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Expression of TLR4 protein is reduced in chronic renal failure: evidence from an experimental model of nephron reduction

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Abstract

Toll-like receptor 4 (TLR4) signaling is involved in various acute and chronic renal lesions and contributes to inflammation and fibrosis in several organs; the latter are important determinants to the progression of chronic kidney disease (CKD). We aimed to assess TLR4 expression in progressive CKD and relate it to severity of kidney damage, using an experimental nephron reduction model. Male Wistar rats were subjected to subtotal nephrectomy using the ligation technique, after 12 weeks of observation, serum creatinine and proteinuria were determined, animals were sacrificed, glomerulosclerosis and interstitial scarring were quantified histologically, and TLR4 expression was assessed by immunohistochemistry. Sham-operated rats served as controls. Case animals had significantly higher creatinine, proteinuria, glomerulosclerosis and tubulointerstitial involvement. TLR4 expression was prominent in proximal tubes, less staining was observed on infiltrating inflammatory cells. Percentage of TLR4-positive tubes was reduced in the subtotal nephrectomy animals, when compared to controls (0.67±0.09 *versus* 0.79±0.07, p=0.003). Percentage of TLR4-positive tubes correlated inversely to markers of kidney damage: to proteinuria (r=-0.55, p=0.02), serum creatinine (r=-0.53, p=0.01); percentage of glomeruli with glomerulosclerosis (r=-0.54, p=0.01) and tubulointerstitial score (r=-0.36, p=0.01). As TLR4 staining appears in tubular casts only in nephrectomy animals, shedding from damaged tubular cells is a very likely explanation for the reduced TLR4 expression in the kidneys of subjects with experimental nephron reduction.

Keywords: chronic kidney disease, toll-like receptor, fibrosis, inflammation, experimental.

Introduction

Toll-like receptors (TLR) are transmembrane proteins involved in the innate immune response. TLR4 was the first (out of 11) TLRs described in humans, and is expressed on monocytes and dendritic cells but was also identified on various other cell types. TLR4 recognizes bacterial lipopolysaccharide and contributes to host defense against Gram-negative bacteria. However, it is currently believed, that endogenous molecules, called danger-associated molecular patterns (DAMPS), that have been altered from their native state, or accumulate in non-physiologic sites or amounts during cellular injury, can also bind to TLR4 [1, 2] and trigger inflammation. TLR4 activation has been involved in many non-infectious inflammatory diseases, including atherosclerosis and diabetes [3].

A pathogenic role of TLR4-related inflammation is confirmed in acute nephrotoxic or sepsis-related kidney injury [1]; but, also, in ischemia-induced injury, including ischemia related graft dysfunction [4]. In chronic renal pathology, a TLR4-related mechanism was also suggested in cyclosporine treatment [5]. Fibrosis and sclerosis are important contributors in both cyclosporine toxicity and chronic allograft dysfunction and furthermore, TLR4 expression was linked to development of fibrotic lesions in the kidney in ureteral obstruction experiments [6, 7], to pulmonary fibrosis [8], hepatic fibrosis [9] and systemic sclerosis [10]. These data suggest a pathogenic link between TLR4 activation and fibrogenesis. As fibrosis consecutive to inflammation is the mainstay in the progression of chronic kidney disease (CKD), the study of a possible role of TLR in progressive CKD is justified. We aimed to describe the expression of TLR4 in the rat remnant kidney model (experimental model of human CKD) and to explore a possible relationship of TLR4 expression to the degree of kidney damage.

Materials and Methods

Experimental model

Three months old male Wistar rats obtained from the Central Animal Facility of the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania, were used for the study. The animals were housed in standard

cages and maintained under controlled room temperature (22±2^{0}C) and humidity (55±5%) with 12:12 hours light and dark cycle. All the rats were provided with commercially available rat normal pellet diet ("Cantacuzino" Institute, Bucharest, Romania: 18% proteins, 1.5% fat, 5% fibers) and water *ad libitum*. The experimental group consisted of 5/6 nephrectomized rats using the ligation technique [11]. Under anesthesia with sodium pentobarbital, 30 mg/kg intraperitoneally, renal ablation was performed by removal of the right kidney and selective infarction of approximately two-thirds of the left kidney by ligation of two or three branches of the renal artery. Only the rats that were alive at 48 hours after surgery were included in the study. Nine sham-operated rats were used as controls. Sham-operation consisted of ventral laparotomy and manipulation of the kidneys and renal pedicle without destruction of renal tissue.

