High-mobility group box 1, early activity marker in lupus nephritis

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Objectives
Systemic lupus erythematosus is an autoimmune disease characterized by the involvement of multiple organ systems. High-mobility group box 1 (HMGB1) is a nuclear nonhistone protein secreted by many cells during activation or cell death. We aim to study the potential pathogenetic role of HMGB1 in lupus and whether urinary, serum, and renal biopsy levels reflect renal inflammation and correlate with disease activity.
Patients and methods
In a case–control study, 61 systemic lupus patients and 18 healthy volunteers were divided into four groups. Group 1 included 21 patients with lupus nephritis (LN). Group 2 included 21 patients with lupus activity without nephritis. Group 3 included 19 patients without activity. Group 4 included 18 healthy volunteers who were age and sex matched. Participants were subjected to assessment of history, physical examination, activity scoring using SLE disease activity index (SLEDAI), and laboratory investigations including plasma and urinary levels of HMGB1 by enzyme-linked immunosorbent assay. Study of the HMGB1 immunohistochemical expression pattern in renal biopsy was carried out in group 1.
Results
Plasma and urinary HMGB1 levels and the renal tissue extranuclear expression (cytoplasmic and extracellular) pattern of HMGB1 were significantly increased in patients with active LN compared with the other groups ($P<0.001$), and were significantly correlated with SLEDAI, suggesting active release of HMGB1. Plasma and urinary levels in patients without active LN were also significantly higher compared with the control group ($P<0.001$).
Conclusion
HMGB1 plays an important role in the pathogenesis of LN and reflects disease activity. Thus, HMGB1 can be utilized as a biomarker for renal disease activity in patients with lupus and the therapeutic value of HMGB1-blocking agents must be investigated.

Keywords:
activity, apoptosis, HMGB1, lupus nephritis, systemic lupus erythematosus

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Introduction

Systemic lupus erythematosus (SLE) is a typical autoimmune disease. Aberrant self-DNA recognition is critical for the initiation of excessive immune responses in lupus [1]. Lupus nephritis (LN) is a severe and frequent manifestation of SLE. Its pathogenesis has not been fully understood, but immune complexes are considered to contribute toward the inflammatory pathology [2].

Mechanisms involved in breaking tolerance against self-components are not clear. However, in the past few years, disturbance in the clearance of apoptotic cells has been reported, and thus apoptotic cells can serve as a source of autoantigens [3].

High-mobility group box 1 (HMGB1), originally recognized as a DNA-binding protein, has been identified recently as a damage-associated molecular pattern [4]. HMGB1 is a nuclear nonhistone protein that is secreted from different types of cells [Lipopolysaccharide (LPS)-activated, tumor necrosis factor-α-activated and interleukin-1-activated monocytes and macrophages] during activation and/or cell death, and may act as a proinflammatory mediator, alone or as part of DNA-containing immune complexes in SLE and participates in many nuclear functions, but once released, it is involved in inflammatory function [3,5].

Although renal biopsy is considered the cornerstone for assessing renal activity, there is a need for new biomarkers

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for the evaluation of disease activity in LN. Therefore, our study was carried out to assess the potential role of HMGB1 in SLE and whether urinary/serum levels of HMGB1 and renal biopsy expression pattern reflect renal inflammation and correlate with disease activity.

Patients and methods

Patients

The present study was carried on 79 participants after approval of the ethical committee of research, faculty of medicine, Cairo university: 61 systemic lupus patients all fulfilling at least four of the criteria of the American College of Rheumatology for SLE diagnosis [6] and 18 healthy controls. They were divided into four groups as follows:

(1) *Group 1*: 21 patients with LN diagnosed by proteinuria exceeding 500 mg/day and/or the presence of cellular casts and confirmed by renal biopsy.
(2) *Group 2*: 21 patients with lupus activity without nephritis as estimated by SLE disease activity index (SLEDAI) of greater than 4.
(3) *Group 3*: 19 patients with lupus without activity as estimated by SLEDAI of less than 4.
(4) *Group 4*: 18 healthy volunteers who were age and sex matched.

