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Renal Expression and Clinical Significance of High Mobility Group Box 1 in Lupus Nephritis

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Abstract

Objective: To evaluate the clinical significance of high mobility group box 1 (HMGB1) expression in nephridial tissues of lupus nephritis (LN). Methods: Sixty-three patients with active LN and 15 systemic lupus erythematosus (SLE) (combined without LN) were included. Renal biopsies were performed in the two groups. The biopsies were evaluated according to the World Health Organization (WHO) classification and renal disease activity was estimated using the British Isles lupus assessment group (BILAG) index. Serum levels of HMGB1 were *analyzed* by western blot. HMGB1 expression in renal tissue was assessed by immunohistochemistry in the two groups. The correlation between HMGB1 and renal active index (AI), chronicity index (CI), pathological type of LN was analyzed. Results: LN biopsies showed WHO class III, IV or V and all patients had high renal disease activity (BILAG A/B). The HMGB1 expression was higher in the LN groups than the control groups ($t = 9.263$, $P < 0.05$). It showed positive correlation between HMGB1 expression and SLE DAI classification ($r = 0.579$, $P < 0.05$), AI ($r = 0.708$, $P < 0.05$) and renal tubule interstitial (TIL) classification ($r = 0.815$, $P < 0.05$), and negative correlation between HMGB1 expression and CI classification ($r = 0.582$, $P < 0.05$). In all patients, serum levels of *HMGB*1 increased only slightly *in the patients only with SLE*; however, in patients with LN WHO class IV a significant decrease was observed ($P = 0.02$). Immunostaining revealed a pronounced extranuclear HMGB1 expression predominantly outlining the glomerular endothelium and in the mesangium. There were significant differences in HMGB1 expression between LN and control biopsies and it existed with apparent association to histopathological classification and clinical outcome. Conclusions: Renal tissue expression and serum levels of HMGB1 were elevated in LN. The unusual elevation of HMGB1 in serum and tissue in LN may reflect persistent inflammatory activity, which clearly indicates a role for HMGB1 in pathogenesis of LN.

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Keywords

Lupus Erythematosus, Systemic, Lupus Nephritis, High Mobility Group Protein 1 (HMGB1)

1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease affecting multiple organs including the kidneys, and lupus nephritis (LN) is a leading cause of death and disability. Therefore, it is very important to explore pathogenesis and search for new *therapeutic* strategies of LN to improve life quality of the patients. At presently, it's been believed that B cells are multi-colonally activated, which *results* in development of many antibodies, increase of immune complex, composure of circulating immune complex and deposition of in situ circulating immune complex in different parts of kidney and eventually injury of renal function. Recent evidence has revealed that many cytokines may be involved in LN, and the balance between proinflammatory cytokines and anti-inflammatory cytokines plays an important role in the immune response. High mobility group protein box 1 (HMGBl), an intranuclear architerctural protein is well known for its nuclear functions. Recently, it has been identified as a potent proinflammatory cytokine. *It has been showed that HMGB*1 *was produced passively by injured or necrotic but not apoptotic cells*, *functions as a major stimulus of necrosis-induced inflammation* [1]. HMGB1 could *also* control the activation and chemotaxis of inflarnmatory cells, and stimulate proinflammatory cytokine synthesis in inflammatory cells. *Normal animals after administrated by HMGB*1 *develop inflammatory responses including fever, weight loss and anorexia, acute lung injury, epithelial barrier dysfunction, arthritis, and even death* [2]. *Anti-HMGB*1 *treatment with antibodies, antisense oligonucleotides or other strategies to inhibit HMGB*1 *may rescue mice from lethal bacterial toxin-induced endotoxemia or sepsis and ameliorates the severity of collagen-induced arthritis.* Recently, it's been reported HMGB1 was involved in pathogenesis of LN [3]. However, the renal expression in kidney tissues of LN and the correlation between HMGB1 and the SLE active index (AI), chronicity index (CI), pathological type of LN is still unclear. The aim of this study was to investigate renal tissue expression of HMGB1 in correlation with renal histopathological and clinical activity in order to further investigate its role in 63 patients with LN.

