Plasma and Urinary Level of High Mobility Group Box 1, Early Marker in Systemic Lupus Erythematosis Activity

AYSHA I. BADAWI, M.D.*; HANAN H. FOUAD, M.D.**; RANDA F. SALAM, M.D.***; AMIRA M. BASSAM, M.D.**** and SAHAR A. AHMED, M.Sc.*
The Departments of Internal Medicine*, Medical Biochemistry** and Pathology***, Faculty of Medicine, Cairo University

Abstract

Introduction: Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disease characterised by involvement of multiple organ systems. Its aetiology is largely unknown; however, genetic and environmental factors are proposed to contribute to breaking tolerance, resulting in the production of a variety of antibodies directed to self-components [1].

Aim of Work: We aim to study the potential role of HMGB 1 in SLE and whether plasma and urinary levels 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 & C4 level, plasma and urinary levels of HMGB 1 by ELISA.

Results: Plasma and urinary HMGB 1 levels were significantly increased in patients with active LN compared to patients without active LN and control (p<0.001), suggesting active release of HMGB 1. Plasma and urinary levels in patients without active LN were also significantly higher compared to control group (p<0.001). Plasma and urinary HMGB 1 levels and renal tissue extra-nuclear expression pattern of HMGB 1 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 activity – HMGB 1 – C3 – C4.

Introduction

SYSTEMIC Lupus Erythematosus (SLE) is a systemic autoimmune disease characterised by involvement of multiple organ systems. Its aetiology is largely unknown; however, genetic and environmental factors are proposed to contribute to breaking tolerance, resulting in the production of a variety of antibodies directed to self-components [2]. Lupus Nephritis (LN) is a severe and frequent manifestation of SLE. Its pathogenesis has not been fully clarified but immune complexes are considered to contribute to the inflammatory pathology in LN [3].

Pathophysiological mechanisms involved in breaking tolerance against self-components are not fully understood. However, in the past few years disturbance in the clearance of apoptotic cells has been reported, and it has been suggested that apoptotic cells can serve as a source of autoantigens [4].

High Mobility Group Box 1 (HMGB 1), originally recognised as a DNA binding protein, has recently been identified as a Damage Associated Molecular Pattern (DAMP) [4]. HMGB 1 is a nuclear non-histone protein which is secreted from different types of cells (LPS-, TNF[α]- and IL-1 activated monocytes and macrophages) during activation and/or cell death and may act as a pro-inflammatory mediator, alone or as part of DNA-containing immune complexes in SLE [8] and participates in many nuclear functions but once released it is involved in inflammatory function [4].

Our study was performed to assess the potential role of HMGB 1 in SLE and whether urinary, plasma levels of HMGB 1 correlate with disease 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, after explaining its nature.

**Inclusion criteria were patients with systemic lupus erythematosus 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.

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.

Plasma and urinary levels of HMGB 1 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 wavelength of 450nm ±2nm. The concentration of HMGB 1 in the samples is then determined by comparing the O.D, of the samples to the standard curve.

**Statistical methodology:**

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 both plasma and urinary levels of HMGB 1 regarding cases of lupus nephritis were detected in comparison to different studied groups as shown in (Table 2). Fig. (1) shows high statistically significant differences between HMGB1 plasma levels in inactive and mild, inactive and moderate, inactive and severe activity groups (**p-value** <0.000 1*) and significant
difference between mild and severe (p-value <0.001) and no statistically significant difference between mild and moderate, moderate and severe activity (p-value 1.000, 0.163) respectively with plasma HMGB 1 level being highest in severe activity. Fig. (2) showed statistically significant differences between HMGB1 urinary levels in inactive and mild, inactive and moderate, inactive and severe activity, mild and severe activity and moderate and severe (p-value <0.000 1*) and between mild and moderate, (p-value <0.001) with urinary HMGB1 level being highest in severe activity.

Plasma and urinary HMGB1 levels were assessed in SLE cases of recent onset and SLE cases of long standing disease. High levels of plasma and 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).

A significant correlation between urinary HMGB 1 with proteinuria was shown in active SLE patients (p<0.001, r=-.529) and plasma HMGB1 with proteinuria (p<0.00 1, r=. 5 5 1).

Comparative study of plasma, urinary and renal tissue levels of HMGB1 in different classes of nephritis shown in (Table 3) showed that there was statistically significant difference only between plasma HMGB 1 level in class II and III nephritis (p-value 0.020).