All participants were recruited from Kasr Al-Aini Hospital, Cairo University. A written consent was obtained from all participants after an explanation was provided on the nature of the study.

Methods

All participants were subjected to the following:

(1) Assessment of medical history.
(2) Physical examination. Clinical disease activity was assessed using SLEDAI.
(3) Laboratory investigations:
    (a) Complete blood count.
    (b) Kidney function tests.
    (c) Urine analysis.
    (d) Erythrocyte sedimentation rate (ESR).
    (e) Antinuclear antibodies.
    (f) Anti dsDNA.
    (g) C3, C4 levels.

Plasma and urinary levels of HMGB1 assessed by enzyme-linked immunosorbent assay (IBL, Hamburg, Germany)

Samples were added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for HMGB1 and avidin conjugated to horseradish peroxidase was added to each microplate well and incubated. Then, a TMB substrate solution was added to each well; only those wells that contained HMGB1 biotin-conjugated antibody and enzyme substrate reaction were terminated by the addition of a sulfuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450±2 nm. The concentration of HMGB1 in the samples was then determined by comparing the OD of the samples with the standard curve.

Study of HMGB1 in renal biopsy in patients with active lupus nephritis

Serial paraffin-embedded sections (4 μm thick) of renal biopsy specimens were obtained from the patients of group 1. Some sections were mounted on glass slides, stained with routine hematoxylin & eosin and Masson trichrome stains, and then reviewed and classified by an experienced nephropathologist. The activity index and the chronicity index were calculated for each specimen, with maximum scores of 24 for the activity and 12 for the chronicity [7]. Other kidney sections were mounted on charged slides. They were deparaffinized, and then antigen retrieval and endogenous peroxidase blocking were performed. Slides were incubated with rabbit anti-HMGB1 antibody (Abcam, Cambridge, UK). Subsequently, slides were incubated with horseradish peroxidase-labeled secondary antibodies (DakoCytomation, Glostrup, Denmark). Next, slides were incubated in diaminobenzidine solution and counterstained with hematoxylin.

Evaluation of HMGB1 staining

The cellular distribution of HMGB1 was determined in the kidney by counting 100 nuclei (glomerular, tubular, and stromal) in three bright fields and scoring both HMGB1-positive (brown) and HMGB1-negative (blue) nuclei. Results are expressed as the percentage of negative cells.

Statistical methodology

SPSS program version 9.0 (IBM corporation, Armonk, New York, USA) was used for analysis of data. Data were summarized as mean±SD. A nonparametric test (Mann Whitney *U*-test) was used for analysis of two quantitative data. The χ^2-test was used for analysis of qualitative data. Analysis of variance was carried out for the analysis of more than two variables, followed by a post-hoc test. A simple linear correlation (Pearson's correlation for quantitative data and Spearman correlation for qualitative data) was performed to detect the relation between HMGB1 with

demographic and laboratory data. A *P*-value was considered significant if less than 0.05, highly significant if *P*-value was less than 0.01, and very highly significant if *P*-value was less than 0.001.

Results

Sixty-one patients with systemic lupus and 18 controls were included in this study. Demographic and clinicopathological data of the study groups are shown in Table 1. According to SLEDAI, six patients showed inactive disease (9.83%), 13 patients had mild activity (21.3%), four patients had moderate activity (6.55%) and 38 patients had severe activity (62.29%). For group 1, eight patients were class II (38.09%), eight patients were class III (38.09%), and five patients were class IV (23.8%).

Significantly higher levels of both plasma and urinary HMGB1 in cases of LN (group 1) were detected in comparison with all the other groups studied as shown in Table 2.

Figure 1 shows highly statistically significant differences between HMGB1 plasma levels in inactive and mild, inactive and moderate, and inactive and severe activity groups ($P<0.0001$), and a significant difference between mild and severe groups ($P<0.001$) and no statistically significant difference between mild and moderate, and moderate and severe activity ($P=1.000$), respectively, with plasma HMGB1 level being the highest in the severe activity group. Figure 2 shows statistically significant differences between HMGB1 urinary levels in inactive and mild, inactive and moderate, inactive and severe activity, mild and severe activity, moderate and severe activity ($P<0.0001$), and between mild and moderate activity ($P<0.001$), with urinary HMGB1 level being the highest in the severe activity group.

