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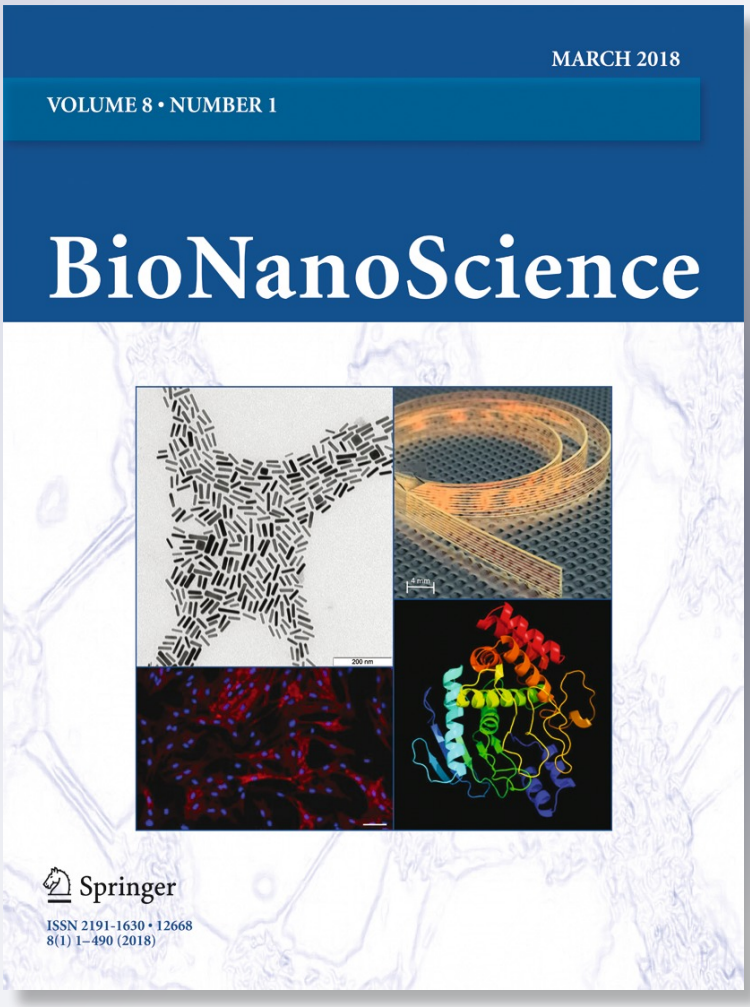

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Cellular Microvesicles in the Blood of Patients with Systemic Lupus Erythematosus

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Abstract
Systemic lupus erythematosus (SLE) is an autoimmune disease with a complex and largely unclear pathogenesis. Cellular phospholipid microvesicles released upon activation and/or death of a cell have been proposed to play a role in inflammatory autoimmune pathologies, including SLE. Here, circulating microvesicles of various cellular origins were marked with fluorescently labeled cell-specific antibodies and enumerated by flow cytometry in platelet-free plasma obtained from the heparinized blood of 29 SLE patients and 19 normal subjects. Significantly higher concentrations of endothelial-, monocyte-, and erythrocyte-derived microvesicles were found in the SLE patients compared to normal subjects with prevalence of microvesicles originating from endothelial cells. No significant difference was found for platelet-derived microvesicles. A correlation analysis of microvesicle counts with laboratory parameters and clinical features of SLE suggest differential implications of various cell-derived microvesicles in the pathogenesis of SLE. These data suggest that SLE is associated with functional alterations of endotheliocytes, monocytes, and erythrocytes followed by enhanced release of microvesicles that may contribute to inflammation and hypercoagulability.

Keywords Microvesicles · Systemic lupus erythematosus · Flow cytometry

1 Introduction

Microvesicles (MVs) are a heterogeneous population of phospholipid vesicles up to about 1 μm in size released from various cells, including blood cells and endothelium, in response to cell activation, aging, and apoptosis [1]. Circulating MVs can play an important physiological role in normal conditions [2, 3] as well as in inflammation, cancer, angiogenesis, thrombosis, etc. [4]. The blood levels of cell-derived circulating MVs of various origins are often increased compared to healthy subjects and MVs contribute to the pathogenesis of various diseases, including inflammatory and autoimmune pathologies [5].