Laboratory assessment

Every two weeks, animals were placed individually in metabolic cages and 24-hour urine was collected; 24-hour protein excretion was measured. At baseline and before sacrifice, blood samples were obtained from the retro-orbital plexus of the rats under light ether anesthesia using capillary tubes. The serum was separated by centrifugation (5 minutes, 5000 rpm) and was analyzed for creatinine. Case and control animals were sacrificed at 16 weeks under general anesthesia with ketamine 10% 80 mg/kg and xylazine 2% 8 mg/kg; kidneys were harvested and conserved in formalin. Ethical guidelines of *Council of International Organization of Medical Sciences* (WHO/UNESCO) as well as national guidelines were applied. The study was approved (No. 497/12.12.2011) by the Ethical Committee on animal experimentation of the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca.

Histology

Each kidney was cut in half and then fixed in 10% neutral buffered paraformaldehyde for 24 hours. The samples were dehydrated in progressive concentrations of ethanol, cleared in xylene and then embedded in paraffin. Sections were cut at 4 μm and then deparaffinized, stained with PAS (Periodic Acid Schiff) (HP01, Farbekit, Roth, Germany), Hematoxylin–Eosin and Trichrome Masson techniques and then examined using an Olympus light microscope (BX41). A number of 100 glomeruli were examined for signs of glomerular sclerosis. Glomerulosclerosis was defined as segmental or global increases in the glomerular matrix, collapse, obliteration of capillary lumina, and accumulation of hyaline material, often with synechial attachment to Bowman's capsule. The percentage of glomeruli displaying glomerulosclerosis was assessed. Presence of interstitial involvement (fibrosis/ inflammatory infiltrate) was graded using a grade scale based on the percentage of affected tissue, as previously described [12]: 0, no evidence of interstitial fibrosis/ inflammation; 1, <25% involvement; 2, 25 to 50% of the interstitial area involved; 3, >50% of interstitial tissue involved. Pictures were taken using the DP 25 camera (Olympus).

TLR4 immunohistochemistry

Four μm tissue sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked using Peroxidase Block for 30 minutes at room temperature. Samples were pretreated by heating in sodium citrate buffer, 10 mM, pH 6.0, using a pressure cooker, then were incubated with primary TLR-4 monoclonal antibody (clone 76B357.1, Abnova, Taiwan) diluted with Dako Antibody Diluent (S0809) for one hour at room temperature, followed by secondary (biotinylated) antibody for 15 minutes and then with ready-to-use Streptavidin–peroxidase conjugate for 15 minutes. Negative control was used, treated only with PBS (phosphate-buffered saline). The immunostaining was revealed using 3,3'-diaminobenzidine tetrahydrochloride for one minute, followed by counterstaining with Hematoxylin. Slides were dehydrated in ethanol and mounted (Neo-Mount, Merck, Darmstadt, Germany).

Statistical analysis

Statistical analysis was performed using SPSS 13.0, Statistica 8.0 and Microsoft Excel programs. Normality was tested using the Kolmogorov–Smirnov test. For comparison of two means of independent samples, *t*-test or Mann–Whitney *U*-test were used. For identifying correlation between two continuous variables, Pearson's correlation coefficient (r) was assessed. Values are expressed as mean±standard deviation. Statistical significance threshold was considered α=0.05.

Results

A total of 20 rats were subjected to nephrectomy. Three of them died within 48 hours because of excessive ischemia of the kidney (one case) and hemorrhage (two cases). Two animals died before completion of the study, at six and eight weeks respectively, of unknown causes. Fifteen animals completed the protocol. All of the control animals completed the study.

