Potential Pathogenic Role of Urinary and Renal Tissue Level of High Mobility Group Box 1 (HMGB1) in Lupus Nephritis


The Departments of Internal Medicine, Medical Biochemistry and Pathology, Faculty of Medicine, Cairo University

Abstract

Introduction: Systemic Lupus Erythematosis (SLE) is a systemic autoimmune disease characterised by involvement of multiple organ systems. HMGB 1 is a nuclear non-histone protein secreted by many cells, during activation or cell death.

Aim of Work: We aim to study the potential pathogenetic role of HMGB 1 in SLE and whether urinary and renal biopsy levels reflect renal inflammation and correlate with disease activity.

Methods: In a case control study, 61 systemic lupus patients and 18 healthy volunteers, were divided in 4 groups. Group 1: 21 patients with lupus nephritis. Group 2: 21 patients with lupus activity without nephritis. Group 3: 19 patients without activity. Group 4: 18 healthy volunteers, age and sex matched. Participants were subjected to history taking, physical examination, activity scoring using SLEDAI, complete blood count, kidney function tests, ESR, ANA, Anti dsDNA, C3 and C4 level urinary levels of HMGB 1 by ELISA. Study of HMGB1 immunohistochemical expression pattern in renal biopsy was conducted in Group 1.

Results: Urinary HMGB 1 levels and renal tissue extra-nuclear expression (cytoplasmic and extra-cytoplasmic) pattern of HMGB 1 were significantly increased in patients with active LN compared to patients without active LN and control (p < 0.001), suggesting active release of HMGB1. Plasma and urinary levels in patients without active LN were also significantly higher compared to control group (p<0.001). Urinary HMGB 1 levels and renal tissue extra-nuclear expression pattern of HMGB1 levels were significantly correlated with SLEDAI.

Conclusion: HMGB 1 plays an important role in pathogenesis of lupus nephritis and reflects disease activity. Thus, HMGB 1 can be utilized as a biomarker for renal disease activity in patients with lupus and the therapeutic value of HMGB 1 blocking agents must be investigated.

Key Words: SLE – Lupus nephritis – HMGB 1 – Apoptosis – Activity.
Patients and Methods

Between October 2013 and June 2014, we prospectively enrolled 61 consecutive patients with systemic lupus at Internal Medicine Department Kasr El-Aini Hospital.

The present study was conducted on 79 participants: 61 Systemic lupus patients all fulfilling at least 4 of the criteria of the American College of Rheumatology for SLE diagnosis [6] and 18 healthy controls. They were divided into four groups:

- **Group 1:** 21 patients with lupus nephritis which was diagnosed by proteinuria exceeding 500mg/d and/or presence of cellular casts (erythrocyte, granular, tubular or mixed) and was confirmed by renal biopsy.
- **Group 2:** 21 patients with lupus activity without nephritis as estimated by SLEDAI >4.
- **Group 3:** 19 patients without activity as estimated by SLEDAI <4.
- **Group 4:** 18 healthy volunteers, age and sex matched.

All participants were chosen from Internal Medicine Department, Kasr Al-Aini Hospital, Cairo University. A written consent was obtained from all subjects participating in this study.

Inclusion criteria were patients with systemic lupus erythematosis with following criteria:
- Lupus nephritis.
- Lupus activity without nephritis as estimated by SLEDAI >4.
- Lupus without activity as estimated by SLEDAI <4.

The exclusion criteria included the following:
- Malignancy.
- Pregnant.
- Overlap syndrome.
- Mixed connective tissue disease.

Methods:

All participants were subjected to the following:
- Detailed medical history taking.
- Complete physical examination. Clinical disease activity of our patients was assessed using SLEDAI.
- Laboratory investigations:
  - Complete blood count.
  - Kidney function tests.
  - Urine analysis.
  - ESR (erythrocyte sedimentation rate).
  - ANA (Anti-Nuclear Antibodies).
  - Anti dsDNA.
  - C3, C4 levels.

