Circulating Endothelial Cells And Cardiovascular Risk In Systemic Lupus Erythematosus

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ABSTRACT: Premature atherosclerosis seen in systemic lupus erythematosus (SLE) patients is not explained by traditional risk factors. Circulating endothelial cells (CECs) have been shown to be a surrogate marker of endothelial dysfunction. The aim of this study was to assess the number of CECs in SLE patients and to determine any potential correlation between CEC count and endothelial function (FMD%), disease activity, organ involvement and therapy used. Also, to investigate VCAM-1and ICAM-1 levels as markers of vascular inflammation and injury. This study was performed on 30 premenopausal female SLE patients and 20 age and sex matched healthy controls (HC). Patients were subjected to full history taking, complete clinical examination and assessment of disease activity using (SLAM) score. For both patients and controls, endothelial function (FMD%), laboratory estimation of CEC count, and serum level of VCAM-1 and ICAM-1 were performed. CEC count was significantly elevated in SLE patients comparing to HC ($P<0.001$). CEC count was positively correlated with SLAM score, while negatively correlated with FMD%. Serum levels of VCAM-1 and ICAM-1 were significantly increased in SLE patients than controls. Moreover, VCAM-1 correlated significantly with disease activity and CEC count while ICAM-1 did not correlate with any of them. There was significant correlation between CEC count and skin vasculitis, renal involvement and anti-malarial medications. In conclusion, increased number of CEC may be a biomarker of disease activity and disseminated vasculopathy occurring in the course of SLE and may represent one of the first specific cellular markers to provide a direct link with the pathophysiology of cardiovascular disease (CVD).VCAM-1 is considered a marker of activation of endothelial cells. Taking together, this may predict patients at increased risk of CVD complications, lupus nephritis or vasculitic skin affection.
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Key words: Circulating endothelial cells (CEC), SLE, adhesion molecules

1-INTRODUCTION:

It has been established that patients with systemic lupus erythematosus (SLE) are at increased risk of cardiovascular (CV) mortality and morbidity (Roman et al., 2003). However, the premature atherosclerosis and endothelial dysfunction in SLE are not solely attributed to traditional risk factors (Edile et al., 2001).

The risk of developing coronary heart disease remains increased 8-10 fold even after adjustment of risk factors identified in the Framingham Heart Study (Edile et al., 2001). This prompted us to investigate additional factors that might be related to disease process itself.

As we all know, the primary pathological findings in SLE patients are those of inflammation, immune complex deposition, altered angiogenesis and vacuities (Robak et al., 2009). Furthermore, the vascular endothelium in general is "primed" for injury by activated leucocytes (Belmont et al., 1997).

Circulating endothelial cells (CEC) are thought to be mature cells that have detached from the intimal mono layer in response to endothelial injury (Boos et al., 2006). Several possibilities can be considered for the mechanism responsible for endothelial detachment. It might be due to apoptosis, mechanical dislodgment of cells, proteolysis of subendothelial matrix proteins, or a consequence of complement dependent injury (6 Hunting et al., 2005).

Endothelial cells (EC) are potential participant in the inflammatory processes that contribute to tissue damage. Furthermore, the activated phenotype of circulating endothelial cells suggests that they may be capable of vascular injury by producing prothrombotic mediators (Robak et al., 2009).

Although endothelial damage due to deposition of immune complexes is considered to be one of the main pathogenetic traits of SLE, other alternative mechanisms should also be taken into account when pondering the etiology of SLE microangiopathy - first and foremost inflammatory immune lesions of endothelial cells (Kluz et al., 2009).

Endothelial damage and dysfunction as well as increased leukocyte migration to loci of inflammation, mediated by adhesion molecules, are

believed to be key factors in the induction of vaculitis (Guillevin et al., 2007). Growing evidence, including increase in the expression of vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) on the endothelial cell surface, speaks for endothelial activation in SLE (Robak et al., 2007 and Constans et al., 2003).

