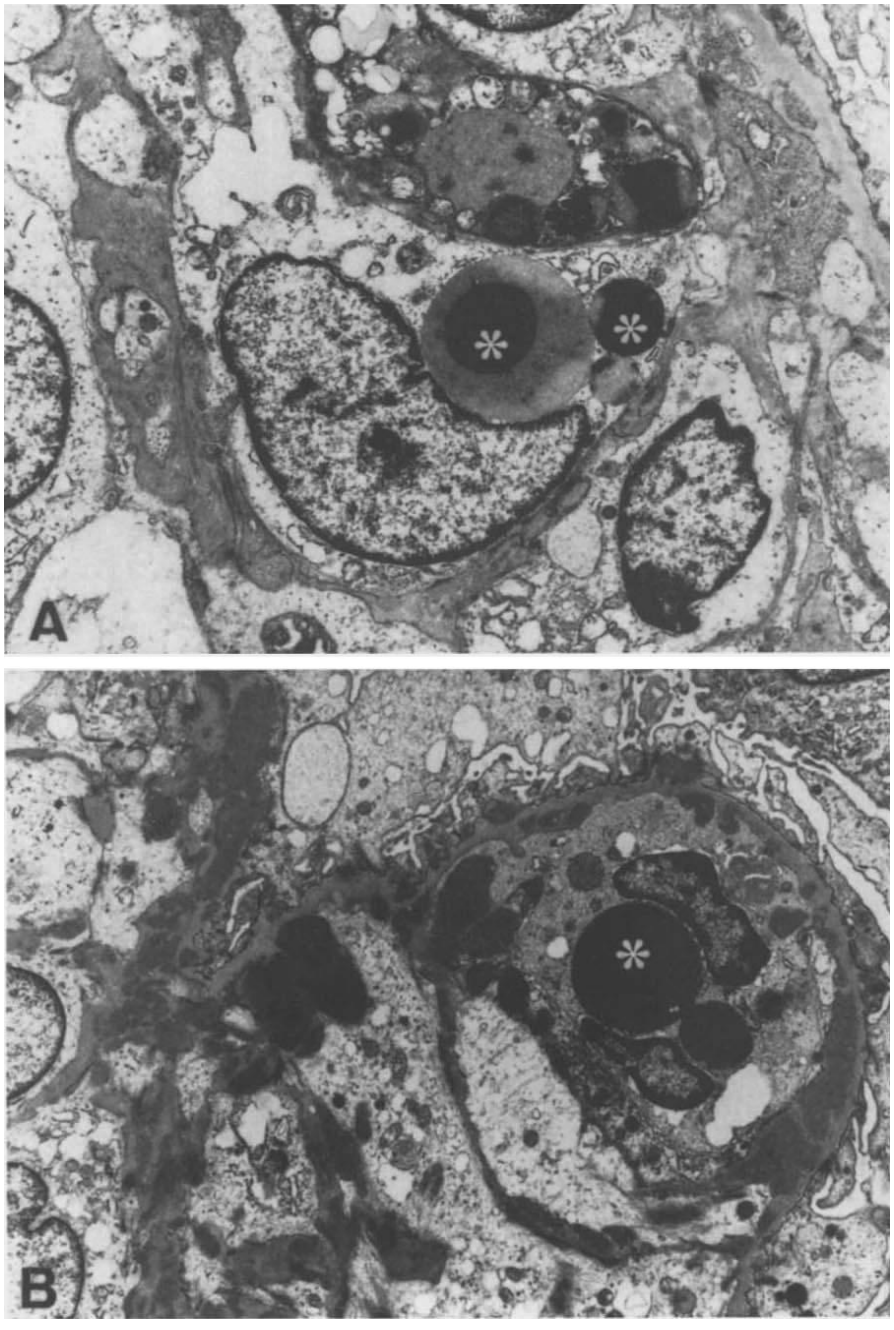

Fig. 3. *Electron microscopy of apoptotic cells and apoptotic bodies in glomeruli of patients with lupus nephritis (WHO Class IV).* Condensed nuclei (asterisks) are present within the mesangial area (**A**) and capillary lumen (**B**). Original magnification: A and B, × 5,000.