2. Materials and Methods

2.1. Patients

Sixty-three patients with biopsy-proven active LN during the period of January 1, 2010 to December 31, 2013 were included in this study. All patients fulfilled the 1982 American College of Rheumatology classification criteria for SLE [4] and participated in a prospective control program for LN at the department of dermatology and venereology of affiliated Hospital, Hebei Engeering University, China. Fifty-five of the 63 patients were female (87.3%) and eight were male (12.7%), and the mean age was 31 years (range of 19 to 56). The biopsy was performed when there was no the history of infection in one month. All patients did not receive intensive treatment by large doses of glucocorticoid (the dose of prednisone > 0.5 mg/kg/d) or cyclophosphamide. Fifteen patients of SLE without LN was included as the control. Twelve of 15 in the control were female and the other 3 were male, and the mean age was 29 years (range of 18 to 45), the history of pathogenesis was for 0.5 - 4 months. There was no genetic connection between the two groups. There was no a significant difference between the two groups in the age and sexuality ($P > 0.05$). All patients had no the history of smoking. The participants tested and their dependents have signed the written informed consent and provide their written informed consent to participate in this study. All clinical investigation have been conducted according to the principles expressed in the Declaration of Helsinki. At both biopsies, clinical data and blood and urinary samples were collected. Serum samples were frozen at −70˚C for the next analysis. The clinical characteristics of patients in the two groups are presented in **Table 1**.

2.2. Evaluation of Renal Function and Renal Activity

At the first and third day before biopsies was performed, the blood sample was collected to assay the blood urea nitrogen (BUN), albumin (Alb), globulin (Glo). The urine in 24 h was collected to investigate the 24-hour proteinuria

Table 1. Clinical characteristics of all patients.

Factors	LN group (n = 63)	Control group (n = 15)	*P* value
Age (year old)	19 - 56	18 - 45	0.49
Sexuality (female/male)	55/8	12/3	

excretion and assess the urinary sediment. Renal function was determined by serum creatinine levels (expressed as micromoles per liter) and estimated glomerular filtration rate (GFR) by using the modification in diet in renal disease (MDRD) formula [5]. Evaluation of renal activity was estimated by using the British Isles lupus assessment group (BILAG) index [6].

2.3. Serology and Complement Measures

Assessments of serum anti-nuclear antibody (ANA) and anti-double-stranded-DNA (anti-dsDNA) antibodies were carried out by immunofluorescence microscopy by using Crithidiae luciliae as a source of antigen. Analyses of complement component C1q were performed by rocket electrophoresis by using rabbit anti-C1q as the antibody. Levels of C1q were expressed as the percentage of the levels of healthy blood donors (normal range of 77% to 139%). C3 (normal range of 0.85 to 1.93 g/L) and C4 (normal range of 0.80 to 1.60 g/L) levels were determined by nephelometry.

2.4. Histopathology and Immunohistochemical Staining of Renal Biopsies

Renal biopsies were performed by *percutaneously* ultrasonography-guided puncture. The renal tissue obtained was evaluated by light microscopy, immunofluorescence, and electron microscopy. The biopsies were graded according to the World Health Organization (WHO) classification of nephritis [7] and additionally scored for activity and chronicity index [8]. Immunohistochemical staining of HMGB1 expression were performed on formaldehyde-fixed paraffin embedded serial 4-μm sections of renal biopsies. Slides were deparaffinized in xylene and rehydrated with ethanol. Antigen retrieval treatment prior to staining was omitted to allow clearer visualization of extranuclear HMGB1. To block endogenous peroxidase activity, sections were treated with 3% H_2O_2 followed by a serum block with 2% human AB sera for 30 minutes and an avidin/biotin-blocking step (Bioworld Technology, Inc., USA). The slides were thereafter incubated overnight with an affinity-purified monoclonal mouse IgG2*β* anti-HMGB1 antibody (concentration of 20 μg/mL; Santa Cruz Biotechnology, Inc., USA). A biotin-labeled horse anti-mouse antibody (Abcam Inc., UK) containing 2% normal horse serum was used for detection. *Staining was* developed by using a DAB kit (Santa Cruz Biotechnology, Inc., USA) in accordance with the instructions of the manufacturer. Sections were counterstained with Mayer's hematoxylin. Phosphate-buffered saline supplemented with 0.1% saponin was used in all subsequent washes and incubation steps in order to permeabilize the cells. In each assay, controls for specificity of the HMGB1 staining were included on the basis of parallel staining studies omitting the primary antibody, and a primary isotype matched immunoglobulin of irrelevant antigen specificity (negative mouse IgG2*β* control; Promega Inc., USA) was used. The specificity of extracellular and intracellular HMGB1 immunoreactivity were further verified by blocking experiments with preabsorption of the HMGB1-specific antibody with recombinant HMGB1 prior to staining. HMGB1 expression was evaluated according to the intracellular staining of brandy amber. The expressing intensity of HMGB1 was graded into four classification, including −, +, + +, + + +. Under the light microscopy of ×400, three different views in every slide were selected to *calculate* the expressing level of HMGB1. The optical density (OD) and total square of positive targets was analyzed by Image-Pro plus Version 6.0 image analyzing system and the integrated optical density (IOD) was used as the statistical data. To ensure the accuracy of obtained data, the parameter in every view field was manipulated by three times and the mean value was collected as the original data to analyze statistically.

