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ORIGINAL ARTICLE

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Apoptosis and proliferation in childhood acute proliferative glomerulonephritis

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Abstract Acute proliferative glomerulonephritis is characterized by glomerular hypercellularity that can be caused by many different etiologies and pathogenetic mechanisms. A balance between cell birth by mitosis and cell death by apoptosis is crucial. In this study, apoptosis and the regenerative activity (Ki67/apoptosis index) were investigated in acute proliferative glomerulonephritis. Thirty-five children with biopsy-proven acute proliferative glomerulonephritis and five controls with MCD were studied retrospectively. According to the clinical outcome, patients were divided into 2 groups: group 1 (n=21) were patients with normal renal functions at follow-up; group 2 (n=8) were patients with end-stage renal failure or those who died. Immunohistochemical staining of proliferating cells (Ki67) was done. In situ end labeling of DNA was used to evaluate apoptosis. Glomerular cell apoptosis was 45% in the patients with acute proliferative glomerulonephritis and 3% in controls (p<0.001). Apoptotic cells were identified in the tubulointerstitial compartment with higher and heavier immunostaining in patients than controls (p=0.001). Tubular proliferative index (=tubular proliferation/tubular apoptosis ratio) was significantly higher in group 1 patients than in group 2 patients (2.03±2% versus 0.32±0.6%, p=0.002). Tubulointerstitial regenerative ratio (=tubular proliferation/interstitial proliferation ratio) was significantly higher in controls than in patients (3.4±1.9 versus 1.52±0.8, p=0.01). In addition, it was significantly increased in group 1 patients when compared with those in group 2 patients (1.89±0.8 versus 0.73±0.2, p=0.001). Since 17 patients presented with postinfectious proliferative glomerulonephritis, which is known to exhibit better course, we also evaluated those parameters in patients with postinfectious proliferative glomerulonephritis separately. We found statistically significant differences only in the tubulointerstitial regenerative ratio, which was higher in postinfectious cases when compared with those in other cases [1.60 interquartile range (IQR) 1.54 versus 1.22 IQR 1.26, respectively, p=0.003]. In conclusion, tubular proliferative index and tubulointerstitial regenerative ratio might be useful parameters for predicting final functional outcome in acute proliferative glomerulonephritis. Further studies, however, are still needed to clarify the importance of these histopathological parameters.

Keywords Apoptosis · Childhood · Acute proliferative glomerulonephritis · Ki67 · Prognosis

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Introduction

Acute proliferative glomerulonephritis is characterized by glomerular hypercellularity due to proliferation of mesangial and endocapillary cells [1]. In the early stage, there is increased nonglomerular cell infiltration consisting of polymorphonuclear leukocytes and monocytes/macrophages. Locally secreted chemokines mediate leukocyte recruitment during the initial and amplification phase of renal inflammation. In turn, the infiltrating leukocytes contribute to renal damage by releasing inflammatory and profibrotic factors [2, 3]. Crescentic glomerulonephritis (CGN) is a morphologic expression of severe glomerular proliferation that can be caused by many different etiologies and pathogenetic mechanisms. The major pathogenic event that causes crescent formation is rupture of glomerular capillaries, which allows cellular and humoral inflammatory mediators to spill into Bowman's space [4]. On the basis of immunopathologic findings, crescentic glomerulonephritis can be classified into three major categories: antiglomerular basement membrane antibody glomerulonephritis, immune-com-

plex glomerulonephritis, and pauci-immune glomerulonephritis [5].

Glomerular crescents are characteristics of severe inflammatory injury and indicators of poor prognosis. Along with its rapidly progressive clinical course, the progression of CGN is associated with fibrosis of the glomerular crescents and the renal interstitium. Several types of cells, such as monocytes, macrophages, epithelial cells, and fibroblasts, have been implicated in the formation of crescents and their evolution to fibrosis [6]. Proliferating macrophages and parietal epithelial cells are thought to be the main contributors to cells in crescents [7]. Excessive glomerular cell proliferation is commonly viewed as an undesirable event that disturbs glomerular function and threatens progression to glomerular scarring and end-stage renal failure [8].