At baseline, both creatinine and proteinuria were similar in the case and control groups. However, in the subtotal nephrectomy group during follow-up proteinuria steadily increased, attaining a maximum at 12 weeks and slightly decreasing thereafter, probably in parallel to decrease in GFR. Mean proteinuria in subtotal nephrectomy animals was 21.30±14.22 mg/dL at two weeks, 40.15± 31.82 mg/dL at four weeks, 54.29±45.08 mg/dL at six weeks, 83.30±79.97 mg/dL at eight weeks, 77.87±87.48 mg/dL at 10 weeks, 161.34±122.56 mg/dL at 12 weeks, 103.55±65.98 mg/dL at 14 weeks, 84.78±54.59 mg/dL at the end of the study, mean proteinuria was 60.69± 43.33mg/dL in case animal. This evolution is depicted in Figure 1. At the end of the study, there was a significant difference in both serum creatinine and 24-hour proteinuria between case and control animals: the animals in the study group displayed higher end of study creatinine and proteinuria when compared to controls. Characteristic of case animals and comparison to controls are presented in Table 1.

Histological evaluation at the end of the study revealed significant changes in the study group in both glomeruli and tubulointerstitial areas: glomerulosclerosis was present

in a diffuse or pattern, the majority (over 70%) of glomeruli in the nephrectomy group with generally global and sometimes focal disposition. Tubulointerstitial changes consisted in interstitial fibrosis together with a significant number of dilated and atrophied tubes; inflammatory tubulointerstitial infiltrate was also present in the nephrectomy group. The latter was represented predominantly by lympho-plasmocytic cells; polynuclear infiltration was virtually absent. Some intra-tubular casts were observed in the nephrectomy group tubular necrosis was not prominent, only a few necrotic aspects were noted. In the control group, histology reflected normal kidney tissue. Histological aspects in case and control animals are presented in Figure 2. Glomerulosclerosis and tubulointerstitial scores computed as described in the methods' section were used to compare extent of chronic damage in case and controls animals. Case animals displayed a significantly increased glomerular and tubulointerstitial score when compared to control animals. Comparison of computed scores is presented in Table 1.

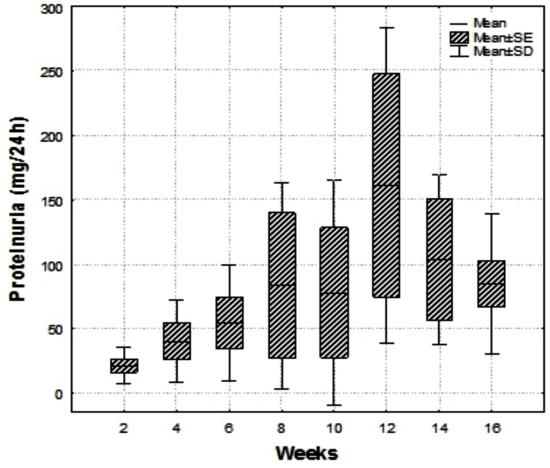

Figure 1 – ***Proteinuria during follow-up in the nephrectomy group.***

Table 1 – ***Comparison of 5/6 nephrectomy subjects and controls***

Parameters	5/6 Nephrectomy	Control	*p*
Baseline creatinine [mg/dL]	0.37±0.03	0.36±0.04	0.52
End of study creatinine [mg/dL]	0.97±0.39	0.46±0.09	0.0001
End of study proteinuria [mg/24 h]	84.78±53.98	0.17±0.16	0.0003
Glomerulosclerosis [%]	0.68±0.13	0.09±0.02	<0.0001
Tubulointerstitial score	0.73±1.10	0.00±0.00	0.03
TLR4 staining [%]	0.67±0.09	0.79±0.07	0.003

Glomerulosclerosis: No. of glomeruli with global or segmental sclerosis lesions in 100 glomeruli [%]; Tubulointerstitial score: Score assigned for evidence of fibrosis and inflammation; TLR4 staining: No. of tubes staining for TLR4 from 100 tubes counted [%]; *p*: Statistical significance level.