Plasma, urinary, and renal tissue HMGB1 (Figs 3 and 4) levels were assessed in SLE patients with recent onset (less than 1 year) and SLE patients with long-standing disease. High levels of plasma and urinary HMGB1 in recent-onset SLE patients (2.76±0.45/41.17 ±7.69) compared with long-standing lupus patients (2.58 ±0.30/37.96±9.12) were found; however, the difference was not statistically significant ($P=0.227$ and 0.266, respectively). HMGB1-negative nuclear count in group 1 was higher in long-standing patients compared with recent-onset patients (93.47±5.9/91.67±6.95), but this was also insignificant statistically ($P=0.557$).

A significant correlation was found between urinary HMGB1 with proteinuria in active SLE patients ($P<0.001$, $r=0.529$) and plasma HMGB1 with proteinuria ($P<0.001$, $r=0.551$).

A comparative study of plasma, urinary, and renal tissue levels of HMGB1 in different classes of

Table 1 Demographic and clinicopathological data of the study groups

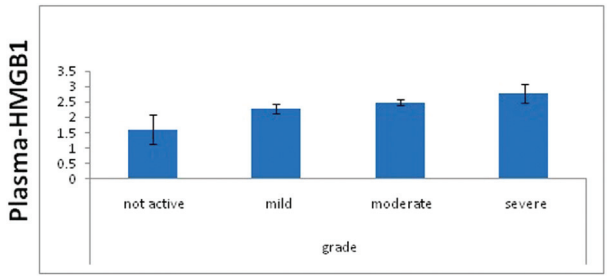
	Group 1 (n=21)	Group 2 (n=21)	Group 3 (n=19)	Group 4 (n=18)
Age (years) (mean±SD)	28.29±8.945	31.81±10.902	31.89±10.939	26.28±9.791
Age at disease onset (mean±SD)	24.13±7.68	27.17±8.58	27.08±9.10	
Sex distribution (F/M)	20/1	21/0	18/1	17/1
Mean ESR	117.10±23.81	105.70±23.258	27.05±17.712	9.56±2.935
Creatinine serum level (mg%)	1.2±0.87	0.842±0.1875	0.7421±0.1865	
Mean 24 h urinary protein (g)	0.48±0.39			
Mean C3 (mg%)	36.73±15.621	36.61±11.916	109.16±7.848	110.89±7.235
Mean C4 (mg%)	4.33±1.287	5.24±1.199	15.16±2.672	16.28±2.58
Anti-dsDNA positive	21	20	10	
Mean HMGB1 in plasma (ng/ml)	2.881±0.334	2.648±0.2136	2.295±0.1353	1.394±0.337
Mean HMGB1 in urine (ng/ml)	45.81±1.030	42.38±3.008	26.42±4.260	3.31±0.877
Mean renal tissue negative nuclear count	92.95±6.14			

ESR, erythrocyte sedimentation rate.

Table 2 Comparative study of plasma and urinary levels of HMGB1 between patients and control groups

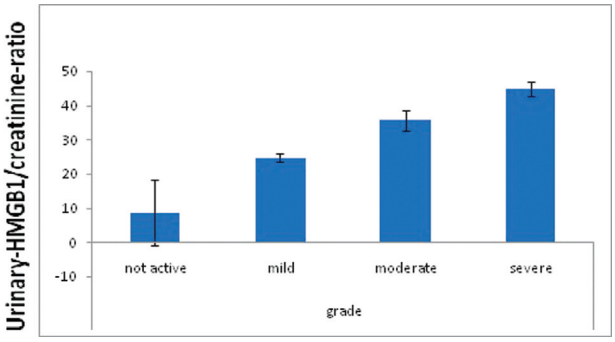
Variable	Group 1	Group 2	Group 3	Group 4	P_1 (I vs. II)	P_2 (I vs. III)	P_3 (I vs. IV)	P_4 (II vs. III)	P_5 (II vs. IV)	P_6 (III vs. IV)
	Lupus nephritis	Systemic lupus with activity	Systemic lupus without activity	Control						
Plasma HMGB1	2.881±0.334	2.648±0.213	2.295±0.135	1.39±0.34	0.037	<0.0001	<0.0001	<0.001	<0.0001	<0.0001
Urinary HMGB1	45.81±1.030	42.38±3.008	26.42±4.260	3.31±0.88	<0.001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Figure 1