2.5. Statistics

For comparisons of variables, the non-parametric Wilcoxon matched pair test was used. For comparisons of variables between *the two* groups, the non-parametric Mann-Whitney test was used. Correlations were calculated by using Spearman's rank correlation. *P* values of less than 0.05 were considered statistically significant.

Statistical evaluation was made with statistical software (STATISTICA 9; StatSoft, Inc., Tulsa, OK, USA).

2.6. Ethics

The participants tested and their dependents have signed the written informed consent and provide their written informed consent to participate in this study, and the study protocol was approved by Hebei Engineering University ethics committee, China.

3. Results

3.1. Histopathology and Renal Activity

All patients *with* LN had an active nephritis *at baseline*, biopsies showed WHO class I *in* 27, class II *in* 22, class III *in* 3, class IV *in* 11. All patients *with LN* had high renal disease activity, 58 out of 63 had renal BILAG A and 5 out of 63 *had renal* BILAG B. *In the control group, follow-up by biopsies* showed WHO II *in* 6, III *in* 3, IV *in* 1, and III/V *in* 5 (**Table 2**). The mean renal activity index was (5.90 ± 3.21) and (3.64 ± 1.15), respectively, and it showed significant *differences* ($P < 0.05$) *between the two groups*; whereas no significant *difference* regarding chronicity index were observed in between the LN (1.94 ± 2.32) and the control group (1.79 ± 1.46) ($P > 0.05$) (**Table 3**).

3.2. HMGB1 Serum Determination and Evaluation of Renal Activity

HMGB1 was significantly elevated in the LN group (mean of (110.1 ± 47.3) ng/mL, median of 119.2, range of 20.2 to 203.9) in comparison with controls (mean of (15.3 ± 9.0) ng/mL, median of 11.8, range of 0 to 37.8) ($P < 0.05$). Serum HMGB1 levels were significantly higher in the patients with WHO class III (mean of (129.8 ± 46.9) ng/mL, median of 129.9, range of 30.1 to 199.8) in comparison with those with class IV (mean of (88.2 ± 40.2) ng/mL, median of 86.9, range of 20.1 to 160.2) ($P < 0.01$). In the patients with WHO class III *by* biopsy, no difference in HMGB1 level was observed at the control group (mean of (137.1 ± 43.2) ng/mL, median of 143.0, range of 39.8 to 184.5), whereas in class IV, a significant decrease was found (mean of (70.9 ± 36.2) ng/mL, median of 72.6, range of 28.3 to 146.1) ($P < 0.03$). There was an inverse correlation between activity index at biopsies and HMGB1 level ($r = -0.59$, $P < 0.05$). HMGB1 showed no significant correlation with complement level, creatinine, GFR, proteinuria, or anti-dsDNA antibody positivity ($P > 0.05$) (**Table 4**). *Data on serum levels of HMGB*1, *WHO classification, proteinuria, and renal function are presented in* **Table 4**.

3.3. Immunohistochemistry

Immunostaining revealed an extranuclear HMGB1 expression found in all of the examined biopsies from the

Table 2. Histopathology and renal activity.

