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Original Article

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Vasculitis in systemic lupus erythematosus (SLE) – assessment of peripheral blood mononuclear cell activation and the degree of endothelial dysfunction: Initial report

Zapalenie naczyń w przebiegu układowego tocznia trzewnego (SLE) – ocena aktywacji jednojądrzastych komórek krwi obwodowej i stopnia dysfunkcji komórek śródbłonka (Doniesienie wstępne)

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Inflammatory-immune changes in the vascular endothelium are one of the main factors initiating vessel wall damage. Enhanced expression of endothelial adhesion molecules and their receptors on the surface of circulating leukocytes seems to play an important role in the pathogenesis of vasculitis. Increasing evidence indicates endothelial cell activation/damage in SLE. In patients with SLE complicated by vasculitis, enhanced expression of integrin activation markers on the surface of peripheral blood mononuclear cells (PBMCs) has been reported. It seems relevant to assess the mechanisms of inflammatory response involving PBMCs and endothelial cells at particular stages of SLE microangiopathy.

Aim: The main aim was to assess the surface expressions of the integrin adhesion molecules VLA-4 (CD49d) and LFA-1 (CD11a) on PBMCs as well as the number of circulating endothelial cells (CECs) in patients with SLE and complications related to inflammatory microangiopathy and to determine whether these parameters vary depending on disease activity.

Patients: Twenty-nine women with SLE (mean age: 38.72±10.23 years) were divided into subgroup I: those with severe disease activity according to the modified disease activity index SLEDAI, characterized by the presence of inflammatory microangiopathy-related complications such as systemic central nervous system affection and/or vasculitis and/or nephritis (15 women, mean age: 38.33±11.02 years), and subgroup II: patients with mild or moderate disease activity according to SLEDAI and without vascular complications (14 women, mean age: 39.14±9.72 years).

Methods: Expressions of VLA-4 and LFA-1 on the surface of peripheral blood lymphocytes and monocytes were assessed by flow cytometry using monoclonal antibodies. CECs (a marker of endothelial damage) were isolated from peripheral blood with anti-CD146(S-Endo 1)-coated immunomagnetic Dynabeads. Tests for the lupus anticoagulant, antinuclear antibody, anti-dsDNA, and anticardiolipin antibody were performed in every study subject by ELISA. Erythrocyte sedimentation rate and serum levels of fibrinogen, C-reactive protein, the complement components C3

and C4, urea, creatinine, and uric acid were determined by standard methods. Peripheral blood counts and a general urinalysis were also performed.

Results: The mean CEC count was significantly higher in SLE patients than in the control group (15.29±12.10 vs. 3.08±1.46 cells/ml, $p<0.001$). CEC counts was notably elevated in patient subgroup II compared with the control group (9.14±5.16 vs. 3.08±1.46 cells/ml, $p<0.05$) and in subgroup I compared with subgroup II (21.03±13.96 vs. 9.14±5.19 cell/ml, $p<0.05$). In patients with severe SLE flares, CEC count visibly correlated with disease activity assessed by SLEDAI score ($R=0.92$, $p<0.001$). The expressions of VLA-4 and LFA-1 on peripheral blood lymphocytes in both patient subgroups were significantly higher than in the control group (subgroup I vs. controls: 1.70±1.56 vs. 0.39±0.26%, $p<0.05$, and 1.97±2.60 vs. 0.67±0.83%, $p<0.05$; subgroup II vs. controls: 1.71±1.04 vs. 0.39±0.26%, $p<0.001$, and 3.32±2.48 vs. 0.67±0.83%, $p<0.05$, for VLA-4 and LFA-1, respectively). There was no significant difference between the two subgroups of patients (1.70±1.56 vs. 1.71±1.04%, $p>0.05$, and 1.97±2.60 vs. 3.32±2.48%, $p>0.05$, respectively). Similarly, the surface expression of LFA-1 on circulating monocytes in patients in both subgroups was notably enhanced over that of the control group (91.44±16.00 vs. 84.95±19.86%, $p<0.05$, and 90.11±10.34 vs. 84.95±19.86%, $p<0.05$, in subgroups I and II respectively) and was comparable in both subgroups of patients (91.44±16.00 vs. 90.11±10.33%, $p>0.05$). The surface expression of VLA-4 on peripheral blood monocytes was considerably higher in patients with severe disease activity than in the control group and in patients with less active disease (77.10±13.56 vs. 64.90±19.13%, $p<0.05$, and 77.10±13.56 vs. 63.40±20.95%, $p<0.05$, respectively). However, there was no significant difference between patients with mild or moderate disease activity and the control group (63.40±20.95 vs. 64.90±19.13%, $p>0.05$).

Conclusions: 1) The number of CECs increases in the course of SLE and correlates with disease activity, indicating progressive endothelial damage.

2) The expressions of VLA-4 and LFA-1 on the surface of peripheral blood lymphocytes as well as that of LFA-1 on circulating monocytes are enhanced in SLE patients regardless of disease activity.

3) The expression of VLA-4 on the surface of circulating monocytes is enhanced only in patients with severe disease activity, characterized by the presence of complications connected with inflammatory microangiopathy, which may indicate that the upregulation of VLA-4 expression in monocytes plays a leading role in the pathogenesis of vasculitis in SLE.