Immunocytochemical staining

Indirect immunofluorescence was performed as described [15, 17]. Section from frozen tissues, 3 μm thick, were reacted with the primary antibody, then incubated with the appropriate secondary antibody which was preabsorbed with normal human plasma. The negative control consisted of substituting the primary antibody with an irrelevant IgG mouse monoclonal antibody, non-immune rabbit serum, and PBS. In each section containing at least 4 glomeruli (the number of glomeruli examined ranged from 4 to 21 with a mean of 10.1), we counted all the glomerular cells immunoreactive for Bcl-2, Fas antigen, and PCNA. The population of positive cells was expressed as the number of reactive cells per glomerular cross section.

Double fluorochrome immunofluorescence was performed as follows [15]: Bcl-2 or Fas antigen was identified with FITC-labeled goat anti-mouse IgG, and type IV collagen was stained with rhodamin-labeled goat anti-rabbit IgG.

To demonstrate the intracellular localization of Bcl-2 protein, immunoelectron microscopy was performed using a pre-embedding method with peroxidase-labeled secondary antibody, as previously described [15]. Control sections were stained with nonimmune mouse serum or an irrelevant mouse monoclonal

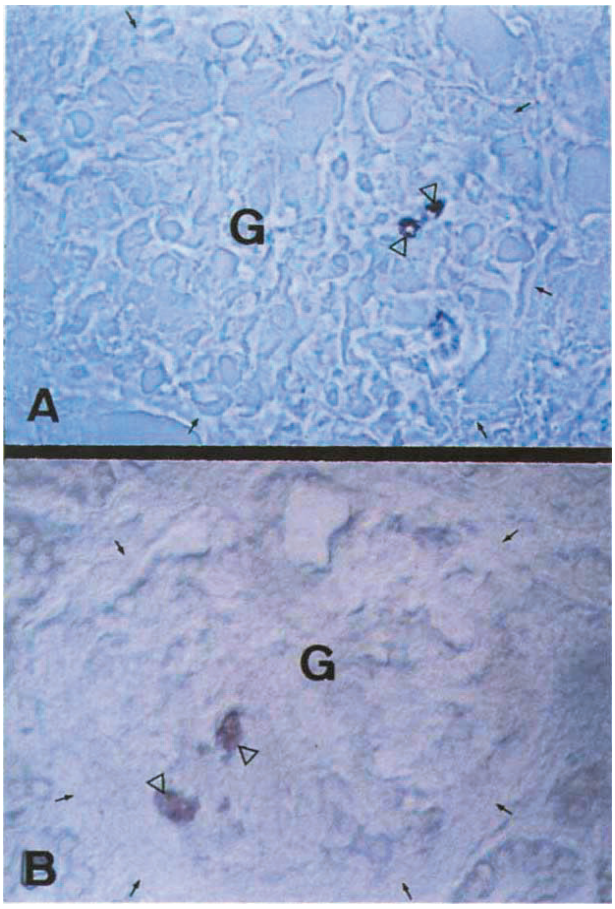

Fig. 4. A. *In situ DNA nick end-labeling in tissue sections from patients with IgA nephritis.* Positive cells are designated by arrowheads. **B.** *In situ* hybridization for Bcl-2 mRNA in tissue sections from patients with IgA nephritis. cDNA probe was labeled with digoxigenin-UTP and localized with anti-digoxigenin antibody conjugated with alkaline phosphatase. The nitroblue tetrazolium color reaction yielded a purple-brown precipitate in the intraglomerular cells (arrowheads). (no counter staining, A and B, × 400). Abbreviation is G, glomerulus. Short arrows denote the Bowman's capsule.

antibody, or secondary antibody alone. These controls were negative.

In situ *DNA nick end labeling*

The sections were fixed in 4% paraformaldehyde-lysine-periodate. DNA was nick end labeled as described by Gavrieli, Sherman and Ben-Sasson [18]. In brief, the slides were washed with distilled water, incubated with 2% H_2O_2 to block endogenous peroxidase, washed with distilled water, and immersed in terminal deoxy-transferase (TdT) buffer (30 mM Tris-HCl buffer, pH 7.2, 100 mM sodium cacodylate, 1 mM cobalt chloride). The tissues were then covered with biotinylated dUTP (0.04 nmol/μl) and TdT (0.3 U/μl) in TdT buffer, and were incubated at 37°C for 90 minutes. After terminating the reaction with 300 mM sodium chloride-30 mM sodium citrate, the slides were washed with distilled water, incubated with 2% bovine serum albumin, and reacted with peroxidase-labeled streptavidin (diluted 1:10 to 1:20 in PBS) at room temperature for 30 minutes. The reaction was visualized using diaminobenzidine-H_2O_2. As a positive control, sections were digested with DNase before processing [18]. The omission of either TdT or its biotinylated substrate gave completely negative results.

