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Severity of tubulointerstitial inflammation and prognosis in immunoglobulin A nephropathy

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Many risk factors for progression in immunoglobulin A nephropathy (IgAN) have been found. We focused on renal leukocyte infiltrations and cytokines in IgAN. The subjects were 204 IgAN patients. Renal histopathological changes were semiquantitatively graded. Expression of tubulointerstitial Leukocyte common antigen (LCA), CD3, CD68, interleukin (IL)-1β, and IL-10 was evaluated by immunohistochemistry. These parameters were correlated with progression of IgAN. The significance of these correlations was tested by a multivariate analysis. Glomerulosclerosis, tubular atrophy, interstitial inflammation, and hyaline arteriolosclerosis correlated with progression in all patients and also in patients with initially normal serum creatinine. Tubulointerstitial LCA, CD3, CD68, and IL-1β expression correlated with progression. CD3 had the strongest correlation. In the multivariate analysis, tubulointerstitial CD3, hypertriglyceridemia, elevated serum creatinine concentration, and interstitial fibrosis were independently associated with progressive disease in all patients, and tubulointerstitial CD3 expression and hyaline arteriolosclerosis in patients with initially normal serum creatinine. We found parameters reflecting tubulointerstitial inflammation to predict deterioration of renal function in IgAN. This was also seen in patients whose serum creatinine was normal at the time of renal biopsy. Our findings show that, an immunohistochemical evaluation of tubulointerstitial inflammation seems to be a useful tool in determining the prognosis in IgAN.

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Immunoglobulin A nephropathy (IgAN) is an immune-mediated glomerulonephritis of unknown etiology and variable outcome.[1,2] Even though the pathogenetic mechanisms are as yet unclear, it is commonly accepted that severe proteinuria, increased serum creatinine concentration, and hypertension predict poor prognosis in IgAN. Recently we also found hyperuricemia and hypertriglyceridemia to be risk factors for the progression of IgAN.[3]

A variety of grading systems have been developed for classifying histopathological changes in renal biopsies from IgAN patients. According to the studies in question severe glomerulosclerosis and chronic tubulointerstitial changes are probably the strongest histopathological risk factors for poor outcome in IgAN. We recently found that high uric acid levels are associated independently with tubulointerstitial damage in IgAN.[4]

Leukocyte common antigen (LCA) is expressed on white blood cells. We recently evaluated immunohistochemically LCA expression to detect leukocyte infiltrations in renal tubulointerstitial tissue in IgAN. The expression strongly correlated with tubular atrophy and interstitial inflammation seen in renal biopsy.[4] An increase in the expression of T-lymphocyte marker, CD3, would appear to be associated with a rapidly progressive course of IgAN.[5] Prominent macrophage infiltrations in human kidney tissue, found by staining CD68, have been reported in the context of aggressive forms of glomerulonephritis.[6] Crescents in IgAN are associated with CD68 positivity of renal tissue.[7]

Interleukin-1β (IL-1β) is a proinflammatory cytokine, which triggers the inflammatory process by activating wide range of inflammatory mediators. Typically IL-1β is produced by activated macrophages.[8] IL-10 is an anti-inflammatory cytokine, which also suppresses IL-1β synthesis.[9]

In this study, we investigated the predictive role of inflammatory parameters as compared with conventional histopathological and clinical factors in IgAN. The main interest focused on tubulointerstitial T lymphocytes (CD3), macrophages (CD68), and proinflammatory and anti-inflammatory cytokines (IL-1β and IL-10) as well as their associations with the natural course of IgAN.

RESULTS

Univariate associations of histopathological parameters with progression in IgAN

Histopathological findings are listed in Table 1. Correlations between histopathological parameters and progression are summarized in Table 2. Glomerulosclerosis, tubular atrophy, interstitial inflammation, interstitial fibrosis (IF), and hyaline arteriolosclerosis were found to be associated with progression in all IgAN patients. All these factors except IF were also associated significantly with progression in the subgroup of patients with normal serum creatinine at the time of renal biopsy.

