

The Blood Cells Ultrastructure Electron Microscopy Changes: Reflection on Systemic Lupus Erythematosus

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Abstract

The sub-laboratory effects of SLE on blood cells were not completely evaluated to explain the thrombotic tendency found in this disease. The present study was conducted to assess the ultrastructural changes of RBCs, WBCs and platelets in SLE and to corelate these changes with the disease activity. Comet assay and 8hydroxydeoguanosine (8-OHdG) were used to confirm cellular dysfunction. Ninety subjects were recruited and equally divided into 3 groups: Group I; SLE (normal CBC), Group II; SLE (abnormal CBC) and Group III; healthy control. Disease activity was evaluated by systemic lupus erythematosus disease activity index score (SLEDAI). Ultrastructure examination of blood cells were done by electron microscope, DNA damage was assessed by Comet assay and serum 8-OHdG levels. CBC and serological tests including serum C3, C4, ANA and anti-dsDNA were evaluated. There was statistically significant negative correlation between RBCs and WBCs elements with SLEDAI score in Group II. There was significant statistical difference in RBCs and WBCs cell membrane defects by electron microscope between Group I and Group II. There was no statistically significant correlation between blood cell membrane defects and SLEDAI score in both Group I and Group II. There was a significant increase in percentage of tail DNA damage (p<0.05) in Comet assay and serum 8-OHdG levels in Group I and Group II. In conclusion, there are ultrastructural changes in blood cells in SLE that could play a crucial role in the thrombotic and inflammatory effects of blood cells. Comet assay can be used as a detectable and reliable method for assessment of other biological genetic research.

Keywords: Systemic lupus erythematosus; Blood cells; Transmission electron microscopy; Comet assay; 8-OHdG

Introduction

Systemic lupus erythematosus (SLE) is a chronic systemic inflammatory autoimmune disease. This clues to the production of proinflammatory cytokines, autoantibodies and immune complexes. Blood cells, kidney and central nervous system are the main target organs for immune complex-mediated inflammation [1,2].

Deposition of complement (C3 and C4) fragments on RBCs membranes during the disease activity leads to cytoskeletal changes and defects in these cells. This augments their liability to destruction, their disturbances in the microcirculation, thrombosis and increase in fibrinogen plasma levels with subsequent erythrocyte aggregation [3,4]. Nitric oxide (NO) plays a crucial role in SLE regarding the pathophysiologic processes of the immune system dysregulation. NO leads to disturbances in the RBCs flow through the capillaries with negative effects on oxygen delivery to the tissues [4,5].

Platelets are other targets for the activated complement system in SLE [6,7]. This leads to the production of anti-phospholipid antibodies which are directed to the phospholipids of the platelets cell membrane leading to its destruction and thrombocytopenia [8]. Defects in the platelets cell membrane predispose to platelets aggregation and venous thrombosis [9].

Autoimmune inflammation also targets white blood cells (WBCs). Circulating anti-neutrophil antibodies lead to destruction of the neutrophils [11]. Neutrophils also plays a leading role in thrombosis because anti-phospholipid antibodies cooperate with neutrophilic cytokines leading to thrombosis [12,13].

The Comet Assay or single cell gel electrophoresis assay is widely used method to assay the microscopically DNA damage in a single cell. The visual scoring of cells is used to determine the DNA damage as classified according to tail length, the shape, size of DNA and its amount, all of above can determine the level of DNA damage [14].

The current study was conducted to assess the ultrastructure of RBCs, WBCs and platelets in SLE to evaluate the sub-laboratory affections of blood cells and to corelate these changes with the disease activity. Comet assay and 8-hydroxydeoguanosine (8-OHdG) were also assessed to confirm cellular dysfunction.

Method

The present cross-sectional study was conducted on 60 SLE patients. Patients were carefully selected and equally divided into two groups: Group I (n=30) SLE patients with normal CBC, Group II (n=30) SLE patients with abnormal CBC. Group III (n=30) included healthy subjects enrolled as control group.

All patients attended to Rheumatology, Rehabilitation and Physical Medicine Department inpatients and outpatients' clinics, Benha

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University Hospitals from September 2015 to May 2017. Written informed consent forms were obtained from all patients. The study complies with the guidelines for human studies and animal welfare regulations. The study protocol has been approved by the institute's ethical committee on human research and meets the standards of the Declaration of Helsinki in its revised version of 1975 and its amendments of 1983, 1989, and 1996 [15].

