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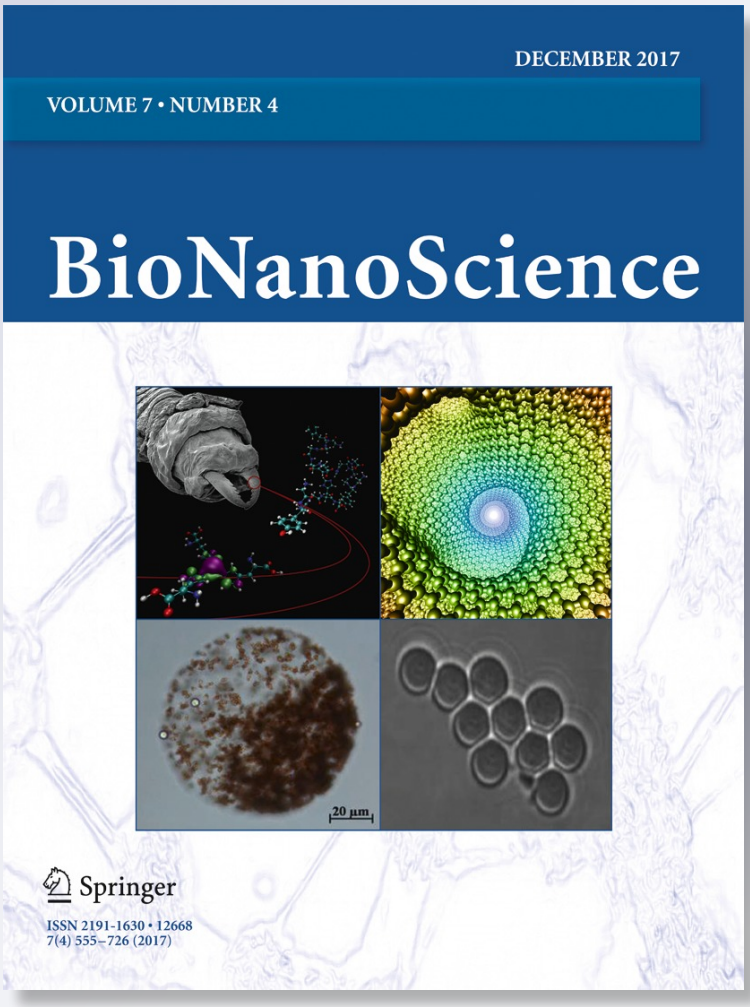

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Structural Alterations of Monocytes in Systemic Lupus Erythematosus

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Abstract Systemic lupus erythematosus (SLE) is an autoimmune disease in which the immune system mistakenly attacks multiple organs and tissues of the body. To elucidate the involvement of blood cells in the pathogenesis of SLE, we used transmission electron microscopy to study ultrastructure of monocytes isolated from the blood of SLE patients. We found that in the SLE patients, a substantial fraction of monocytes had abnormal morphology that corresponded to the structural signs of either necrosis or apoptosis. The number of altered monocytes in the SLE patients was significantly higher than in healthy subjects and related directly with the level of anti-dsDNA autoantibodies in the blood. Our results suggest that monocytes are involved in the pathogenesis of SLE and undergo adverse necrotic and/or apoptotic changes, likely induced by autoantibodies.

Keywords Systemic lupus erythematosus · Monocyte · Transmission electron microscopy

1 Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by systemic inflammation, affecting various organs and systems, including the joints, kidney, skin and central nervous system. Inflammation in SLE is associated with production of autoantibodies, generation of circulating immune complexes and activation of the complement system [1]. Monocytes play a pathogenic role in this disease as a source of secreted immunomodulators that promote activation of the key immunoreactive T and B cells [2]. A high level of interferon I, which correlates directly with the disease activity [3], can promote maturation of monocytes into dendritic cells, whose primary function is to process and present antigens to T cells [4]. In addition, monocytes can play a role in promoting thrombotic complications of SLE. It is known that SLE patients are predisposed to venous or arterial thrombosis [5] and activated monocytes that express tissue factor on their surface can contribute to the (peo)thrombotic status [6]. Also, monocytes from the blood of SLE patients have been shown to expose high levels of the procoagulant phosphatidylserine on the cell surface that also may enhance the prothrombotic tendency in SLE [7].

To provide a structural basis for the monocyte alterations in SLE, we investigated morphology of monocytes isolated from the blood of SLE patients and healthy donors using transmission electron microscopy.