Urinary levels of HMGB1 assessed by ELISA (IBL, Hamburg, Germany): Samples are added to the appropriate microtiter plate wells with a biotin conjugated polyclonal antibody preparation specific for HMGB 1 and avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well only those wells that contain HMGB 1 biotin conjugated antibody and enzyme substrate reaction are terminated by the addition of asulphuric acid solution and the color change is measured spectrophotometrically at a wave length of 450nm±2nm. The concentration of HMGB1 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Study of HMGB1 in renal biopsy samples in patients with active lupus nephritis: Serial paraffin-embedded sections (4 µm thick) of renal biopsy specimens were obtained from Group 1 patients. Some sections were mounted on glass slides, stained with routine Hematoxylin and Eosin and Masson Trichrome stains then reviewed and classified by an experienced nephropathologist according to the revised criteria for LN. The Activity Index (AI) and Chronicity Index (CI) were calculated for each specimen with maximum scores of 24 for the AI and 12 for the CI [7]. Other kidney sections were mounted on charged slides for immunohistochemical staining. They were deparaffinized, then antigen retrieval and endogenous peroxidase blocking was performed. Slides were incubated with rabbit anti-HMGB 1 antibody (Abcam, Cambridge, UK). Subsequently, slides were incubated with HRP-labeled secondary antibodies (DakoCytomation, Glostrup, Denmark). Next, slides were incubated in diaminobenzidine solution and counterstained with hematoxylin.

Evaluation of HMGB1 staining: Cellular distribution of HMGB 1 was determined in the kidney by counting one hundred nuclei (glomerular, tubular, and stromal) in three bright field pictures and scoring both HMGB1-positive (brown) and HMGB 1-negative (blue) nuclei. Results are expressed as the percentage of negative cells.
Statistical analysis:

Statistical Package for Social Science (SPSS) program version 9.0 was used for analysis of data. Data was summarized as mean ± SD. Non parametric test (Mann Whitney U) was used for analysis of two quantitative data. While Chi square test was used for analysis of qualitative data. ANOVA was done for analysis of more than two variables followed by post Hoc test for detection of significance. Simple linear correlation (Pearson's correlation for quantitative data and spearman correlation for qualitative data) was done to detect the relation between HMGB 1 with all other demographic and laboratory data. p-value is considered significant if <0.05*. p<0.01 is Highly Significant (HS). p<0.001 is Very Highly Significant (VHS).

Results

61 patients with systemic lupus patients compared to 18 controls were included in the study. Demographic and clinical data of the study groups are shown in (Table 1). We used SLEDAI to assess disease activity, 6 cases showed inactive disease (9.83%), 13 cases with mild activity (21.3%), 4 cases with moderate activity (6.55%) and 38 cases with severe activity (62.29%). As regards Group 1, 8 cases with class II (38.09%), 8 cases with class III (38.09%) and 5 cases with class IV (23.8%) but there was no statistically significant difference between the classes (p-value 0.557).

Significantly higher levels of Urinary levels of HMGB 1 regarding cases of lupus nephritis were detected in comparison to different studied groups. Fig. (1) showed statistically significant differences between HMGB 1 urinary levels in inactive and mild, inactive and moderate, inactive and severe activity, mild and severe activity and moderate and severe (p-value <0.0001 *) and between mild and moderate, (p-value <0.001) with urinary HMGB 1 level being highest in severe activity.

Urinary and renal tissue HMGB 1 levels were assessed in SLE cases of recent onset and SLE cases of long standing disease. High levels of urinary HMGB 1 in recent onset SLE (2.76 ±0.45/41.17±7.69) compared to long standing lupus (2.58 ±0.30/37.96±9.12) were found, however the difference is not statistically significant (p-value=0.227 and 0.266 respectively). However HMGB1 negative nuclear count was higher in long standing cases as compared to recent (93.47±5.9/91.67±6.95), but as well insignificant statistically (0.557).

A significant correlation between urinary HMGB 1 with proteinuria was shown in active SLE patients (p<0.001, r=.529).

High renal tissue levels of negative nuclear expression and positive cytoplasmic and extracellular staining for HMGB 1 in long standing lupus (93.47±5.9) was found in comparison to recent onset lupus (91.67±6.95) in Group 1 (active lupus nephritis).