All patients were diagnosed according to Systemic Lupus International Collaborating Clinics (SLICC) criteria for diagnosis of SLE patients [16]. Clinical history and clinical examination were obtained from all patients. SLE disease activity was assessed according to systemic lupus erythematosus disease activity index score (SLEDAI) to mild (score: 0-10), moderate (score: 11-20), severe (score: 21-45) and very severe (score >45) [17].

Venous blood samples were collected from all subjects and the following parameters measured: complete blood count, electron microscope analysis, serum creatinine, creatinine clearance, Antinuclear antibody (ANA) by immunofluorescence technique, Antidouble stranded DNA (dsDNA) antibody by indirect fluorescent antibody test, complement C3 and C4, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), Comet assay, and 8hydroxydeoxyguanosine (8-OHdG). Twenty-four hours' urine and spot urine sample were collected to estimate urinary protein excretion.

Ultrastructure analysis by transmission electron microscope

Blood samples were collected from all groups in heparinized tubes (2 mL of blood) then centrifuged at 500 g for 5 min at room temperature. A layer of white blood cells and thrombocytes plus a small portion of the red blood cells layer were removed and fixed in a 2% glutaraldehyde solution in phosphate buffered saline (PBS) for 90 min. The fixed cells sediment was centrifuged at 1000 rpm for 10 min and washed with PBS three times for 15 minutes. The samples were added to buffered 2% osmium tetroxide as secondary fixative for 4 hours then were washed three times in distilled water for 15 minutes and left 4 hours in 1% uranyl acetate solution. Samples were dehydrated in ethanol (20%, 40%, 60%, 70%, 100% series) and passed through ethanol-propylene oxide, propylene oxide and propylene Oxide-Embed 812 resins. Samples were polymerized in fresh Embed resin, and were cut on an ultramicrotome (Reichert, Bensheim, Germany). Finally, the sections were stained with uranyl acetate. Sections were examined with transmission electron microscope and images were taken with an EM digital camera system (MegaView, analysisc docu 3.2, Olympus Soft Imaging Systems GmbH, Munster, Germany) according to Winey et al. [18].

Serum 8-OHdG Levels measurement

Serum 8-OHdG levels were measured using a commercially available enzyme-linked immunosorbent assay kit (Abcam, England, catalogue No. ab201734).

Single cell gel electrophoresis assay (Comet assay)

The DNA damage was detection done by using the single-cell gel electrophoresis (Comet assay) "a sensitive and powerful method for determining DNA strand breakage" [19].

In Eppendorf tubes 1 ml of lymphocyte and WBC suspensions were transferred and centrifuged at $2000 \times g$ for 5 min. The supernatant was cleaned, and the cell pellet was mixed with 500 ml of low-melting-

point (LMP) agarose in phosphate-buffered saline (PBS) at 37°C. Then this mixture was applied onto a glass microscope slide. Whole blood was used directly for comet assay, in which 20 ml of whole blood was mixed with 150 ml of LMP agarose, and 140 mL of the mixture was applied onto a glass microscope slide pre-coated with a layer of 1% normal melting-point agarose (150 ml). After application of a third layer of 1% normal-melting point agarose (150 ml), the slides were immersed in ice-cold-lysing solution (2.5 mg sodium chloride, 10 ml Tris, 100 mL Ethylenediaminetetraacetic acid (EDTA), 10% dimethyl sulfoxide, 1% Triton X-100, pH 10.0) for at least 2 hours. The slides were then incubated in ice-cold electrophoresis solution (0.3 mg sodium chloride, 1 ml EDTA, pH 13.0) for 20 min, followed by electrophoresis at 15V for 25 min allowing the DNA to unwind for 15 min in the alkaline solution (300 mm NaOH and 1 mm Na₂EDTA). After electrophoresis, the slides were neutralized and stained with ethidium bromide. Fluorescence microscope (Leica DM-2500) was used to analysis of comets, and the percentage tail DNA was calculated based on (% tail DNA = 100 - % head DNA) for the quantification of DNA damage and the assessment of length of the DNA migration (i.e., diameter of the nucleus plus migrated DNA) was measured using image analysis Axiovision 3.1 software (Carl Zeiss, Canada) [20].

Collection of whole blood and isolation of lymphocytes

The 10 ml blood was obtained from all groups in tube containing K_{3^-} EDTA. Lymphocytes were isolated by a modified procedure previously described. Briefly, 3 mL of whole blood was diluted 1:1 with RPMI and carefully layered on the top of lymphocyte separation medium in a centrifugation tube in a ratio of 1:1 for 15 min. The white layer of lymphocytes between plasma and the medium was then transferred by Pasteur pipette in 5ml of culture media, RPMI. Then lymphocytes were then washed twice with RPMI and centrifuged at 250 × g for 10 min. Then the cell pellet was put in 6 mL of RPMI containing no FBS (ca. 5.2×10^5 lymphocytes/ml medium), and 1ml of the suspension was used for comet assay or for in vitro incubation. Cell viability determined using the Trypan blue assay [21].

