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Original Article

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Clinicopathological and immunohistological features in childhood IgA nephropathy: a single-centre experience

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Abstract
Background. IgA nephropathy is a glomerular disease diagnosed by renal biopsy and is characterized by a highly variable course ranging from a completely benign condition to rapidly progressive renal failure. We aimed to evaluate the clinical, histopathological and inflammatory characteristics of children with IgA nephropathy.
Methods. Data of 37 patients with IgA nephropathy diagnosed between the years 1980 and 2008 were retrospectively reviewed. Immunohistochemistry was performed in 24 patients. Expression of CD3, CD4, CD8, CD20, CD68, IL-1β, IL-10, IL-17, TGF-β, TNF-α and the newly proposed tubulointerstitial fibrosis marker nestin were evaluated.
Results. The median age at diagnosis was 10 years. Recurrent macroscopic haematuria (66%) was the most common clinical manifestation, and 35% of the patients had synpharyngitic presentation. A significant correlation was found between proteinuria and increase in mesangial matrix ($r = 0.406$, $P = 0.013$). The presence of CD4+ T lymphocytes and CD68+ macrophages were also significantly associated with proteinuria >1 g/day. While cytokines IL-1β, IL-10 and TNF-α were mainly expressed in tubular epithelial cells, TGF-β was evident in glomeruli but they had no correlation to clinical features and severity of the disease. Nestin was detected at the tubules in almost half of the patients with no correlation to proteinuria and tubulointersititial fibrosis.
Conclusions. We found a correlation between proteinuria and mesangial matrix expansion. The presence of CD4+ T-lymphocytes and CD68+ macrophages were also significantly associated with proteinuria >1 g/day. Although there are many evidences, for immunological basis of IgA nephropathy, the immunological markers were not fully expressed in children to evaluate glomerular and tubulointerstitial inflammation, and progression of the disease. Further studies with the extended number of children are needed to shed light on the immunological basis of the disease.

Keywords: cytokines; childhood IgA nephropathy; histopathology; inflammatory cells; nestin

Introduction

Immunglobulin A nephropathy (IgAN) is the most common form of primary glomerulonephritis, seen in adults and children. The diagnosis is based on the occurrence of mesengial IgA deposits in the glomeruli and the presence of recurrent episodes of macroscopic haematuria with upper respiratory tract infection or microscopic haematuria and/or proteinuria. The basic defect is impaired O-glycosylation of serum IgA1 that leads to mesengial deposition of abnormal IgA1 [1–3]. In the pathogenesis, the role of mucosal pathogens has been proved in different experimental models [3, 4]. The mucosal immune system deals with continuous antigenic challenge that triggers nephritogenic IgA. This polymeric IgA activates Th1, Th2 and Th17 and their related cytokines in patients [5–9]. Most recently, the role of innate immunity in the pathogenesis of IgA has been proposed [10]. Innate immunity acts through the recognition of pathogen-associated molecular patterns (PAMPs) by macrophages and dendritic cells. Macrophages and mature dendritic cells interact with lymphocytes leading to activation of specific T cells, antibody synthesis and subsequently inflammation. Production and local release of cytokines, interleukin-1β (IL-1β), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), growth factors, platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and angiotensin II by renal resident cells such as dendritic cells and by circulating inflammatory cells leading to inflammatory injury resulting in characteristic histopathological features of mesangial cell proliferation and mesangial matrix deposition [5, 11]. It has been shown that PDGF and TGF-β may have a key role in the induction and progression of mesangial injury [5, 11, 12]. In addition to T cells and cytokines, activation of complement proteins and Toll-like receptors (TLR) through innate immunity and C_3 deposition in mesangium may mediate glomerular injury [10, 13].

Several studies in regard to the immunological basis of the disease and role of immunological markers in progression of IgAN have been reported in adults [5, 11, 12]. Tubulointerstitial CD3, IL-1β expression and the glomerular membrane protein of 17 kDa (GMP-17)-positive cytotoxic T lymphocytes in renal tubules were found to be predicting factors of progression. In addition to these studies, CD4+ T cells, CD8+ T cells, CD20, lL-11, IL-17, TNF-α and TGF-β have been studied in IgAN [5, 7–9, 12, 14]. Furthermore, the intermediate filament protein nestin, which is expressed in podocytes in human mature glomerulus, has been shown as a potential marker for peritubular endothelial cell injury and tubulointerstitial fibrosis in IgAN [15].