Table 1 | Histopathological findings in 204 patients with IgAN and in 166 IgAN patients with initially normal serum creatinine

Finding	All patients, *n* (%)	Patients with initially normal serum creatinine, *n* (%)
Glomeruli		
Normal morphology or minimal lesions	12 (6)	10 (6)
Mesangial cellularity		
Mild hypercellularity	94 (47)	81 (49)
Marked hypercellularity	46 (23)	42 (25)
Glomerulosclerosis		
Mild	126 (62)	104 (63)
Marked	52 (26)	38 (23)
Tubulointerstitial tissue		
Normal morphology	92 (46)	88 (53)
Tubular atrophy		
Mild	59 (29)	47 (28)
Marked	21 (10)	10 (6)
IF		
Mild	39 (19)	26 (16)
Marked	22 (11)	12 (7)
Interstitial inflammation		
Mild	45 (22)	33 (20)
Marked	7 (3)	1 (1)
Vascular tissue		
Normal morphology	80 (40)	69 (42)
Hyaline arteriolosclerosis[a]	84 (42)	66 (40)
Arterial intimafibrosis[a]	81 (40)	63 (38)

IF, interstitial fibrosis; IgAN, IgA nephropathy.
[a]Mild, moderate, or marked.

Univariate associations of tubulointerstitial LCA, CD3, and CD68 expressions with progression and clinical parameters in IgAN

Correlations between immunohistochemically studied parameters and progression are summarized in Table 3. Tubulointerstitial CD3 and LCA expressions correlated significantly with the degree of urine protein excretion (UPE). Tubulointerstitial CD3 also correlated with serum levels of uric acid and creatinine, but not with blood pressure. Tubulointerstitial CD68 expression also correlated significantly with UPE and serum creatinine. We found the level of tubulointerstitial CD3 expression to be associated most strongly with progression in all patients with IgAN (Figure 1), but also in the subgroup of patients with initially normal serum creatinine. Likewise tubulointerstitial LCA, also CD68 (Figure 2) correlated significantly with progression.

Univariate associations of tubulointerstitial IL-1β and IL-10 immunoreactivity with progression, histopathological, and clinical parameters in IgAN

Cytokines IL-1β and IL-10 were mainly expressed in tubular epithelial cells. Expression of IL-10 was markedly more prominent than the IL-1β expression ($P<0.001$). Both IL-1β and IL-10 correlated significantly with CD3 ($P<0.001$, $P<0.01$) and CD68 ($P<0.001$, $P<0.001$) expression. Immunoreactivity of IL-1β also correlated with LCA expression ($P<0.05$), and equally with IF ($P<0.05$).

Initial serum creatinine concentration correlated with IL-1β expression ($P<0.01$). Patients with progressive disease had a more prominent IL-1β expression than patients with stable disease ($P<0.01$) (Table 3) (Figure 3). A positive association with progression was also present when investigating the subgroup of patients with initially normal serum creatinine. We found no correlation between initial serum creatinine or progression and IL-10 expression.

Table 2 | Correlations of histopathological parameters with progressive disease in patients with IgAN

Histopathological parameter	All patients, *n*=204		Patients with initially normal serum creatinine, *n*=166	
	Progressive disease, *n*=41	Stable disease, *n*=163	Progressive disease, *n*=27	Stable disease, *n*=139
Mesangial cellularity, No/Mi/Ma (%)	34/37/29	29/49/22	22/41/37	27/50/23
Glomerulosclerosis, No/Mi/Ma (%)	5/46/49	14/66/20**	7/41/52	16/67/17***
Tubular atrophy, No/Mi/Ma (%)	39/37/24	66/27/7**	48/37/15	69/27/4*
Interstitial inflammation, No/Mi/Ma (%)	56/34/10	79/19/2**	63/33/4	83/17/0*
IF, No/Mi/Ma (%)	46/27/27	76/17/7***	63/22/15	80/14/6
Hyaline arteriolosclerosis, No/Mi/Ma (%)	49/7/44	61/25/14***	52/4/44	62/27/11***
Arterial intimafibrosis, No/Mi/Ma (%)	51/22/27	62/19/19	44/26/30	66/20/14

IF, interstitial fibrosis; IgAN, IgA nephropathy; No/Mi/Ma, normal/mild/marked.
*$P<0.05$, **$P<0.01$, ***$P<0.001$.