Key words: **systemic lupus erythematosus (SLE) • circulating endothelial cells (CECs) • vasculitis • VLA-4 • LFA-1 • monocytes • lymphocytes**

Streszczenie

Jednym z głównych czynników inicjujących uszkodzenie ściany naczyniowej są zmiany immunologiczno-zapalne śródbłonka naczyń krwionośnych. Zwiększona ekspresja śródbłonkowych molekuł adhezyjnych oraz odpowiadających im receptorów na powierzchni krążących leukocytów wydaje się odgrywać podstawową rolę w patogenezie zapalenia naczyń. Wiele dowodów wskazuje na to, iż w przebiegu SLE dochodzi do aktywacji i uszkodzenia komórek śródbłonka. U pacjentów z SLE wikłanym zapaleniem naczyń opisywano zwiększoną ekspresję integrynowych markerów aktywacji na powierzchni mononuklearów krwi obwodowej. Stąd celowa wydaje się ocena zjawisk odpowiedzi zapalnej z udziałem jednojądrzastych komórek krwi obwodowej i komórek śródbłonka na poszczególnych etapach rozwoju mikroangiopatii toczniowej.

Zasadniczym celem pracy była ocena powierzchniowej ekspresji integrynowych molekuł adhezyjnych VLA-4 (CD49d) i LFA-1 (CD11a) na jednojądrzastych komórkach krwi obwodowej oraz ilościowa ocena krążących komórek śródbłonka (CECs) u chorych na SLE wykazujących obecność powikłań na podłożu zapalnej mikroangiopatii. Ponadto, określenie czy ekspresja wymienionych integryn i liczba CECs zmieniają się w zależności od aktywności choroby.

Pacjenci: 29 kobiet z rozpoznaniem SLE, w wieku 17–54 lat (średnia wieku 38,72±10,23 lat). Podgrupa I: pacjentki z dużą aktywnością kliniczną choroby wg zmodyfikowanej skali aktywności choroby SLEDAI, wykazujące obecność powikłań na podłożu zapalnej mikroangiopatii o typie układowego zajęcia CUN i/lub *vasculitis* i/lub *nephritis* – 15 osób (średnia wieku 38,33±11,02 lat). Podgrupa II: pacjentki z łagodną i umiarkowaną kliniczną aktywnością choroby wg zmodyfikowanej skali aktywności choroby SLEDAI, niewykazujące obecności ww. powikłań – 14 osób (średnia wieku 39,14±9,72 lat).

Metody: ekspresję VLA-4 i LFA-1 na powierzchni limfocytów i monocytów krwi obwodowej określono metodą cytofluorymetrii przepływowej z zastosowaniem zestawu przeciwciał monoklonalnych. Za wykładnik stopnia uszkodzenia śródbłonka przyjęto liczbę CECs, którą oznaczono metodą separacji immunomagnetycznej z użyciem przeciwciała monoklonalnego S-Endo 1. U każdej osoby wykonano badania w kierunku obecności antykoagulanta toczniowego oraz przeciwciał przeciwjądrowych (ANA), przeciwciał przeciwko dwuniciowemu DNA (dsDNA) i przeciwciał antykardiolipinowych (ACA) [metoda immunoenzymatyczna rutynowo stosowana w klinicznym laboratorium naukowym]. Dodatkowo metodami standardowymi oznaczono stężenie fibrynogenu, białka C-reaktywnego, składowych C3 i C4 dopełniacza, mocznika, kreatyniny i kwasu moczowego w surowicy, odczyn opadania krwinek czerwonych oraz wykonano badanie morfologii krwi obwodowej i badanie ogólne moczu.

Wyniki: U chorych na SLE, w porównaniu z grupą kontrolną, wykazano znamiennie wyższą liczbę CECs (15,29±12,10 vs 3,08±1,46 [kom./ml]; p<0,001). Liczba CECs była istotnie podwyższona w podgrupie II, w porównaniu z grupą kontrolną (9,14±5,16 vs 3,08±1,46 [kom./ml]; p<0,05) oraz w podgrupie I, w porównaniu z podgrupą II (21,03±13,96 vs 9,14±5,19 [kom./ml]; p<0,05). W grupie chorych z aktywnym SLE liczba CECs w znaczący sposób korelowała z klinicznym nasileniem choroby, ocenianym za pomocą punktacji w skali SLEDAI (R=0,92, p<0,001). Powierzchniowa ekspresja VLA-4 i LFA-1 na limfocytach krwi obwodowej w obu podgrupach chorych była znamiennie wyższa, niż w grupie kontrolnej (odpowiednio: 1,71±1,04 vs 0,39±0,26 [%]; p<0,001 oraz 3,32±2,48 vs 0,67±0,83 [%]; p<0,05/1,70±1,56 vs 0,39±0,26 [%]; p<0,05 oraz 1,97±2,60 vs 0,67±0,83 [%]; p<0,05), bez istotnych statystycznie różnic pomiędzy podgrupą I i II (odpowiednio: 1,70±1,56 vs 1,71±1,04 [%]; p>0,05 oraz 1,97±2,60 vs 3,32±2,48 [%]; p>0,05). Podobnie powierzchniowa ekspresja LFA-1 na krążących monocytach u pacjentek z małą i umiarkowaną kliniczną aktywnością choroby oraz u pacjentek z dużą kliniczną aktywnością choroby była znacząco wyższa, niż u osób zdrowych (odpowiednio: 90,11±10,34 vs 84,95±19,86 [%]; p<0,05 oraz 91,44±16,00 vs 84,95±19,86 [%]; p<0,05) i przedstawiała się porównywalnie w obu podgrupach chorych (91,44±16,00 vs 90,11±10,33 [%]; p>0,05). Powierzchniowa ekspresja VLA-4 na monocytach krwi obwodowej była istotnie wyższa u pacjentek z wysoką kliniczną aktywnością choroby, w porównaniu z grupą kontrolną oraz pacjentkami z chorobą mniej aktywną (odpowiednio: 77,10±13,56 vs 64,90±19,13 [%]; p<0,05 oraz 77,10±13,56 vs 63,40±20,95 [%]; p<0,05). Nie stwierdzono natomiast znamiennych statystycznie różnic pomiędzy pacjentkami z małą i umiarkowaną kliniczną aktywnością choroby, a osobami z grupy kontrolnej (63,40±20,95 vs 64,90±19,13 [%]; p>0,05).