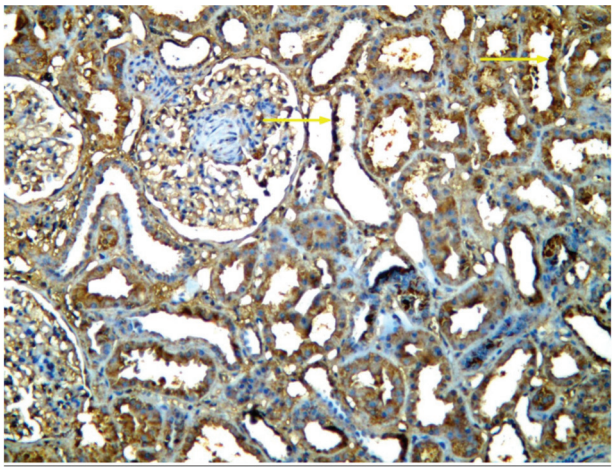
Comparative study of plasma levels of HMGB1 in different grades of disease activity of systemic lupus erythematosus (SLE) patients.

Figure 2



Comparative study of urinary HMGB1 levels in different grades of disease activity of systemic lupus erythematosus (SLE) patients.

Figure 3

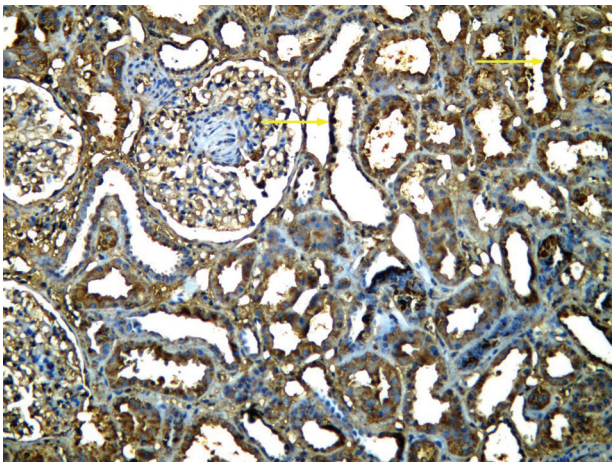


Lupus nephritis class IV-S (A/C) showed negative nuclear staining for HMBG1 in 89% of counted nuclei, with dense cytoplasmic staining, in all cellular elements. (IHC ×200). Arrow points to positive nuclei.

nephritis shown in Table 3 showed that there was a statistically significant difference only between plasma HMGB1 levels in class II and III nephritis (2.71±0.14/3.14±0.39) (P=0.020).

The incidence of anti-dsDNA positivity in recent-onset patients was 75% as opposed to 100% positivity of plasma,

Figure 4



Lupus nephritis class IV-S (A/C) showed negative nuclear staining for HMBG1-A1 in 46% of counted nuclei, with positive intense cytoplasmic staining, in all cellular elements of the core (IHC ×200).

urinary, and tissue for HMGB1. A comparative study of plasma, urinary levels of HMGB1, and anti-dsDNA positivity in different groups showed that there was a statistically significant correlation, where plasma and urinary levels in anti-dsDNA-positive cases were higher (2.92±0.40/45.22±1.39) compared with anti-dsDNA-negative cases (2.27±0.12/29.00±4.58) (P=0.0001).