Table 3 | Correlations of tubulointerstitial expressions of CD3, CD68, LCA, IL-1β, and IL-10 with progressive disease in patients with IgAN

Histopathological parameter	All patients, *n*=176		Patients with initially normal serum creatinine, *n*=146	
	Progressive disease, *n*=34	Stable disease, *n*=142	Progressive disease, *n*=24	Stable disease, *n*=122
CD3, mean (2 s.d.)	99.5 (208.8)	34.4 (132.8)***	86.7 (200.5)	22.1 (55.2)***
CD68, mean (2 s.d.)	290.2 (351.1)	195.7 (334.3)**	288.0 (360.4)	164.1 (230.9)**
LCA, mean (2 s.d.)	18.5 (34.3)	8.3 (17.1)***	20.6 (38.7)	7.30 (13.8)***
IL-1β, mean (2 s.d.)	1.62 (2.70)	0.95 (1.90)**	1.52 (2.72)	0.83 (1.72)*
IL-10, mean (2 s.d.)	2.74 (3.28)	2.26 (2.56)	2.80 (3.10)	2.19 (2.52)

IgAN, IgA nephropathy; IL, interleukin; LCA, leukocyte common antigen; s.d., standard deviation.
*$P<0.05$, **$P<0.01$, ***$P<0.001$.

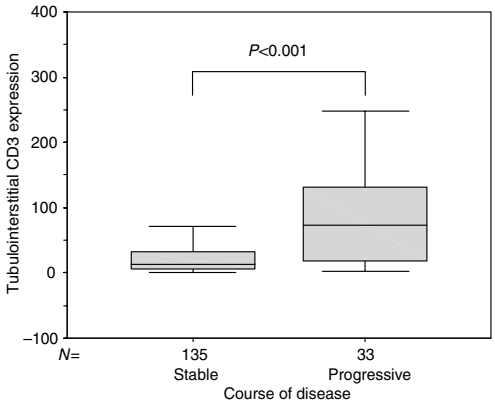

Figure 1 | Tubulointerstitial CD3 expression in patients with IgAN with stable or progressive course of renal disease. The line across the box indicates the median. The box represents the interquartile (IQ) range, which contains the middle 50% of the records. The whiskers are lines extending from the upper and lower edge of the box to the highest and lowest values, which are no greater than 1.5 times the IQ range.

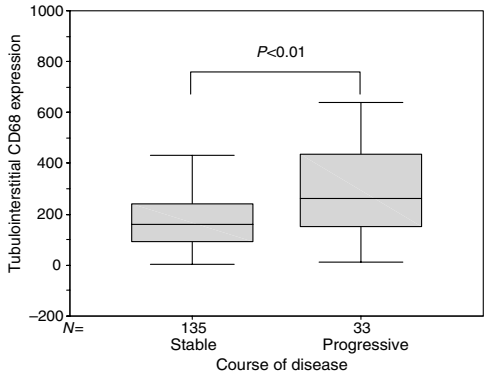

Figure 2 | Tubulointerstitial CD68 expression in patients with IgAN with stable or progressive course of renal disease. The line across the box indicates the median. The box represents the interquartile (IQ) range, which contains the middle 50% of the records. The whiskers are lines extending from the upper and lower edge of the box to the highest and lowest values, which are no greater than 1.5 times the IQ range.

Associations of histopathological and immunohistochemical parameters with progression of IgAN in multivariate analysis

To find histopathological and immunohistochemical parameters most strongly associated with progression in IgAN, we included all the aforementioned univariately correlating factors in the same multivariate analysis model. We found glomerulosclerosis ($P<0.05$), IF ($P<0.01$) and tubulointerstitial CD3 ($P<0.001$) to be most strongly associated with progression in all IgAN patients and hyaline arteriolosclerosis ($P<0.05$), tubulointerstitial CD3 ($P<0.001$), and IL-1β ($P<0.05$) expressions in the case of patients with initially normal serum creatinine.

