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ORIGINAL STUDY

VIA MEDICA

Tubular NF-κB is overexpressed in proteinuric patients with IgA nephropathy

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Abstract: Increasing evidence suggests that nuclear factor κB (NF-κB) plays a pivotal role in many glomerulopathies. Therefore, the aim of the present study was to determine the tubular immunoexpression of NF-κB in non-proteinuric (n = 22) and proteinuric patients (n = 16) with IgA nephropathy (IgAN). Another purpose of this study was to examine the possible relationship between NF-κB immunoexpression and proteinuria, interstitial fibrosis as well as interstitial infiltrates. Tubular immunoexpression of NF-κB, interstitial monocytes/macrophages, T lymphocytes, B lymphocytes and interstitial area were determined using a computer image analysis system. The mean values of the tubular immunoexpression of NF-κB, interstitial area and interstitial monocytes/macrophages were in proteinuric IgAN patients significantly increased compared to non-proteinuric IgAN cases, whereas interstitial T and B lymphocytes did not differ between these groups. In proteinuric patients, tubular immunoexpression of NF-κB was highly significantly positively correlated with the degree of proteinuria. Moreover, in both the non-proteinuric and the proteinuric groups with IgAN, tubular immunoexpression of NF-κB was positively correlated with the interstitial area and interstitial monocytes/macrophages. Our findings raise the possibility that proteinuria causes tubular overexpression of NF-κB and, in the process, recruitment of monocytes/macrophages and tubulointerstitial injury in IgAN patients. *(Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 1, 93–98)*

Key words: IgA nephropathy, NF-κB, interstitial infiltrates, interstitial fibrosis

Introduction

Nuclear factor κB (NF-κB) is a protein present as a homodimer or heterodimer of five members of the NF-κB/Rel family. The commonest dimer in many cell types is composed of subunits p50 and p65 [1–4]. This dimer is retained in an inactive form within the cytoplasm through non-covalent binding to inhibitory proteins called inhibitory κB (IκB). When activated by cytokines, mitogens, viruses or cell injury it moves to the nucleus, binds DNA and influences the transcription of specific genes involved in inflammation, such as cytokines and adhesion molecules; hence, it is present in a variety of chronic inflammatory disorders [5, 6]. Increasing data also suggests that NF-κB plays a pivotal role in many glomerulopathies [7, 8], especially immune-mediated with prominent tubulointerstitial injury [9, 10]. It has also been shown that high albumin concentration may induce NF-κB activation and in the process tubular injury in proteinuric states [1, 11, 12]. On the other hand, tubular epithelial cells are known to play a central role in initiating and amplifying tubulointerstitial inflammation via cross-talk with inflammatory cells by the production of a variety of inflammatory mediators [9, 13, 14].

IgA nephropathy (IgAN), the commonest glomerulonephritis worldwide, is known as a disease with prominent tubulointerstitial injury [15–17]. Although most of the patients with IgA nephropathy present with hematuria, cases with proteinuria or nephrotic syndrome have also been noted [17].

In view of the above, the aim of the present study was to determine the tubular immunoexpression of

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NF-κB (nuclear translocation of p65) in non-proteinuric and proteinuric patients with IgA nephropathy. Another purpose of this study was to examine a possible relationship between NF-κB immunoexpression and proteinuria, interstitial fibrosis as well as interstitial infiltrates.

Material and methods

Patients. Twenty two patients with idiopathic IgAN presenting with hematuria (mean age 37.4 ± 10.5 years), and 16 IgAN participants presenting with proteinuria or nephrotic syndrome (mean age 44.6 ± 9.6 years) were examined via percutaneous renal biopsy. For the present study, only cases with diffuse mesangial proliferation were selected. In all cases, a diagnosis of IgAN was based on characteristic findings by light microscopy (sections stained with hematoxylin and eosin, Masson-Trichrome, Jones' silver impregnation and periodic acid-Schiff followed by Alcian Blue) as well as immunofluorescence (using antibodies against IgA, IgG, IgM, C3, C1q and light chains lambda and kappa). Moreover, in all patients, electron microscopy was performed using standard protocols. The thickness of each section was controlled according to the method described by Weibel [18]. Most of our patients were middle-aged. Male predominance was noticeable in both IgAN groups. At the time of renal biopsy, all patients with non-proteinuric IgAN showed hematuria. In the proteinuric group, two participants had nephrotic syndrome, whereas hematuria accompanied proteinuria in three cases. Clinical renal impairment (serum creatinine greater than 1.5 mg/dl) was noted only in two non-proteinuric and two proteinuric IgAN patients. Elevated blood pressure was found in three non-proteinuric and two proteinuric IgAN patients. As a control, 12 biopsy specimens of the kidneys removed because of trauma were used (the male to female ratio was 8:4, the mean age was 39.1 ± ± 8.1 years). None of the persons from whom renal tissue originated were known to have had previous or current renal disease. Before the quantitative examinations were carried out, all control specimens were histologically examined by a nephropathologist and found to be normal renal tissue.