A significant inverse correlation was found between plasma, urinary, and renal tissue levels of HMGB1 in different groups with serum levels of C3 and C4 (P<0.001, r=-0.727 for C3 and -0.844 for C4) and a significant positive correlation was found with ESR (P<0.001).

A nonsignificant correlation was found between plasma, urinary, and renal tissue levels of HMGB1 in different groups with serum creatinine (P=0.768, 0.809, and 0.799), respectively.

A significant positive correlation was found between HMGB1 in renal tissue (group 1) and serum HMGB1 (P<0.003, r=0.612), but no correlation with urinary HMGB1 (P=0.951, r=014). However, in all study groups, a significant positive correlation was found between plasma and urinary HMGB1 levels (P<0.001).

Discussion

HMGB1 has been recognized as an important inflammatory mediator in SLE. Both HMGB1 and anti-HMGB1 antibodies were associated in some studies with SLE disease activity, decreased complement levels, and proteinuria [8].

Table 3 Comparative study of plasma, urinary, and renal tissue HMGB1 levels in different classes of nephritis in group 1

Variables	Class II (n=8)	Class III (n=8)	Class IV (n=5)	P_1 (II vs. III)	P_2 (II vs. VI)	P_3 (III vs. IV)
Plasma HMGB1	2.71±0.14	3.14±0.39	2.74±0.21	0.020	1.000	0.064
Urinary HMGB1	46.12±0.83	45.25±1.16	46.20±0.84	0.270	1.000	0.316
Renal tissue HMGB1	93.12±5.82	93.12±8.29	92.40±2.88	1.000	1.000	1.000

An important role for HMGB1 in the pathogenesis of SLE has been described by Voll *et al.* [9]. They reported that this protein is tightly attached to chromatin released from late apoptotic cells. These complexes can induce inflammatory and immune responses.

Li *et al.* [1] showed that HMGB1 inhibited phagocytosis of apoptotic neutrophils by macrophages through binding to phosphatidylserine, which moves from the inner to the outer membrane leaflet of cells undergoing apoptosis. Zickert *et al.* [10] provided evidence implicating that enhanced expression of HMGB1 is perhaps crucial in the pathogenesis of a very complicated disease. In addition, Ma *et al.* [11] showed a positive correlation between HMGB1 and peripheral blood neutrophils in SLE patients. These data, together with previous reports, imply that apoptotic neutrophils may be an important source of the increased serum HMGB1 in SLE.

In our study, plasma and urinary levels of HMGB1 were significantly increased in patients with active LN compared with the other three groups, with a *P*-value of less than 0.001. Plasma and urinary levels of HMGB1 in SLE patients with activity but without nephritis were significantly higher compared with controls, with *P*-value less than 0.001. Similarly, renal tissue of active LN patients showed strong expression of HMGB1 at cytoplasmic and extracellular sites, suggesting active release of HMGB1 from nuclear localization.

Our study is in agreement with Zickert *et al.* [10] and Ma *et al.* [11], who found that plasma levels of HMGB1 were significantly higher in SLE patients with active nephritis compared with those with inactive nephritis or inactive disease and controls. According to the findings of Li *et al.* [8], the increased serum HMGB1 concentration of SLE patients might be either the product of peripheral blood mononuclear cell activation or the product of uncleared apoptotic cells.

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 and/or increased levels of plasma HMGB1 might lead to urinary excretion of HMGB1 in the absence of nephritis.

A significant correlation was found between urinary HMGB1 with proteinuria in active SLE patients ($P<0.001$, $r=0.529$) and plasma HMGB1 with proteinuria ($P<0.001$, $r=0.551$), and this finding is in line with Abdulahad *et al.* [5].

We found a strong association between plasma and urinary levels of HMGB1 reactivity and disease activity assessed by SLEDAI. This is in line with Abdulahad *et al.* [5], Li *et al.* [8], Ma *et al.* [11], and David [12]. In a study carried out by Abdulahad *et al.* [5], serum and urinary HMGB1 levels were correlated with SLEDAI and concluded that urinary HMGB1 might be an additional biomarker for the assessment of renal disease activity in SLE. Ma *et al.* [11] found that, in SLE patients, particularly those with active LN, plasma and urine levels of HMGB1 were increased and correlated with SLEDAI scores. In a study carried out by Li *et al.* [8], HMGB1 levels were correlated positively with SLEDAI, but did not show an association with specific organ involvement.