Wnioski: 1) liczba CECs wzrasta w przebiegu SLE oraz koreluje z aktywnością choroby, wskazując na postępujące uszkodzenie śródbłonka naczyniowego.

2) ekspresja VLA-4 i LFA-1 na powierzchni limfocytów krwi obwodowej oraz ekspresja LFA-1 na powierzchni krążących monocytów wzrasta w przebiegu SLE niezależnie od aktywności choroby.

3) ekspresja VLA-4 na powierzchni krążących monocytów wzrasta jedynie u pacjentek z chorobą wysoce aktywną, wykazujących obecność powikłań na podłożu zapalnej mikroangiopatii, co może wskazywać, iż zwiększona ekspresja tej integryny na monocytach odgrywa główną rolę w patogenezie zapalenia naczyń w przebiegu SLE.

Słowa kluczowe: **układowy toczeń trzewny • krążące komórki śródbłonka • zapalenie naczyń • VLA-4 • LFA-1 • monocyty • limfocyty**

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Introduction

Systemic lupus erythematosus (SLE) is a systemic connective tissue disease linked with the development of inflammatory lesions in cutaneous and internal organ vessels, including perivasculitis of the small arteries and periarteriolitis [8]. The hitherto unexplained pathogenic processes underlying the formation of inflammatory vascular lesions in SLE continuously prompt the search for new factors involved in the development of its vascular complications.

According to the latest views, the vascular inflammatory process is deemed to be connected with the deposition of complement-activating immune complexes within vessel walls as a result of auto-aggression. Although tissue deposition of immune complexes is considered to be one of the basic pathomechanisms in SLE, other possibilities should also be taken into account, first and foremost inflammatory-immune lesions of the vascular endothelium [8,24]. The pathological activation of peripheral blood leukocytes as well as endothelial cell damage/activation are believed to be key factors in the induction of this process [22].

Ample evidence, including increased expression of VCAM-1 (vascular cell adhesion molecule-1), ICAM-1 (intercellular adhesion molecule-1), and E-selectin on the endothelial cell surface, speaks for endothelial activation in SLE [8,19,26]. The enhanced expression of endothelial adhesion molecules and their surface receptors on circulating leukocytes seems to be of prime significance in both the pathogenesis of vasculitis and in its systemic exacerbation [8,24].

In patients with SLE complicated by vasculitis, enhanced expressions of VLA-4 (very late activation antigen) and LFA-1 (leukocyte function-associated antigen) have been observed on the surface of peripheral blood lymphocytes. In patients without vascular complications, only an increase in LFA-1 expression was noted, while VLA-4 expression remained similar to that found in a control group [23]. VLA-4 and LFA-1 are receptors for the corresponding endothelial surface adhesion molecules (VCAM-1 and ICAM-1). Interactions between VLA-4 and its endothelial ligand VCAM-1 determine a strong leukocyte (lymphocyte and monocyte) adhesion to the activated endothelial surface as well as their migration to loci of inflammation. It seems that enhanced surface VLA-4 expression in peripheral blood mononuclear cells (PBMCs) may play a significant role in the pathogenesis of inflammatory vascular lesions in SLE [23].

Compared with the general population, patients with SLE have an increased risk of cardiovascular disease development. The pathomechanisms of accelerated atherosclerosis and its dynamic evolution in these patients have not yet been fully explained. The presence of classical atherosclerosis risk factors does not elucidate higher cardiovascular incidence in SLE. It has become evident that "SLE diagnosis" remains the strongest predictor of cardiovascular disease, also in those patients in whom classical risk factors remain well under control [10].

In light of the latest studies, atherosclerosis has been defined as a chronic inflammatory-proliferative process. Presumably, monocytes/macrophages infiltrating the tunica intima play a key role in atherogenesis. The vascular inflammatory process leads to functional changes and endothelial damage, initiating the atherogenic transformation of arteries. On the other hand, endothelial dysfunction secondarily induces inflammatory processes within the vessel wall, thus closing the vicious circle.

Publications concerning monocyte involvement in the development/progression of vascular complications in SLE are scant. Part of the studies on monocytes in the pathogenesis of vasculitis and vascular proliferative lesions were limited to *in vitro* adhesion tests. In this context, attempts were made to evaluate the immune response involving peripheral blood monocytes in patients with SLE at different clinical stages of disease progression.

Desquamated CECs, present in peripheral blood, are markers of endothelial damage. Several studies show that CEC numbers are higher in active SLE and may reflect progressive vascular lesions, also in patients without clinical symptoms of vasculitis [5,20].

The principal aim of the study was to assess the surface expressions of the integrin adhesion molecules VLA-4 (CD49b) and LFA-1 (CD11a) on PBMCs as well as quantitative analysis of CEC numbers in patients with SLE and complications related to inflammatory microangiopathy. In this study it was demonstrated that CEC counts increase in SLE and correlate with disease activity. Furthermore, surface expressions of VLA-4 and LFA-1 in lymphocytes as well as LFA-1 expression in monocytes were enhanced in patients with SLE in comparison with healthy controls, regardless of disease activity, while surface VLA-4 expression in monocytes increased only in patients with a severe disease activity index and microangiopathic complications. The results may indicate that enhanced surface VLA-4 expression in monocytes could play a key role in the pathogenesis of vasculitis in SLE.