The aim of this study was to assess the number of CECs in SLE patients and to determine any potential correlation between CEC count and endothelial function (FMD%), disease activity, organ involvement and therapy used. Also, to investigate VCAM-1 and ICAM-1 levels as markers of vascular inflammation and injury.

2-SUBJECTS AND METHODS

This study was conducted on 30 premenopausal SLE female patients and 20 age and sex-matched healthy controls (HC). They were recruited from the Internal Medicine and Rheumatology & Rehabilitation Departments, Tanta University Hospital, Egypt. All patients met the American College of Rheumatology (ACR) criteria for SLE (Tan et al., 1982). Assessment of disease activity was achieved by use of (SLAM) score (Liang et al., 1989). Clinical assessment included physical examination and laboratory investigations; also a complete medication history was obtained.

A written consent prior to participation in the study was taken from all patients and controls.

Exclusion criteria:

1- Coronary artery disease, myocardial infarction and cardiac insufficiency which affect CEC count (Robak et al., 2009).

2- Patients received heamoperitoneal dialysis or had undergone kidney transplantation(de Groot et al., 2005)

3- Patients with clinical signs of infection or neoplastic disease(3 Robak et al., 2009)

4- Diabetic patients(Deng et al., 2009)

5- Patients with evidence with other disease known to cause endothelial dysfunction.

6- Patients received other medications for at least 4 weeks before blood donation (Robak et al., 2009).

Assessment of endothelial function by flow mediated dilatation (FMD %):

Endothelial function was assessed with high-resolution B-mode Doppler (ATL HDI 5000 with a 7.4 – MHz linear –array transducer) examination of the brachial artery using the protocol described by Rajagopalan et al. (2002). Briefly, the test was reformed in the morning in quiet, low light room; subjects had fasted and not smoked for at least the preceding 12hs. The brachial artery was scanned 5-15 cm above cubital fossa. Resting diameter was measured. Then blood pressure cuff inflated to 300 mmHg around forearm and further scan was done 1 minute during occlusion then after occlusion (cuff release) by 1 minute.

FMD was calculated as follows:

[(post deflation diameter - resting diameter) / resting diameter] X 100.

Sampling:

Venous blood samples (10 ml) were taken from each patient and controls and separated into two tubes: one tube (5ml) was collected into ethylene diaminetetra acetic acid (EDTA) and immediately transferred into citrate-theophylline-adenosine-dipyridamole (CTAD) anticoagulant, which recently has been shown to maximize antigen stabilization on leucocytes. Anticoagulated blood samples were kept at 48C and analyzed by flow cytometry within 4 hr of venesection (Macey et al., 2003).

The remaining 5 ml were left to clot at room temperature for 30 min in the second tube then centrifuged at 1500 rpm for 15 min and serum was separated stored at -80° C till time of assay of serum ICAM-1, VCAM-1, anti-phospholipid antibodies, C3, C4, ANA, anti-dsDNA, urea, creatinine, lipid profile and blood glucose

24 hours urine was collected from patients for creatinine clearance, urinary protein and complete urinalysis.

Study measurements:

- Complete blood count using Advia 60 Cell Counter (Bayer),
- Erythrocyte sedimentation rate by Westergren method.
- Serum ANA was assessed by indirect immunofluorescence using Hep-2 cells and anti-double-stranded DNA by indirect immunofluorescence on Crithidia luciliae (Sanofi Diagnostics Pasteur Inc, Minnesota, USA) (Fritzler 1992).
- Complement 3 (C3) and complement 4 (C4) were assayed by nephelometry (Behringwerke, Marburg, Germany)(Virella 1980). Antiphospholipid (APL) antibodies were measured by ELISA technique (HM007, Technoclone Diagnostics Ltd., UK).
- Quantitative sandwich ELISA technique was used to measure serum concentration of ICAM-1 (BMS201CE human sICAM-1, Bender Biosystem, Vienna, Austria, Europe) and VCAM-1 (BMS232CE human sVCAM-1, Bender Biosystem, Vienna, Austria, Europe). The standard range of sICAM-1

was 6.25 - 100 ng/ml and sVCAM-1 was 3.2 - 100 ng/ml (Robak et al., 2007).