In situ *hybridization*

Hybridization with a digoxigenin-labeled Bcl-2 cDNA probe (Oncogene Science, Inc.) proceeded as described [19]. In brief, tissues fixed in 4% paraformaldehyde were cut into 4 μm thick sections, and mounted on slides coated with 3-aminopropyltriethoxysilane (Merck, Darmstadt, Germany). Endogenous alkaline phosphatase was inactivated with 0.2 N HCl, then the sections were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamide, pH 8.0 for 10 minutes, followed by two 5-min rinses with 0.1 M phosphate buffer (PB). They were finally dehydrated with an ethanol series and dried. Prehybridization and hybridization proceeded in 50% formamide, 2 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate), 1 mg/ml *E. coli* tRNA, 1 mg/ml sonicated salmon sperm DNA, 0.1% bovine serum albumin, and 10% dextran sulfate. After prehybridization at 42°C for 30 minutes, a hybridization solution containing 1 to 5 ng/ml of cDNA probe was applied to the section, which was then covered with Parafilm and hybridized at 42°C overnight in a moist chamber. The next day, the slides were washed briefly with 5 × SSC, followed by 50% formamide, 2 × SSC at 42°C for 20 minutes, incubated at 37°C for 30 minutes in 20 mg/ml RNase A to remove unbound probe, then stringently washed in 0.2 × SSC at 42°C for 20 minutes twice. Hybridized digoxigenin-labeled probe was identified using the Nucleic Acid Detection Kit (Boehringer Mannheim GmbH) according to the manufacturer's instructions. Sections were mounted in glycerin and coverslips were applied. Controls included: (i) RNase A digestion (20 μg/ml) prior to hybridization for 30 minutes at 37°C; (ii) preincubation with tenfold excess of unlabeled probe prior to hybridization; (iii) the absence of labeled cDNA probe and the antidigoxigenin antibody. There were no positive signals from any of the controls.

Statistical analysis

The data were analyzed using the Mann-Whitney test, the Kruskal-Wallis test, and Spearman's rank co-efficients. $P < 0.05$ was considered statistically significant.

Results

Fas antigen expression and apoptosis

Only small number of glomerular cells were labeled for Fas antigen in normal kidney specimens (Fig. 1). Tissues from patients with glomerular diseases showed various number of glomerular cells positive for Fas antigen. By double staining or staining using serial sections, Fas antigen-positive cells were present in the mesangial area and within the glomerular capillary walls (Fig. 2 A, B). These cells were occasionally labeled for LCA.

Electron microscopy was performed upon 12 tissues (8 with IgA nephritis and 4 with lupus nephritis), which were selected because Fas-positive cells were detected by immunofluorescent staining. Condensed nuclei and apoptotic bodies were observed within the mesangial areas and the glomerular capillaries (Fig. 3).

In situ nick end labeling was carried out in 24 tissues (Fig. 4A). The data correlated well with the glomerular population of Fas-positive cells (Fig. 5A).