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that affects multiple tissues and organs, leading to relatively high morbidity and mortality rates [6]. The pathogenesis of SLE is complicated and, despite numerous studies, remains largely unclear [7]. The role of MVs in the mechanisms of SLE has been a matter of controversy. Some studies reported elevated levels of circulating MVs in SLE [8], while others found that the number of MVs in the blood is either unchanged or even decreased [9]. The cellular origin of circulating MVs as well as their functional importance in SLE remains vague, although potentially, the content and cellular origins of circulating MVs may shed light on the pathogenesis of SLE and serve as biomarkers of disease activity and progression.

The aim of this study was to determine the levels of cell-derived circulating MVs in SLE patients with respect to their origin and reveal associations between clinical features and the levels of circulating MVs.

2 Materials and Methods

2.1 SLE Patients and Healthy Subjects

The study was approved by the Ethical Committee of Kazan State Medical Academy (Kazan, Russian Federation) and performed in accordance with the Declaration of Helsinki. Patients with SLE enrolled in this study were from the

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Hospital of Kazan Federal University and the Republican Clinical Hospital, Kazan, Russian Federation. SLE was documented based on the criteria of the American College of Rheumatology. Twenty-nine patients with the active form of SLE having the SLE Disease Activity Index (SLEDAI) ≥ 4 were included in the study and 19 healthy donors comprised a control group. The groups of SLE patients and control donors were comparable by age (35 ± 9 and 28 ± 9 years, respectively) and by gender (females comprised 80 and 64%, respectively). The donors did not take any medications at least 2 weeks prior to blood withdrawal; they did not have any autoimmune conditions in the past and had no close relatives with autoimmune diseases.

2.2 Blood Collection and Processing

Venous blood was drawn into vacuum containers with heparin (Improve, Germany) and centrifuged at 200 g for 10 min at room temperature to obtain platelet-rich plasma that was further centrifuged at 1500 g for 15 min to remove platelets. The platelet-poor plasma was spun at 10,000 g for 5 min at room temperature to obtain platelet-free plasma that was immediately used for detection of MVs.

2.3 Labeling of MVs with Cell-Specific Antibodies

Platelet-free plasma was diluted 100-fold with a buffer (10 mM HEPES, 0.14 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) that was freshly pre-filtered through a 0.1-μm pore size filter [10]. To track the cellular origin of MVs, 150-μl aliquots of the diluted plasma samples were incubated simultaneously for 20–60 min in dark with the following mouse anti-human monoclonal cell-specific antibodies. MVs derived from red blood cells were labeled by adding 0.2 μl allophycocyanin (APC)-conjugated anti-CD235 antibodies (BioLegend, USA). MVs derived from monocytes were labeled by adding 5 μl peridinin chlorophyll protein complex (PerCP)-conjugated anti-CD14 antibodies (BioLegend, USA). MVs derived from endothelial cells were labeled by adding 5 μl Alexa Fluor-conjugated anti-CD146 antibodies (BioLegend, USA) and MVs derived from platelets were labeled by adding 0.5 μl phycoerythrin (PE)-conjugated anti-CD41 (Invitrogen, USA). A control plasma sample was incubated in the same conditions without labeling.

2.4 Flow Cytometry of MVs

Quantification of MVs in the diluted and labeled platelet-free plasma samples was performed with a FacsCalibur flow cytometer (BD Biosciences, USA). The gate of MVs in FSC/SSC dot plots was determined based on fluorescence of MVs stained with calcein AM in FSC/FL1 dot plots [11] (Fig. 1). The upper limit of the MVs quadrant gate was established using 1.0 μm latex beads (ThermoFisherSci, USA); the lowest level was indeterminate and based on the capacity of the instrument (around 300 nm). MVs were identified and quantified as the events that contained specific CD markers registered as fluorescent signals with various emission wavelengths and the fluorescence threshold levels were set by analysis of unstained MVs. The MVs were analyzed with a flow of buffer at 200 events/s and a sample flow rate of 12 ± 3 μl/min. Total events were collected during 60 s per sample using a CellQuest software; the absolute total number of particles analyzed was (1.0–4.8) × 10^6/μl and MVs in the established gate comprised 98–100%. The analysis was performed using a FlowJo software (Scripps Research Institute). Enumeration of MVs was based on the MV counts per time unit, the flow rate in the cuvette of the cytometer with correction for a sample dilution.

