Tubular expression of Toll-like receptor 9 in lupus and primary membranous nephropathy

Expression tubulaire du récepteur Toll-like 9 dans la Glomérulonéphrite extramembraneuse lupique et la glomérulonéphrite extramembraneuse primitive

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Abstract

Background: Toll-like- receptors (TLR) control important aspects of innate and adaptive immune responses. Renal cells are among the non-immune cells that express (TLR). Therefore, their activation might be implicated in renal tubulo-interstitial injury.
Aim: The study aimed to compare TLR9 expression in patients with primary membranous nephropathy (MN) to patients with lupus membranous nephropathy.
Methods: Kidney sections from 10 Lupus nephritis (LN) patients and ten patients with primary MN were analyzed by immunohistochemistry using anti-human TLR9 antibody.
Results: Results showed that TLR9 expression was weak and exclusively tubular in primary MN patients' biopsies. There was a significant difference between LN patients' biopsies and primary MN patients' biopsies. TLR9 expression was more diffused in LN patients' specimen than in those with primary MN.
Conclusion: This study focuses on molecular level pathogenesis of MN. The data suggest that the receptors TLR9 may play role in tubulointerstitial injury in the pathogenesis of LN but not primary membranous nephropathy.

Key words: Membranous nephropathy, Lupus nephritis, TLR9, Kidney biopsies, Tubular expression, Tubulointerstitial injury

Résumé

Introduction: Les récepteurs Toll-like (TLR) contrôlent des aspects importants des réponses immunitaires innées et adaptatives. Les cellules rénales font partie des cellules non immunitaires qui expriment ces TLR, leur activation pourrait donc être impliquée dans des lésions tubulo-interstitielles rénales.
Objectif: Comparer l'expression de TLR9 chez les patients atteints de glomérulonéphrite extramembraneuse primitive (GNEM) à celle des patients atteints de glomérulonéphrite extramembraneuse lupique.
Méthodes: L'expression du TLR9 au niveau des biopsies rénales de 10 patients atteints de GNEM lupique et de dix patients atteints de GNEM primaire a été analysée par immunohistochimie en utilisant un anticorps anti-TLR9 humain.
Résultats: L'expression de TLR9 a été faible et exclusivement tubulaire dans les biopsies des patients atteints de GNEM primaire. Il y avait une différence significative entre les biopsies des patients atteints de lupus et celles des patients atteints de GNEM primaire. L'expression de TLR9 a été plus diffuse sur les biopsies des patients atteints de GNEM lupique par rapport à ceux atteints de GNEM primaire.
Conclusion: Ces données suggèrent que les récepteurs TLR9 pourraient jouer un rôle dans les lésions tubulo-interstitielles associées à la GNEM lupique mais pas dans la GNEM primitive.

Mots clés: Glomérulonéphrite extra membraneuse (GNEM), Néphropathie lupique, TLR9, Biopsies rénales, Expression tubulaire, Lésions tubulo-interstitielles

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LA TUNISIE MEDICALE-2024; Vol 102 (04): 241-244

Introduction

Toll-like receptors (TLRs) are a family of transmembrane proteins expressed by various immune (e.g., dendritic cells, macrophages, and B lymphocytes) and non-immune cells (e.g., epithelial cells, endothelial cells, and fibroblasts)(1–3). Currently, at least ten TLRs have been identified in humans(4). They can recognize exogenous pathogen-associated molecular patterns (PAMPs) and/or tissue injury through the recognition of endogenous danger-associated molecular patterns (DAMPs)(2). This ligation by exogenous and endogenous ligands triggers a pro-inflammatory signaling cascade in various cells (5). TLR activation allows the host to develop innate and subsequent adaptive immune responses to defend against invading microbes and to repair the damaged tissues.

Among these receptors, TLR9 has been identified in experimental and human models as a receptor implicated in the initiation and progression of kidney diseases such as crescentic, lupus nephritis, and IgA nephropathy(6–8). Dysregulated TLR9 signaling could disrupt the immune system and induce inflammatory and autoimmune diseases.

Several studies support their role, especially in lupus nephritis (LN)(9,10). TLR 9 has been implicated in the activation of immune cells in lupus. In a previous study, we showed that TLR9 is not expressed in normal kidney glomeruli and is weakly expressed in LN glomeruli (11). However, the tubular expression of TLR 9 was more pronounced in LN than normal Kidneys. In this study (11), TLR9 was more expressed in lupus membranous nephropathy (MN) than in other forms of LN. However, we do not know if this overexpression in MN is specific to lupus MN or may be the trigger for any MN including primary MN.

To our knowledge, no immunohistochemistry study of TLR9 on primary MN kidney biopsy has been carried out to date. Thus, in this report we aim to evaluate the expression of TLR9 in patients with primary MN and patients with lupus MN, and we compare TLR9 expression between the two above-mentioned membranous nephropathies.