Study Population and Methods

Twenty-nine women with SLE aged 17 to 54 years (mean: 38.72±10.23 years), meeting the 1997 American College of Rheumatology diagnostic criteria, were enrolled in the study. The study group was divided into two subgroups:

I) patients with severe disease activity, a modified Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) of >12 points, and inflammatory microangiopathy-related complications such as systemic CNS affection and/or vasculitis and/or nephritis (15 women, mean age: 38.33±11.02 years).
II) patients with mild or moderate disease activity and an SLEDAI of >3 points (14 women, mean age: 39.14±9.72 years).

The control group included 12 healthy female volunteers aged 25 to 49 (mean: 37.17±9.56 years).

Venous blood samples were collected from all the study subjects in order to determine surface VLA-4 and LFA-1 expressions in peripheral blood lymphocytes and monocytes by flow cytometry as wells as CEC numbers using an immunomagnetic method. Moreover, all blood samples were tested for lupus anticoagulant, antinuclear antibodies (ANAs), anti-dsDNA antibodies, and anticardiolipin antibodies (ACAs) using commercial ELISA kits routinely used in clinical laboratories.

Determination of CEC counts

To isolate CECs, 4-ml venous blood samples were collected by atraumatic puncture on heparin (0.4 ml heparin to 4 ml blood). The first 5 ml were excluded from testing in order to avoid false positives due to the presence of endothelial cells resulting from vein traumatization. Immunomagnetic

Dynabeads (4.5 µm, Dynal Biotech, Norway) with anti-mouse IgG immunoglobulin (Dynabeads Pan Mouse IgG, Dynal Biotech, Norway) covalently bound to the bead surface were coated with S-Endo 1 (Mouse Mab anti-human CD146 endothelial cells, clone F4-35H7, Biocytex, France) against human umbilical vein endothelial cells. The antibody does not interact with erythrocytes, lymphocytes, monocytes, granulocytes, platelets, or with cultured fibroblasts and smooth muscle cells. PBS (875 µl) was added to a 125-µl magnetic bead suspension and subsequently washed with PBS four times in a magnetic separator (MPC-S, Dynal Biotech, Norway). Then 100 µl of S-Endo monoclonal antibody was added to the bead suspension and incubated for 1h at 4°C with continuous mixing. The suspension was then washed four times with PBS, as before. Thus prepared, the beads were suspended in 1000 µl of PBS and stored at 4°C. One hundred µl of the magnetic bead suspension sensitized with S-Endo 1 antibodies was added to diluted blood (4 ml blood + 4 ml PBS) and incubated for 1 h at 4°C with continuous mixing. After washing with PBS as before, the beads were suspended in 50 µl of PBS. The number of endothelial cells forming rosettes with the magnetic beads was determined in a Nageotte counting chamber (Paul Marienfeld, Germany) under fluorescence microscope using the cells' autofluorescence phenomenon to allow identification. The rosette suspension was synchronically stained with acridin orange, a fluorescent stain. As the criteria for endothelial cell identification, at least four magnetic beads bound to a cell with a diameter of minimum 20 µm were required.

Intra- and inter-assay variations were 7% and 11%, respectively. Results are expressed quantitatively as the number of cells per milliliter of whole blood.

Determination of surface VLA-4 and LFA-1 expression in peripheral blood monocytes and lymphocytes

1) Initial isolation of mononuclear cells

To isolate mononuclear cells from peripheral blood, 5 ml of venous blood was collected on heparin (0.5 ml heparin to 5 ml blood). The samples were diluted with Mg^{2+}- and Ca^{2+}-free PBS (1:1 ratio) and gently mixed. 1.25 ml Gradisol and 2.5 ml diluted blood were then layered in four plastic test tubes and centrifuged (3000 × *g*, 20 min, at room temperature: 18–25°C). The interphase was collected into a clean test tube, re-diluted with Mg^{2+}- and Ca^{2+}-free PBS (1:1 ratio), shaken, and centrifuged (1000 × *g*, 3 min, 4°C). The supernatant was then decanted, the sediment was gently shaken, 3 ml of erythrocyte lysing solution was added, and the sample was centrifuged (1000 × *g*, 3 min, 4°C). The supernatant was then re-decanted, the sediment shaken, and centrifuged in PBS as above. The obtained sediment was suspended in Mg^{2+}- and Ca^{2+}-free PBS, filled up to 0.2 ml. Cellularity was then determined in a Burker chamber, diluting the sample with Turk's solution (1:20 ratio, 10 µl sample + 190 µl Turk's solution) in an Eppendorf test tube.

2) Incubation with monoclonal antibodies

An adequately diluted sample was pipetted into four plastic test tubes, 50 µl per tube. 2.5 µl of each MoAb was then added according to the protocol:
Test tube 1: CD14/LFA-1/CD45 (test sample).
Test tube 2: CD14/VLA-4/CD45 (test sample).
Test tube 3: CD14/IgG2A/CD45 (isotype control).
Test tube 4: CD14/IgG1/C45 (isotype control).

After mixing, the samples were incubated for 30 min in the dark at 4°C, then washed by centrifuging with 1.5 ml of Mg^{2+}- and Ca^{2+}-free PBS (1500, 3 min, 4°C). The samples were then suspended in 1.5 ml of Mg^{2+}- and Ca^{2+}-free PBS and analyzed by three-color flow cytometry (DAKO Galaxy Flow Cytometer, Partec GmbH, Germany) to determine the percentage of monocytes and lymphocytes showing surface expression of VLA-4 and LFA-1. Monocytes were identified by staining for CD14 and CD45 antigens and by forward and side light scattering. Lymphocytes were stained for CD45 and identified by forward and side light scattering. A negative control for a nonspecific flow cytometric background was performed with an isotype-matched, fluorescent, non-binding monoclonal antibody.