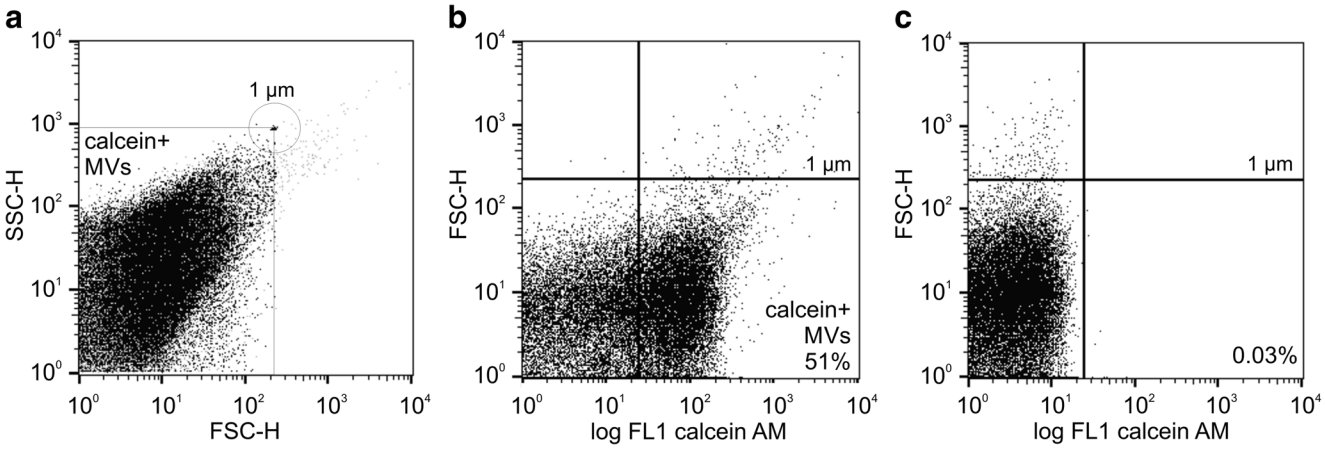

Fig. 1 Flow cytometry dot plots of MVs in platelet-free plasma. **a** Unlabeled MVs (black dots in the gate of MVs) in a two-channel FSC vs SSC mode (distribution by size and granularity, respectively), showing a 1-μm gate corresponding to calcein + MVs, **b** MVs smaller than 1 μm labeled with calcein AM (log FL1 calcein AM, gate of calcein + MVs), **c** untreated plasma in a two-channel FSC vs log FL1 calcein AM mode (control)

2.5 Statistical Analysis

Microsoft Excel and GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA) were used for the statistical analysis. MV data are presented as a median (Interquartile Range (IQR) and percentiles. Differences between the groups were determined with a Mann-Whitney *U* test. Correlations between MV levels and clinical/laboratory parameters were determined using a Spearman's rank-order correlation coefficient. Differences and correlation coefficients were considered statistically significant at $P \leq 0.05$.

3 Results

The total number of MVs ranged from $\sim 1.0 \times 10^6$ to 4.8×10^6/μl (median 3.8×10^6/μl) in the plasma samples of SLE patients and from $\sim 1.0 \times 10^6$ to 4.7×10^6/μl (median 3.1×10^6/μl) in the plasma samples of healthy donors. The scatter of the data was high and there was no significant difference in the average or median concentrations of total MVs between the two investigated groups.