Methods

Patients

This study was approved by the ethical committee of the Habib Bourguiba University Hospital of Sfax, Tunisia (Protocol number: 4/12), and written consent was obtained before collecting samples. We carried out a retrospective case-control study. All patients underwent native kidney biopsy in the department of Nephrology at Hedi Chaker University Hospital of Sfax, and screened in the Anatomopathology department at Habib Bourguiba Hospital. All patients with isolated class V LN were included. Age matched (+/- 10 years) primary MN were recruited from the same population and during the same period. The classification of lupus nephritis was performed according to the International Society of Nephrology/Renal Pathology Society classification of lupus nephritis (ISN/RPS) 2003 revised classification system (12).

Exclusion criteria were secondary non-lupus membranous nephropathy, other class of lupus nephritis or lupus MN associated with another class, and biopsies of renal grafts. The patients who received corticoids (>10mg) or immunosuppressive drugs during the last 3 months were also excluded from the study. Patients were evaluated regarding demographic data (age, gender); clinical presentation and laboratory evaluation (serum creatinine, estimated glomerular filtration rate (eGFR) calculated by the Chronic Kidney Disease Epidemiology (CKD-EPI) equation, complete blood count, plasma proteins, albumin, and 24 hours urinary protein excretion). Immunohistochemistry (IH) for TLR 9 expression examination, was performed as described previously (11).

Scoring of kidney biopsies

The interpretation of renal biopsies was carried out during an anatomic-clinical confrontation session, carried out by a nephrologist and an anatomopathological.

For every biopsy section, TLR9 expression score was assigned by giving a score ranging from 0 to 12 resulting of the product: intensity score X diffusion score.

- The tubular intensity score: 0 for negative, 1 for weakly positive, 2 for moderately positive, and 3 for strongly positive in tubular cells.
- The tubular diffusion score is the ratio of the stained tubuli to the total observed tubuli of each specimen: 0 for 0%, 1 for 0–25%, 2 for 25–50%, 3 for 50-75%, and 4 for >75%.

Statistical analysis

Statistical analysis was performed using SPSS software (Statistical Package Social Sciences) version 2.0. The Mann–Whitney U-test was used to analyze the differences in TLR9 expression in renal tissues between controls the two patients' group. The t-test was used for the association studies. The significance level of $p<0.05$ and Odds ratios (OR) with 95% confidence intervals (CI) was chosen for all sets. The significance level of $p<0.05$ was chosen for all sets.

Results

Ten patients with lupus MN (control group) and 10 age-matched patients with primary MN confirmed by kidney biopsy were assigned to the study groups. As shown in table 1 there was no difference regarding neither demographic parameters, nor clinical or biological characteristics between the two groups.

Anatomopathological study

Sampling for light microscopy included a mean of 10, 75 ±

Table 1. Demographic, clinical, and biological data of the patients

	Lupus MN patients (N=10)	Primary MN patients (N=10)
Age (years)	37 ± 11,2	37,7 ± 11,8
Sexe (M/F)	0/10	2/8
Mean SBP (mmHg)	126± 25	124 ±20
Mean DBP (mmHg)	72 ±16	75 ± 12
Oedema (N, %)	8 (80 %)	8 (80 %)
Hypertension at the time of presentation	3 (30%)	4 (40%)
MicroscopicHematuria(N,%)	4 (40%)	2 (20%)
Median proteinuria (g/d)	3,6 [1,2; 18 g/24 Hours]	6 [1; 34 g/24 Hours
Median serum creatinine (µmol/L)	64 [44, 711 µmol /L]	76[50, 146 µmol/L]
Median eGFR CKD-EPI (ml/mn/1,73m2)	103 [6, 134]	86 [48, 129]
Median serum protein (g/l)	56 [40, 68 g/L]	48 [33, 78 g/L]
Median serum albumin (g/L)	18,9 g/l [11; 30 g/l]	21g/l [10,5; 48 g/l]
Anemia (N, %)	8 (80%)	5 (50%)

eGFR: Estimated glomerular filtration rate, **CKD-EPI**: Chronic Kidney Disease Epidemiology, **SBP**: systolic blood pressure

9 glomeruli. A mean of 5,8 ± 8 % of glomeruli was globally sclerotic. Endo and extra-capillary proliferation were absent in all patients. Interstitial fibrosis was minimal in 2 biopsies (10%) and moderate in 1 patient (5%).
No patient presented a vascular lesion on the biopsy.
Regarding TLR9 expression site, no primary MN patient had TLR9 glomerular expression. Hence, the TLR9 scores in glomerular cells were all zero. The TLR9 staining was exclusively observed in tubuli.
This tubular expression was detected in the majority of biopsies of lupus MN and primary MN patients (80%and 60% respectively) with a significant difference between the 2 groups (p< 0,05). In primary MN group, the expression was weak on 3 biopsies (30%) (Figure1A), moderate on 2 biopsies (20%) (Figure 1B), strong on 1 biopsy (10%) (Figures 1C), and negative for the rest. On biopsies showing lupus MN, the expression was; weak on 20%, of the biopsies, one moderate stained biopsy (10%), and strong on 5 biopsies (50%) and negative for the rest of biopsies.