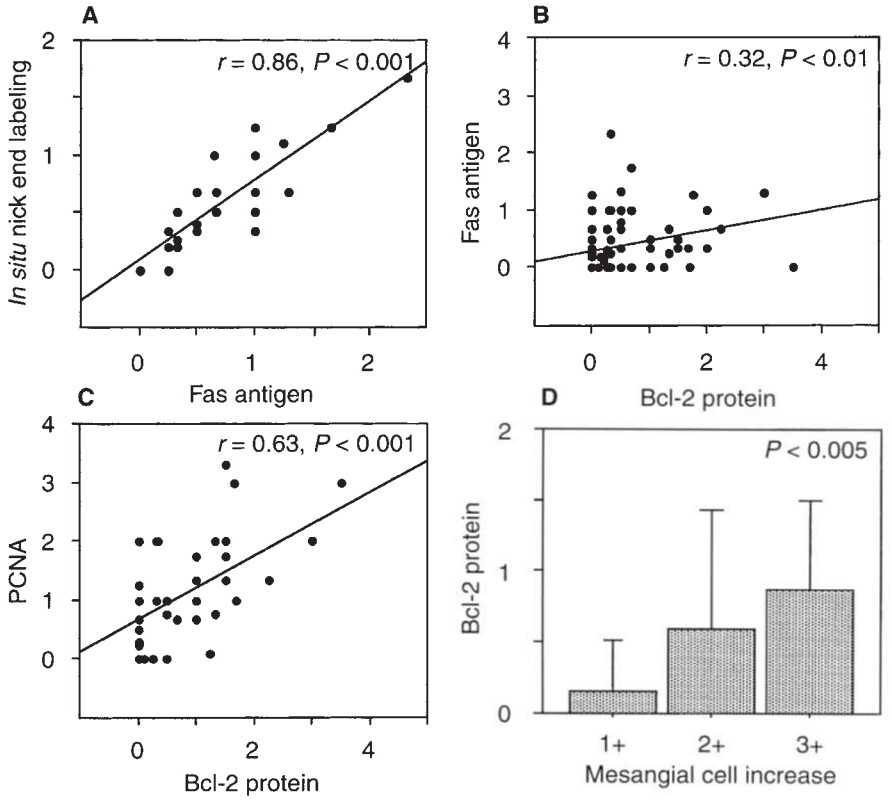

Fig. 5. A. *Correlation between the number of Fas antigen-positive cells per glomerular cross-section (horizontal axis) and that of glomerular cells stained by in situ nick end labeling (vertical axis) in tissues from patients with various types of glomerular diseases.* Each circle represents a single patient. **B.** Correlation between the Bcl-2 positive cells per glomerular cross section (horizontal axis) and Fas antigen-positive glomerular cells (vertical axis) in tissues from patients with various types of glomerular diseases. **C.** Correlation between Bcl-2 positive glomerular cells (horizontal axis) and PCNA-positive glomerular cells (vertical axis) in tissues from patients with mesangial proliferative types of glomerulonephritis (IgA nephritis, IgA-negative mesangial proliferative glomerulonephritis, and Henoch-Schönlein purpura nephritis). **D.** The relationship between Bcl-2-positive glomerular cells (vertical axis) and the mesangial hypercellularity (graded semiquantitatively upon light microscopy, see **Methods**) (horizontal axis) in tissues from patients with mesangial proliferative types of glomerulonephritis (Kruskal-Wallis test).

Expression of Bcl-2

Normal kidney specimens contained only small number of Bcl-2-positive cells within the glomeruli (Fig. 1). Double staining or staining on serial sections showed that Bcl-2-positive cells were localized mostly in the mesangial area (Fig. 2C, D). They were occasionally present within the glomerular capillary lumens (Fig. 2 C, D), and labeled for LCA. Immunoelectron microscopy showed that the Bcl-2 protein was localized in intracellular organelles of the mesangial cells (Fig. 6A) and leukocytes infiltrating glomeruli (Fig. 6B). Bcl-2 mRNA was detected in glomerular cells by *in situ* hybridization (Fig. 4B). The number of intraglomerular cells positive for Bcl-2 protein weakly correlated with that of intraglomerular cells stained for Fas antigen (Fig. 5B). Staining using serial sections showed that Bcl-2-positive cells generally differed from cells expressing Fas antigen.

Fas antigen or Bcl-2 expression and type of glomerular disease

Among disease categories, the number of Fas antigen-positive intraglomerular cells was high in glomeruli of patients with Henoch-Schönlein purpura nephritis and lupus nephritis, compared with that in the glomeruli of normal kidneys and of patients with other types of glomerular diseases. That of Bcl-2-positive intraglomerular cells was high in lupus nephritis, IgA nephritis, and focal glomerular sclerosis (Fig. 1).

Correlation of Bcl-2 or Fas antigen expression with renal pathology and clinical findings

A statistical analysis was performed upon data from patients with mesangial proliferative types of glomerulonephritis, including IgA nephritis, IgA-negative mesangial proliferative glomerulonephritis, and Henoch-Schönlein purpura nephritis. The population of Bcl-2-positive glomerular cells was significantly correlated with the number of PCNA-positive intraglomerular cells ($P < 0.001$, Fig. 5C), the grade of the mesangial cell increase ($P < 0.005$, Fig. 5D), and the magnitude of proteinuria ($P < 0.01$), but not with the grade of hematuria. The relationship between Fas antigen-positive glomerular cells and those parameters was not statistically significant.