Apoptosis, programmed cell death, is an established form of regulation of cell population in both glomerular [9] and tubulointerstitial areas [10]. Apoptosis may be an important mechanism affecting remodeling and removal of injured and inflammatory cells. It speeds up the resolution of harmful inflammation through the death of infiltrating inflammatory cells [11] but also contributes to the progressive loss of glomerular and tubular cells, leading to atrophy and sclerosis in human kidney diseases [10, 11]. It appears that a crucial factor is the balance between cell proliferation by mitosis and cell death by apoptosis.

In this study, we aimed to investigate the balance between apoptosis and proliferation in childhood acute proliferative glomerulonephritis.

Subjects and methods

Patients and renal biopsy specimens

From 1980 to 2000, 35 patients (23 boys, 12 girls) aged 10±3 (range 5–16) years with biopsy-proven acute proliferative glomerulonephritis accompanied by various degrees of epithelial crescents and five controls with MCD were studied retrospectively. If the percentage of crescents was higher than 50, then these patients were considered as crescentic glomerulonephritis (CGN). The histopathological diagnoses of the patients were as follows: 17 postinfectious glomerulonephritis, 3 Henoch Schönlein purpura nephritis, 2 microscopic polyangiitis, 2 lupus nephritis, 5 membranoproliferative glomerulonephritis (MPGN), and 6 idiopathic crescentic glomerulonephritis. At admission, most of the patients (62.9%) presented with nephritic–nephrotic syndrome. Twelve (34.3%) and 1 (2.9%) out of 35 presented with only nephritic and only nephrotic syndrome, respectively. Thirteen (37%) (5 postinfectious, 3 idiopathic, 2 lupus nephritis, 1 Henoch Schönlein nephritis, 1 microscopic polyangiitis, 1 membranoproliferative glomerulonephritis) had received either corticosteroid alone or corticosteroid plus cyclophosphamide in another center before renal biopsy.

According to the clinical outcome, patients were divided into 2 groups: group 1 (n=21) were patients with normal renal functions at the end of the follow-up period; group 2 (n =8) were patients with end-stage renal failure or those who had died. Six were lost to follow-up. Since 17 patients presented with acute postinfectious glomerulonephritis, which is known to exhibit better course, we also evaluated these patients separately. All available renal biopsy materials were reexamined under a light microscope by three observers with no knowledge of patients' clinical course to establish the diagnosis by standard pathological methods. The biopsy specimens were evaluated for the number of glomeruli exhibiting different types of glomerular crescents, which were classified into 3 categories, as follows: (1) cellular crescents containing 3 or more layers of cells, with most cells having a round morphology; (2) fibrocellular crescents, defined as cellular crescents containing fibroblasts with some collagen; and (3) fibrous crescents having fibrous appearance with few cells. The percentage of glomeruli exhibiting cellular, fibrocellular, or fibrous crescents was calculated by dividing each of them into the total number of glomeruli. Severity of the renal histological lesions (tubular atrophy, interstitial fibrosis, and inflammation) was scored according to a semiquantitative arbitrary score from 0–3 and was expressed as absent (0) and present (1–3) based on their previous reports [12].

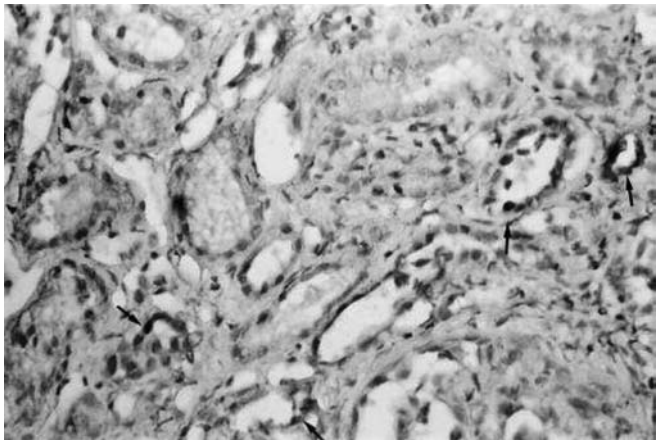

Fig. 1 Ki67 expression in proximal and distal tubular cells (*arrow*) (×400)