2 Material and Methods

The study was approved by the Ethical Committee of Kazan State Medical University. SLE patients were included in the study based on the diagnostic criteria of the American College of Rheumatology. Citrated blood was collected by venipuncture from six SLE patients and three healthy aspirin-free donors and centrifuged at 200*g* for 10 min at room temperature. Platelet-rich plasma (PRP) was removed and the pellet was resuspended in a Tyrode-EDTA buffer added in the volume

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equal to the removed supernatant. The suspension of red and white blood cells was diluted 1:1 with the Tyrode-EDTA buffer and layered on top of Ficoll-Urografin (ρ = 1.067 g/cm^3). After centrifugation at 1100*g* for 20 min at room temperature, the peripheral blood mononuclear cells were collected, then washed twice in the Tyrode-EDTA buffer (300 g; 10 min; 4 °C) and re-suspended in the same buffer. Next, monocytes were isolated by negative immune separation using Dynabeads® Untouched™ Human Monocytes Kit (Invitrogen, USA) according to the manufacturer's instructions.

Purity of the isolated monocytes was confirmed with flow cytometry (FACSCalibur, BD Biosciences, USA) using fluorescently labeled antibodies against monocyte-specific CD14 (BioLegend, USA). The preparations contained more than 96% monocytes of all mononuclear cells. Viability of the isolated monocytes assessed by exclusion of trypan blue was 95–99%.

The isolated monocytes (~ 1.5×10^6/ml) were fixed in a 2% glutaraldehyde solution in phosphate-buffered saline (PBS) for 90 min at the room temperature. The fixed monocytes were pelleted by centrifugation at 300*g* for 5 min. The pellet was washed once with PBS, and the samples were postfixed with 1% osmium tetroxide in the same buffer with addition of sucrose (25 mg/ml) for 2 h. The samples were dehydrated serially in 30, 50, 70, 50, 90 and 95 vol% alcohol and then in acetone and propylene oxide. Epon 812 was used as the embedding resin. Samples were polymerized for 3 days under increasing temperature from 37 to 60 °C. Sections were obtained on an LKB-III ultramicrotome (Sweden). The sections were contrasted with saturated aqueous solution of uranyl acetate for 10 min at 60 °C and then with an aqueous solution of lead citrate for 10 min. The preparations were examined using a Jem1200EX electron microscope (Jeol, Japan).

The level of anti-dsDNA antibodies was determined using a standard enzyme-linked immunosorbent assay (ELISA). dsDNA was absorbed on a 96-well plate followed by the incubation first with a serum sample and then with anti-IgG monoclonal antibodies conjugated with horseradish peroxidase. 3,3′,5,5′-tetramethylbenzidine (TMB) was added as a chromogenic substrate followed by addition of sulfuric acid to stop the reaction. Results of ELISA were read by an automated microtiter plate reader at λ = 450 nm.

3 Results and Discussion

All the SLE patients had normal red blood cell counts [$(4.2 \pm 0.7) \times 10^{12}$/l] and normal platelet counts [$(277 \pm 49) \times 10^9$/l]. One patient with a high leukocyte count had laboratory signs of hypercoagulability (shortened activated partial thromboplastin time) associated with the antiphospholipid syndrome. A titer of anti-dsDNA Abs in the blood serum was determined with a standard ELISA. Three SLE patients had a high level of anti-dsDNA Abs exceeding 200 IU/ml, and the other three patients had a low level of anti-dsDNA Abs <25 IU/ml.

Using transmission electron microscopy, we examined the morphology of 154 individual monocytes from the SLE patients (105 cells from patients with a high level of anti-dsDNA Abs and 49 cells from patients with a low level of anti-dsDNA Abs) and 22 monocytes from healthy donors. Monocytes with the normal morphology were characterized by a ruffled cell surface, formation of filopodia, a bean-shaped nucleus and uniform chromatin distribution (Fig. 1a). Such normal cells comprised 95% of monocytes from the healthy donors, 84% of monocytes from the SLE patients with a low level of anti-dsDNA Abs and 54% of monocytes from the SLE patients with a high level of anti-dsDNA Abs (Fig. 2a). The differences were statistically significant ($p < 0.001$) based on a χ^2 test.