<table>
<thead>
<tr>
<th>Table (1): Demographic and clinical data of the study groups.</th>
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<tbody>
<tr>
<td>Group (1) n=21</td>
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<tr>
<td>Mean of age in years ± SD.</td>
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<tr>
<td>Mean of age at disease onset ± SD.</td>
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<td>Sex distribution F/M.</td>
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<tr>
<td>Treatment (no of patients):</td>
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<tr>
<td>Steroids.</td>
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<tr>
<td>Antimalarial.</td>
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<tr>
<td>Azathioprine.</td>
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<tr>
<td>Cyclophosphamide.</td>
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<tr>
<td>Creatinine serum level (mg%).</td>
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<td>Mean 24 hour urinary protein (g).</td>
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<td>C3 (mg%).</td>
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<tr>
<td>C4 (mg%).</td>
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<tr>
<td>ADNA positive.</td>
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<tr>
<td>HMGB 1 in urine (ng/ml).</td>
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</tbody>
</table>
Table (2): Comparative study of urinary and renal tissue HMGB 1 levels in different classes of nephritis.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Class II n=8</th>
<th>Class III n=8</th>
<th>Class IV n=5</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
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<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>II vs III</td>
<td>II vs VI</td>
<td>III vs IV</td>
</tr>
<tr>
<td>Urinary HMGB 1</td>
<td>46.12±.83</td>
<td>45.25±1.16</td>
<td>46.20±.84</td>
<td>0.270</td>
<td>1.000</td>
<td>0.316</td>
</tr>
<tr>
<td>Renal tissue HMGB 1</td>
<td>93.12±5.82</td>
<td>93.12±8.29</td>
<td>92.40±2.88</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
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Fig. (1): Comparative study of urinary HMGB1 in different grades of disease activity of SLE patients included in the study.

**Discussion**

HMGB 1 has been recognized as a new autoantigen and an important inflammatory mediator in SLE as exemplified by increased serum levels and presence of antibodies against this protein. Both HMGB 1 and anti-HMGB 1 antibodies have been shown to be associated with SLE disease activity, decreased complement levels, and proteinuria [8].

An important role for HMGB 1 in the pathogenesis of SLE has been described by Vollet et al., [9]. They demonstrated that this protein is tightly attached to chromatin released from late apoptotic cells. These complexes are able to induce inflammatory and immune responses and as such form an important factor in the pathogenesis of SLE.

Liu et al., [1] showed that HMGB1 inhibited phagocytosis of apoptotic neutrophils by macrophages through binding tophosphatidylserine, which was redistributed from the inner to the outer membrane leaflet of cells undergoing apoptosis. Zickert et al., [10] provided important new evidence implicating High-Mobility Group Box 1 protein (HMGB 1) as a mediator of lupus nephritis, and the enhanced expression of HMGB1 is perhaps a smoking gun in the pathogenesis of a very complicated disease. In addition Ma et al., [11] showed a positive correlation between HMGB 1 and peripheral blood neutrophils in SLE patients but not in healthy controls. These data together with previous reports imply that apoptotic neutrophils may be an important source of the increased serum HMGB 1 in SLE.

In our study, urinary levels of HMGB1 were significantly increased in patients with active LN compared to patients without active LN and control groups with p-value <0.001. Urinary levels of HMGB 1 in SLE patients with activity without nephritis were also significantly higher compared to control with p-value <0.001. Similarly, renal tissue of active LN patients showed strong expression of HMGB 1 at cytoplasmic and extracellular sites suggesting active release of HMGB 1 from nuclear localization.

Urinary excretion of HMGB 1 might reflect renal inflammatory injury. Thus urinary levels of HMGB 1 were increased in patients with active LN. Urinary HMGB1 levels were also detectable, but at a lower level, in patients without active LN. This might be explained in two ways. A possibly on-going low grade renal inflammatory activity could contribute to the release of HMGB 1 and/or increased levels of plasma HMGB 1 might lead to urinary excretion of HMGB 1, particularly in patients with a history of LN and slight persisting proteinuria.

A significant correlation between urinary HMGB1 with proteinuria was shown in active SLE patients (p<0.001, r=.529) this finding is in line with Abdulahad et al., [5].
We found a strong association between urinary levels of HMGB1 reactivity and disease activity assessed by SLEDAI. This is in line with Abdulahad et al., [5] Ma et al., [11], Li et al., [8] and David, [15]. In study done by Abdulahad et al., [5] plasma and urinary HMGB1 levels were correlated with SLEDAI, furthermore, urinary levels of HMGB1 were inversely correlated with complement levels. Nevertheless, increased urine levels of HMGB1 might indicate that HMGB1 is an important inflammatory mediator and that urinary HMGB1 might be an additional biomarker for assessment of renal disease activity in SLE. Ma et al., [11] found that, in SLE patients, particularly in those with active lupus nephritis, not only plasma but also urinolevels of HMGB1 were increased and correlated with SLEDAI scores. In study done by Li et al., [8], HMGB1 levels were correlated positively with SLEDAI, but did not demonstrate an association of HMGB1 with specific organ involvement.