TLR4 protein expression was confined to the tubes and was significantly less prominent in experimental animals when compared to controls (Figure 3). No TLR4 staining could be identified on endothelial, mesangial or glomerular epithelial cells, neither in case nor in control animals. Only scarce positivity of inflammatory cells for TLR4 was noted (Figure 4). Interestingly, TLR4 positive casts were observed in 5/6 nephrectomy animals, but not in controls.

TLR4 expression was quantified as percentage of positive tubes in both case and control animals; the percentage of proximal tubes that stained positively for TLR4 was higher in control animals than in the remnant kidney group; comparison of TLR4-positive tubes is presented in Table 1. The percentage of TLR4-positive tubes correlated significantly inversely to markers o kidney damage: end of study creatinine, end of study proteinuria, and with glomerulosclerosis and tubulointerstitial score; results of simple regression analysis are shown in Table 2.

Table 2 – ***Correlations between TLR4 staining and parameters of kidney damage***

Parameters	End of study proteinuria		End of study creatinine		Glomerulosclerosis		Tubulointerstitial score	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
End of study creatinine	0.84	<0.0001						
Glomerulosclerosis	0.80	<0.0001	0.79	<0.0001				
Tubulointerstitial score	0.86	<0.0001	0.75	<0.0001	0.49	0.01		
TLR4 staining	-0.55	0.02	-0.53	0.01	-0.54	0.01	-0.36	0.01

Glomerulosclerosis: No. of glomeruli with global or segmental sclerosis lesions in 100 glomeruli [%]; Tubulointerstitial score: Score assigned for evidence of fibrosis and inflammation; TLR4 staining: No. of tubes staining for TLR4 from 100 tubes counted [%]; *r*: Correlation coefficient; *p*: Statistical significance level.

Discussion

Inflammation and fibrosis are major determinants for the progression of CKD regardless of the underlying etiology; TLR4-mediated mechanisms are susceptible to contribute to these processes. It is thought that TLR4 can be activated by endogenous ligands (DAMPs) exposed because of cellular injury and increased matrix turnover: extracellular matrix breakdown products, cellular debris, heat shock proteins [2, 13, 14]. During progressive renal fibrosis and tubulointerstitial injury, endogenous TLR4 ligands such as fibrinogen, heparan sulfate, hyaluroran, and fibronectin are produced in excess and are susceptible to bind to TLR4 on macrophages. A TLR4-triggered intracellular cascade may eventually result in activation of antigen presenting cells and in NF-κB-dependent gene expression [15]. This, in turn, would be followed by additional leukocyte recruitment to the kidney supporting sustained interstitial inflammation; the latter eventually results in fibrogenesis.

It has, however, to be emphasized that the definite role of TLR4 stimulation by endogenous cellular debris as a component of inflammation in non-sepsis associated conditions has been questioned: in an interesting review, the possibility of such substances to merely act as co-stimulatory or sensitizing molecules for the classical ligand of TLR4 (lypopolysaccharide) is discussed [16].