In this study, we aimed to evaluate the association of T lymphocytes (CD3, CD4 and CD8), B lymphocytes (CD20), macrophages (CD68), proinflammatory cytokines (IL-1β, IL-17, TNF-α), anti-inflammatory cytokines (IL-10), TGF-β and nestin with clinical and conventional histopathological features and to assess the predictive role of these markers in the progression of IgA nephropaty in children.

Materials and methods

Patients

Medical reports of 37 children (12 females, 25 males; 18 ± 6 years) with biopsy-proven IgAN from 1980 to 2008 were retrospectively reviewed. Clinical data including age, gender, history of the disease, age at onset, age at diagnosis and history of upper respiratory tract infection were obtained. The criteria used in the decision to biopsy paediatric patients were as follows: (i) recurrent macroscopic haematuria, (ii) persistent proteinuria of >0.5 g/day and (iii) persistent microscopic haematuria. The diagnosis of IgAN was based on the demonstration of an IgA sole or predominant glomerular immunofluorescence finding in the biopsy and the lack of clinical evidence for Henoch–Schöenlein purpura. The ethical committee of Hacettepe University Faculty of Medicine has approved the study. All clinical parameters were reviewed at the time of biopsy. Blood pressure was measured after rest and was evaluated according to age, gender and height [16]. Urine protein excretion was measured in a 24-h collection of urine. Patients were divided into two groups according to daily protein excretion as patients with >1 g/day proteinuria and patients with <1 g/day of proteinuria for the statistical analysis.

Renal pathological evaluation

Haematoxylin–eosin, periodic acid-Schiff, Masson's trichrome and Jones' silver-stained 3 μm thick paraffin sections were examined by light microscopy. Mesangial hypercellularity, increase in mesangial matrix, glomerulosclerosis, extracapillary lesions (crescent formation), interstitial fibrosis and inflammation, tubular atrophy, tubulitis and arterial hyalinosis were evaluated. Grading of these features was done by one masked observer (D.O.). Mesangial hypercellularity was graded into four groups as normal (<4 cells per mesangial zone), mild (4–5 cells per mesangial zone), moderate (6–7 cells per mesangial zone) and marked (≥8 cells per mesangial zone) according to Oxford classification [17]. The percentage of global and segmental sclerotic glomeruli was noted for each biopsy. The percentage of crescentic glomeruli was also recorded. Mesangial matrix, tubular atrophy, tubulitis, interstitial inflammation and fibrosis were semiquantitatively graded into four groups (normal, mild, moderate and marked). This grading was modelled on the grading system used by Myllymaki *et al.* [18]. According to this grading, tubular atrophy was scored as normal (no tubular atrophy), mild (tubular atrophy in <25% of cortical tubules), moderate (tubular atrophy in 25–50% of cortical tubules) and marked (tubular atrophy in >50% of cortical tubules). Tubulitis was classified as absent (no inflammatory cells in tubules), mild (1–4 inflammatory cells per tubular cross section), moderate (5–10 inflammatory cells per tubular cross section) and marked (>10 inflammatory cells per tubular cross section). Interstitial fibrosis and inflammation were graded as normal (<5% of the cortical area is involved in interstitial fibrosis/inflammation), mild (<25% of the cortical area is involved in interstitial fibrosis/inflammation), moderate (25–50% of the cortical area is involved in interstitial fibrosis/inflammation) and marked (>50% of the cortical area is involved in interstitial fibrosis/inflammation) [18].