Immunohistochemistry. Paraffin sections were mounted onto superfrost slides, deparaffinized, then treated in a microwave oven in a solution of citrate buffer, pH 6.0 for 20 min and transferred to distilled water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in distilled water for 5 min, and then sections were rinsed with Tris-buffered saline (TBS, DakoCytomation, Denmark) and incubated with: polyclonal rabbit-anti-human NFkB p65 (Immuno-Biological Laboratories Co., LTD., dilution 5 μg/mL), monoclonal mouse anti-human CD68 antibody (Clone KP-1, DakoCytomation, Denmark, dilution 1:100), monoclonal mouse anti-human CD3 T cell antibody (Clone PC3/188A, DakoCytomation, Denmark, dilution 1:50) and monoclonal mouse anti-human CD20cy B cell antibody (Clone L-26, DakoCytomation, Denmark, dilution 1:200). Afterwards, LSAB$^+$/HRP Universal kit (DakoCytomation, Denmark) prepared according to the instructions of the manufacturer was used. Visualization was performed by incubating the sections in a solution of 0.5 mg 3,3'-diaminobenzidine (DakoCytomation, Denmark), per ml Tris-HCl buffer, pH 7.6, containing 0.02% hydrogen peroxide, for 10 min. After washing, the sections were counter-stained with hematoxylin and coverslipped. For each antibody, and for each sample, a negative control was processed. Negative controls were carried out by incubation in the absence of the primary antibody, and always yielded negative results.

Morphometry. Histological morphometry was performed by means of an image analysis system consisting of a PC computer equipped with a Pentagram graphical tablet, Indeo Fast card (frame grabber, true-color, real-time) produced by Indeo (Taiwan), and color TV camera by Panasonic (Japan), coupled to a Carl Zeiss microscope (Germany). This system was programmed (MultiScan 8.08 software, produced by Computer Scanning Systems, Poland) to calculate the number of objects (semiautomatic function) and the surface area of a structure using stereological net (with a regulated number of points). The colored microscopic images were saved serially in the memory of a computer, and then quantitative examinations were carried out.

The interstitial area in sections stained with Masson trichrome was measured using the point-counting method, which is an adaptation of the principles of Weibel [18], the point spacing being 16 μm. The total number of the points of a net was 169, and the total area was 36,864 mm^2. Under the net described above, 8–10 randomly selected adjacent fields of the renal cortex were investigated. Glomeruli and large blood vessels were neglected. The percentage interstitial area was an expression of the number of points overlying renal cortical interstitium as a percentage of the total points counted.

Tubular immunoexpression of NF-kB, interstitial monocytes/macrophages, T lymphocytes and B lymphocytes were determined by counting p65$^+$, CD68$^+$, CD3 and CD20cy$^+$ cells (semiautomatic function) in a sequence of ten consecutive computer images of 400 × high power fields — 0.0047 mm^2 each. The only adjustments of field were made to avoid glomeruli and large vessels. The results were expressed as a mean number of immunopositive cells per mm^2.

Statistical methods. Differences between groups were tested using unpaired Student's *t*-test preceded by evaluation of normality and Levene's test. The Mann–Whitney U test was used where appropriate. Correlation coefficients were calculated using Spearman's method. Results were considered statistically significant if $p < 0.05$.

Table 1. Clinical and laboratory findings at the time of biopsy in non-proteinuric and proteinuric patients with IgAN

Group	Gender (M/F)	Micro -hematuria	Gross hematuria	Proteinuria			Renal function impairment[1]	Hypertension (> 90/160)
				< 1 g/24 h	1–3,5 g/24 h	> 3,5 g/24 h		
Non-proteinuric IgAN (n = 22)	14/8	15	7	0	0	0	2	3
Proteinuric IgAN (n = 16)	9/7	3	0	4	8	2	2	2

