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Original Article

Erythrocyte and glomerular C4d deposits as a biomarker for active lupus nephritis

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ABSTRACT

Introduction: Lupus nephritis (LN) is a serious manifestation of systemic lupus erythematosus (SLE) and histologically evident even in those without clinical manifestations of renal disease.
Aim of the work: To assess C4d on erythrocytes (E-C4d) and glomerular deposits (G-C4d) in SLE patients and study its association to LN and disease activity. Patients and methods: 61 subjects were enrolled including 15 with LN (study group); 15 with renal disease not due to SLE (control A group); 16 SLE patients with no renal affection (control B group) and 15 healthy individuals (control C group). Flow cytometry system was used for C4d immunohistochemical staining. SLE disease activity index (SLEDAI) was assessed for SLE patients.
Results: The age was comparable among groups; for LN patients was 28.3 ± 8.2 years; group A (35.9 ± 13.3); group B (27.1 ± 8.8) and group C (29.4 ± 7.1) ($p = .06$). Patients were mostly females. The disease duration of LN patients was 1–2 years; group A (3–5 years) and group B (5–10 years). E-C4d and G-C4d deposits were significantly higher in LN patients (8.08 ± 2.93 and 2.3 ± 0.97) in comparison to the control groups (A/B/C) (A: 3.78 ± 0.38 and 0.6 ± 1.12; B: 3.72 ± 0.32; C: 3.55 ± 0.44 $p < .001$, $p < .001$, $p < .001$ MFI respectively). E-C4d and G-C4d significantly correlated with LN activity ($r = 0.8$, $p < .001$ and $r = 0.7$, $p = .005$) and with SLEDAI ($r = 0.9$, $p = .005$ and $r = 0.8$, $p = .002$, respectively).
Conclusion: Erythrocytic C4d correlated significantly with the LN activity which might serve as a potential biomarker for renal activity in the future instead of biopsy and may further help in the optimum follow up of LN patients.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that chiefly influences women. It has a myriad of clinical manifestations, yet the disease outcome and organ involvements remain unpredictable. In spite of the advances in the survival of SLE patients, the pathogenesis is still unknown. Comorbidities due to both disease and treatment, and the many faces of SLE require intensive investigations [1]. Lupus nephritis (LN) is one of the most frequent and serious complications in SLE patients. Autoimmune-mediated inflammation in both renal glomerular and tubulointerstitial tissues is the major pathological finding of LN. In clinical practice, increased anti-double stranded deoxyribonucleic acid (anti-dsDNA) antibody titer and consumed complement C3 and C4 levels are potential disease-activity surrogate biomarkers in LN [2]. LN usually arises within 5 years of diagnosis and is histologically evident in most SLE patients, even in those without clinical manifestations of renal disease [3]. Renal biopsy is still the gold standard for deciding therapy in LN but its invasive nature prevents it from being used repetitively. Since histopathology remains the cornerstone for deciding treatment in LN, the ultimate goal of 'the quest for a biomarker of LN' is to find a marker that could reflect the in vivo events obviating the need for renal biopsy [4].

More evidences suggest that autoantibodies other than anti-double stranded deoxyribonucleic acid (anti-dsDNA) antibodies, as anti-nucleosome, anti-C1q, anti-C3b, anti-cardiolipin (aCL), anti-ribonuclear proteins, and anti-glomerular matrix antibodies, may also be involved in LN [2]. An ideal biomarker for LN would be the one which reflects renal disease activity better, correlates with renal histology, predicts flares, is easily measurable, not affected by age, gender and ethnicity, is specific to SLE and renal involvement for making an early diagnosis of LN. However, no biomarker seems to have all the desired qualities [5]. Several biomarkers have been proposed in studies on Egyptian SLE

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patients [6–10] with preference to LN [11–15].

Systemic lupus erythematosus is characterized by a vicious cycle maintaining systemic inflammation. It starts by autoantibody production, immune complex formation and complement activation that contribute to inflammation, tissue damage and further autoantibody production [16]. Future in-depth studies on the association of oxidative stress to complement pathways in SLE pathogenesis are encouraged [17]. Complement factors (C3 and C4) are traditional markers of disease activity. Their low levels signify consumption in the clearing process and correlate with disease activity. Apart from C3 and C4, their degradation products also have been tested as biomarkers in LN [5].

C4d is now increasingly recognized as a potential biomarker in fields where antibodies can cause tissue damage, as systemic autoimmune diseases and pregnancy. C4d holds promise to detect patients at risk for the consequences of antibody-mediated disease. Moreover, the emergence of new therapeutics that inhibits complement activation makes C4d a marker with potential to identify patients who may benefit from these drugs [18]. Serum C4d detected by quantitative micro-assay plate enzyme immunoassay is more sensitive than C3, C4 and C5b-9 in SLE patients with moderate-to-severe disease activity [19]. In LN, Glomerular-C4d (G-C4d) deposition can be detected in the majority of cases with an immune-fluorescence pattern, as a result of immune complex deposition. Biopsies of LN patients with prominent diffuse G-C4d staining had detectable glomerular microthrombi significantly more than in patients with focal/mild C4d staining. The extent of G-C4d deposition correlates with LN activity and with thrombotic microangiopathy (TMA). Also TMA correlate with LN activity [20].

The aim of the present work was to assess C4d on circulating erythrocytes (E-C4d) and deposits in renal glomerular tissues (G-C4d) and study their correlation with LN activity.