Immunohistochemistry

Ki67 staining

The paraffinized tissue blocks were sectioned at 5 μm and stained using standard immunoperoxidase-staining technique. Briefly, sections were deparaffinized and rehydrated in descending grades of alcohol. All immunostaining steps were performed at room temperature. Sections were treated with 3% H_2O_2 in methanol in order to block endogenous peroxidase activity. Primary antibody was added to the sections for 30 min at room temperature (Ki67 clone: KiS5, DAKO, Denmark). After washing with phosphate-buffered saline (PBS), the biotinylated secondary antibody (anti-mouse + rabbit, Histostain-plus, 85–9043, CA, USA) was added at a concentration of 5% for 10 min followed by addition of streptavidin-conjugated peroxidase (Histostain-plus, 85–9043) for 10 min and washed with PBS. Ultraclean diluent (TA-125-UC-ready-to-use, Lab Vision) in 1:25 dilution was employed to remove primary antibody background staining. Sections were treated with diaminobenzidine (DAB) chromogen solution for 10 min and washed with distilled water followed by addition of Harris hematoxylin for 20 s. Results were evaluated by light microscopy under a magnification of ×1,000/immersion oil unless indicated otherwise (Fig. 1).

Some sections were incubated in the absence of the primary antibody as a negative control.

In situ detection of apoptosis

Apoptosis was detected by in situ end labeling (ISEL) of fragmented DNA using a commercial Apoptag kit (Intergen, New York, NY, USA). Briefly, sections were deparaffinized and stripped

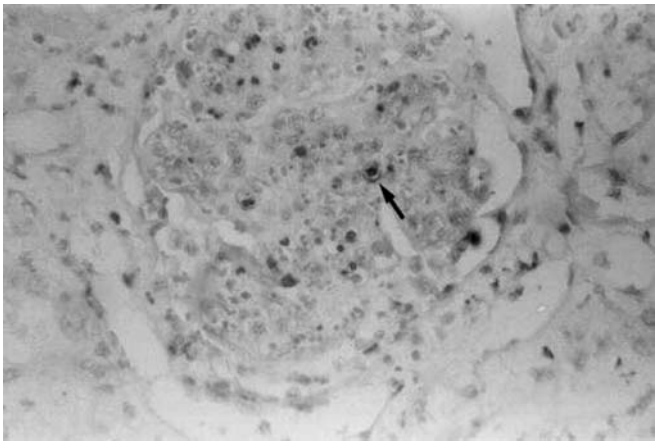

Fig. 2 Apoptotic cells within glomerulus (*arrow*) [in situ end labeling (ISEL) ×400]

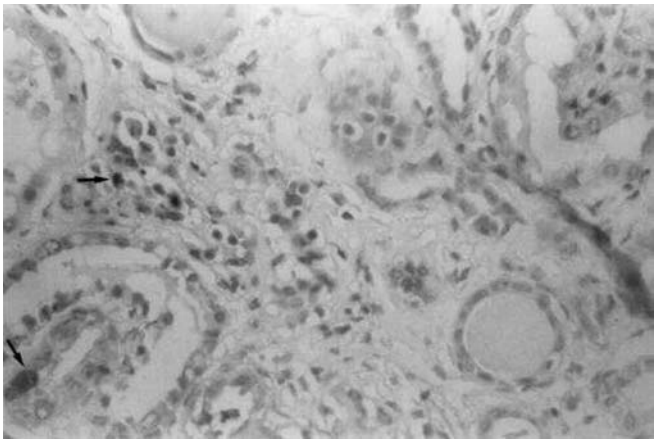

Fig. 3 Apoptotic cells within tubulointerstitial infiltration and in some tubular epithelial cells (*arrows*) [in situ end labeling (ISEL ×400]