- Serum creatinine, lipid profile, blood urea and glucose concentration were measured by using standard laboratory techniques on Synchron CX7 autoanalyzer (Beckman Instruments, CA, USA).
- Complete microscopic urine analysis for WBCs, RBCs, and casts, 24-hour urinary protein excretion (UPE) by the turbidimetric method using TP Kit supplied by Stanbio (Stanbio Laboratory Inc., San Antonio, USA) and creatinine clearance were measured for renal assessment.

Immunophenotyping of CECs Flow cytometry (Goon et al., 2006):

Freshly isolated peripheral blood mononuclear cells (PBMCs) were washed and separated from blood of patients and healthy control using 1X FACs lysis solution (BD) for erythrocytes lysis then PBMCs were resuspended in phosphate buffered saline (PBS, pH 7.4) containing 20 uL of the appropriate antibody and cells were double stained with mouse anti-human flurescein isothiocynat (FITC) conjugated CD45 antibody and mouse anti-human phycoerythrin conjugated CD 146 antibody (BD Biosciences) to identify CD45- and CD146+ respectively. The isotype control was used to determine nonspecific binding of the lymphocyte subset-specific antibodies and to set the cut-off between fluorescence-negative and fluorescence-positive staining. Stained cells were washed three times with 1% bovine serum albumin BSA-PBS, pH 7.2, and then 7AAD was added to stain dead cells. The cells were analyzed within 15 minutes after addition of 7AAD using a fluorescence-activated cell scanner and Cell Quest software [FACS Caliber, Becton-Dickinson (BD)]. Cells were plotted according to forward scatter (FSC) and side scatter profiles (SSC) and a region was drawn around the small, live cell population containing the lymphocyte. The cell population data obtained from the quadrant statistics (2- color staining) was standardized for the number of mature CEC using the sum of CD45-, CD146+ and 7-AAD negative (Live) cells within this region (i.e., CD45-, CD146+ and 7-AAD+ cells were not accounted). Normal CEC count by flow cytometry was < 20 cells/ml (Woywodt et al., 2006).

Statistical analysis:

Data were analyzed using SPSS version 11.5. Descriptive statistics were done by number and percent as well as mean, median and range. Unpaired student's t-test was used for comparison between groups. Correlation between variables was calculated using Spearman's correlation coefficient. $P < 0.05$ was considered statistically significant.

3RESULTS:

The cardiovascular (CV) risk profile regard to obesity, smoking, hypertension, hyper lipidaemia and diabetes mellitus did not differ significantly between patients and controls (Table 1).

Circulating endothelial cells are elevated in SLE patients:

Circulating endothelial cells (CEC) count in peripheral blood was significantly elevated in SLE patients than HC $p<0.001$ {39.1 (22.5 - 55.7) vs. 7.8 (0.9 - 14.7)} and in active than in non active disease $p<0.001$ {42.6 (29.5 - 55.7) vs. 25.7 (22.5 - 28.9)} (table 2, figure 1). CEC count from patients with vasculitic skin lesion and renal manifestations was significantly higher than patients without these manifestations ($p< 0.01$).

Impaired endothelial function (FMD %) is linked to CEC count:

FMD% was significantly reduced in SLE patients than HC [3.8 (0.5 - 7.25)] versus [8.45 (4.50 - 12.40)] respectively ($P<0.05$), and in the active than in non active disease [2.55 (0.60 - 4.50)] versus [4.2 (1.20 - 7.20)] respectively ($P<0.05$) (Table2).

There was a significant negative correlation between CEC count and FMD% ($r=-0.942$, $P<0.001$) (Table 3).

VCAM-1 and ICAM-1were significantly increased in SLE patients than HC [276.5 (103 -450)] and [149.5 (103-196)] versus [66 (37- 95)] and [69.5 (57- 82)] $P<0.05$ respectively.