Values in the table are number of cases. [1]Serum creatinine > 1.5 mg/dl

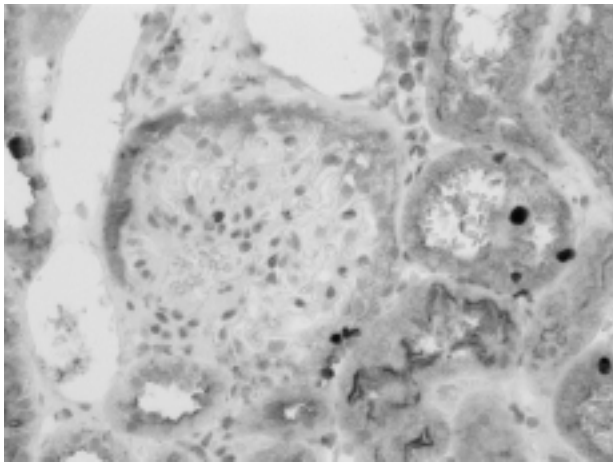

Figure 1. Weak nuclear immunoexpression of NF-κB in non-proteinuric IgAN patient. Magnification × 200

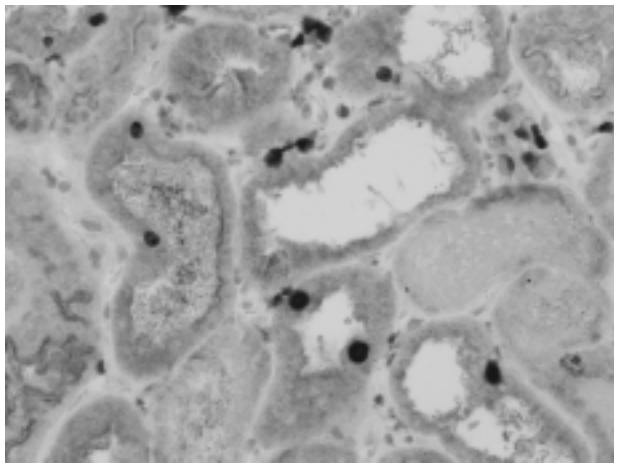

Figure 2. Intense nuclear immunoexpression of NF-κB in proteinuric IgAN patient. Magnification × 200

Results

Clinical features of the patients at the time of biopsy are given in Table 1. The tubular immunoexpression of NF-κB in both the non-proteinuric and the proteinuric groups with IgAN was exclusively nuclear (Figures 1, 2). In controls, nuclear tubular immunoexpression of NF-κB was almost negative (Figure 3). Nuclear immunoexpression of NF-κB was also seen in some glomerular cells and interstitial infiltrates, but for the present study it was not taken into consideration. A morphometric comparison of the interstitial area, tubular immunoexpression of NF-κB and interstitial infiltrates in non-proteinuric and proteinuric patients with IgAN as well as in controls is presented in Table 2. The mean values of the tubular immunoexpression of NF-κB, interstitial area and interstitial infiltrates ($CD68^+$, $CD3^+$ and $CD20^+$ cells) were significantly increased in both non-proteinuric and proteinuric IgAN groups in comparison with controls. The mean values of the tubular immunoexpression of NF-κB, interstitial area and interstitial monocytes/macrophages (Figure 4) were in proteinuric IgAN patients significantly increased compared to non-proteinuric IgAN cases. Interstitial T and B lymphocytes (Figures 5, 6) did not differ in these groups. In proteinuric patients, tubular immunoexpression of NF-κB was highly significantly positively correlated with the degree of proteinuria. Moreover, in both the non-proteinuric and the proteinuric groups with IgAN, tubular immunoexpression of NF-κB was positively correlated with the interstitial area and interstitial monocytes/macrophages; however, these correlations were statistically significant only in the proteinu-

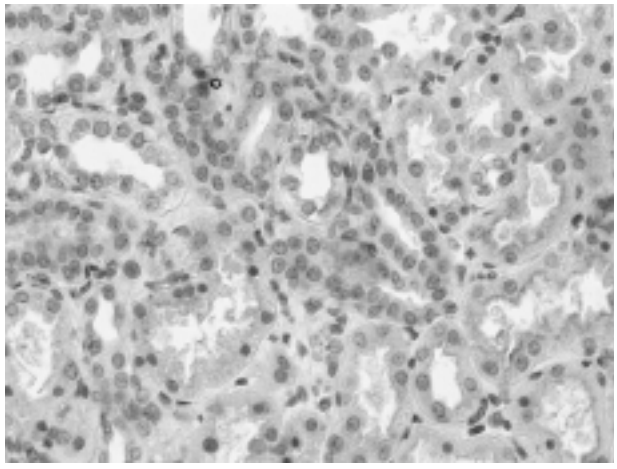

Figure 3. Almost negative nuclear staining for NF-κB in controls — only very weak focal staining can be seen (asterisk). Magnification × 200