Immunohistochemistry

Three-micrometre-thick paraffin sections of archival kidney biopsies were dewaxed, rehydrated and microwaved (700 W, 15 min). Detection of the primary antibodies was obtained by performing indirect immunohistochemistry using the streptavidin–biotin peroxidase method (Zymed Laboratories, Histostain Plus Kit). After inhibition of endogeneous peroxidase activity, the primary antibodies against CD3 (Invitrogen, 1:100), CD4 (Invitrogen, 1:100), CD8 (Novocastra Laboratories, prediluted), CD20 (Novocastra Laboratories, prediluted), CD68 (Abcam, 1:200), IL-1β (Novus Biologicals, 1:100), IL-10 (Novus Biologicals, 1:10), IL-17 (R&D Systems, 1:20), TNF-α (Abcam, 1:100), TGF-β (Chemicon, 1:500) and nestin (Chemicon, 1:100) were applied and incubated for 1-h at room temperature. Normal renal tissue from nephrectomies of patients who had undergone surgery for Wilms' tumours was used as control for the immunostaining.

Quantification of immunohistochemically stained cells

Grading of the degree of staining was performed by one masked observer (D.O.) with an Olympus BX51 microscope at ×400 magnification. For each section stained for TNF-α, TGF-β, IL-1β, IL-10 and IL-17, positive immunostaining was graded using a three-point scale: 0 (no immunoreactivity), 1 (faint immunoreactivity—single positive cell), 2 (scattered moderately intense immunoreactivity—numerous positive cells) and 3 (dense intense immunoreactivity—clusters of positive cells). Tubular and glomerular cells showing immunostaining were scored separately as T0 (negative staining) or T1 (positive staining) for tubular cells and G0 (negative staining) or G1 (positive staining) for glomerular cells. Immunostaining with nestin in glomeruli was graded as normal, increased or decreased.

Tubular staining with nestin was also recorded. Sections were examined for CD3, CD4, CD8, CD20 and CD68 staining with an ocular grid of 0.25 mm^2 at ×400 magnification. Only nucleated cells were counted and the number of positive cells per square millimetre was calculated.

Statistics

Results were analysed using the SPSS version 11.5 and were expressed as median (minimum–maximum) for non-normally distributed data and as mean ± SD for normal distributed data. Mann–Whitney *U* test was used for comparison of patient and control groups for numerical variables. Extension of Fisher's exact test to r × c tables was used to evaluate two groups (>1 g/day proteinuria versus <1 g/day proteinuria) with histological parameters. Friedman test was used to compare immunohistochemical staining of tubulointerstitial CD3, CD4, CD8, CD20 and CD68 positive cells. χ^2 test was used to evaluate glomerular and tubular staining of IL-1β, IL-10, TNF-α and TGF-β. Correlation analysis was done by Spearman non-parametric correlation analysis. $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics

Clinical characteristics of the study population are presented in Table 1. The median age at the time of diagnosis was 10 years (range 5–14 years) and renal biopsy was performed about 8 months (ranging 0–108) after the detection of urine abnormalities. Sixty-six per cent of the patients had recurrent macroscopic haematuria. Proteinuria was >500 mg/day in 51% of the patients and >1 g/day in 32% of the patients. Twenty-four per cent of them had elevated creatinine levels up to 3.9 mg/dL during macroscopic haematuria attacks. Thirty-five per cent of patients had synpharyngitic presentation. Thirteen per cent of the patients had high blood pressure. High serum IgA levels were found in 60% of the study group.

Renal histology

The most common finding in biopsies of our paediatric patients was mild glomerular hypercellularity with mild mesangial matrix expansion observed in 68 and 78%, respectively. In addition to these observations, mild tubular atrophy was seen in 59.5%, mild interstitial inflammation in 24%, segmental glomerulosclerosis in 29.9% and global sclerosis in 29.7% of the patients. Arteriosclerosis was seen in only one biopsy. None of the biopsies revealed tubulitis (Table 2). The patients were divided into two groups as daily protein excretion <1 g ($n = 25$, 68%) and more than 1 g ($n = 12$, 32%). Mesangial matrix expansion was significantly increased in the group with >1 g protein excretion ($P < 0.05$) (Table 2). A significant correlation was found between proteinuria and mesangial matrix expansion ($r = 0.406$, $P = 0.013$). Segmental sclerosis, global sclerosis, interstitial inflammation and tubular atrophy were not statistically different between the groups with >1 and <1 g/day of proteinuria (Table 2).