The following antibodies were used: anti-CD14 mouse IgG2ak FITC (Becton Dickinson, USA), MONOCYTES A-HU CD45/RPE-Cy5, T29/33 (DakoCytomation, Denmark), antibodies to LFA-1 mouse IgG2ak PE (Becton Dickinson, USA), antibodies to VLA-4 mouse IgGk PE (Becton Dickinson, USA), isotype control for LFA-1: IgG2ak PE (Becton Dickinson, USA), and isotype control for VLA-4: IgG1k PE (Becton Dickinson, USA).

Additionally, erythrocyte sedimentation rate, blood count, urinalysis, and serum levels of fibrinogen, C-reactive protein, complement components C3 and C4, urea, and creatinin were routinely evaluated using standard methods.

Statistical methods

Results are expressed as the mean ± standard deviation. Normality was tested using the Shapiro-Wilk test. Comparisons between groups were performed using the Wilcoxon signed rank test and the Welch two-sample *t*-test. Independence of variables was tested using the chi-squared test. For subgroup comparisons involving additional qualitative parameters, ANOVA was used. Correlations were tested using either Pearson's method or Spearman's rank correlation. For all analyses, significance was defined as a p value of less than 0.05.

Results

A juxtaposition of selected parameter levels in the study subjects and controls shows significantly higher acute-phase reactant levels (fibrinogen, C-reactive protein, and ESR) in patients with SLE. Moreover, significantly lower levels of hemoglobin and complement components C3 and C4 were observed in the patients. Mean leukocyte and platelet counts did not significantly differ in patients and controls (Table 1). Fibrinogen concentration positively correlated with CRP level and ESR in the patients with SLE. A similar interdependence was observed between CRP level and ESR. However, no significant correlation was established between antiphospholipid antibodies and CECs or anti-dsDNA antibodies.

In further analysis, patients with severe disease activity (SLEDAI >12) were integrated into subgroup I (n=15) and

Table 1. Biochemical patient profile: patients with severe and with mild and moderate disease activity

Parameters (mean)	Disease activity		Difference significance
	Severe (N=15)	Mild to moderate (N=14)	
C3 concentration (g/l)	0.96±0.31	1.05±0.27	SI
C4 concentration (g/l)	0.17±0.07	0.20±0.11	SI
Fibrinogen concentration (g/l)	3.94±1.45	3.89±1.33	SI
ESR (mm/h)	29.40±18.87	21.57±21.37	SI
CRP concentration (mg/l)	6.79±7.20	4.79±5.11	SI
Leukocyte count (×1000/mm^3)	5840±2190	5280±1510	SI
Hemoglobin concentration (g/l)	12.17±1.29	12.36±0.92	SI
Platelet count (×1000/mm^3)	255,000±2190	249,780±95,770	SI
ANA titer (IU/ml)	240.50±181.78	310.66±635.03	SI

SI – statistically insignificant difference.

Table 2. Clinical profile of patients with severe disease activity

Clinical manifestation (complications connected with inflammatory microangiopathy)	Patient count (N=15)	Patient percentage
Systemic vasculitis	7	46.67%
Nephritis (proteinuria >0.5 g/24h)	2	13.33%
CNS affection, including:	6	40.00%
– seizure	1	6.67%
– organic brain syndrome	2	13.33%
– cranial nerve disorder	1	6.67%
– lupus headache	2	13.33%

those with low or moderate disease activity (SLEDAI >3) formed subgroup II (n=14). The clinical profile of the patients with severe disease activity is presented in Table 2 and a comparison of immune test results in the two patient groups can be found in Table 3.

In subgroup I, ANA and ACA (including IgM ACA) as well as antiphospholipid antibodies (repeatedly positive test result for ACA and/or lupus anticoagulant) were detectable significantly more often than in subgroup II.

However, no significant differences were found in the subgroups regarding the occurrence of anti-dsDNA antibodies, IgG ACA, LA, anemia, leukopenia, and decrease in complement component C3 and C4 levels (Table 4). Mean ANA titers in both subgroups did not significantly correlate with disease activity.

Higher mean levels of CRP, fibrinogen, and ESR, and lower mean levels of complement components C3 and C4 were found in subgroup I compared with subgroup II without, however, reaching statistical significance. Similarly, mean hemoglobin levels did not significantly vary between the patient subgroups (Table 5).

CECs

Significantly higher CEC counts were demonstrated in patients with SLE than in controls. CEC numbers did not correlate with age in either the study and control groups. Moreover, significantly higher numbers of CECs were found in patients with low or moderate disease activity than in the healthy controls. A similar trend was noted in patients with severe disease activity and microangiopathic complications versus those with lesser disease activity (Table 6). In the group with severe SLE flares, the CEC count significantly correlated with disease activity assessed according to SLEDAI.

Flow cytometric analysis of VLA-4 and LFA-1 expression:

A significant increase in surface VLA-4 and LFA-1 expression in peripheral blood lymphocytes was observed in the patients with low and moderate disease activity in comparison with the healthy controls. A similar dependence was noted between patients with severe disease activity and controls. However, no significant differences were found between these parameters in the patient subgroups depending on disease activity.