Our study showed a significant inverse correlation between plasma, urinary, and renal tissue levels of HMGB1 in different groups with serum levels of C3 and C4 ($P<0.001$) and no correlation was found with serum creatinine levels, which was exactly the same result obtained by Abdulahad *et al.* [5]. The significant positive correlation of serum HMGB1 and ESR is in line with Schaper *et al.* [13].

The mechanism of action of HMGB1 in the development of LN may include the following factors: first, the interaction between HMGB1 and various factors in the system of clearing apoptotic cells, thus reducing the clearance of dead cells, second, when cells in the patients with SLE develop apoptosis, HMGB1 combines with nucleosome and develops an immune complex that stimulates antigen-presenting cells to break the immunologic tolerance against DNA, and third, activated immune cells in SLE secrete HMGB1. Extracellular HMGB1 promotes the production and activation of various inflammatory factors such as tumor necrosis factor-α and interleukin-1β, and then

contributes toward the development and progression of LN [14–16].

In the current study, the renal tissue of patients with active LN showed absence of nuclear staining for HMBG1 in high percent, with a high intensity for cytoplasmic staining, in all cellular elements of the core as well as and extracellular sites, suggesting active release of HMGB1 in the proinflammatory processes within the kidney.

Zickert and colleagues reported that renal tissue staining for HMGB1 was detected in LN, whereas the staining was absent in control renal tissue. There was no distinct difference in the 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 origin of the increased renal expression of HMGB1 is not fully understood [17]. 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 endothelial staining and expression in the mesangium suggest a colocalization for HMGB1 and immune depositions in LN. However, further studies with other methodologies are required to address this issue.

Li *et al.* [1] concluded that extracellular, but not intracellular HMGB1, facilitates auto-DNA-induced macrophage activation by promoting DNA accumulation in endosomes and contributes toward the pathogenesis of LN.

We could not definitely identify the cells releasing HMGB1. HMGB1 release could result from infiltrating inflammatory cells as indicated by immunohistochemical staining and could also result from either activation or cell death of constitutive renal tissue. Also, there is a possibility that at least some of the urinary HMGB1 might have emerged from systemic inflammation. This might explain the lack of correlation between urinary HMGB1 and HMGB1 released from nuclei in renal biopsy. Our results are in line with Abdulahad *et al.* [5].

Our results still showed no significant correlation between HMGB1 tissue expression and pathological classes of LN, in agreement with Li *et al.* [1], who suggested that the pathogenesis of HMGB1 involved 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 SLE patients presenting with lupus nephritis, core biopsy to test the level of HMGB1 expression should be performed early to determine the severity of injury of renal interstitium and clinical treatment should be focused toward reducing the development of complications [1,18].

Comparing the incidence of anti dsDNA antibodies positivity (75%) and high plasma/urinary levels of HMGB1 and renal tissue HMGB1expression (100%) in recent-onset cases, a finding suggests that plasma, urinary levels of HMGB1, and renal tissue HMGB1 appear earlier than anti-dsDNA antibodies. Plasma, urinary levels of HMGB1 and renal tissue HMGB1 can be used to diagnose SLE early in the course of the disease even before other antibodies are evident.

Conclusion

The present study shows increases in plasma, urine, and renal tissue extranuclear expression of HMGB1 levels in SLE patients, especially in active LN. An increase in HMGB1 levels correlated to the SLEDAI. Thus, HMGB1 plays an important role in pathogenesis and activity in LN. Plasma, urinary levels of HMGB1 and renal tissue HMGB1 can be used to diagnose SLE early even before other antibodies are evident.

To our knowledge, the current study is the first to assess the correlation between the levels of both plasma and 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 HMGB1 as a biomarker for renal disease activity in patients with lupus and to evaluate the therapeutic value of HMGB1-blocking agents.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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