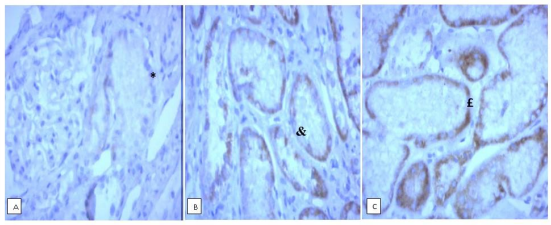

Figure 1. TLR9 expression in tubular cells.
(A) Weak expression of TLR9 on tubules *, **(B)** Moderate expression of TLR9 on tubules & **(C)** Strong of TLR9 on tubules £. Magnification (×400)

The means of diffusion and expression scores were 1,1 and 2,3 in primary MN and, 2,9 and 7,1 lupus MN respectively. A significant difference was noted in the diffusion score (pdiffusion=0,029), however and the expressing score tend to reach significance pscore =0,063) (Figure 2). We did not report any significant correlation between TLR9 expression and the serological and biological markers (data not shown).

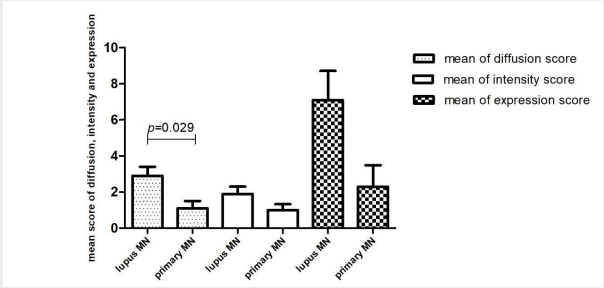

Figure 2. Histogram according to expression, intensity and diffusion score

Discussion

Membranous nephropathy is an immune-related disease that is the most common cause of idiopathic nephrotic syndrome in adults. About 80% of cases are renal limited (primary MN) and 20% are associated with other diseases such as viral hepatitis B, cancer and systemic lupus erythematosus(13). Certainly, the understanding of the pathogenesis and immunogenic mechanisms in these MN nephropathies may lead to the identification of new diagnostic targets and refined therapeutic modulation.
Several studies have suggested a role for TLRs in the pathogenesis of lupus and lupus nephritis (14–17). The severity of tubule-interstitial lesions is strongly associated with a less favorable renal prognosis (18). Functional evaluations of these TLRs in mediating the tubulointerstitial pathology of LN are now required
Genetic studies have demonstrated a relation between some TLR9 nucleotide polymorphisms and lupus MN (11,19). On the other side, to our knowledge, only one genetic study examining the relationship of TLR9 gene polymorphisms to primary MN disease was reported in the literature(20). Furthermore, expression of TLR-9 has been detected in the tubulointerstitium of patients with LN (11,21). However, no immunohistochemistry study of TLR9 on primary MN has been carried out to date. We investigated TLR9 expression on renal biopsies of patients with primary MN by comparing this expression with control patients having lupus MN. TLR9 immunohistochemical staining in our study revealed that this receptor is only expressed by tubuli and that it is undetectable in glomerular cells in primary MN groups. Besides, we noted that a part of our patients did not present a tubular expression of TLR9. This result can be due to the reduced size of the kidney specimen with an expression of weak extent in the same patients. Moreover, findings indicated a wider distribution of TLR9 within Lupus MN biopsies compared to primary MN biopsies. Actually, TLR9 was found to be more extensive in the renal tubules of lupus nephritis (LN) patients than in those with primary membranous nephropathy (MN).
Indeed, emerging evidence reports that, TLR-9 has been implicated in the dysregulated immunity of LN, either responding to self-nucleic acids alone or in ICs(10) and a significant correlation between the levels of TLR-9 expression in renal tubular epithelial cells (RTECs) and tubulointerstitial damage have been reported in NZB/

NZW lupus mice and LN patients(22). Autoantibody (anti-DNA)-producing plasma-cells/plasma blasts are found expanded in the periphery and kidneys of patients and mice with lupus renal disease (23,24). Those anti-double-stranded DNA (dsDNA) antibodies play a critical role in the pathogenesis, through their direct binding to cross-reactive antigens on resident renal cells or indirect binding through chromatin material to extracellular matrix components, resulting in complement activation, cell activation and proliferation, and induction of inflammatory and fibrotic processes and contribute to renal inflammation(25,26)

We speculate that, kidney-infiltrating anti-DNA antibodies in human LN overexpress TLR9 receptors which are rare / absent in the case of primary MN

Indeed, a previous study found that sera or ICs from SLE patients significantly increased TLR-9 expression in human RTECs when compared to healthy controls or undifferentiated connective tissue disease patients (22). This increased RTEC expression of TLR-9 suggests that the DNA component within ICs in LN plays an important stimulatory role (27).

Data in this report consolidate the hypothesis that TLR9 overexpression in MN is lupus-specific and do not include primary MN, and provide deeper insights into the expression profile of TLR9 to interrupt underlying disease mechanisms in immune-mediated renal injury. There are some limitations to our study that could be exploited in future research. A larger number of renal biopsies are needed to expand the results, and autoantibodies profile could help to draw a definite conclusion.

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