Interstitial inflammatory cells and their correlation with clinical parameters

The number of interstitial CD3+ T cells [median 14 (range 9–23)] and CD68+ cells [median 10.5 (range 1–58)] (Figure 1a and b) were higher than the number of other inflammatory cells (CD4-, CD8-, CD20-positive cells) (Figure 1c and d). Additional colour images are available as Supplementary material. There was a statistically significant difference in CD4 and CD68 staining between the patients with proteinuria >1 g/day versus the patients with proteinuria <1 g/day ($P = 0.024$, <0.001), respectively. There was no statistical difference between the groups regarding the tubulointerstitial CD3-, CD8- and CD20-positive cells ($P = 0.175$, $P = 0.168$, $P = 0.541$), respectively (Table 3). The correlation was observed between the

Table 1. Clinical characteristics of IgA patients

Age (mean ± SD)	18 ± 6 year
Gender (F/M)	12/25
Age at biopsy (median)	10 (5–14 year)
Time from onset of symptoms t0 biopsy (median)	8 (0–108 months)
Recurrent macroscopic haematuria	66%
Coexisting infections	35%
High blood pressure	13%
Proteinuria (mg/day) (median)	475 (30–11 000)
Proteinuria >1 g/day	32%
Proteinuria <1 g/day	68%
[a]e-Glomerular filtration rate (mL/dk/1.73 m^2) (median)	98 (16–239)
[b]High IgA level	60%

[a]e-GFR is calculated by Schwartz formula.
[b]Normal serum levels of IgA 70–378 mg/dL.

Table 2. Histopathological findings in patients with IgA nephropathy and in patients with proteinuria

Finding	Proteinuria <1 g/day (n = 25) %	Proteinuria >1 g/day (n = 12) %	All patients (n = 37) %	P-value
Glomeruli				
Mesangial cellularity				
Normal	4.3	0	2.7	0.079
Mild	78.3	50	67.6	
Moderate	17.4	35.7	24.3	
Marked	0	14.3	5.4	
Mesangial matrix expansion				
Normal	8.7	0	5.4	0.03
Mild	87	64.3	78.4	
Moderate	4.3	35.7	16.2	
Marked	0	0	0	
Glomerulosclerosis				
Global				
Present	26.1	35.7	29.7	0.093
Absent	73.9	64.3	70.3	
Segmental				
Present	17.4	50	29.9	0.063
Absent	82.6	50	70.3	
Crescent				
Present	21.7	50	32.4	0.79
Absent	78.3	50	67.6	
Tubulointerstitial				
Tubular atrophy				
Normal	47.8	14.3	35.1	0.081
Mild	47.8	78.6	59.5	
Moderate	4.3	7.1	5.4	
Marked	0	0	0	
Interstitial inflammation				
Normal	73.9	64.3	70.3	0.418
Mild	17.4	35.7	24.3	
Moderate	8.7	0	5.4	
Marked	0	0	0	