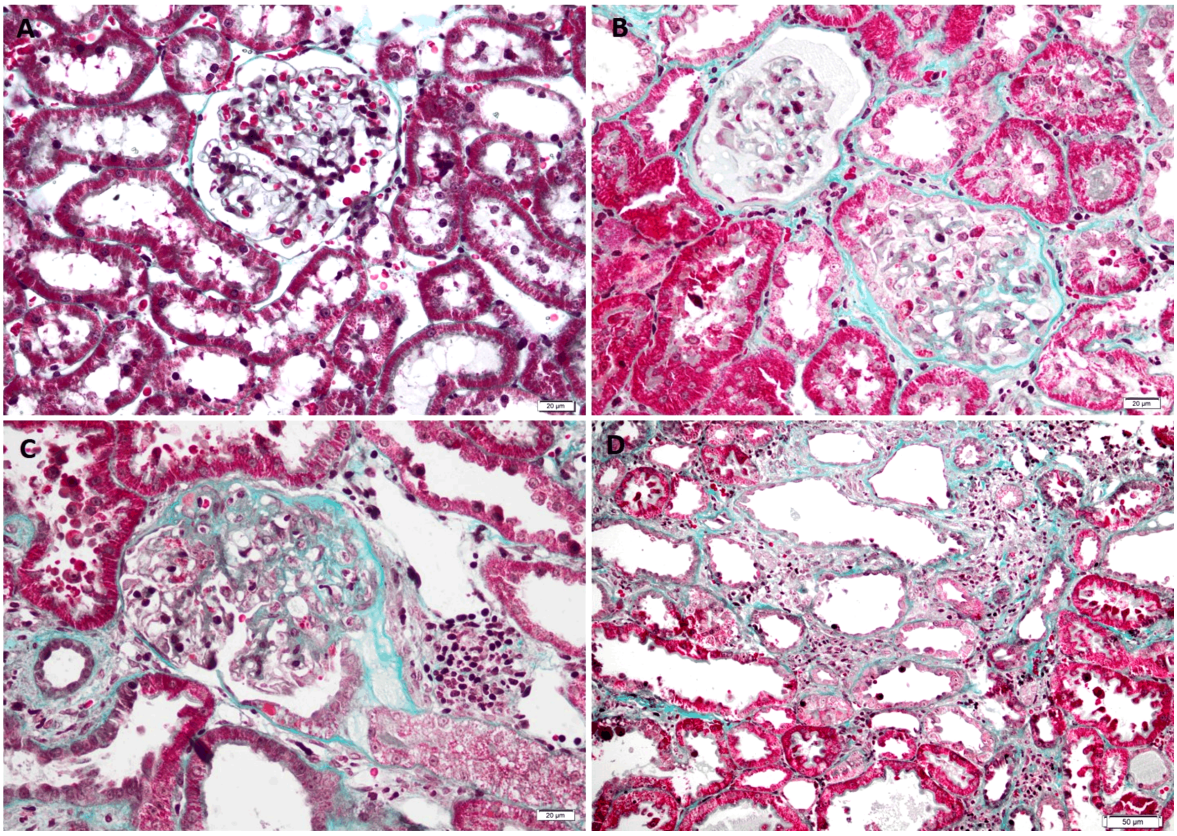

Figure 2 – ***Renal cortical histology in case and control subjects (Trichrome staining): (A) Normal renal cortex in control animals (×400); (B and C) Renal cortex from a nephrectomy subject. Note the advanced stage of segmental glomerular sclerosis and also the inflammatory infiltrate in the interstitium (×400); (D) Renal interstitial fibrosis from an individual case (×200).***