2. Patients and methods

A total of 61 subjects were enrolled in this study and included 15 patients with LN (study group); 15 with renal disease not due to SLE (control A group); 16 SLE patients without renal affection (control B group) and 15 healthy individuals (control C group). All SLE patients met the systemic lupus international collaborating clinics (SLICC) classification criteria [21]. This study was conducted at Internal Medicine Hospital Kasr Al-Ainy, Faculty of Medicine, Cairo University. The study conforms to the 1995 Helsinki declaration and was approved by Cairo University Hospitals' ethical committee. Informed consent was obtained from all patients.

Patients were subjected to history taking, thorough clinical examination and the following laboratory investigations: complete blood count (CBC), erythrocyte sedimentation rate (ESR), serum albumin, serum creatinine, urine analysis, anti-nuclear antibody (ANA), anti-dsDNA, serum C3 and C4, aCL (IgG and IgM) and urine albumin/creatinine ratio. E-C4d was assayed by flow cytometry in all subjects. SLE disease activity index (SLEDAI) was assessed in SLE patients [22]. Renal biopsy was done for LN patients and control A group and histopathological evaluation by immunohistochemistry staining for G-C4d. Renal specimens were processed for light microscopy and classified according to the 1982 modified world health organization (WHO) morphologic classification of LN and renal activity and chronicity were considered [23].

Flow cytometry system (EPICS XL, Bickman Coulter) was used (Primary anti body: Anti C4d Ab (ab36075). Vial 50 micro Lat 0.2 mg/ml. Secondary Antibody: Goat Anti Rabbit IgG H & L (AleXa Flour ®488) (ab150077) vial 500 µg at 2 mg/ml**).** Venous blood was taken using sterile tubes containing an EDTA salt as the anti coagulant. The samples were kept at room temperature (18–25 °C) and not shaken. The samples were homogenized by gentle agitation and were analyzed within 24 h of venipuncture. Surface expression of C4d on gated cells was expressed as specific mean fluorescence intensity (MFI) (C4d-specific mean the isotype control mean fluorescence).

Table 1
Demographic, laboratory and activity features and medications received by lupus nephritis patients.

Variable mean ± SD or n (%)	LN patients (n = 15)
Age (years)	28.3 ± 8.2
Sex F:M	12 (80): 3 (20)
Hemoglobin (g/dl)	9.9 ± 1.9
TLC ($\times 10^3$/mm^3)	5.8 ± 1.9
Platelets ($\times 10^3$/mm^3)	218 ± 71.5
sCreatinine (mg/dl)	1.4 ± 0.61
ESR (mm/1st hr)	100.2 ± 30.6
A/C	2.04 ± 1.1
sAlbumin (mg/dl)	2.6 ± 0.5
E-C4d (MFI)	8.1 ± 2.9
Complement C3 (mg/dl)	32.5 ± 10.3
C4 (mg/dl)	12.9 ± 4.5
SLEDAI score	13.9 ± 0.9
Renal activity score	4.3 ± 3.7
Medications	
Steroids	15 (100)
Azathioprine	15 (100)
Mycophenolate mofetil	9 (60)
Cyclophosphamide	6 (40)

LN: lupus nephritis, TLC: total leucocytic count, ESR: erythrocyte sedimentation rate, A/C: albumin-creatinine ratio, E-C4d: C4d on erythrocytes, MFI: mean fluorescence intensity, SLEDAI: systemic lupus erythematosus disease activity index.

2.1. Statistical analysis

Data were coded and entered using the statistical package SPSS version 23. Data was summarized using mean ± standard deviation for quantitative variables and frequencies (number of cases) and relative frequencies (percentages) for categorical variables. Comparisons among groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test. For comparing categorical data, Chi square ($\chi 2$) test was performed. Exact test was used instead when the expected frequency is < 5. Spearmans correlation test was used. P-values < .05 was considered significant.

3. Results

The age was comparable among groups; for LN patients was 28.3 ± 8.2 years; group A (35.9 ± 13.3 years); group B (27.1 ± 8.8 years) and group C (29.4 ± 7.1 years) (p = .06). The frequency of female patients was significantly higher in LN patients (80%) and controls B (100%) and C (73.3%) compared to control A (40%) (p = .001). The disease duration of the patients with LN was 1–2 years, of group A was 3–5 years and of group B was 5–10 years. Table 1 shows the demographic, clinical, laboratory and activity features of the study groups as well as the medications received.

The SLEDAI score was significantly higher in SLE patients with LN patients compared to those without patients (p = .03). Serum creatinine levels of LN patients were significantly higher than control B and C (p < .001) but lower than control A (p = .7). The ESR levels were significantly higher in LN patients than control (A/B/C) (p < .001). A/C ratio in LN patients was significantly higher than control B and C (p < .001) but lower than control A (p = .9). Serum C3 and C4 were significantly lower in LN patients (p < .001) compared with the 3 control groups (A/B/C).

MFI was significantly higher in LN patients (8.08 ± 2.93) in comparison to the control groups (A/B/C) (A: 3.78 ± 0.38; B: 3.72 ± 0.32; C: 3.55 ± .0.44 p < .001, p < .001, p < .001 respectively). (Fig. 1).

Renal biopsy results for LN and renal-non nephritis groups showed that the activity and chronicity indices in LN group were 4.27 ± 3.7 and 1.73 ± 2.3, respectively, while in renal group they were