of proteins by incubation with proteinase K in PBS at room temperature for 15 min. Following washing in distilled water, endogenous peroxidase activity was quenched by 3% H_2O_2 for 5 min. After incubation with the equilibration buffer, the samples were incubated with terminal deoxyribonucleotidyl transferase (TdT) in reaction buffer (containing digoxigenin nucleotide) at 37°C for 60 min. The reaction was terminated using a stop buffer. Following rinsing with PBS, the tissue sections were covered with antidigoxigenin peroxidase for 30 min at room temperature, washed in PBS, stained with DAB, and counterstained with 0.5% methyl green. Negative controls were included in each run in the form of omission of TdT in reaction buffer. Results were examined by using light microscopy under a magnification of ×400 (Figs. 2, 3).

Apoptotic cells were identified by both positive staining with the ApopTag immunostain as well as the characteristic morphology of apoptotic cells: condensed chromatin, fragmented nuclei, and perinuclear halo.

Quantitation of tissue staining

All sections were evaluated in a blinded fashion. In order to quantify the amount of apoptotic and proliferating cells in both glomerular and tubulointerstitial compartments, all available glomeruli in the biopsy and 12 consecutive nonoverlapping fields of interstitium were examined under high power (×400) light microscopy. Apoptotic and nonapoptotic cells were counted. Percentage of total glomerular apoptosis (TGA) was calculated by dividing apoptotic cells to total glomerular cells (apoptotic and nonapoptotic). Mean apoptotic cell number per glomerulus was calculated by dividing the total number of glomerular apoptotic cells into the number of glomeruli for each specimen.

Tubular (TA) and interstitial (IA) apoptosis were counted in the same manner. Percentages of TA and interstitial IA apoptosis were calculated by dividing apoptotic cells into total cells (apoptotic and nonapoptotic) in each compartment.

A comprehensive proliferative index for glomerulus, tubulus, and interstitium was derived from the ratio between Ki67-positive cells and apoptotic cells in each compartment.

Statistical analysis

Results are given as mean ± SD for normal distributed data and median interquartile range (IQR) (minimum–maximum) for the others. Differences between groups were determined by the Mann—Whitney *U* test. Correlations between different parameters were performed by using Spearman's rho for numeric variables and Kendall's tau-b for ordinal variables. Logistic regression analysis using a computer-based program (SPSS) was performed to determine the risk factors for poor prognosis. The Kaplan—Meier method was used to evaluate cumulative survival for poor prognosis determined as death and/or end-stage renal failure. Results were considered significant when the p value was below than 0.05.

Results

Clinical findings

Patient clinical characteristics, including subgroups, are given in Table 1. At admission, significantly reduced glomerular filtration rate (GFR) and serum albumin along with an increased diastolic blood pressure were found in group 2 patients when compared with those patients in group 1. Four out of 29 patients (13%) died due to complications of end-stage renal disease at a median of 30 months following the diagnosis. Initial diagnoses were as follows: 1 lupus nephritis, 1 MPGN, 1 microscopic polyangiitis, 1 idiopathic crescentic glomerulonephritis. While 2 showed nephritic–nephrotic syndrome, 1 was nephritic and 1 was nephrotic at the beginning of the disease. All needed renal replacement therapy initially and had reached end-stage renal failure at the end of 1 year. When all patients were taken into consideration, regardless of subgroups, 17% at 1 year, 30% at 2 years, and 42% at 5 years reached end-stage renal disease and/or died.

Histological findings

In total, 716 glomeruli (median 20) were obtained from 35 patients. Detailed histological parameters are given in Table 2. The percentage of crescents, which were predominantly fibrous, was significantly higher in group 2 than in group 1, in which it was predominantly cellular (p=0.003) (Table 2). Interstitial fibrosis and tubular at-

Table 1 Clinical features of all the patients and the subgroups. *GFR* glomerular filtration rate