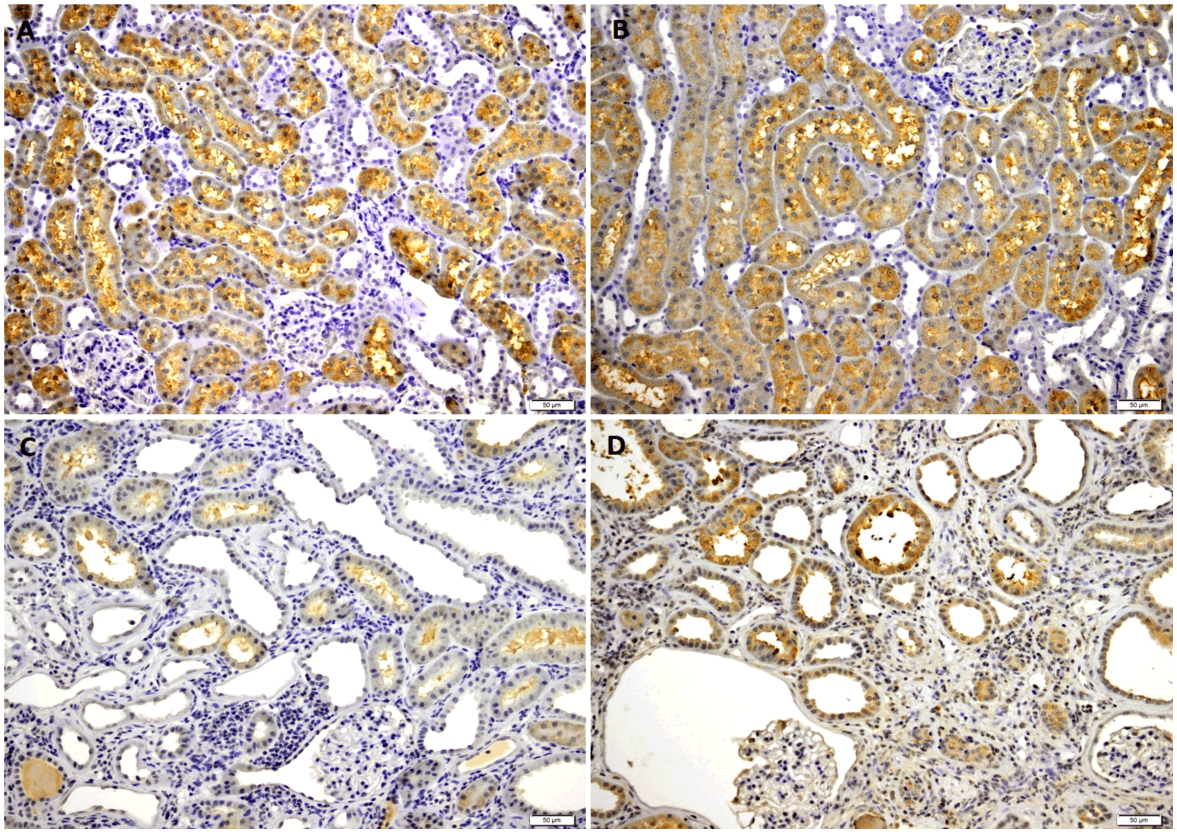

Figure 3 – ***TLR4 expression in proximal tubes of case and control subjects: (A) Normal renal cortex showing intense TLR4 expression in proximal tubes (×200); (B) Normal renal cortex (×200) showing particularly high TLR4 expression of the brush border of proximal tubules, and no expression in glomeruli; (C and D) Renal cortex of 5/6 nephrectomy rat showing TLR4 depletion in proximal tubes. Note the appearance of TLR4 expression in tubular lumina in the form of casts (×200).***

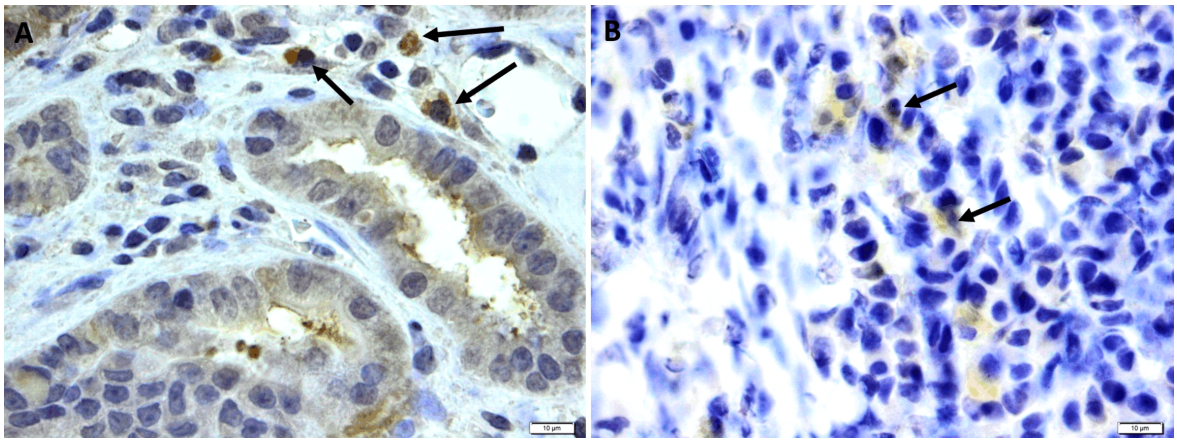

Figure 4 – ***TLR4 expression on infiltrating inflammatory cells in case subjects: (A and B) TLR4 expression in inflammatory mononuclear cells (arrows), ×100.***

Based on the above-mentioned data, we wanted to explore TLR4 expression in progression of CKD. Noteworthy, previous clinical and/or experimental data identified a potential role for TLR4 stimulation in several models of chronic nephropathies: experimental immuno-mediated glomerulonephritis [17–19], IgA nephropathy [20], chronic allograft rejection [21–23] and cyclosporine treatment [5], but its role in progression of CKD in a remnant kidney model was not yet studied to our knowledge.