Isolation of white blood cell from fresh blood

WBCs were separated using a modified version of the procedure [22]. In which 3 ml of whole blood collected in K3-EDTA-containing was centrifugation at 2000 × g for 5 min and diluted 1:3 with RBC lysis buffer (0.15 M NH₄Cl, 12 mM NaHCO₃, 0.16 mM Na₂EDTA, pH 7.0). The tube was inverted to mix well and incubated 5 min at room temperature then centrifuged (2000 × g, 5 min), and the supernatant was removed, leaving only the visible WBC pellet. Five milliliters of RBC lysis solution were then added to resuspend the WBCs, the last step was repeated at least twice to clear all RBCs. After centrifugation, the cells were suspended in 6ml of RPMI containing no FBS (ca. 9 × 10⁶ cells/mL). For comparison between groups we used slide prepared from damaged cells that known to containing amount of DNA damage to avoid variation that can occur during the measurements of DNA damage.

Statistical analysis

Statistical analyses were carried out using Microsoft excel 2013 (Microsoft Egypt, Cairo, Egypt). Clinical data are expressed as mean and standard deviation (SD), T-test was used to determine statistical differences, Chi-squared test for proportion correlations and a p-value <0.05 was considered statistically significant.

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Results

Our subjects were all females and assembled to group I included 30 SLE patients without laboratory evidence of haematological manifestation (mean age 26.97±6.44), group II included 30 SLE patients with laboratory evidence of haematological manifestation (mean age 27.37±5.90), and group III healthy control group included 30 healthy females (mean age 26.5±4.5). The clinical and laboratory characteristics are presented in Table 1.

Our data revealed statistically significant negative correlation between CBC and WBCs counts with SLEDAI score in Group II whereas, there was no statistically significant correlation regarding platelets count. Moreover, there was no significant correlation between SLEDAI and blood elements counts in Group I (Table 2).

Parameter	Group I	Group II	
Disease duration	1.5 ± 0.8	3.27± 2.67	
mucocutaneous manifestation	90%	57%	
Synovitis	15%	10%	
Serositis	83.3%	30%	
Renal	10%	86.6%	
Neurologic	0%	3.3%	
anemia	0%	90%	
Leukopenia	0%	50%	
Thrombocytopenia	0%	16.6%	
ANA	100%	100%	
Anti-dsDNA	3.3%	86.6%	
Low complement	0%	20%	
	70% mild	16.6% mild	
SLEDAI Score		50% moderate	
	30%moderate		
		33.3% severe	
	-		

ANA: antinuclear antibody, Anti-DNA: Anti-double stranded deoxyribonucleic acid, SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

Table 1: Clinical and serological characteristics of patients at the time of the study.

Parameter	SLEDAI		
	Group I	Group II	
	r=0.44822	r=-0.90	
RBCS	p=0.19839	p <0.001*	
	r=-0.03712	r=-0.8530	
WBCs	p=0.9137	p <0.001*	
	r=0.43021	r=-0.9	
Platelets	p=0.18659	p=0.2	
SLEDAI: Systemic Lupus Erythematosus Disease Activity Index, RBCs: Red Blood Cells, WBCs: White blood cells.			

Table 2: Correlation between CBC elements and SLEDAI score in group I and II.

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Electron microscopic results

Ultrastructure of blood cells from control group revealed normal appearance of the blood cells in which Neutrophil showed many granules within the cytoplasm and lobed nucleus (Figure 1a). A basophil showed irregular round nucleus with sparse chromatin and eccentric nucleoli with heterogenous granules in the cytoplasm (Figure 1b). Whereas, an eosinophil cell showed segmented nucleus with condensed chromatin and its cytoplasm embedded with enormous number of granules of different shapes, electron- density crystalloid structure was easily identified (Figure 1c). A monocyte showed large indented nucleus and less electron dense cytoplasm (Figure 1d). A lymphocyte revealed nuclear indentation (Figure 1e). Platelet contains intracellular organelles in its cytoplasm and contains granules, but no nucleus (Figure 1f).