Table 2. Morphometric analysis of the interstitial area, tubular immunoexpression of NF-κB and interstitial infiltrates in non-proteinuric and proteinuric patients with IgAN as well as in controls

Number of cases	Interstitial area (%)	Number of immunopositive cells per area (1 mm^2)			
		NF-κB$^+$	CD68$^+$	CD3$^+$	CD20$^+$
Non-proteinuric IgAN (n = 22)	14.08 ± 6.25	51.27 ± 18.06	103.86 ± 57.91	118.35 ± 57.05	10.21 ± 4.52
Proteinuric IgAN (n = 16)	20.12 ± 8.45	75.16 ± 29.25	158.25 ± 86.41	136.41 ± 66.52	12.25 ± 7.91
Controls (n = 12)	9.18 ± 1.22	0.26 ± 0.22	27.88 ± 16.25	35.27 ± 16.25	1.12 ± 0.55
p	< 0.02*	< 0.004*	< 0.03*	= 037*	= 0,32*
	< 0.02**	< 0.001**	< 0.001**	< 0.001**	< 0.001**
	< 0.001***	< 0.001***	< 0.001***	< 0.001***	< 0.001***

Data is expressed as mean ± standard deviation. *Between non-proteinuric IgAN and proteinuric IgAN; ** Between non-proteinuric IgAN and controls; *** Proteinuric IgAN and controls

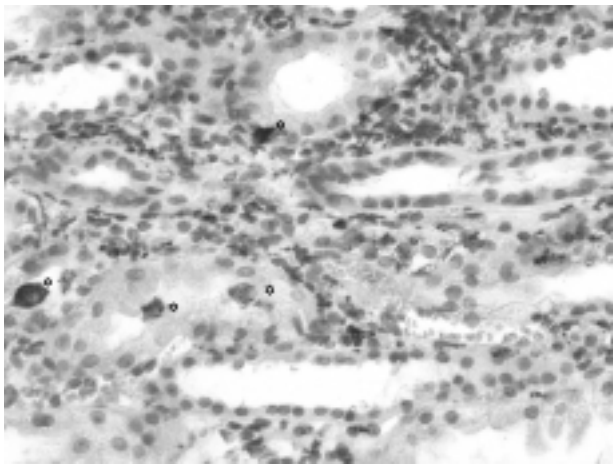

Figure 4. Infiltrates of CD68$^+$ cells in non-proteinuric IgAN patient (asterisks). Magnification × 200

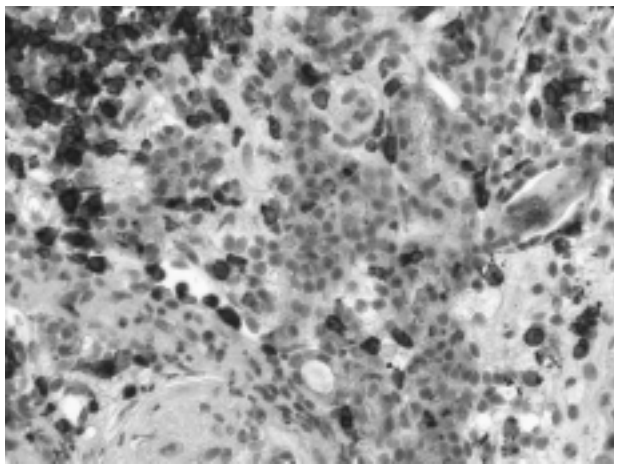

Figure 5. Numerous CD3$^+$ cells in proteinuric IgAN patient. Magnification × 200

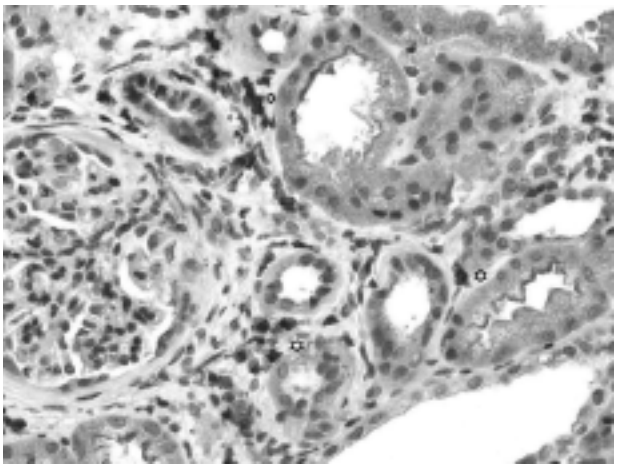

Figure 6. Infiltrates of CD20$^+$ cells in non-proteinuric IgAN patient (asterisks). Magnification × 200

ric patients. The correlations between tubular immunoexpression of NF-κB and T and B lymphocytes were weak and not significant (Table 3).