Next, we analyzed a difference between the plasma samples of SLE patients and healthy subjects in the content of subsets of MVs of distinct cellular origins. Therefore, we compared concentrations of MVs derived from platelets (PMVs), endothelial cells (EMVs), monocytes (MMVs), and erythrocytes (ErMVs). We found that the average levels of EMVs, MMVs, and ErMVs were significantly higher in SLE, while there was no difference in the average counts of PMVs (Fig. 2). Then we asked which cell types released more MVs into the blood in SLE, and the results showed the following order: EMVs > MMVs > ErMVs > PMVs (Fig. 2) with a respective median concentration of MVs equal to 2.4×10^6/μl, 1.2×10^6/μl, 1.0×10^6/μl, and 0.1×10^6/μl.

Finally, we performed a correlation analysis of the total number of MVs and the levels of subpopulations of MVs with clinical characteristics of SLE and laboratory parameters. The *total* number of MVs in SLE patients had a significant negative correlation with the erythrocyte sedimentation rate and a positive correlation with a lymphocyte count (Table 1). The levels of *PMVs* in SLE patients correlated positively with a monocyte count, fibrinogen, and albumin concentrations; a significant negative correlation was found between concentrations of PMVs versus serum β1-globulin and circulating immune complexes. Also, a negative correlation between PMVs and SELENA-SLEDAI was observed. The *EMV* levels correlated negatively with concentrations of fibrinogen and the erythrocyte sedimentation rate. There was a positive correlation between *MMVs* and lymphocyte counts; also, the number of MMVs correlated negatively with the SELENA-SLEDAI and erythrocyte sedimentation rate.

ErMVs correlated positively and significantly with monocyte counts and with fibrinogen concentration. These observations suggest distinct implications for different cell-derived MVs in the course of SLE.

4 Discussion

Existing data on the levels of circulating MVs in SLE are controversial. An increase in the level of circulating MVs has been observed in SLE patients in a number of studies [8], while other investigators did not observe a significant

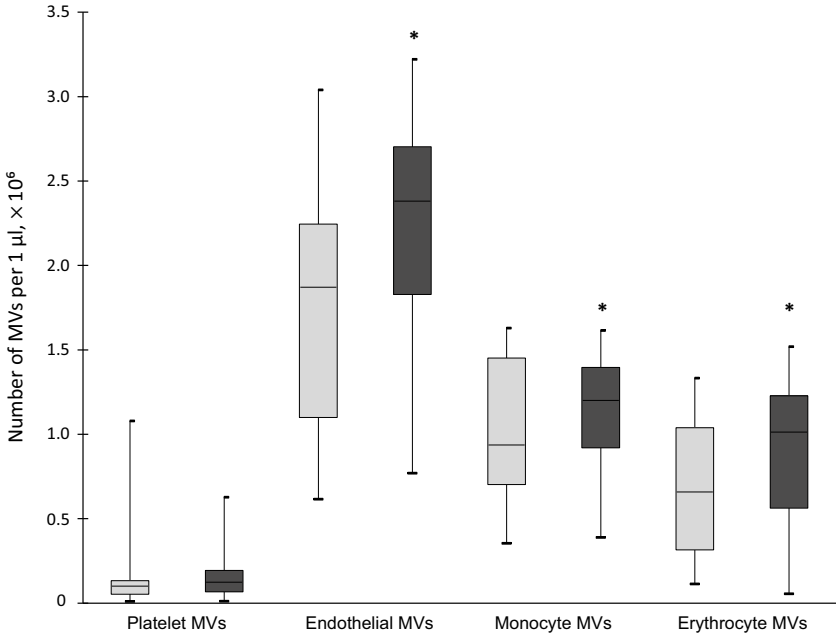

Fig. 2 The levels of circulating MVs positive for various cell-specific surface markers in platelet-free plasma of patients with SLE and healthy subjects. Bars represent the median values, the bottom and top edges of boxes indicate interquartile range (25th and 75th percentile, respectively), and the whiskers are 2.5 and 97.5 percentiles. *$P \leq 0.05$, evaluated by a Mann-Whitney *U* test, compared to the control group

Table 1 Spearman correlation coefficients of the MV counts with laboratory parameters and clinical characteristics of the SLE patients