Parameters	All patients' median (min–max) (n=35)	Group 1 median (min–max) (n=21)	Group 2 median (min–max) (n=8)	p value*
Age at presentation (years)	10 (5–16)	10 (6–14)	11 (8–16)	0.5
Duration of disease at admission (days)	30 (5–1825)	30 (5–540)	37.5 (7–1825)	0.6
Timing of renal biopsy (days)	43(15–210)	39 (15–210)	60 (35–127)	0.06
Serum creatinine (mg/dl)	2.4 (0.5–10.4)	1.6 (0.5–10.4)	4.5 (1.6–10.4)	**0.03**
Serum albumin (g/dl)	2.8 (1.7–5.6)	3.1 (2–5.6)	2.2 (1.7–4.6)	**0.035**
Estimated GFR (ml/min per 1.73 m^2)	40.5(5–150)	47 (6–150)	14 (5.4–43)	**0.005**
Systolic blood pressure (mmHg)	130 (100–190)	130 (100–190)	150 (120–180)	0.08
Diastolic blood pressure (mmHg)	85 (50–120)	80 (50–140)	105 (70–135)	**0.04**
Urinary protein/creatinine ratio	6.8 (0.8–32)	11 (0.8–21.5)	6.7 (3.1–32)	0.9
Follow-up period (months)	36 (2–240)	60 (12–240)	18 (2–60)	**0.02**

* Group 1 versus group 2, $p<0.05$ is significant (in bold)

Table 2 Detailed histological parameters of patients' crescents

	All patients (%)	Group 1 (%)	Group 2 (%)	p value*
Percentage of crescent	56±25	43±18	79±24	0.003
Contents of crescent				0.036
Cellular	23	29	-	
Fibrocellular	68	67	75	
Fibrous	9	4	25	

* Group 1 versus group 2

Table 3 Histological parameters of patients' tubulointerstitial areas

	All patients (%)	Group 1 (%)	Group 2 (%)	p value*
Interstitial infiltration	88.6	85.7	100	0.06
Intensity				0.5
1+	50.6	47.6	66.5	
2+	35.1	38.1	25	
3+	2.9	-	12.5	
Interstitial fibrosis	48.6	42.9	62.5	0.3
Intensity				0.2
1+	25.7	23.9	-	
2+	14.3	19	-	
3+	8.6	-	25	
Tubular atrophy	60	57.1	75	0.3
Intensity				0.1
1+	38.1	47.6	37.5	
2+	6.2	9.5	-	
3+	15.7	-	37.5	

* Group 1 versus group 2

rophy were not statistically different between groups 1 and 2. However, the percentage of interstitial infiltration was significantly higher in group 2 patients than in group 1 patients. Although statistically not significant, we observed that intensity of interstitial infiltration was higher in group 2 patients when compared with those in group 1 patients (Table 3).

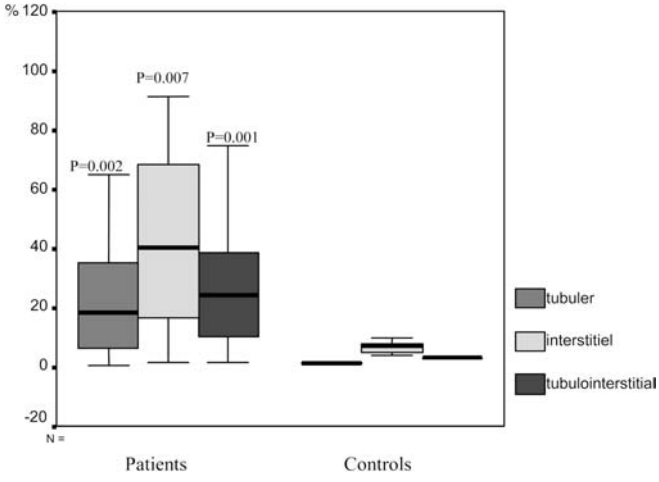

Fig. 4 Tubular, interstitial and tubulointerstitial apoptotic cells in patient and control groups, respectively. Significantly increased apoptotic cells in all nonglomerular compartments in the patient group were noted

In situ end labeling (apoptosis staining)

Median glomerular cell apoptosis was 45% in the patient group and only 3% in the control group ($p<0.001$). In patient groups, apoptosis within the crescents and the glomerular tufts were 17.5% and 27.9%, respectively. There was no statistically significant difference between group 1 (11%) and group 2 (21%) for glomerular cell apoptosis. Apoptotic cells were identified in the tubulointerstitial compartment, with higher and heavier immunostaining in patients than in controls (24 IQR 28% versus 3 IQR 0.9%, p=0.001) (Fig. 4). However, percentages of the tubular and interstitial apoptotic cells between groups 1 and 2 were similar (Table 4).