Moreover, VCAM-1 was significantly increased in the active than in non active disease, with a significant correlation with CEC count [$r=0.917$, $P<0.001$] (Table 2, 3), while there was no significant variation in ICAM-1 during SLE flare or any correlation with CEC count.

Analyzing the relationship between CEC count and the presence of particular clinical and laboratory parameters of the disease, organ involvement and therapy used, revealed significant positive correlation between CEC count and SLAM score, vasculitic skin lesion and renal involvement, but there was significant negative correlation between CEC count and low complement and antimalarial medications. On the other hand, we did not find any correlation between CEC count and joint involvement, CNS involvement or steroid medication.

Table (1): Characteristics of SLE patients and controls

	SLE (n=30)	HC(n=20)	P
Demographics			
Age (years mean ±SD)	25.68±7.78	23 ± 5.8	NS
Disease duration (years mean ±SD)	9 ± 3.7	-	
Cardiovascular risk factors			
BMI (kg/m^2)	7%	8%	NS
Smoking (%)	0%	0%	NS
Hyperlipidaemia (%)	6%	4%	NS
Hypertension (%)	19%	15%	NS
Diabetes mellitus **(%)**	2%	0%	NS
Clinical & laboratory features of patients (%)			
Active (%)	57%		
Inactive (%)	43%		
Low C3&C4 (%)	45%		
ANA +ve (%)	100%		
Anti-ds DNA +ve (%)	60%		
Anti-phospholipids antibody positive (%)	50%		
Medication usage (%)			
Antimalarials (%)	69%		
Steroids (%)	45%		
Cyclophosphamide (%)	13%		
Steroids + immunosup-pressant (%)	34%		
Organ involvement (%)			
Joint involvement (%)	56%		
Renal involvement (%)	48%		
Cerebral involvement (%)	11%		
Skin involvement (%)	28%		

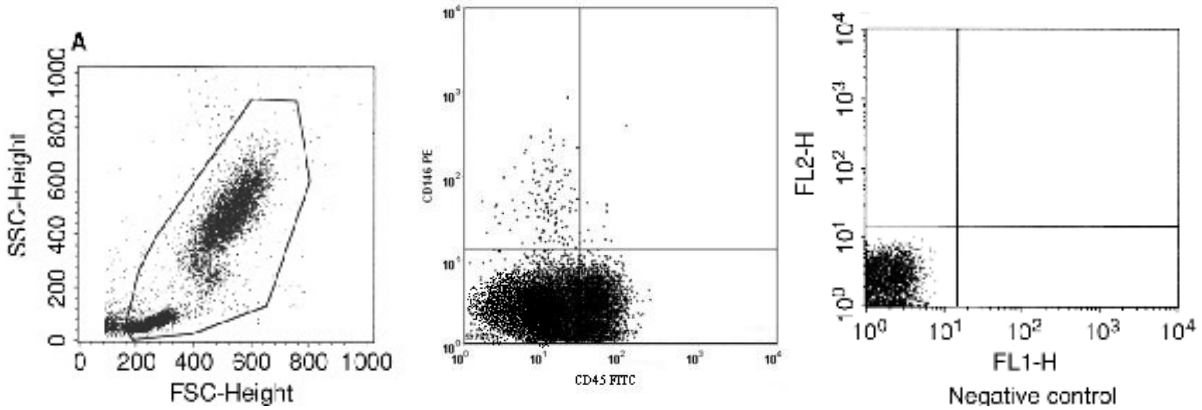
Table (2): CEC count and other parameters in SLE patients and controls.