IF, glomerulosclerosis, and tubulointerstitial CD3 were included in the same multivariate analysis model together with proteinuria (>1g/24 h), hypertension, increased serum creatinine concentration, hyperuricemia, and hypertriglyceridemia when investigating all patients. We found hypertriglyceridemia ($P<0.05$), increased serum creatinine concentration ($P<0.05$), IF ($P<0.05$), and tubulointerstitial CD3 expression ($P<0.001$) to be independently associated with progression.

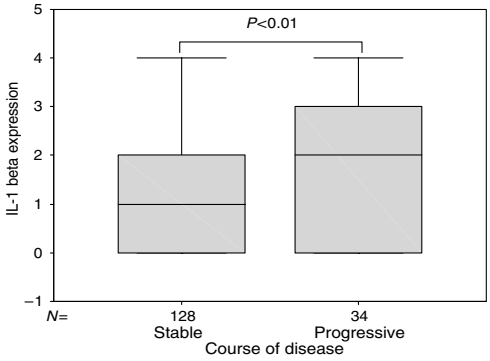

Figure 3 | Tubulointerstitial IL-1β expression in patients with IgAN with stable or progressive course of renal disease. The line across the box indicates the median. The box represents the interquartile (IQ) range, which contains the middle 50% of the records. The whiskers are lines extending from the upper edge of the box to the highest values, which are no greater than 1.5 times the IQ range.

To annul the influence of initial renal insufficiency in the risk factor analysis, we studied patients with initially normal serum creatinine separately. Tubulointerstitial CD3, IL-1β,

hyaline arteriolosclerosis, hypertension, proteinuria, hypertriglyceridemia, and hyperuricemia were parameters in the model for multivariate analysis. Tubulointerstitial CD3 expression ($P<0.001$) and hyaline arteriolosclerosis ($P<0.05$) were found to be independently associated with progression in that subgroup of patients.

DISCUSSION

Previously many clinical and histopathological risk factors have been found to predict poor prognosis in IgAN. Recently published review articles provide summaries of these factors.[1,10] Severe glomerulosclerosis and especially severe tubulointerstitial damage are histopathological parameters most powerfully associated with progression. In this study, we found parameters reflecting tubulointerstitial inflammation to be strongly associated with progression of renal disease in IgAN.

In addition to glomerular, chronic tubulointerstitial changes such as tubular atrophy and IF are often seen in renal biopsies from patients with IgAN.[11] However, inflammatory cell infiltrations, which have been classified as a marker of an active phase in renal disease, may also be present in biopsy specimens from a patient with IgAN.[12] In this study, half of the patients had significant tubulointerstitial histopathological lesions in renal biopsy. A significant extent of interstitial inflammation was found in 25% of specimens. Chronic tubulointerstitial lesions have usually been associated with poor prognosis in IgAN.[10] Much less attention has been paid to inflammatory cell infiltrations, even though Freese *et al.*[13] found the presence of interstitial cellular infiltrates to be a high-risk factor in IgAN.

In this study, we found the severity of interstitial inflammation to be associated with progression of IgAN. Active inflammation in inflammatory diseases is commonly depressed by corticosteroids. Corticosteroid treatment has been found to improve the clinical picture in IgAN.[14,15] These findings may be attributable especially to the positive effect of corticosteroids to patients with active inflammation in renal tissue.

LCA has an important role in the functions of inflammatory cells.[16] By staining LCA (CD45) in a tissue sample the localization of white blood cells can be established. Study of LCA alone cannot differentiate the levels of chronic and acute inflammation. In this study, we made quantitative determinations of renal inflammation by staining LCA in the tubulointerstitium of patients with IgAN. Tubulointerstitial LCA correlated significantly with the prognosis of renal function.