Renal histology	LN group (n = 63)	Control group (n = 15)
Class I	27	
Class II	22	6
Class III	3	3
Class IV	11	1
Class III/V		5
Class V		

Table 3. Comparison of renal activity index and chronicity index in between LN and control groups.

Factors	LN group (n = 63)	Control group (n = 15)	*P* value
Activity index	5.90 ± 3.21	3.64 ± 1.15	0.04
Chronicity index	1.94 ± 2.32	0.79 ± 1.46	0.21

Table 4. The serum levels of HMGB1, WHO classification, proteinuria, and renal function and correlation between the two groups.

Factors	Normal reference value	LN (n = 63)	Control (n = 15)
Blood urea nitrogen (mmol/L)	2.86 - 7.14	9.32 ± 7.54	3.29 ± 2.61
*Serum HMGB*1 (ng/mL)	2.43 ± 0.27	110.1 ± 47.3	15.3 ± 9.0
Creatinine (μmol/L)	79.56 - 132.60	161.26 ± 129.84	129.52 ± 13.74
Proteinuria (g/24h)	0 - 0.08	3.96 ± 1.27	0.22 + 0.17
Albumin (Alb) (g/L)	35 - 50	31.52 ± 13.45	33.16 ± 12.34
Globulin (Glo) (g/L)	20 - 35	19.81 ± 1.32	18.67 ± 1.59
C3 (g/L)	0.85 - 1.93	0.37 ± 0.29	0.76 ± 0.39
C4 (g/L)	0.80 - 1.60	0.24 ± 0.13	0.55 ± 0.21
C1q (%)	77 - 139	0.36 ± 0.50	0.27 ± 0.32
Anti-nuclear-antibody (ANA)	<0.10	1.83 ± 0.92	1.64 ± 0.27
Anti-dsDNA-antibody	<0.20	2.61 ± 0.54	2.35 ± 0.67
Glomerular filtration rate (mL/min)	80 - 120	81.3 ± 29.5	124.6 ± 0.7
U-erythrocytes (/high power field)	0 - 2	3 - 5+	0 - 1+

Note: Values are presented as mean (standard deviation) unless otherwise indicated. [a]Anti-double-stranded DNA (anti-dsDNA) raised to at least 1:25.

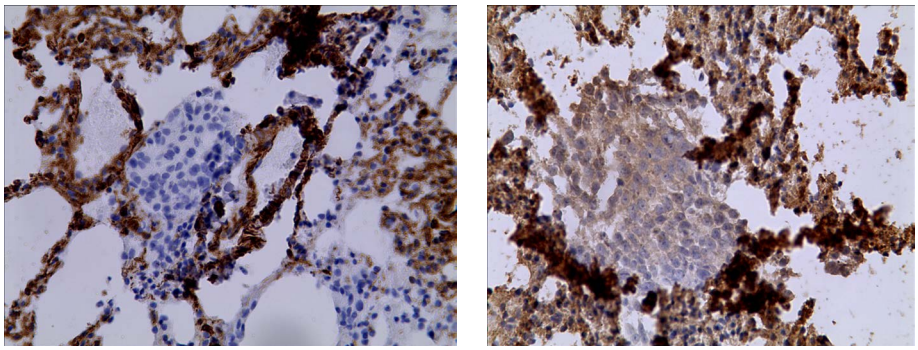
patients with LN. The staining was predominantly found outlining the glomerular endothelium and was also documented in the mesangium. A less pronounced HMGB1 expression was documented in vessels and tubular cells. To investigate the amount of HMGB1 expression, an arbitrary scale was used for comparison between biopsies. Five patients had unchanged amounts, eight had a decrease, and 12 had more abundant amounts of HMGB1 staining. Overall, no difference in the comparison of first and second biopsies was demonstrated, and no clear correlation with histopathological classification or clinical outcome could be documented. HMGB1 is abundantly present in all cell nuclei, thus generating a strong expression that might interfere with visualization of a cytoplasmatic and extranuclear staining, which most often is weaker. By omission of antigen retrieval treatment prior to staining, a clearer visualization of extranuclear HMGB1 expression is allowed. In kidney biopsies from control renal tissue, HMGB1 expression was predominantly negative or, if present, was restricted to the cell nuclei (**Figure 1**). *It showed significant difference of HMGB*1 *expression in the patients between LN and control group* ($P < 0.05$) (**Figure 2**).