Table 3. Comparison of immune tests in the patient subgroups

Parameters	Study group (Patients with SLE) (N=29)	Disease activity	
		Severe (N=15)	Mild and moderate (N=14)
Number/percentage of patients with positive ANA	26 (89.66%)	15 (100%)	11 (78.57%)
Mean ANA titer (IU/ml)	274.37±452.80	240.50±181.78	310.66±635.03
Number/percentage of patients with positive dsDNA	7 (24.14%)	5 (33.33%)	2 (14.28%)
Number/percentage of patients with doubtful dsDNA	2 (6.90%)	2 (13.33%)	0 (0%)
Number/percentage of patients with positive LA	7 (24.14%) including: – 2 weak (28.57%) – 4 moderate (57.14%) – 1 strong (14.28%)	4 (26.67%) including: – 3 moderate (75%) – 1 strong (25%)	3 (21.43%) including: – 2 weak (66.67%) – 1 moderate (33.33%)
LA index	1.81±0.50	2.01±0.60	1.54±0.18
Number/percentage of patients with positive ACA	9 (31.03%) including: – 5 IgG ACA (55.56%) – 7 IgM ACA (77.78%) – 3 IgG + IgM ACA (33.33%)	8 (53.33%) including: – 4 IgG ACA (26.67%) – 6 IgM ACA (40%) – 2 IgG + IgM ACA (13.33%)	1 (7.14%) including: – 1 IgG + IgM ACA
Mean ACA IgG titer (GPL-U/ml)	25.16±12.03 including: – 2 weakly positive (40%) – 2 moderately positive (40%) – 1 strongly positive (20%)	26.64±13.35 including: – 1 weakly positive (25%) – 2 moderately positive (50%) – 1 strongly positive (25%)	1 weakly positive
Mean ACA IgM titer (MPL-U/ml)	29.14±25.56 including: – 4 weakly positive (57.14%) – 1 moderately positive (14.28%) – 2 strongly positive (28.57%)	30.49±27.73 including: – 4 weakly positive (66.67%) – 2 strongly positive (33.33%)	1 moderately positive
Number/percentage of patients with positive LA and ACA (IgM ACA)	2 (6.90%)	2 (13.33%)	0 (0%)
Number/percentage of patients with positive LA and/or ACA	14 (48.28%)	10 (66.67%)	4 (28.57%)

Analogously, surface LFA-1 expression in peripheral blood monocytes was significantly enhanced in both subgroups in comparison with controls, without significant differences between subgroups I and II.

Regarding surface VLA-4 expression in peripheral blood monocytes, no significant differences were noted between patients with low and moderate disease activity and the control group. However, there was a significant increase in this expression in subgroup I of patients compared with controls and subgroup II of patients.

A juxtaposition of the selected parameter levels in the controls and the patient subgroups is presented in Tables 7–9.

Discussion

As mentioned above, vascular endothelial damage develops in the course of numerous inflammatory diseases. Identifying reliable markers of the progressive endothelial damage remains an issue of primary clinical significance. Results of studies conducted on patients with vasculitis suggest that the number of circulating, mature, desquamated endothelial cells may be representative of endothelial damage. These cells also seem to be markers of disease activity, useful in monitoring the disease and predicting the risk of relapse [3,4,5,7,11,18,25].

According to reports by Clancy et al. [5], in patients with SLE the number of CECs increased in SLE flares, acting as a predictor of disease severity and outcome. In contrast to clinically active disease, the number of CECs during remission remained only slightly elevated. These observations concur with our own data showing that CEC counts increase in the course of lupus and are considerably higher in patients with severe disease activity (according to SLEDAI score) than in patients with less active disease, and may reflect the degree of endothelial damage.

In healthy people, the CEC count is usually very low, not exceeding 5 cells/ml. Detection methods must therefore

Table 4. Comparison of patient subgroups: patients with severe vs. patients with mild and moderate disease activity

Parameters Number (percentage) of patients with:	Study group (patients with SLE) (N=29)	Disease activity		Difference significance
		Severe (N=15)	Mild and moderate (N=14)	
Positive ANA	26 (89.66%)	15 (100%)	11 (78.57%)	p<0.001
Positive dsDNA	7 (24.14%)	5 (33.33%)	2 (14.28%)	p>0.05
Positive LA	7 (24.14%)	4 (26.67%)	3 (21.43%)	p>0.05
Positive ACA	9 (31.03%)	8 (53.33%)	1 (7.14%)	p<0.05
Positive IgG ACA	5 (55.56%)	4 (26.67%)	1 (7.14%)	p>0.05
Positive IgM ACA	7 (77.78%)	6 (40.00%)	1 (7.14%)	p<0.05
Positive LA and/or ACA	14 (48.28%)	10 (66.67%)	4 (28.57%)	p<0.05
Decrease in C3 concentration	11 (37.93%)	8 (53.33%)	3 (27.27%)	p>0.05
Decrease in C4 concentration	3 (10.34%)	2 (13.33%)	1 (7.14%)	p>0.05
Increase in ESR	23 (79.31%)	14 (93.33%)	9 (60.00%)	p>0.05
Increase in CRP concentration	13 (44.83%)	8 (53.33%)	5 (35.71%)	p>0.05
Leukopenia	6 (20.69%)	4 (26.67%)	2 (14.28%)	p>0.05
Anemia	10 (34.48%)	6 (40.00%)	4 (28.57%)	p>0.05

Table 5. Comparison of test results in the study and control groups

Parameters (mean)	Study group (patients with SLE) (N=29)	Control group (N=12)	Difference significance
C3 concentration (g/l)	1.00±0.29	1.19±0.08	p<0.001
C4 concentration (g/l)	0.18±0.09	0.26±0.02	p<0.001
Fibrinogen concentration (g/l)	3.92±1.37	3.29±0.43	p<0.05
ESR (mm/h)	25.62±20.15	8.17±3.78	p<0.001
CRP concentration (mg/l)	5.82±6.25	3.44±3.32	p<0.05
Leukocyte count (×1000/mm^3)	5570±1880	5720±720	SI
Hemoglobin concentration (g/l)	12.26±1.11	13.17±0.44	p<0.05
Platelet count (×1000/mm^3)	252 480±84 300	250 830±54 700	SI

SI – statistically insignificant difference.