		CEC count (cells/ml)	FMD%	VCAM-1 (ng/ml)	ICAM-1 (ng/ml)
(a)	**SLE=30 Median (Range)**	39.1 (22.5-55.7)	3.8 (0.5-7.25)	276.5 (103-450)	149.5 (103-196)
(b)	**Active =17 Median (Range)**	42.6 (29.5-55.7)	2.55 (0.60-4.50)	288 (126-450)	156 (116-196)
(c)	**Inactive=13 Median (Range)**	25.7 (22.5-28.9)	4.2 (1.20-7.20)	149.5 (103-196)	123 (103-143)
(d)	**Control =20 Median (Range)**	7.8 (0.9-14.7)	8.45 (4.50-12.40)	66 (37-95)	69.5 (57-82)
P		(a)vs(d)P<0.001 (b)vs(c)P<0.001	(a)vs(d)P<0.05 (b)vs(c)P<0.05	(a)vs(d)P<0.05 (b)vs(c)P<0.05	(a)vs(d)P<0.05 (b)vs(c)P>0.05

CEC: circulating endothelial cells; FMD: flow mediated dilatation; VCAM-1: vascular cell adhesion molecule; ICAM-1 intracellular adhesion molecule

Table (3): Correlation between CEC count and different parameters

	CEC count	
	r	p
FMD%	-0.942	<0.001
SLAM score	0.966	<0.001
Low complement	-0.384	<0.05
VCAM-1 ng/ml	0.917	<0.001
ICAM-1 ng/ml	0.201	>0.05
Steroid therapy	0.101	>0.05
Antimalarial	-0.451	<0.05
Vasculitic skin lesion	0.662	<0.01
Renal involvement	0.541	<0.01
Joint involvement	0.132	>0.05
CNS involvement	0.168	>0.05



(A) **(B)** **(C)**

Figure (1): Flow cytometry evaluation of circulating endothelial cells (CECs). (A) Representative panel showing the analysis gate used to exclude platelets and debris. (B) Representative histogram of CECs cells (CD45- and CD146+). (C) Representative panels showing the negative control. PE, phycoerythrin; FITC, fluorescein isothiocyanate.

4-DISCUSSION

Although premature atherosclerosis and endothelial dysfunction are well known co-morbidities associated with SLE, their underlying cause is not fully explained by traditional risk factors (Lee et al., 2007).

Circulating endothelial cells (CECs) predict vascular function and serve as a surrogate marker of endothelial dysfunction and cumulative CV risk (Lee et al., 2007). CEC activation, described in patients with SLE, was suggested to be a potential inflammatory process mediator, able to induce progressive vascular damage on the vicious circle principle (Clancy et al., 2001).

Our results showed significant elevation in CEC count in SLE patients than healthy controls. This result was in agreement with those reported by Clancy et al. (2001) Woywodt et al.(2003) Robak et al.(2009) and Kluz et al. (2009). This suggests that increased number of CEC may be a marker of disseminated vasculopathy occurring in the course of SLE (Robak et al.2009).

In the contrary to our data, however, two other reports showed significant deficiency of CEC count in their SLE patients (Lee et al., 2007 and Westerweel et al., 2007). The explanation of these discrepancies may be due to the fact that the population studied here comprised totally Egyptian, whereas the study of Lee et al. (2007) involved Africans, Americans, whites and others to nearly equal parts. In their study the ethnic distribution among controls was not matched to that among patients. Also those involved in Westerweel et al. (2007) study were in clinical remission but our patients include active and inactive disease.

Although elevated CEC count are observed mostly in conditions linked with endothelial damage, It seems that the dissociation of mature endothelial cells (ECs) from the vascular wall due to its damage is not the sole reason for increasing CEC numbers noted in those patients. It seems more probable that

extensive vascular involvement, resulting in the release of "desquamated" EC into peripheral blood, also mobilizes medullary endothelial progenitor cells (EPCs) reserves, as well as stimulates their differentiation into mature endothelial cells as a compensatory response to its damage (Kluz et al., 2009).

In our study, there was strong positive correlation between CEC count and disease activity. This observation matches with those reported by Elshal et al.(2009), Kluz et al.(2009), Sesin et al.,(2005) and Clancy et al.(2001), but, contrasting that reported by Robak et al. (2009).