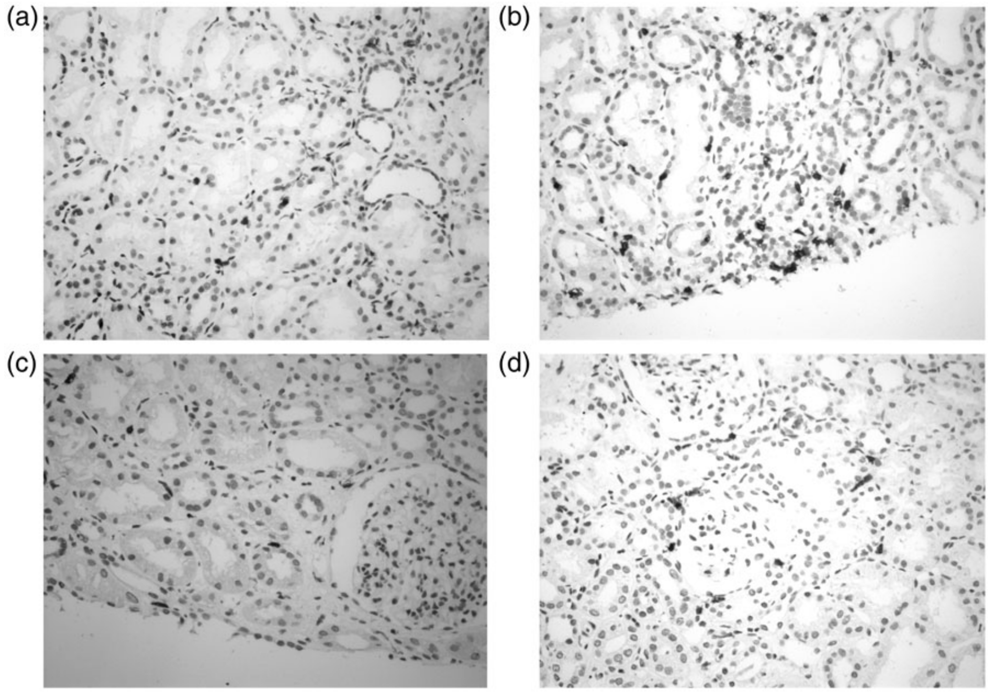

Fig 1. (**a**) CD3-positive T cells in the interstitium (**b**) Numerous CD68-positive macrophages are observed between tubuli (**c**) CD4-positive cells in the interstitium (**d**) CD20-positive B cells between tubuli. For a colour version of this figure please see supplementary data.

Table 3. The number of tubulointerstitial CD3-, CD4-, CD8-, CD20-, CD68-positive cells

	Patients without proteinuria <1 g/day (n = 17)	Patients with proteinuria >1 g/day (n = 7)	P-value
CD3 (cell/mm^2) (median)	13 (7–19)	12 (8–23)	0.175
CD4 (cell/mm^2) (median)	3 (1–8)	6 (3–10)	0.024
CD8 (cell/mm^2) (median)	1 (0–7)	2 (1–4)	0.168
CD20 (cell/mm^2) (median)	2 (0–16)	1 (0–17)	0.541
CD68 (cell/mm^2) (median)	8 (1–19)	22 (15–62)	<0.001

number of CD68+ cells and proteinuria ($r = 0.713$ $P < 0.001$) (Figure 2).

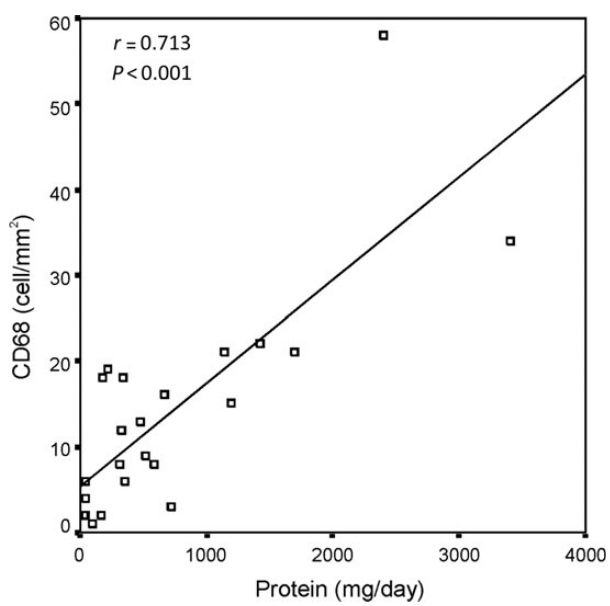

Fig 2. Correlation between proteinuria and tubulointerstitial CD68 staining.

Expression of inflammatory markers

There was no glomerular cell staining with IL-1β, IL-10 and IL-17. Tubular IL-1β and IL-10 staining was observed in 75 and 60.7% of the biopsies, respectively. Sixty-five per cent of biopsies showed tubular staining with TNF-α while glomerular staining was observed in 10.7% of the biopsies. On the other hand, 84% of biopsies showed glomerular staining with TGF-β while tubular staining was observed in 14.3% of the biopsies. No staining was observed with IL-17 in tubular cells. There was no correlation between the immunostaining findings and conventional histological data of 24 patients.

Nestin expression

In normal kidney tissues, nestin expression was limited to podocytes in glomeruli (Figure 3a). Tubular epithelial cells never expressed nestin in normal kidneys. Glomerular nestin expression varied: normal in 9 biopsies, decreased in 12 and increased in 3. There was nestin expression in tubular epithelial cells in 10 biopsies. Nestin expression in tubular cells was localized to the tubular injury areas (Figure 3b).