3.4. Correlation between HMGB1 Expression and the Renal Index in LN

It showed positive correlation between HMGB1 expression and SLEDAI score in LN patients ($r = 0.579$, $P < 0.05$) and positive correlation between HMGB1 expression and AI score ($r = 0.708$, $P < 0.05$), negative correlation between HMGB1 and CI score and positive correlation between HMGB1 expression and TIL score ($r = 0.815$, $P < 0.05$).

3.5. HMGB1 Expression in the Different Pathological Type of LN Patients

According to the revised standard of the 2003 International Society of Nephrology and Renal Pathology Society Classification, the pathological type was graded into six types. In the present study, 3 of 63 patients with LN were I type (Mesangial slight change disease) and one patient expressed HMGB1, accounting for 3.33%; 13 were II type (Mesangial hyperplastic) and 3 patients expressed HMGB1, accounting for 23.08%; 22 were III type (Focal with lupus nephritis) and 9 patients expressed HMGB1, accounting for 40.91%; 19 were IV type (Diffuse with lupus nephritis) and 6 patients expressed HMGB1, accounting for 31.58%; 5 were V type (Membranous lupus nephritis) and 3 patients expressed HMGB1, accounting for 60.00%; 1 were VI (End-stage sclerosing with lupus nephritis) and patients expressed no HMGB1. It showed no correlation between the HMGB1 expression and pathological type ($P > 0.05$) (**Table 5**).



(a) (b)

Figure 1. HMGB1 expression by immunohistochemistry (×400). (a) HMGB1 expression in extranuclear in patients with LN; (b) HMGB1 expression in cell nuclei of renal tissue in control group.

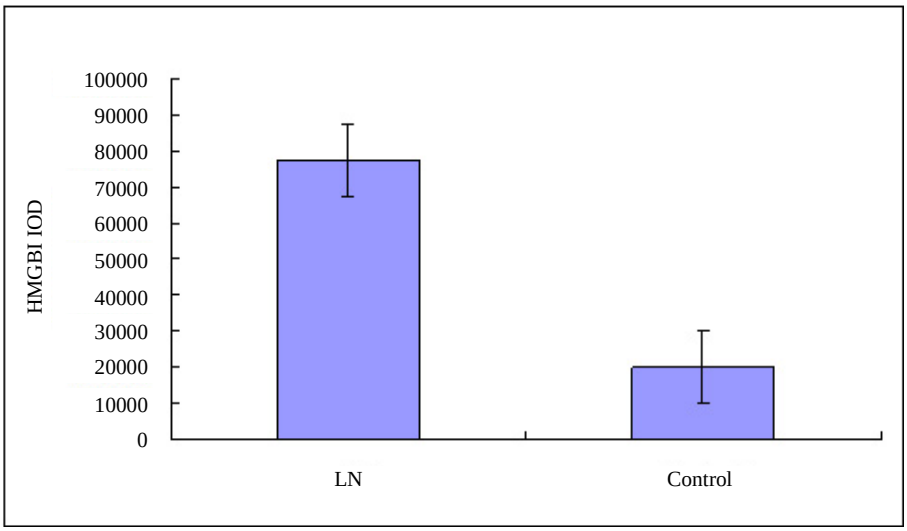


Figure 2. The difference of HMGB1 expression in between LN and control.

Table 5. HMGB1 expression in the different pathological type of LN patients.