**Table (1): Demographic and clinical data of the study groups.**

<table>
<thead>
<tr>
<th></th>
<th>Group (1) n=21</th>
<th>Group (2) n=21</th>
<th>Group (3) n=19</th>
<th>Group (4) n=18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of age in years ± SD.</td>
<td>28.29±8.945</td>
<td>31.81±10.902</td>
<td>31.89±10.939</td>
<td>26.28±9.791</td>
</tr>
<tr>
<td>Mean of age at disease onset ± SD.</td>
<td>24.13±7.68</td>
<td>27.17±8.58</td>
<td>27.08±9.10</td>
<td>–</td>
</tr>
<tr>
<td>Sex distribution F/M.</td>
<td>20/1</td>
<td>21/0</td>
<td>18/1</td>
<td>17/1</td>
</tr>
<tr>
<td>Treatment (no of patients):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steroids.</td>
<td>21</td>
<td>21</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Antimalarial.</td>
<td>21</td>
<td>21</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Azathioprine.</td>
<td>21</td>
<td>15</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide.</td>
<td>13</td>
<td>10</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Creatinine serum level (mg%).</td>
<td>1.2±0.87</td>
<td>0.842±0.1875</td>
<td>0.7421±.1865</td>
<td></td>
</tr>
<tr>
<td>Mean 24 hour urinary protein (g).</td>
<td>0.48±0.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3 (mg%).</td>
<td>36.73±15.621</td>
<td>36.61±11.916</td>
<td>109.16±7.848</td>
<td>110.89±7.235</td>
</tr>
<tr>
<td>C4 (mg%).</td>
<td>4.33±1.287</td>
<td>5.24±1.199</td>
<td>15.16±2.672</td>
<td>16.28±2.58</td>
</tr>
<tr>
<td>ADNA positive.</td>
<td>21</td>
<td>20</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>HMGB 1 in plasma (ng/ml).</td>
<td>2.881±0.334</td>
<td>2.648±0.2136</td>
<td>2.295±0.1353</td>
<td>1.394±0.337</td>
</tr>
<tr>
<td>HMGB 1 in urine (ng/ml).</td>
<td>45.81±1.030</td>
<td>42.38±3.008</td>
<td>26.42±4.260</td>
<td>3.31±0.877</td>
</tr>
</tbody>
</table>
between HMGB 1 and peripheral blood neutrophils.

Phagocytosis of apoptotic neutrophils by macrophages is known to be tightly associated with the 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 [7].

An important role for HMGB 1 in the pathogenesis of SLE has been described by Vollet et al., 2008 [8]. 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., [9] showed that HMGB 1 inhibited phagocytosis of apoptotic neutrophils by macrophages through binding to phosphatidylserine, which was redistributed from the inner to the outer membrane leaflet of cells undergoing apoptosis. In addition Ma et al., [10] 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 increased plasma HMGB 1 in SLE.

In our study, plasma and urinary levels of HMGB 1 were significantly increased in patients with active LN compared to patients without active LN and control groups with p-value <0.001. Plasma and urinary levels of HMGB 1 in SLE patients with activity without nephritis were also significantly higher compared to control with p-value <0.001.

Table (2): Comparative study of plasma and urinary HMGB1 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 II vs III</th>
<th>P2 II vs VI</th>
<th>P3 III vs IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma HMGB1</td>
<td>2.71±14</td>
<td>3.14±39</td>
<td>2.74±21</td>
<td>0.20</td>
<td>1.00</td>
<td>0.064</td>
</tr>
<tr>
<td>Urinary HMGB1</td>
<td>46.12±83</td>
<td>45.25±1.16</td>
<td>46.20±84</td>
<td>0.270</td>
<td>1.00</td>
<td>0.316</td>
</tr>
</tbody>
</table>

Table (3): Comparative study of plasma and urinary levels of HMGB 1 between patients and control group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group (1)</th>
<th>Group (2)</th>
<th>Group (3)</th>
<th>Group (4)</th>
<th>Control</th>
<th>P1 I vs. II</th>
<th>P2 I vs. III</th>
<th>P3 I vs. IV</th>
<th>P4 II vs. III</th>
<th>P5 H vs. IV</th>
<th>P6 III vs. IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma HMGB1</td>
<td>2.88±0.334</td>
<td>2.64±0.213</td>
<td>2.29±0.135</td>
<td>1.39±34</td>
<td>.037</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Urinary HMGB1</td>
<td>45.81±1.030</td>
<td>42.38±3.008</td>
<td>26.42±4.260</td>
<td>3.31±88</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

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 [7].

Urinary excretion of HMGB1 might reflect renal inflammatory injury. Thus urinary levels of HMGB1 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 HMGB1 and/or increased levels of plasma HMGB1 might lead to urinary excretion of HMGB1, 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 ($p<0.001$, $r=.529$) and plasma HMGB1 with proteinuria ($p<0.001$, $r=.551$) and this finding is in line with Abdulahad et al., [5].