Clinical data	*n*	Total MVs	CD41+ platelet-derived MVs	CD146+ MVs from endothelial cells	CD14+-monocyte-derived MVs	CD235+ MVs from erythrocytes
SELENA-SLEDAI	19		− 0.48*		− 0.42*	
Lymphocytes	21	0.54**			0.44*	
Monocytes	21		0.43*			0.40*
Fibrinogen	17		0.43*	− 0.49*		− 0.46*
β1-globulin	18		− 0.48*			
Albumin	20		0.59**			
Erythrocyte sedimentation rate	22	− 0.57**		− 0.53**	− 0.40*	
Circulating immune complexes (CIC)	18		− 0.57**			

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

increase in the total concentration of MVs [9]. Our observations are consistent with the latter observation because we did not reveal a significant difference in the total number of MVs on the blood of healthy donors and SLE patients. It should be noted that the lack of the difference might be due to a high data variability as well as to inherent limitations of flow cytometry that misses a substantial fraction of MVs smaller than 0.4–0.5 μm [12]. Flow cytometry is the most common method to detect, quantify and characterize MVs in different samples, but it has limitations related to the lowest size of MVs, which is around 300 nm. It is noteworthy that the difference in MV content was earlier established for phosphatidylserine-expressing MVs, while in our study, MVs were identified using a calcein dye [11] without segregation into phosphatidylserine-positive and phosphatidylserine-negative particles [13].

Despite the absence of a difference in the content of total MVs, there was a distinct content of MVs of a different cellular origin. In particular, the number of EMVs, MMVs, and ErMVs were significantly elevated in patients with SLE compared to control healthy subjects with no difference in the content of PMVs. These variations reflect differential MV production and distinct recruitment of various parental cells in the pathogenesis of SLE. In addition, a decrease or increase in the number of EMVs, MMVs, and ErMVs in SLE may result from an increased or decreased clearance of MVs bearing markers of their parental cells [14]. Notably, the number of detectable EMVs in SLE may depend on CDs used as the markers for detection [15]. Moreover, different stimuli that induce MVs formation cause the release of phenotypically distinct EMVs. Different surface markers can distinguish these subgroups of EMVs and these EMVs can be associated with various pathological mechanisms.

Our study confirms other reports that EMVs prevail in SLE [16], suggesting pronounced endothelial damage in these patients. In addition, SLE patients exhibit a population of EMVs, not present in healthy subjects, which contain apoptosis-modified chromatin [16].

High levels of MMVs reflect activation of monocytes during inflammatory response in SLE similar to the increased MVs formation after stimulation with lipopolysaccharide [17]. MMVs express tissue factor that can determine their procoagulant activity and thrombogenicity. Activation of monocytes may secondarily promote activation of endotheliocytes, thus enhancing production of endothelial MVs.

An increased release of ErMVs probably has a role in the predisposition of SLE patients to prothrombotic conditions [17, 18]. ErMVs enhance blood coagulation, so they can amplify inflammation-associated intravascular thrombin generation [18].

Correlation analysis revealed several important mechanistic links related to the role of cell-derived MVs in SLE. First,

there was a direct correlation between the production of PMVs and signs of systemic inflammation, such as monocyte count and fibrinogen concentration (Table 1) which belongs to "acute phase" proteins. Unexpectedly, there was a negative correlation between the content of MVs (total, EMVs and MMVs) and disease activity, erythrocyte sedimentation rate, circulating immune complexes and β1-globulins (Table 1), suggesting an increased consumption or clearance of MVs during progression of the inflammatory response in SLE.

5 Conclusion

The number of circulating MVs of different cellular origin is elevated in the blood of SLE patients compared to healthy subjects in the following order: EMVs > MMVs > ErMVs > PMVs. Variations of the levels of MVs in blood correlate inversely with the disease severity and are associated with laboratory parameters that characterize changes in blood composition, suggesting an importance of cell-derived MVs in the pathogenesis of SLE.

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Author Contributions The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Compliance with Ethical Standards The study was approved by the Ethical Committee of Kazan State Medical Academy (Kazan, Russian Federation) and performed in accordance with the Declaration of Helsinki.

Conflict of Interest The authors declare that they have no conflict of interest.

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