Figure 1: A transmission electron micrograph from a control group presenting A) a neutrophil with lobulated nucleus (n) and cytoplasmic granules(g) TEM ×17500. B) A basophil showing round eccentric nucleus covered by cytoplasmic granules (g) TEM ×8780. C) An eosinophil with a cytoplasm filled with crystal-containing granules (g), and a segmented nucleus with heavily condensed chromatin, (n) TEM ×17500. D) A monocyte showing deep indented nucleus (arrow) and less electron dense cytoplasm. E) A lymphocyte revealed nuclear indentation (n) TEM ×17500. F) platelets contain cytoplasm with some intracellular organelles (arrow). Note no nucleus. TEM ×17500.

The ultrastructure of blood cells that obtained from SLE patient in group I showed moderate changes in the blood cells in which there is less electron dense cytoplasm in neutrophil (Figure 2a), a basophil showing vacuolated cytoplasm (Figure 2b), Eosinophil showing irregular membrane with heterochromatic nuclei and cytoplasmic granule (Figure 2c), Monocyte showing less electron density with less distinct organelles (Figure 2d), Lymphocyte showing irregular nucleus with irregular membrane (Figure 2e). Loss of discoid shape of platelets, vacuolated cytoplasm, and prominent Golgi structures (Figure 2f).

The ultrastructure of blood cells that obtained from SLE patient in Group II revealed marked alterations in the morphology of all blood elements. The nuclei showed less heterochromatin content and several nuclear envelope blebs. Vacuolated less electron dense cytoplasm was noticed and slightly irregular outline with less distinct organelles. (Figure 3).

There was significant statistical difference in RBCs and WBCs cell membrane defects by electron microscope between Group I and Group II (Table 3). There was no statistically significant correlation between blood cell membrane defects and SLEDAI score in both Group I and Group II (Table 4).



Figure 2: A transmission electron micrograph from SLE patients in group I show A) a neutrophil showing less electron dense cytoplasm (C). Note absence of microvilli (arrow). B) A basophil showing vacuolated cytoplasm (v) TEM ×17500. C) eosinophilia showing irregular membrane with heterochromatic nuclei(g). Note cytoplasmic granule (arrow). TEMx17500. D) monocyte showing less electron density with less distinct organelles (arrow). TEM ×17500. E) A lymphocyte irregular nucleus with irregular membrane (arrow). TEM ×17500. F) loss of discoid shape of palette, vacuolated cytoplasm, (arrow). Note presence of Prominent Golgi structures (G). TEM ×17500.



Figure 3: A transmission electron micrograph from SLE patients in group II showing A) a neutrophil showing less electron dense cytoplasm (arrow) with less heterochromatic nuclei(n) B) A basophil showing vaculations (arrow) and sparse cytoplasmic granules (g) TEM ×17500.C) eosinophil showing vacuolated (V) cytoplasm containing specific granules without central crystals (arrow). TEMx17500. D) monocyte showing less electron density with less distinct organelles (arrow). TEM ×8780. E) A lymphocyte irregular nucleus (arrow). TEM ×8780. F) Irregular membrane, vacuolated cytoplasm, disappearance of cytoplasmic granules (arrows head) TEM ×17500.

EM changes	Group I (30)	Group II (30)	p value	
RBCS	(50%)	(83.3%)	0.006	
WBCs	(33.3%)	(66.7%)	0.01	
Platelets	(23.3%)	(33.3%)	0.4	
EM: electron microscope, RBCs: Red Blood Cells, WBCs: White blood cells.				

Table 3: comparison between the studied groups according to EMfindings and the number of patients.

Groups	SLEDAI	ЕМ	p-value
Group I	Mild 70%	50%	0.11
	Moderate 30%	23.3%	0.56
Group II	Mild 16.6%	13.3%	0.72
	Moderate 50%	40%	0.44
	Severe 33.3%	26.6%	0.57
SLEDAI: Systemic Lupus Erythematosus Disease Activity Index, EM: electron			

 Table 4: Correlation between EM blood cells changes and score in group I and II.

Serum 8-OHdG level

microscope

There was no statistically significant difference between group I and group II, but there was a significant difference between each patient group and the control group (Table 5).



Figure 4: DNA migration (mm) in the blood cells assessed by Comet assay in group I and group II (A). Data are means \pm SEM (n=5). B) Representative images illustrating the quantification of the DNA migration by the Comet assay. There were significant differences in Group I and group II DNA strand breaks between lymphocytes and whole blood in control group (1 and 2), group I(3 and 4) and group II(5 and 6).

Parameter	Group I	Group II	Control (III)
8-OHdG ng/mL	295.5±34.7	343.5 ±39.6	95.5 ± 32.1
Note: P I&III < 0.0001, P II&III < 0.0001 and P I& II 0.295. 8-OHdG: 8-hydroxydeoguanosine.			