Proliferative indices

Glomerular, tubular, and interstitial proliferative indices were 0.16±0.2, 1.40±1.8, and 0.90±1.2, respectively, in patients whereas they were 0.20±02, 0.53±0.1, and 0.17±0.1, respectively, in controls ($p>0.05$). However,

Table 4 Percentage of tubular and interstitial apoptosis in the groups. *IQR* interquartile range

	Group 1	Group 2	
	Median IQR (min–max)	Median IQR (min–max)	*p* value*
Tubular apoptosis (%)	15 IQR 30 (0.7–65)	21 IQR 52 (3.5–96)	0.6
Interstitial apoptosis (%)	41 IQR 43 (2.5–83)	24 IQR 62 (1.5–87)	0.8

* Group 1 versus group 2

tubular proliferative index (=tubular proliferation/tubular apoptosis; TPI) was significantly higher in group 1 than in group 2 (2.03±2% versus 0.32±0.6%, p=0.002). Tubulointerstitial regenerative ratio (=tubular proliferation/interstitial proliferation; TP/IP) was significantly higher in controls that patients (3.4±1.9 versus 1.52±0.8, p=0.01). It was also significantly increased in group 1 when compared with group 2 (1.89±0.8 versus 0.73±0.2, p=0.001). TP/IP ratio negatively correlated with both tubular atrophy and interstitial fibrosis (r −0.45, p 0.008 and r −0.47, p 0.006, respectively). However, we failed to detect any relationship between glomerular, tubular, interstitial proliferative indices, and histopathologic findings. Increased percentage of crescents and decreased TP/IP ratio were related to increased risk of chronic renal failure. Each 10% increase in the percentage of crescents and 0.1 decrease in the TP/IP ratio was found to increase the risk five-fold and 1.07-fold, respectively, for poor prognosis [p=0.002, RR 1.07, 95% CI (1.02–1.12); p=0.001, RR=5, 95% CI (4.5–5.7)].

Since postinfectious proliferative glomerulonephritis has been known to exhibit a relatively better clinical course than other causes, we also evaluated apoptotic and regenerative parameters in postinfectious cases (n =17) separately. We only found statistically significant difference in the TP/IP ratio, which was higher in postinfectious cases when compared with those in other cases (1.60 IQR 1.54 versus 1.22 IQR 1.26, respectively; p = 0.003).

Discussion

This is the first study describing apoptosis and proliferation in pediatric patients with acute proliferative glomerulonephritis. Apoptosis has been an established form of regulation of cell population in both the glomerulus and tubulointerstitial areas [9, 10]. It has been implicated as a cell deletion mechanism for removal of excess mesangial, endothelial, and epithelial cells during progressive glomerulonephritis in many experimental models and human glomerulonephritis [13, 14, 15, 16]. In the present study, we clearly demonstrated excess glomerular cell apoptosis in patients with proliferative glomerulonephritis when compared with those in patients with MCD. Qiu et al. [17] recently demonstrated in human IgA nephropathy that increased apoptotic activity in glomeruli in advanced-stage disease is highly correlated with the overexpression of pro-proliferative cell-cycle proteins. We therefore suggest that increased apoptotic activity in our patients was stimulated by the inflammation, which occurred in acute proliferative glomerulonephritis, since we never observed such a high degree of apoptosis in the control group.