Discussion

The Fas antigen-Fas ligand system mediates apoptotic cell death in several types of cells [20]. In contrast, the Bcl-2 gene promotes cell survival by blocking programmed cell death and its morphological equivalent apoptosis [7–9]. Here we described the local expression of Fas antigen and Bcl-2 in human glomerulonephritis for the first time. We found that the mesangial cells as well as infiltrating leukocytes express these apoptosis-related proteins. Among the disease categories studied, the population of Fas antigen-positive cells was high in tissues from patients with Henoch-Schönlein purpura nephritis and lupus nephritis, compared with that in normal specimens and tissues from patients with other types of glomerular diseases. The number of Bcl-2 positive cells was high in lupus nephritis, focal glomerular sclerosis, and IgA nephritis. Previously, Harrison [3] briefly described the presence of apoptotic bodies in glomeruli of patients with proliferative glomerulonephritis. Observations from experimental models of glomerulonephritis [4, 5, 21] have indicated that infiltrating neutrophils and mesangial cells undergo apoptosis. It

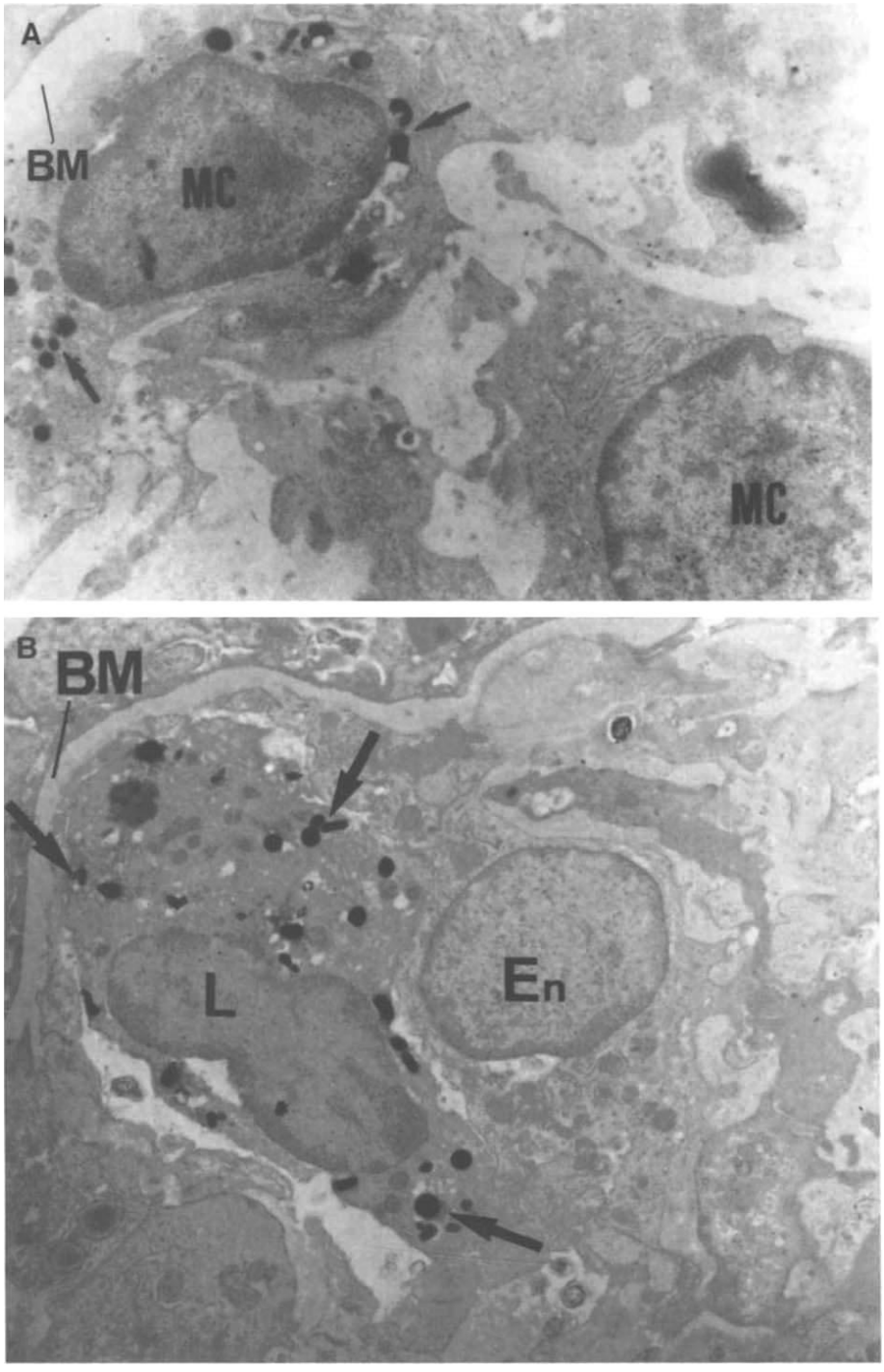

Fig. 6. *Ultrastructural observation of Bcl-2 protein in glomeruli of a patient with IgA nephritis, stained with monoclonal antibody to Bcl-2 protein and peroxidase-conjugated secondary antibody.* Reaction products are seen in intracellular organelles of one mesangial cell (**A**) and a leukocyte infiltrating a glomerulus (**B**). Original magnification: × 8,000. Abbreviations are: BM, basement membrane in the paramesangial zone (A) and the glomerular capillary (B); En, glomerular endothelial cell; L, leukocyte; MC, mesangial cell.

appears that these processes play a role in limiting neutrophil-mediated glomerular injury and in recovery from mesangial cell proliferation.

In glomerulonephritis induced by anti-thymocyte antibodies, mesangial cell proliferation and extracellular matrix accumulation are transient, and mesangial lesions in most glomeruli resume their original structures around 6 to 10 weeks after disease induction [4]. Shimizu et al [5] have indicated that proliferated glomerular cells regress by apoptosis in the recovery phase of this model. In contrast, prolonged mesangial cell proliferation with increased mesangial matrix is a key feature of chronic forms of human glomerulonephritis. We speculate that there is a mechanism which suppresses the apoptosis, leading mesangial cells to continuous proliferation. The Bcl-2 gene is representative of a category of protooncogenes that function in the regulation of programmed cell death or apoptosis.