Table 6. Comparison of circulating endothelial cell counts in study and controls groups

	Study group (patients with SLE) (N=29)	Control group (N=12)	Difference significance
CEC count	15.29±12.10	3.08±1.46	p<0.001
	Patients with mild and moderate disease activity (N=14)	**Control group (N=12)**	**Difference significance**
CEC count	9.14±5.16	3.08±1.46	p<0.05
	Patients with severe disease activity (N=15)	**Patients with mild and moderate disease activity (N=14)**	**Difference significance**
CEC count	21.03±13.96	9.14±5.19	p<0.05

Table 7. Flow cytometric analysis: patients with severe disease activity vs. controls

Parameters	Patients with severe disease activity (N=15)	Control group (N=12)	Difference significance
M VLA-4%	77.10±13.56	64.90±19.13	p<0.05
M LFA-1%	91.44±16.00	84.95±19.86	p<0.05
L VLA-4%	1.70±1.56	0.39±0.26	p<0.05
L LFA-1%	1.97±2.60	0.67±0.83	p<0.05

Table 8. Flow cytometric analysis: patients with low and moderate disease activity vs. controls

Parameters	Patients with mild and moderate disease activity (N=14)	Control group (N=12)	Difference significance
M VLA-4%	63.40±20.95	64.90±19.13	SI
M LFA-1%	90.11±10.34	84.95±19.86	p<0.05
L VLA-4%	1.71±1.04	0.39±0.26	p<0.001
L LFA-1%	3.32±2.48	0.67±0.83	p<0.05

SI – statistically insignificant difference.

Table 9. Flow cytometric analysis: patients with severe vs. patients with mild and moderate disease activity

Parameters	Patients with severe disease activity (N=15)	Patients with mild and moderate disease activity (N=14)	Difference significance
M VLA-4%	77.10±13.56	63.40±20.95	p<0.05
M LFA-1%	91.44±16.00	90.11±10.33	SI
L VLA-4%	1.70±1.56	1.71±1.04	SI
L LFA-1%	1.97±2.60	3.32±2.48	SI

SI – statistically insignificant difference.

encompass sensitive isolation techniques and subsequent identification using specific endothelial markers. In most studies, CECs were isolated using the immunomagnetic method, with magnetic beads coated with anti-endothelial CD146 antibodies [3]. CD146 antigen occurs mostly on the surface of endothelial cells, with only traces of surface expression in megakaryocytes and certain cancer cell lines, and is thus considered a specific endothelial marker and, therefore, a marker of CECs [11].

CEC activation, as described in patients with SLE, suggests they may be potential inflammatory mediators, able to induce progressive vascular damage on the vicious circle principle [5]. It seems that the Schwartzmann model best illustrates the pathological endothelial activation in patients with SLE. The activation of complement components C3 and C4 is an important step in the development of this type of endothelial lesion. Simultaneously, through the influence of numerous immune stimuli, surface expression of adhesion molecules on endothelial cells is enhanced. Synchronic activation of circulating leukocytes and local endothelial cells as well as an increase in activated CEC numbers are meant to promote the formation of leukocyte aggregates, leading to small-vessel occlusion.

In this study, patients diagnosed with SLE had significantly lower mean hemoglobin and complement component C3 and C4 levels as well as considerably higher acute-phase parameters (ESR, CRP, and fibrinogen levels) than in the control group. However, no significant differences were noted in these parameters between the subgroups. Analogously, mean ANA titers were not found to depend on disease activity, although positive tests for ANA were significantly more common in patients with severe disease activity. The subgroups, however, differed in respect to CEC count, which was significantly more elevated in patients with flares.

These data suggest that in patients with SLE, the number of CECs in the peripheral blood may constitute a reliable marker of disease activity. Regardless of its prognostic worth, the number of CECs seems to be an additional diagnostic tool in situations where conventional tests prove insufficient.

SLE patients are not uncommonly diagnosed with hypercoagulability, which clinically manifests as recurring incidents of arterial and/or venous thrombosis [9]. Thrombotic vascular occlusion may be induced both by direct endothelial lesion and by pathological platelet activation in response to various agonists, e.g. antiphospholipid antibodies. Antiphospholipid antibodies may activate endothelial cells. The anti-endothelial activity observed in some patients with antiphospholipid syndrome largely depends on the presence of serum anti-B2-glycoprotein (B2-GP I) antibodies, which react with the B2-GPI molecule, adhering to the endothelial surface [17].

Comparing both patient subgroups, significantly higher occurrences of both fractions of ACA, IgM ACA and antiphospholipid antibodies (repeatedly positive test for serum ACA and/or lupus anticoagulant) was determined in patients with severe disease activity and microangiopathic complications compared with those with less active disease. No direct correlation was noted between the presence of antiphospholipid antibodies and the number of CECs in patients with SLE. In half of the patients the presence of antiphospholipid antibodies remained asymptomatic, while in the remaining patients it manifested clinically as antiphospholipid syndrome with cerebral stroke (3 patients), lower-limb deep-vein thrombosis (2 patients), recurrent miscarriages (1 patient), and peripheral thrombosis (1 patient) in their histories.