Pathological type	Number (n = 63)	HMGB1 expression	
		Yes	No
I	3	1	2
II	13	3	10
III	22	9	13
IV	19	6	13
V	5	3	2
VI	1		1

4. Discussion

Recently, the role of HMGB1 and its receptors in the pathogenesis of autoimmune diseases have appealed to extensive attention. In the patients with rheumatoid arthritis (RA) and the biopsy sample of synovium from experimental arthritis, the extracellular expression of HMGB1 significantly elevated [9]. Pullerits *et al*. [10] injected HMGB1 into the articular cavity of mice, HMGB1 induced the development of arthritis. However, pre-

treated rodents with collagen-induced arthritis (CIA) by HMGB1 neutralizing antibody could improve the symptom of arthritis. Yoshizaki *et al*. [11] found the serum level of HMGB1 in the patients with systemic sclerosis (SSc) significantly elevated, and demonstrated positive correlation with the severity of disease, which suggested the HMGB1 played a role in the local inflammation and tissue injury induced by autoimmune diseases.

Our results clearly show an increased expression of HMGB1 in active disease in the LN group. HMGB1 tissue staining was predominantly found outlining the glomerular endothelium but also were expressed in the mesangium. Moreover, the positive correlation between HMGB1 and SLE DAI score was observed, which suggested HMGB1 related with the activity of disease. Our results were consistent with that of Urbonaviciute [12]. The expression of HMGB1 in glomerulus was significantly higher in the patients with LN than that of the control. As for the expressing location of HMGB1, compared to the control, the distribution of HMGB1 in the LN group had changed. Some cells expressed HMGB1 in the cytoplasm or extracellular region, but in the control, HMGB1 were expressed only in the nucleus.

The mechanism of action of HMGB1 in the development of LN was still unclear, it may include the following factors: 1) HMGB1 reduces the clearance of dead cells in patients. The process of macrophages recognizing and endocytosing apoptotic cells was complicated and regulated by several products of apoptotic cells, scavenger receptors and soluble molecules. The link between phagocytes and apoptotic cells was established. The interaction between HMGB1 and varied factors in the system of clearing apoptotic cells could reduce the clearance for dead cells [13]. Moreover, HMGB1 combined with phosphatidylserine locating in the outside of apoptotic cells to inhibit the phagocytosis for macrophages. Results of *in vivo*- and *in vitro*-experiment have proved the C-tail of HMGB1 could reduce the role of clearing the apoptotic cells mediated by receptors for advanced glycation end-products(RAGE). The increase of apoptotic cells and attenuation of the ability of clearing HMGB1, and eventually exceeding the ability of clearing HMGB1 cause the release of lots of anti-nuclear antibody and secondary necrosis of lots of cells and then aggravate the development of SLE [14]; 2) When cells in the patients with SLE develop apoptosis, HMGB1 combines with nucleosome and developes complex and is released, stimulates antigen presenting cells to break the immunologic tolerance against DNA, generates the anti-double-strand DNA antibody with high affinity; 3) Varied immune cells are activated in the patients with SLE. Activated immune cells secrete HMGB1 to extracellular by the way of active secretion. Extracellular HMGB1 promotes the produce and activation of varied inflammatory factors such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8) and then contributes to the development and progress of LN [15].

Our results also showed positive correlation between the level of HMGB1 and active index (AI) score, negative correlation and chronicity index (CI) score, positive correlation and renal tubule interstitial (TIL) in the patients with LN. The AI stands for the active degree of renal disease, and the higher score of AI suggests more active and severe disease. The CI is related with the reversible degree of renal disease and long-term renal function, which suggests the patients with high expressing HMGB1 should be given the therapy by the appropriate immunosuppressor. The low level of HMGB1 suggests the less possibility of reversibility of renal disease. Our results still showed no significant correlation between the HMGB1 expression and pathological type of LN, which suggested the pathogenesis of HMGB1 was multiple-pathway and multiple-targeted sites. HMGB1 causes the development of the disease in not only glomerulus but also kidney tubules and renal interstitium. Therefore, as for the SLE patients combined with LN, the biopsy to test the level of HMGB1 expression should be performed as early as possible to determine the severity of injury of renal interstitium and direct the clinical treatment to reduce the development of complications as possible.

5. Conclusion

In a word, the level of HMGB1 was significantly elevated in the SLE patients combined with LN. The increased tissue staining for HMGB1, most pronounced outlining the endothelium and the mesangium, clearly indicates a role for HMGB1 in the inflammatory process of LN. Serum levels of HMGB1 were significantly increased in patients with LN, possibly reflecting persistent inflammatory activity and severity of the injury of renal interstitium in patients with LN.

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