T lymphocytes typically express CD3 antigen. By evaluating the level of CD3+ cell infiltration, it is possible to approximate the intensity of inflammation in human tissue. Infiltrations of CD3+ cells have been linked to the development of IF in glomerulonephritis and to significant elevation of serum creatinine concentration in IgAN.[17,18] Falk *et al.*[5] found that prominent expression of CD3 in renal tissue from patients with IgAN occurred commonly in the rapidly progressive type of disease. However, the follow-up time in aforementioned study was quite short and the number of patients rather small. In the present study, with longer post-biopsy follow-up and a larger patient population, we found a particularly strong correlation between the amount of CD3+ cell infiltrations and progression of IgAN. We also found tubulointerstitial CD3 expression to be independently associated with progression when studying in the same multivariate analysis model with the most essential clinical, biochemical, and histopathological parameters. Hypertriglyceridemia, initially elevated serum creatinine concentration and IF were also found to be independent risk factors in all IgAN patients. When studying patients with initially normal serum creatinine, hyaline arteriolosclerosis, and tubulointerstitial C3D expression were independently associated with progression.

Prominent infiltrations of macrophages with significant proliferation seem to be more common in the aggressive type of glomerulonephritis (Yang, 1998 No.264). In pediatric IgAN patients the level of CD68+ macrophage infiltrations in the interstitium correlates significantly with urinary macrophage count, which further correlates with the activity of renal disease and UPE.[19] In this study, with mainly adult patients, expression of CD68 in the tubulointerstitium correlated significantly with the level of UPE and serum creatinine concentration, but also with progression.

Cytokine IL-1β promotes inflammation by activating a wide range of chemokines, enzymes, other cytokines and leukocyte adhesion molecules.[8] Activated macrophages are the main source of IL-1β. We found IL-1β expression to correlate with the amount of tubulointerstitial CD3+ and CD68+ cells. However, intrinsic cells seem to be the main source of IL-1β in kidney, where it seems to be involved in the disease processes in many levels.[20,21] We found IL-1β expression mainly in tubular epithelial cells. This expression correlated significantly with progression rate. It was also associated with previously established high-risk factors, IF and initial serum creatinine concentration. As an anti-inflammatory cytokine, IL-10 suppresses inflammation among other mechanisms by decreasing IL-1β synthesis. Intrarenal IL-10 expression in IgAN patients occurs mainly in tubular regions.[22] This was also the case in our study. Although IL-10 was more prominently expressed than IL-1β, we found no correlation between IL-10 expression and clinical or histopathological risk factors for progression or with progression of IgAN.

In summary, this results show that in addition to risk factors previously found, inflammation also predicts a poor course of renal disease in IgAN. Immunohistochemistry, especially investigating tubulointerstitial CD3+ T-lymphocyte infiltrations and the expression of IL-1β, proved to be a useful tool in predicting the prognosis in these patients. By evaluating the level of inflammation in renal tissue the medical treatment may be more accurately targeted to those patients who may derive the most significant benefit.

MATERIALS AND METHODS

Patients

The original patient population was the same as in our previous studies.[4,23] It comprised all 223 IgAN patients diagnosed in Tampere University Hospital during a period of 11 years from January 1980 to December 1990. The renal biopsy specimens from 204 patients contained four or more glomeruli and were considered representative for the present analyses. IgAN was diagnosed when IgA was the sole or predominant glomerular IF finding in the biopsy. Of all IgAN cases 131 (64%) were men and 73 (36%) women. Their median age was 41 years (range, 16–78) at the time of renal biopsy; 166 patients had normal serum creatinine at the time of renal biopsy. The Ethical committee of Tampere University Hospital has approved this study.

Clinical definitions

Serum creatinine values $\leqslant 125\,\mu$mol/l in men or $\leqslant 105\,\mu$mol/l in women were considered normal. Progression in renal disease was defined as an elevation of serum creatinine value above the normal limit and over 20% from baseline.[3] Blood pressure was measured by sphygmomanometer after rest. Hypertension was defined as systolic blood pressure over 140 mmHg and/or diastolic blood pressure over 90 mmHg or usage of antihypertensive medication. UPE measurements were based on 24 h collection of urine. Proteinuria was defined as UPE 1g/24 h or more. Serum uric acid was measured in 172 (84%) patients. Hyperuricemia was defined as serum uric acid >0.45 mmol/l in men and >0.34 mmol/l in women. Two patients were using allopurinol for gout. Serum triglyceride values were studied in 173 (85%) and were measured enzymatically after an overnight fast at the time of biopsy. Hypertriglyceridemia was defined as serum triglyceride concentration over 1.7 mmol/l. None of the patients was using lipid-lowering medication at the time of biopsy.