The mechanism of action of HMGB1 in the development of LN is still unclear, it may include the following factors: First; the interaction between HMGB1 and varied factors in the system of clearing apoptotic cells could reduce the clearance for dead cells [12], second; when cells in the patients with SLE develop apoptosis, HMGB1 combines with nucleosome and develops 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 [13,14], third, 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 production and activation of varied inflammatory factors such as tumor necrosis factor-α (TNF-α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and interleukin-8 (IL-8) and then contributes to the development and progress of LN [15].

In the current study, the renal tissue of patients with active LN showed absence of nuclear staining for HMGB1 in high percent, with dense intensity for cytoplasmic staining, in all cellular elements of the core as well as and extracellular sites suggesting active release of HMGB1 in pro-inflammatory processes within the kidney.

Zickert et al., [16] stated that clear tissue staining for HMGB1 was detected in LN. Whereas, the staining was absent in non-lupus control renal tissue. There was no distinct difference in expression of HMGB1 either in the proliferative glomerular lesions or in sites with infiltrates of inflammatory cells in comparison with less affected glomeruli, and the exact origin of the increased renal expression of HMGB1 is not fully clear. The staining outlining the glomerular endothelium could emanate from local extracellular release but also may reflect capture from circulating HMGB1. Thus, one may speculate that the findings of increased serum levels as well as tissue expression of HMGB1 reflect both systemic and local inflammation within the kidney. In glomeruli, the pronounced endothelial staining and the increased expression in the mesangium suggest a co-localization for HMGB1 and immune depositions in LN. However, further studies with other methodologies are required to address this issue.

Liu et al., [1] concluded that extracellular, but not intracellular HMGB1, facilitates self-DNA induced macrophage activation via promoting DNA accumulation in endosomes and contributes to the pathogenesis of lupus nephritis.

We were not able to definitely identify the cells releasing HMGB1. HMGB1 release could result from infiltrating cells as indicated by immunohistochemical staining, but could also result from either activation or cell death of constitutive renal tissue. Also, we cannot exclude the possibility that at least some of urinary HMGB1 might emerge from systemic inflammation. This might explain the lack of correlation between urinary HMGB1 and HMGB1 released from nuclei in the kidney; our results are in line with Abdulahad et al., 2015 [5] who found lack of correlation between urinary HMGB1 and HMGB1 released from nuclei in the kidney.

Our results still showed no significant correlation between the HMGB1 expression and pathological classes of Lupus nephritis in line with Liu et al., 2015 [1] who 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 [1-17].

Comparing the incidence of anti dsDNA antibodies (75%) and urinary levels of HMGB1 and renal tissue HMGB1 (100%) in recent onset cases (onset of disease <1y), a finding suggesting that urinary levels of HMGB1 and renal tissue HMGB1
appear earlier than anti dsDNA antibodies. Urinary levels of HMGB 1 and renal tissue HMGB 1 can be used to diagnose SLE early in the course of the disease even before other antibodies are evident as anti dsDNA.

Conclusion:
The present study demonstrates increase in urine and renal tissue extra-nuclear expression of HMGB1 levels in SLE patients, especially in active LN. Increase in HMGB1 levels correlated to SLE Disease Activity Index (SLEDAI). Thus, HMGB 1 plays an important role in pathogenesis and activity in lupus nephritis. Urinary levels of HMGB1 and renal tissue HMGB1 can be used to diagnose SLE early even before other antibodies are evident.

Recommendations:
To our knowledge the current study first one to assess correlation between the levels of urinary levels of HMGB1 and renal tissue expression of HMGB1 in SLE patients, as our study was of limited size, additional extended studies will be required to study the role of HMGB 1 as a biomarker for renal disease activity in patients with lupus and to evaluate the therapeutic value of HMGB 1 blocking agents.

References
الملخص العربي

ذاك لزيادة احتمال حدوث اضطرابات بالكلى في هؤلاء المرضى.