Many previous studies concluded that endothelial function (estimated by FMD) serves as a better marker of vascular reactivity than traditional risk factors (Lee et al., 2007).Also, Moreover, endothelial dysfunction is the key point in both the development of vascular inflammation and atherosclerosis (Deng et al., 2009). In our study FMD% was significantly reduced in SLE patients than HC with significant negative correlation between CEC count and FMD. Soltecz et al. (2010) found that the endothelium dependent vasodilation (FMD) was significantly impaired in patients with MCTD, as compared to controls and concluded that FMD is a reliable sensitive marker of endothelial cell dysfunction in MCTD.

In analyzing the relationship between CEC count and organ involvement, we found that CEC count from patients with vasculitic skin lesion and renal manifestations was significantly higher than patients without these manifestations, a result which matches with that of Elshal et al. (Elshal et al., 2009). Also, we found a significant positive correlation between CEC count and both of skin vasculitis and renal involvement. This indicates that endothelial injury, as a part of immune mediated vascular damage, could play a crucial role in the pathogenesis of these manifestations. Sesin et al. (2005) showed that decreased expression of endothelial protein c receptor in CEC of SLE patients may predict and/or reflect vasculopathy and renal injury in SLE. Moreover, we found a significant correlation between CEC count and low complement, a finding which is in consistent with the previous study of Clancy et al. (2001).

Regarding the relationship between CEC and therapy used, we found a significant negative correlation between CEC count and antimalarial medications, but no correlation with steroids. Jung et al. (2010) demonstrated that antimalarial drugs are thromboprotective in SLE with a risk reduction of thrombovascular events of 68%. However, Lee et al.(2007) and Robak et al.(2009) concluded that there was no correlation between CEC count and steroids, anti- malarials or cytotoxic agents.

It has been established that leukocyte stimulation due to complement activation (C3 and C4) is an important step in the development of endothelial dysfunction. Simultaneously, through the influence of numerous immune stimuli such as cytokines, immune complexes, antiendothelial or antiphospholipid antibodies, surface expression of adhesion molecules in endothelial cells is enhanced (Clancy et al., 2001).

In glomerulonephritis murine models, increased expression of VCAM-1 and ICAM-1 was found in renal tissue (McHal et al., 1999). Moreover, greater survival rates were observed in mice deprived of ICAM-1, suggesting that their enhanced expression may play a considerable role in SLE pathogenesis (Kevil et al., 2004). Furthermore, in numerous patients with active vasculitis, no circulating or deposited immune complexes are found. Similarly, in many instances, such a deposition within vessel wall does not lead to inflammatory infiltration or fibrotic necrosis development. Hence the suggestion that alternative mechanisms might be involved in the pathogenesis of inflammatory vascular lesions, such as interaction between VCAM-1 and very late activation antigen (VLA-4) as well as ICAM-1 and leukocyte function-associated antigen (LFA-1), determining the adhesion of leukocytes to endothelial cells and their subsequent damage linked with cytokines and neutrophils (Guillevin et al., 2007).

In this study, there was significant elevation of VCAM-1 and ICAM-1 in SLE patients than HC, but regarding their relations to disease activity and CEC count, VCAM-1 significantly increased with disease activity and correlate positively with CEC count, which led to the suggestion that increased expression of VCAM-1 plays a leading role in the pathogenesis of SLE and is a direct cause of enhancing activated leukocytes migration, responsible for inflammatory tissue lesions. A similar result was reported by Kluz et al.(2009), but opposite result was reported by Robak et al.(2009). Additionally, there was no interrelationship between ICAM-1 and CEC count or disease activity, implying that increased expression of this molecule seems to be primarily an indicator of a generalized EC dysfunction rather than a marker reflecting the degree of endothelial damage. This observation agrees with that reported by Kluz et al. (2009). However, the pathogenesis of SLE is complex, and it is based on several overlapping regulatory loops; so, further studies are needed to determine the relationship between CEC and angiogenic proteins and inflammatory cytokines.

CONCLUSION:

*Increased circulating endothelial cells number constitute a reliable marker of disease activity in SLE, reflecting endothelial damage and thus enabling the distinction of a patient group running a higher risk of vascular lesion development.

* Progressive increase in serum VCAM-1 concentration is linked with progression of SLE activity and development of lupus angiopathy.

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