We found a strong association between plasma and urinary levels of HMGB 1 reactivity and disease activity assessed by SLEDAI. This is in line with Abdulahad et al., [5]: Ma et al., [10] and Li et al., 2010 [7]. In study done by Abdulahad et al., 2012 [5] plasma and urinary HMGB 1 levels were correlated with SLEDAI, furthermore, urinary levels of HMGB 1 were inversely correlated with complement levels. Nevertheless, increased urine levels compared to those with inactive nephritis or inactive disease. These results are in agreement with Zickert et al., [11] in which plasma levels of HMGB 1 were significantly increased in active LN patients compared to patients without active renal disease and controls. According to findings of Li et al., [7], the increased HMGB 1 concentration, found in serum of SLE patients, is unlikely to be linked to increased transcription of the gene. Thus, it might be either the product of peripheral blood mononuclear cell activation, resulting in its increased extracellular release, or it might be the product of un-cleared apoptotic cells.
of HMGB 1 might indicate that HMGB 1 is an important inflammatory mediator and that urinary HMGB 1 might be an additional biomarker for assessment of renal disease activity in SLE. Ma et al., [10] found that, in SLE patients, particularly in those with active lupus nephritis, not only plasma but also urine levels of HMGB 1 were increased and correlated with SLEDAI scores. In study done by Li et al., [7], HMGB 1 levels were correlated positively with SLEDAI, but did not demonstrate an association of HMGB1 with specific organ involvement.

Liu et al., [9] 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.

Comparing the incidence of anti dsDNA antibodies (75%) and plasma and urinary levels of HMGB1 (100%) in recent onset cases (onset of disease <1y), a finding suggesting that plasma, urinary levels of HMGB 1 appear earlier than anti dsDNA antibodies. Plasma and urinary levels of 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 plasma and urine HMGB 1 levels in SLE patients, especially in active LN. Increase in HMGB 1 levels correlated to SLE Disease Activity Index (SLE-DAI). Thus, HMGB1 plays an important role in pathogenesis and activity in lupus nephritis. Plasma and urinary levels of HMGB 1 can be used to diagnose SLE early even before other antibodies are evident.

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.

Recommendations:

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

داخله نسبة في الدم البول في إظهار الكلى المصاصب لمرض الذببة الحمراء الجهازى وفي حالات الذببة الحمراء غير مصحوبة بالتهاب البول.

أجريت هذه الدراسة لتقييم وجود HMGB1 ونسبة في الدم والبول في إظهار الكلى المصاصب لمرض الذببة الحمراء الجهازى وفي حالات الذببة الحمراء غير مصحوبة بالتهاب بالكلى.

استخدمت هذه الدراسة على واحد وستين مريضاً يعانون من مرض الذببة الحمراء الجهازى ومنهم واحد وعشرون مريضاً يعانون من اصابات بالكلى ناتجة عن المرض نفسه كما تضمنت الدراسة ثمانية عشرة من الأصحاء في نفس المجموعة العمرية كمجموعة ضابطة.

ELISA وقد تم قياس HMGB1 في الدم والبول عن طريق الكلى.

وقد أثبت النتائج وجود علاقة طردية ذات دلالة إحصائية هامة بين نسبة HMGB1 ونسبة في الدم ودرجة نشاط المرض.

كما أشارت النتائج إلى أن نسبة وجود HMGB1 في الأشخاص حديثي العهد بمرض الذببة الحمراء الجهازى تصل إلى (70%) وفي نسبة تعتبر أعلى إحصائياً من نسبة حدوث الأجسام المضادة للحمض النووي ثانياً الشريط الوراثي والتي تصل إلى (60%) في نفس المرض.

وفي ضوء هذه النتائج يتبين أن مستوى HMGB1 يرتفع في الدم والبول في مرحلة مبكرة من المرض حتى قبل ظهور الأجسام المضادة للحمض النووي ثانياً الشريط الوراثي ومن هنا يتبين أن HMGB1 هو المحفز الذاتي الأساسي في مرض الذببة الحمراء الجهازى.

وقد أشارت نتائج هذه الدراسة إلى أن HMGB1 تتواجد بنسبة أعلى في مرض الذببة الحمراء الجهازى المصابين باضطرابات في الكلى عن مرضى الذببة الحمراء الجهازى بدون إصابات بالكلى.

وبهذا يوجد علاقة عكسية ذات دلالة إحصائية هامة بين نسبة HMGB1 ونسبة في الدم البول في (0.03 و0.04) ونسبة طردية ذات دلالة إحصائية هامة بين نسبة HMGB1 ونسبة في الدم البول في 24 ساعة.

وبهذا نستطيع أن نستنتج من هذه الدراسة أن نسبة HMGB1 تعتبر وسيلة نافعة لتشخيص وقياس مرض الذببة الحمراء الجهازى ودرجة نشاط المرض.

وفي ضوء ذلك يوصى بالمتابعة المستمرة الدقيقة لظواهر الكلى لمرضى الذببة الحمراء الجهازى الذين لديهم نسبة عالية من إصابات الحمراء الجهازى بالكلى في هؤلاء المرضى.