The principal product of the Bcl-2 gene is a 26 kD protein that resides primarily in the inner mitochondrial membrane, nuclear

envelope, and parts of the endoplasmic reticulum [8, 22]. The Bcl-2 gene is normally expressed in long lived or proliferating cell zones of apoptotic tissues such as the thymus, skin, intestines, and brain [12, 23]. Studies using leukemia or lymphoma cell lines have shown that Bcl-2 interferes with the apoptotic process mediated by the Fas antigen and the TNF receptor [24, 25]. Immunohistochemical studies show that Bcl-2 expression is up-regulated in the human fetal kidney, and suggest that a potential role for Bcl-2 expression during morphogenesis and differentiation of the developing nephron [10]. In fact small kidneys with polycystic kidney-like change of renal tubules is observed in Bcl-2 knock-out mice [26]. Bcl-2 expression in the normal adult kidney is not a consistent finding [10, 23]. The results of this study provided a detailed description of the distribution of Bcl-2 protein in tissues from patients with glomerular diseases. Bcl-2 mRNA was identified within the glomeruli, and its protein was detected within mesangial cells and occasionally infiltrating leukocytes. Bcl-2 expression correlated well with PCNA staining and glomerular hypercellularity, indicating an association between Bcl-2 expression and glomerular cell proliferation. These data suggest that a Grant-in-aid of the Japanese Medical Association (K.Y), a Grant-in-aid for Scitentific Project (A07770623) from the Ministry of Education (H.M.), Bcl-2 plays a role in the persistent proliferation of intrinsic glomerular cells and prolonged survival of infiltrating leukocytes in human glomerulonephritis. Manipulations of Bcl-2 expression in glomerular cell with gene technology could reveal the exact role of this gene in the pathogenesis of glomerulonephritis.

Recently, a protein with a partial sequence homology with Bcl-2, termed Bax, has been described. This protein seems to oppose the action of the Bcl-2 protein. Further studies to elucidate the exact role of Bcl-2 overexpression and also the functional association between Bcl-2 and Bax in glomerulonephritis are necessary.

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Reprint requests to Kazuo Yoshioka, M.D., Department of Pediatrics, Kinki University School of Medicine, 377-2 Ohno-higashi, Osaka-sayama 589, Japan.

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