In kidney diseases, apoptosis has been shown to have both beneficial and detrimental effects. It speeds up the resolution of harmful inflammation through the infiltrating inflammatory cells [11, 14] but also contributes to the progressive loss of glomerular and tubular cells, leading to atrophy and sclerosis [10, 11, 18]. This could be a reason for progressive glomerular cell loss in our group 2 patients. However, this change failed to reach statistical significance, most likely due to the small number of group 2 patients.

The fine balance between apoptosis and mitosis determines prognosis of glomerular diseases. Proliferative index (mitosis/apoptosis ratio) is the comprehensive marker of regenerative activity in the injured kidney [8]. In an experimental Thy1.1 model, it was shown that a rebound proliferation became apparent to the response of injury and that apoptosis was stimulated at the same time. However, although mitosis returned to normal in the second week, it was still continuing. At these late stages of the Thy1.1 model, proliferation within glomeruli decreased [13]. In our study, since renal biopsy timing was late (median 43 days), a decreased glomerular proliferative index could be explained by persisting increased apoptosis or decreased proliferation rates that were expected at this stage.

The importance of tubulointerstitial factors in progressive glomerular injury has long been recognized in different nephropathies [19, 20]. Marked tubulointerstitial apoptosis was previously shown in an experimental model of glomerulopathy [22]. However, the mechanisms by which inflammatory events in the glomerulus involve the tubulointerstitium remain poorly understood. In the acute phase, tubular epithelial injury and interstitial inflammation occur in glomerulonephritis. Injured tubuli are either regenerated or undergo apoptosis or necrosis and eventually tubular atrophy becomes evident. In the later stages of renal fibrogenesis, tubular epithelial changes assist permanent impairment of renal functions [19, 20]. Muchenate-Kubara and El-Nahas [22] have shown inadequate proliferation in addition to excess apoptosis in tubular cells, leading to tubular atrophy in a progressive renal scar model. In the present study, we showed much more intense apoptosis in the tubulointerstitial compartment in patients than controls. In addition, the TP/IP ratio was significantly decreased in the patient group when compared with the control group. Moreover, the lowest value was observed in group 2 patients. These findings might be secondary to inadequate tubular regeneration following initial apoptosis or increased interstitial infil-

tration or both in our patients. Since we showed a significant negative correlation between TP/IP ratio and tubular atrophy, as well as interstitial fibrosis, the lowest value in group 2 patients could be related to both insufficient tubular regeneration leading to tubular atrophy and excess interstitial infiltration leading to interstitial fibrosis. TPI was significantly decreased in group 2 patients. As tubular apoptosis was similar between group 1 and 2 patients, decreased TPI could be explained by insufficient tubular proliferation. Based on these findings, we speculate that low TP/IP ratio in patients was due to insufficient tubular regeneration and that the low ratio in group 2 patients was most likely due to tubular atrophy.

It is very well known that postinfectious glomerulonephritis exhibits a better clinical course than other acute proliferative glomerulonephritis. For this reason, we also evaluated this group separately. We showed a significant increase in TP/IP ratio in this group. We also found that each 0.1 decline in TP/IP ratio was related to a five-fold increase for poor prognosis. Taken together, we suggest that balance between apoptosis and proliferation is much more important than apoptosis alone and that tubulointerstitial regeneration following apoptosis is an important process for healing.

A weakness of our study was the inability to control for the effects of therapy on apoptotic and proliferative events. Thirteen patients received either corticosteroid alone or corticosteroid plus cyclophosphamide in another hospital prior to renal biopsy. This therapy might have affected apoptotic and proliferative events in the kidney. All renal biopsies were performed in our hospital before switching or starting a treatment. Since this study covered a 20-year period and treatments were not uniform from one center to another in this time period, we were unable to determine the relationship between treatment and clinicopathologic features. We therefore cannot exclude the impacts of treatment on histopathologic findings and clinical course.

In conclusion, glomerular proliferative index is likely to be more important than apoptosis alone in the course of childhood acute proliferative glomerulonephritis. TPI and TP/IP ratio seem to be useful parameters to predict final outcome. Further studies are needed to uncover the importance of balance between proliferation and apoptosis in the pathogenesis and prognosis of acute proliferative glomerulonephritis.

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