The follow-up ended at the time of a control visit performed during years 1996–1997 or if the patient died. The median follow-up time after renal biopsy was 10 years (range, 0.2–17).

Renal pathological evaluation

Paraffin sections for light microscopy were stained by the hematoxylin–eosin, periodic acid-Schiff reaction, Masson's trichrome, and periodic acid silver methenamine methods. Mesangial cellularity, glomerulosclerosis, tubular atrophy, IF and inflammation, hyaline arteriolosclerosis, and arterial intimafibrosis were evaluated and semiquantitatively graded into three groups as normal, mild and marked as in our previous study.[4] Histopathological evaluation was made by one investigator who was not aware of the clinical data.

Immunohistochemistry

Tissue specimens were available from 176 patients for immunohistochemistry, 146 of them having initially normal renal function. Renal tissue was available from 163 patients for IL-1β and IL-10 studies. For light microscopic immunoperoxidase staining, 3-μm paraffin sections were cut onto ChemMate™ capillary gap microscope slides (DakoCytomation, Denmark A/S). Inflammatory cells were investigated by staining the CD45 (DakoCytomation, Denmark A/S, leukocyte common antigen, LCA, clone 2B11 + PD7/26) (1:2000), CD3 (Novocastra Laboratories Ltd, clone NCL-CD3-PS1) (1:100) and CD68 (DakoCytomation, Denmark A/S, clone PG-M1) (1:150) antigens identifying lymphocytes and macrophages, respectively. Monoclonal mouse antibodies to IL-1β (Abcam, Cambridge, UK, clone 11E5) (1:200) and IL-10 (Serotec, Oxford, UK, clone B-S10) (1:100) were used to study the intrarenal expression of these cytokines.

Antigen retrieval was performed on re-hydrated sections in a microwave oven at 850 W for two 7-min cycles using Tris-ethylenediaminetetraacetic acid buffer (pH 9.0) as retrieval solution with CD45, CD3, and CD68 antibodies. Enzymatic digestion with 0.01% trypsin (Sigma, St Louis, MO, USA) in phosphate-buffered saline (pH 7.4) was used with IL-1β and IL-10 antibodies for 10 min at 37°C. Immunostaining was carried out in a TechMate™ 500 Immunostainer (DakoCytomation, Denmark A/S) using the En-Vision™ polymer technique (DakoCytomation, Denmark A/S). Diaminobenzidine was used as chromogen and hematoxylin as nuclear stain. The specificity of immunohistochemistry was controlled by omitting the primary antibodies or replacing them with irrelevant antisera. Known positive tissue samples were also used to confirm the staining reliability of all separate staining batches.

Quantification of immunohistochemically stained cells

Immunoperoxidase staining results were investigated at $\times 400$ magnification with an ocular grid (0.0625 mm^2). The area of tubulointersitium was determined by point counting using a 100-point square lattice in the eyepiece. Tubulointerstitial LCA, CD3, and CD68-positive cells per mm^2 were counted from cortical area in 10 adjacent fields. Fields presenting cortical scarring were excluded.

The expression of IL-1β and IL-10 was graded from 0 to 5 according to the following scale: 0 = no immunoreactivity/no positive cells; 1 = faint immunoreactivity in single positive cells; 2 = clear immunoreactivity in single positive cells; 3 = scattered moderately intense reactivity/numerous positive cells; 4 = dense intense immunoreactivity/focal clusters of positive cells; 5 = dense intense immunoreactivity/numerous clusters of positive cells.

Statistics

Mann–Whitney U or Kruskall–Wallis test was used to find significant associations of histopathological or clinical factors with immunohistochemical parameters when appropriate. Differences between categorical variables were tested by χ^2-test or Fisher's exact test when appropriate. Multivariate stepwise Cox proportional hazard regression analysis was used in detecting independent risk factors for progression. $P<0.05$ was considered significant for all tests. The software used for statistical analysis was SPSS for Windows 9.0.

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