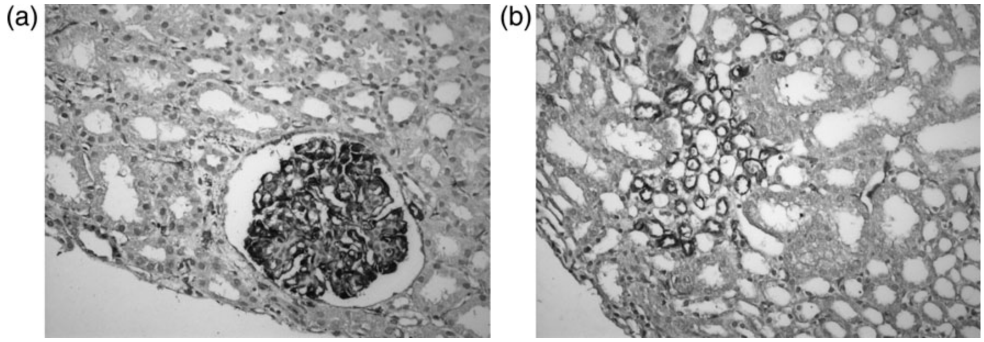

Fig 3. (**a**) Nestin positivity in glomerulus is seen in a control case. No tubular staining is observed (**b**) Nestin positivity in tubular epithelial in IgAN. For a colour version of this figure please see supplementary data.

Discussion

IgAN is an immune-mediated glomerulonephritis seen both in adults and in children. The most frequent form of presentation in children and young adults is the episodes of gross haematuria coincident with an upper respiratory tract infection. The episodes usually last a day or two. A smaller number of patients may present clinical sings of nephritic or nephrotic syndrome [19, 20]. Up to 20% of paediatric patients with IgAN have progressive disease leading ultimately to end-stage renal failure [20].

Most of our patients (66%) presented with haematuria and 35% had synpharyngitic presentation similar to others [19, 20]. Clinical parameters, which indicate poor prognosis, are severe proteinuria (>1 gr/day), renal insufficiency and hypertension [18, 21, 22]. In our group, 13% of the patients have had high blood pressure, and 32% ($n = 12$) had proteinuria >1 g/day who may have relative risk of progression and chronicity. Twenty-four per cent had elevated serum creatinine levels up to 3.9 during haematuria attacks. Only one of our patients presented with NS and one with rapidly progressive glomerulonephritis.

In regard to conventional renal biopsy features, interstitial fibrosis, tubular atrophy, interstitial infiltrate and glomerular sclerosis are known as poor prognostic factors [14, 22, 23]. In our study, the most common conventional histopathologic findings were mild mesangial hypercellularity and mild mesangial matrix expansion. Mild and moderate interstitial inflammation, segmental and global glomerulosclerosis were less commonly encountered in our patients, which was compatible with the previous studies including the new Oxford classification (Table 2) [19, 20, 23]. As the poor prognostic factors, tubulointerstitial inflammation was seen in 30% of the cases, whereas mild and moderate tubular atrophy were seen in 65% of the cases but both of them were not significantly different between the groups with >1 and <1 g proteinuria. None of our patients had tubulitis.

It is suggested that mesangial matrix expansion is the dominating finding in adults while mesangial proliferation is characteristic of the early lesion in paediatric IgAN [19, 24, 25]. However, in our study, mesangial matrix was significantly increased in the group with >1 g daily protein excretion compared with the group with <1 g daily protein excretion ($P < 0.05$) (Table 2). A significant correlation was found between proteinuria and mesangial matrix expansion ($r = 0.406$, $P = 0.013$) (Figure 2).

From the immunological point of view, one of the common histopathological features of IgA nephropathy is the presence of CD68 + macrophages and CD3+ T cells within the glomeruli and tubulointerstitial area in both paediatric and adult IgAN. Glomerular macrophage and T-cell infiltrates and their relationship with clinical parameters vary widely, nevertheless the intensity of the interstitial macrophages and T cells correlates with the degree of proteinuria and renal function [19]. Es van *et al*. [14] showed that the histological scores for tubulitis in intact and atrophic tubules was not associated with progression but GMP-17-positive cytotoxic T lymphocytes in intact renal tubules and B cells in the interstitium were significantly associated with progression. In our study, we have evaluated tubulointerstitial CD3+, CD4+, CD8+ T cells, CD20+ B cells and CD68+ macrophages in patients with proteinuria above 1 g/day and in patients <1 g/day of proteinuria (Table 3). We found a correlation between tubulointerstitial expression of CD4+, CD68+ cells and proteinuria, which was compatible with the previous studies [11, 19].