Table 5: Levels of Serum Levels of 8-OHdG in all groups.

Comet assay of DNA (tail DNA migration)

There was significant remarkable increase in percentage of tail DNA damage in Comet assay in Group I and Group II in comparison to

control (p<0.05). The percentage of tail DNA damage was significantly elevated in Group II in comparison to Group I (p< 0.01) (Figure 4).

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Discussion

Despite of several treatment strategies used in SLE, recurrent exacerbation attacks supervene all through patients' lives. Various continuous symptoms frequently retard the patient daily activities. Headache, pain, fatigue, delirium, and many constitutional symptoms are unbounded complains in lupus patients [23]. SLE is characterized by ongoing complement activation followed by deposition of complement fragments on circulating blood cells. The deformed blood cells by complement fragment have limited circulation and functions [23]. The ongoing pathogenesis in blood cells may contribute to all these symptoms. So, the present study was conducted to investigate the ultrastructure and cytogenetic changes of blood elements in SLE patients.

Focusing on the blood cells function, we used electron microscopy to detect the sub laboratory changes of blood cells which we considered that it is more accurate methodology in assessing the cell membrane changes as a site of the pathogenesis rather than assessing only blood cells counts. Also, we used Comet assay to notice DNA abnormalities and measured 8-OHdG serum levels to illuminate the defects of oxygen delivery by the distorted cells.

Ultrastructure results demonstrated noticeable alterations in the morphology of blood cells, in which the nuclei showed less heterochromatin content and several nuclear envelope blebs. Vacuolated less electron dense cytoplasm was revealed and slightly irregular outline. These findings were in group I and group II with more obvious changes on group II but no significant differences between the disease groups whereas there was statistically significant difference between both patient groups in comparison to the control group. SLEDAI score was not sensitive enough to reveal the sub laboratory changes of blood cells in Group I with normal CBC because it depends on the number of the blood cells and not the function. Whereas, significant negative correlation was noticed between blood element defects and SLEDAI score in Group II. Our findings agreed with previous studies which revealed that the deposition of C3 and C4 complement fragments on the erythrocyte membranes is a link between decreased membrane deformability, tissue hypoxia and SLEspecific organ damage [24]. It was stated in another study that SLE patients have inflammatory ultrastructural changes in their blood elements [4]. Deformed platelets show blebbing, formation of microparticles and fusion with the erythrocyte membranes. Platelets interact actively with other inflammatory cells as white blood cells (WBCs). Concerted interactions of platelets, WBCs and erythrocytes inside the inflammatory fibrin networks predispose to the prothrombotic states in SLE patients [9].

As regards serum 8-OHdG level, there was statistically significant elevation between both patient groups in comparison to the control group. Comet assay revealed that the degradation of nuclear DNA in lymphocytes of SLE was seen to occur at elevated percentage. Comet test and 8-OHgG serum levels results confirmed the study hitting point where findings in group II were significantly higher than results of group I.

These observations could be explained by the fact that defects in the cell membrane lead to decreased ability of RBCs to deliver oxygen to the tissues [25]. Our findings in SLE patients of the diminished membrane deformability in RBCs, in association with chronic anemia, could explain some of the constitutional symptoms of patients with SLE such as chronic fatigue [26]. Another explanation is that the damaged blood cells lead to the release free radicals which cause damage vascular endothelial cells and smooth muscle cells [27]. Accumulation of the altered base, 8-OHdG, was proved to be a sensitive biomarker of reactive oxygen species-induced damage [28]. It was found that 8-OHdG accumulate in DNA and associate with the circulating immune complexes. Oxygen radicals induce chronic inflammatory state in SLE by maintaining the presence of the damaged antigenic form of DNA in the circulation [29].

Moreover, free radicals were also identified as a significant factor in inducing mitochondrial proliferation with hyperpolarization and induction of abnormal signaling pathways in T cells [30,31]. Higher oxidative stress with deficiency in DNA repair system, antioxidant enzymes and mitochondrial biogenesis may be implicated in SLE deterioration [32].

Conclusion

The current study described the ongoing pathological cytogenetic effect of SLE on blood cells with sub laboratory ultrastructure changes that could explain several pathogenic aspects of SLE manifestations. Therefore, blood cells changes are not only considered as SLE biomarker for early detection of subtle disease activity, but also an important therapeutic target for intercepting abnormal signaling. Limitations of the present study are: the small sample size, the missed declaration of our findings with different treatment lines and the non-quantitative data obtained from EM findings as there was no scoring methods used to interpret these findings.

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