In our study, we identified prominent TLR4 expression on tubular epithelial cells, the majority of staining being confined to proximal tubules. There was only scarce staining on infiltrating inflammatory cells and none could be identified on endothelial, mesangial or glomerular epithelial cells. This is in contradiction with the fact that in certain specific kidney disease models TLR4 expression has been identified on endothelial [24], mesangial cells [17] or podocytes [17, 18]. However, in the majority of experimental studies, only expression of TLR4 on tubular epithelial cells [25, 26] or on infiltrating inflammatory cells was proven [27, 28], which is in agreement with our findings.

We quantified TLR4 expression and related it to the degree of kidney damage. In spite of the fact that, as expected, in the experimental group renal histological damage as reflected by glomerulosclerosis and interstitial involvement, was evident, an increase in TLR4 staining in these animals could not be demonstrated. On the contrary, in parallel to biochemical and histological evidence of renal damage, TLR4 expression was significantly reduced. This could be confirmed by counting positive TLR4 tubes and relating the results to markers of kidney damage (Table 2). Of note, because in the CKD group a significant number of tubes displayed atrophy and dilation, we did not use as a measure the number of positive tubes/field, but the percentage of TLR4-stained tubes. The inverse relationship of TLR4 staining to evidence of chronic renal histological lesions group was statistically significant even in our small cohort (Table 1) and inversely linked to severity of kidney damage (Table 2).

Whereas in models of acute nephrotoxic, ischemic as well as sepsis-related kidney injury [25–28, 29–31] or in acute ureteral obstruction [6, 7], increased TLR4 expression is related to negative renal outcome, and in agreement with our current findings, our study suggests that this does not seem to be the case in progressive CKD. Our results are in agreement with the only other study that we are aware of, relating renal TLR4 expression to kidney histology in chronic non-transplantation related kidney disease [32], where a negative influence of TLR4 expression on renal function and outcome could not be demonstrated. However, in contrast with our findings, in this histological study in humans, the authors found a relationship of TLR4 expression to inflammatory markers but none to glomerulosclerosis or renal function. Noteworthy, heterogeneous types of nephropathies were included in this study with different propensities for progression.

The most likely explanation for the decreased TLR4 staining in our remnant kidney model is shedding of TLR4 from injured and modified tubular epithelial cells. This was previously reported for acute kidney injury: in a very well designed and convincing experimental study [26], it was shown that while upregulation of TLR4 at mRNA level is evident in acute kidney injury, at the level of protein expression TLR4 tubular epithelial staining is decreased. The authors explain "shedding and depletion" of tubular TLR4 as a consequence (and hence marker) of kidney injury, and not necessary as a pathogenic contribution to the latter. Of note, in some of the above-mentioned studies that report TLR4 upregulation in different acute or chronic kidney disease models, only mRNA level was quantified but protein expression was not measured [17, 22, 23]. In light of these findings, it would be conceivable that at least in some experimental settings, TLR4 expression at the protein level could be reduced secondary to tubular damage and only mRNA expression would be increased, possibly as a feedback mechanism. Our study has the shortcoming of not having measured mARN levels, but TLR4 expression was clearly reduced – and not increased – in our remnant model experiment. Therefore, TLR4-mediated mechanisms are not likely to have a significant contribution to progression of chronic kidney disease.

TLR4 staining in rodent models in normal and pathologic conditions should, however, not be directly extrapolated to humans as differences between rodent

models and human disease recently outlined in a comparative study [33]. With the exception of leukocyte expression, in normal humans TLR4 expression was confined to the endothelium whereas in rodents tubular epithelial cells express TLR4. Further histological evidence in humans is necessary.

Conclusions

TLR4 expression in the remnant kidney model is identified mainly on proximal epithelial tubular cells and is clearly reduced in comparison to that of healthy animals. TLR4 staining is inversely related to markers of kidney damage. As TLR4 staining appears in tubular casts, shedding from damaged tubular cells is very likely.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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