On the other hand, glomerular macrophages may play a role in glomerular lesions through production of TGF-β, which may stimulate glomerular matrix expansion. Furthermore, some subsets of macrophages present antigen to T cells which subsequently promote T-cell-mediated injury [7, 8, 19]. Activated T cells produce cytokines leading to glomerular and tubulointerstitial injury in IgA nephropathy. In light of these findings, we have evaluated several pro-inflammatory cytokines such as IL-1β, TNF-α and anti-inflammatory cytokine IL-10 along with TGF-β.

TGF-β has been shown to be involved in the excessive deposition of extracellular matrix leading to glomerulosclerosis [26]. In our study, glomerular TGF-β staining was prominent (84% of the patients) and 14.3% of the biopsies showed tubular TGF-β staining. While 25% of the biopsies with TGF-β staining in tubular epithelial cells showed segmental and global glomerulosclerosis, 33% of the biopsies with TGF-β positivity in glomeruli had segmental or global glomerulosclerosis. Crescents were observed in 50% of the cases with tubular TGF-β staining and in 41.6% of cases with glomerular TGF-β positivity. However, there was not a significant correlation between crescent formation, glomerulosclerosis and tubular or glomerular TGF-β expression in our cases.

It has been shown that the proinflammatory cytokines TNF-α and IL-1β increase the expression of Fc α receptors in mesangial cells. By interaction with IgA, these receptors lead to increased expression and synthesis of monocyte chemotactic peptide-1 and IL-8 [27, 28]. On the other hand, IL-10 is an anti-inflammatory cytokine, which suppresses IL-1β synthesis [11, 29]. In our study, IL-10, IL-1β and TNF-α immunostainings were localized to the tubular epithelial cells in concordance with the literature [30]. None of them correlated with proteinuria and severity. We also did not find any relationship between IL-10 and IL-1β expression.

The discovery of Th17 cells shed a new light on our understanding of inflammation. We have also evaluated Th17 response and IL-17 expression in our patients. To our knowledge, this is the first study evaluating IL-17 expression in childhood IgA nephropathy. Th17 cells mainly produce IL-17 [31]. Besides Th17 cells, IL-17 is also produced by CD4+ activated memory cells [32–34]. IL-17 is an important activator of proinflammatory cytokine production from monocytes/macrophages. Although it has been shown that IL-17 may augment renal inflammatory responses in adult IgA nephropathy, there was neither glomerular nor tubular staining of IL-17 in our patients [9,35].

We also evaluated nestin expression, which is an intermediate filament and identified as a marker of neural progenitor cells but also is transiently expressed in the progenitors of glomerular endothelial cells and epithelial cells of immature proximal tubules [36, 37]. Although it is proposed that nestin expression is limited to podocytes in the mature kidney, recently increased nestin expression has been shown at tubulointerstitial areas of adult patients with IgA nephropathy [37–39]. In these patients, the degree of tubulointerstitial nestin expression was positively correlated with tubulointerstitial fibrosis [15]. In parallel to these findings, we also found tubular epithelial nestin expression in 10 biopsies localized to the tubular injury areas; however, we could not find any correlation with proteinuria and tubulointerstitial fibrosis.

In conclusion, we observed a correlation between proteinuria and mesangial matrix expansion as well as proteinuria and the expression of tubulointerstitial CD4+ and CD68+ cells. However, other studied immunological markers were not fully expressed in the patients. The difference may be explained by the presence of early glomerular lesions at the time of renal biopsy in children. Further studies with extended number of the patients and follow-up biopsies could be more elucidative in immunopathogenesis and prognosis of the disease and may lead to more precise treatment modalities in childhood IgAN.

Supplementary data

Supplementary data are available online at http://ckj.oxfordjournals.org.

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Conflict of interest statement. None declared.

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