A number of adhesion molecules are involved in intercellular adhesion and in adhesion to the interstitium. Among these are molecules belonging to the B1-integrin subfamily, such as VLA-4, and those belonging to the B2-integrin subfamily, LFA-1 being their main representative. ICAM-1 and fibrinogen are the main ligands for LFA-1, while VLA-4 is the main receptor for VCAM-1 and extracellular matrix proteins such as endothelial base membrane fibronectin. VLA-4 mediates in the process of B2-integrin activation, homotypical aggregation, and at the "rolling leukocytes" stage and in their strong adhesion to VCAM-1 molecules on the endothelial cell surface [16]. As demonstrated, interactions between VLA-4 and VCAM-1 and between LFA-1 and ICAM-1 are the main mechanisms behind increased lymphocyte T and monocyte adhesion to the endothelial cell surface. They are, moreover, involved in transendothelial leukocyte migration, although proper VLA-4 function requires additional exogenous cytokines and endothelial cell activation.

In many patients with active vasculitis but no circulating immune complexes or those deposited within the vascular wall are detected. Similarly, in many instances their presence does not lead to inflammatory infiltration and the development of fibrinoid necrosis [6]. Hence the suggestion that other, alternative mechanisms might be involved in the pathogenesis of inflammatory vascular lesions, i.e. Schwartzmann's reaction. In this model, interactions between LFA-1 and ICAM-1 are of prime significance, determining the adhesion of leukocytes to endothelial cells and their subsequent damage linked with cytokines and neutrophils [23]. Considering that LFA-1/ICAM-1 and VLA-4/VCAM-1 interactions activate different pathways of the adhesion and transendothelial migration of mononuclear cells, it seems relevant to assume that to induce a vascular inflammatory reaction, the involvement of both integrins is necessary [21].

In the available publications, isolated reports were found concerning the surface expression of integrin adhesive molecules on peripheral blood mononuclear cells in SLE-associated vasculitis. Takeuchi et al. [23] described an enhanced expression of VLA-4 and LFA-1 on lymphocytes in patients with SLE complicated by vasculitis. In patients without signs of vasculitis, upregulated expression was noted only in the case of LFA-1, leading to the hypothesis that increased surface VLA-4 expression in lymphocytes may play a key role in the pathogenesis of vascular lesions in SLE. Similarly, our own observations showed an enhanced surface expression of VLA-4 and LFA-1 in peripheral blood mononuclear cells in patients with symptoms of SLE-related vasculitis. Surface VLA-4 and LFA-1 expression, however, did not just increase in the presence of inflammatory microangiopathic complications (systemic CNS affection and/or vasculitis and/or nephritis), but also in patients with lower disease activity, without these vascular complications. Analogously, surface LFA-1 expression in peripheral blood monocytes was increased in both study groups, regardless of disease activity (in comparison with controls). In patients with severe disease activity and microangiopathic complications, upregulated surface expression of VLA-4 on circulating monocytes was also noted, a fact not observed in patients with lower disease activity.

Publications regarding monocyte involvement in the development/progression of vascular complications in SLE are very scant, although their engagement in the pathogenesis of inflammatory and proliferative vascular lesions is unquestionable, especially with regard to atherogenesis. The authors quoted above focused solely on integrin expression in lymphocytes, without analyzing whether similar effects may concern peripheral blood monocytes. According to their results, enhanced VLA-4 expression in lymphocytes occurred only in patients with vasculitis. Our observations, however, showed an increase in VLA-4 expression in lymphocytes in all patients, regardless of the degree of vascular lesions, a fact which rather speaks for a global activation of monocytes, which is connected with the presence of the basic disease. The upgraded surface expression of VLA-4 in monocytes in patients with microangiopathic complications additionally supports the idea that enhanced VLA-4 expression in peripheral blood mononuclear cells may play a significant role in the pathogenesis of vascular lesions in SLE.

Animal models provide further data concerning the pathogenesis of vasculitis in patients with SLE. For instance, James et al. [13] noted that a systemic inflammatory response in MRL/fas(lpr) mice with a genetic propensity to develop SLE leads, among others, to increased adhesion of leukocytes to the endothelium in cerebral microcirculation, a process mediated mainly by $\alpha 4\beta 1$-integrin/VLA-4 interactions. These data are of importance in light of the latest studies, which demonstrate that natalizumab (anti-$\alpha 4$-inte-

grin monoclonal antibody) may bring therapeutic benefits, among others in auto-aggressive disease [1]. Great hopes are also connected with VLA-4 inhibition in patients with SLE and CNS-localized vasculitis, especially since no effective treatment is currently available [12].

Kevil et al. [14] also demonstrated that there is no incidence of SLE in LFA-1-free genetically SLE-prone mice, a fact further showing that LFA-1 might be a potential therapeutic target in SLE patients.

In vitro studies have demonstrated that mophetil mycophenolate considerably reduces monocyte adhesion to human umbilical vein endothelium, down-regulates ICAM-1 and MHC II surface expression in monocytes, and prevents lymphocyte adhesion and penetration through the endothelial surface, probably by inhibiting ICAM-1/LFA-1, VCAM-1/VLA-4, and P-selectin/PSGL-1 (P-selectin glycoprotein ligand-1) interactions [15]. In SLE patients at risk of flare recurrence, administration of mophetil mycophenolate resulted in decreased anti-dsDNA titers and inhibited lymphocyte B activation, thus preventing a clinical exacerbation of the disease [2].

In summary, investigating the pathogenic mechanisms underlying vascular complications in some SLE patients may result in the development of new treatment methods in the future.

Conclusions

1) CEC counts increase in SLE and correlate with disease activity, indicating progressive endothelial damage.

2) Surface expressions of VLA-4 and LFA-1 on peripheral blood lymphocytes as well as of LFA-1 on circulating monocytes are enhanced regardless of disease activity.

3) Surface expression of VLA-4 on circulating monocytes is upgraded only in patients with severe disease activity and microangiopathic complications, which indicates that this integrin's enhanced expression on monocytes may play a key role in the pathogenesis of vasculitis in SLE.

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