Discussion

Recent research has shown that abnormal glomerular permeability to proteins causes proximal tubular cell dysfunction and tubular activation of transcription factors including NF-κB [19, 20]. NF-κB regulates the gene expression of several cytokines and matrix proteins that are involved in inflammation [6, 19, 21, 22]. In the present study, we found that tubular immunoexpression of NF-κB was significantly increased in proteinuric IgAN patients in comparison with the non-proteinuric IgAN group and significantly positively correlated with proteinuria in proteinuric individuals. To the best of our knowledge, this is the first study on NF-κB immunoexpression in proteinuric and non-proteinuric patients presenting morphologically the same type of glomerulopathy. Our results are in concordance with the data of Mezzano et al. [19] who found immunoexpression of NF-κB mainly in tubules of proteinuric patients with membranous glomerulopathy and minimal change disease, but rare-

Table 3. Spearman rank order correlations between tubular immunoexpression of NF-κB and proteinuria, interstitial area as well as interstitial infiltrates in non-proteinuric and proteinuric patients with IgAN

Pair of variables	Non-proteinuric IgAN (n = 22)	Proteinuric IgAN (n = 16)
Tubular immunoexpression of NF-κB and proteinuria	–	r = 0.71, p < 0.003
Tubular immunoexpression of NF-κB and interstitial area	r = –0.41, p = 0.06	r = 0.56, p < 0.03
Tubular immunoexpression of NF-κB and interstitial $CD68^+$ cells	r = 0.32, p = 0.15	r = 0.67, p < 0.005
Tubular immunoexpression of NF-κB and interstitial $CD3^+$ cells	r = –0.12, p = 0.59	r = 0.23, p = 0.39
Tubular immunoexpression of NF-κB and interstitial $CD20^+$ cells	r = 0.26, p = 0.24	r = 0.33, p = 0.21

ly in non-proteinuric IgA nephropathy subjects. In this study, patients with minimal change disease had a significantly higher NF-κB tubular activation than those with membranous glomerulopathy. In both abovementioned glomerulopathies there was, as in our study, a significant positive relationship between the intensity of proteinuria and NF-κB activation. Extensive upregulation of NF-κB in renal tubular cells has also been observed in lupus nephritis, as compared with normal controls and minimal change disease [9]. Moreover, it has been noted that NF-κB is activated in tubules in various experimental models of renal injury with protein-overload proteinuria [23, 24]. In particular, activation of NF-κB in renal cortex has been shown in adriamycin-induced nephrosis [25].

Furthermore, our study revealed that interstitial fibrosis was significantly increased in the proteinuric IgAN group compared to the non-proteinuric IgAN patients. Additionally, this parameter correlated positively with the tubular immunoexpression of NF-κB, in the proteinuric group significantly. The mechanisms by which proteinuria could cause interstitial inflammation and fibrosis are still not fully understood [19, 26]. NF-κB activation in renal tubular cells has been implicated in tubulointerstitial injury in proteinuria-induced rat models [23, 27] and has been suggested to play a role in tubulointerstitial injury in human membranous glomerulopathy, IgA nephropathy, lupus nephritis, minimal change disease and diabetic nephropathy [9, 19, 28, 29]. The results of our present study support these suggestions.

Finally, we found that interstitial monocytes/macrophages in proteinuric IgAN patients were significantly more numerous than those in non-proteinuric cases, whereas lymphocytes T and B did not differ significantly in these groups. Moreover, the immunoexpression of NF-κB correlated positively with interstitial infiltrations of $CD68^+$ cells, in proteinuric individuals significantly. A similar relationship was observed by Zheng et al. in human lupus nephritis [9]. Monocytes/macrophages are believed to be involved in an interplay through a network of inflammatory mediators, which is crucial for the progression of tubulointerstitial injury [14].

In conclusion, although we are aware that a morphometric analysis does not lend itself to establish such causal associations, our findings raise the possibility that proteinuria causes tubular overexpression of NF-κB and, in the process, recruitment of monocytes/macrophages and tubulointerstitial injury in IgAN patients.

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