Monocytes with abnormal morphology could be segregated into two groups. Group I abnormal monocytes displayed the ultrastructural feature characteristic of an apoptotic cell. In particular, they had the smooth cell surface, electron-dense cytoplasm, mitochondrial swelling, the spherical nucleus with a perinuclear accumulation of condensed chromatin or condensation of chromatin into dense spherical particles; nuclear fragmentation was often seen (Fig. 1b). Percent of the apoptotic monocytes in the SLE patients with a high level of anti-dsDNA Abs was significantly greater than in the SLE patients with a low level of anti-dsDNA Abs (Fig. 2c, d). Such cells were not seen among the monocytes isolated from the blood of healthy donors. The presence of apoptotic monocytes in active SLE was previously shown by Richardson and et al. [8], and our results confirm that a substantial fraction of monocytes is prone to apoptosis in the blood of SLE patients.

In addition to apoptosis, we revealed group II abnormal monocytes undergoing necrosis that comprises an alternative mode for cell death. Group II monocytes had structural features of necrotic cells characterized by the smooth cell surface without or with quite a few filopodia (not shown), low electron-density cytoplasm, small electron-dense mitochondria and spherical pyknotic nucleus with more diffuse euchromatin (Fig. 1c). The fraction of necrotic monocytes in the SLE patients with a high level of anti-dsDNA Abs was significantly greater than in the SLE patients with a low level of anti-dsDNA Abs and in the healthy donors (Fig. 2b–d). Importantly, the incidence of both apoptotic and necrotic monocytes in SLE associated directly with a high level of anti-dsDNA Abs in the blood, suggesting a pathogenic link between the severity of the autoimmune reactions and the structural and functional status of monocytes.

In addition to multiple pathogenic consequences of monocyte dysfunction, the apoptotic and necrotic monocytes can be an additional source of auto-antigens during SLE that can aggravate the disease activity. Apoptotic bodies and necrotic

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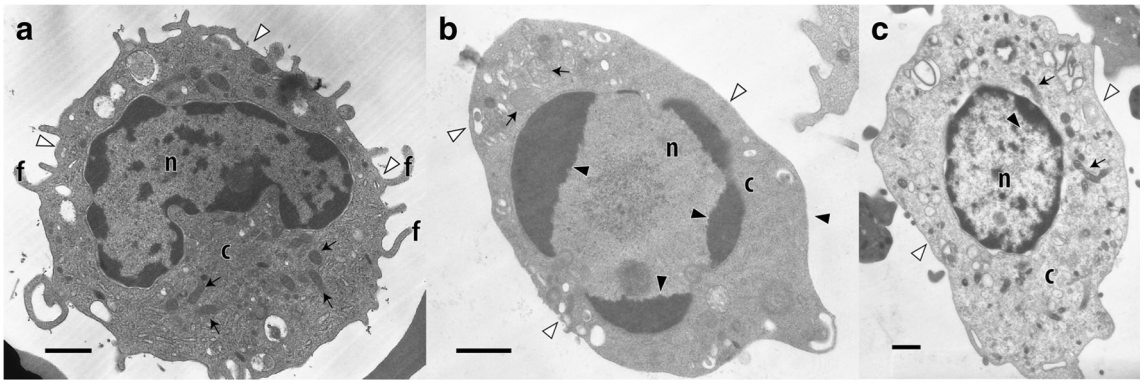

Fig. 1 Representative transmission electron micrographs of the main types of monocytes. **a** Ultrastructure of a normal monocyte with a ruffled plasma membrane and a normal nucleus isolated from the blood of a healthy donor. **b** An apoptotic monocyte from an SLE patient that has the smooth cell surface, electron-dense cytoplasm, mitochondrial swelling and the spherical nucleus with a perinuclear accumulation of condensed chromatin. **c** A necrotic monocyte from a SLE patient that has the smooth plasma membrane, low electron-density cytoplasm, small electron-dense mitochondria and the spherical pyknotic nucleus with more diffuse euchromatin. Designations: 'n' stands for nucleus, 'c' for cytoplasm and 'f' for filopodia. White arrows indicate the plasma membrane, black arrows without a tail indicate chromatin abnormalities and black arrows with a tail indicate mitochondria. Scale bars = 1 μm

particles derived from dying or dead monocytes can lead to an increase of presentation of the auto-antigens by dendritic cells during SLE [9]. Moreover, apoptotic monocytes may be a source of phosphatidylserine [10] which can enhance intravascular activation of the blood clotting cascade and promote the prothrombotic status. Microparticles generated by apoptotic

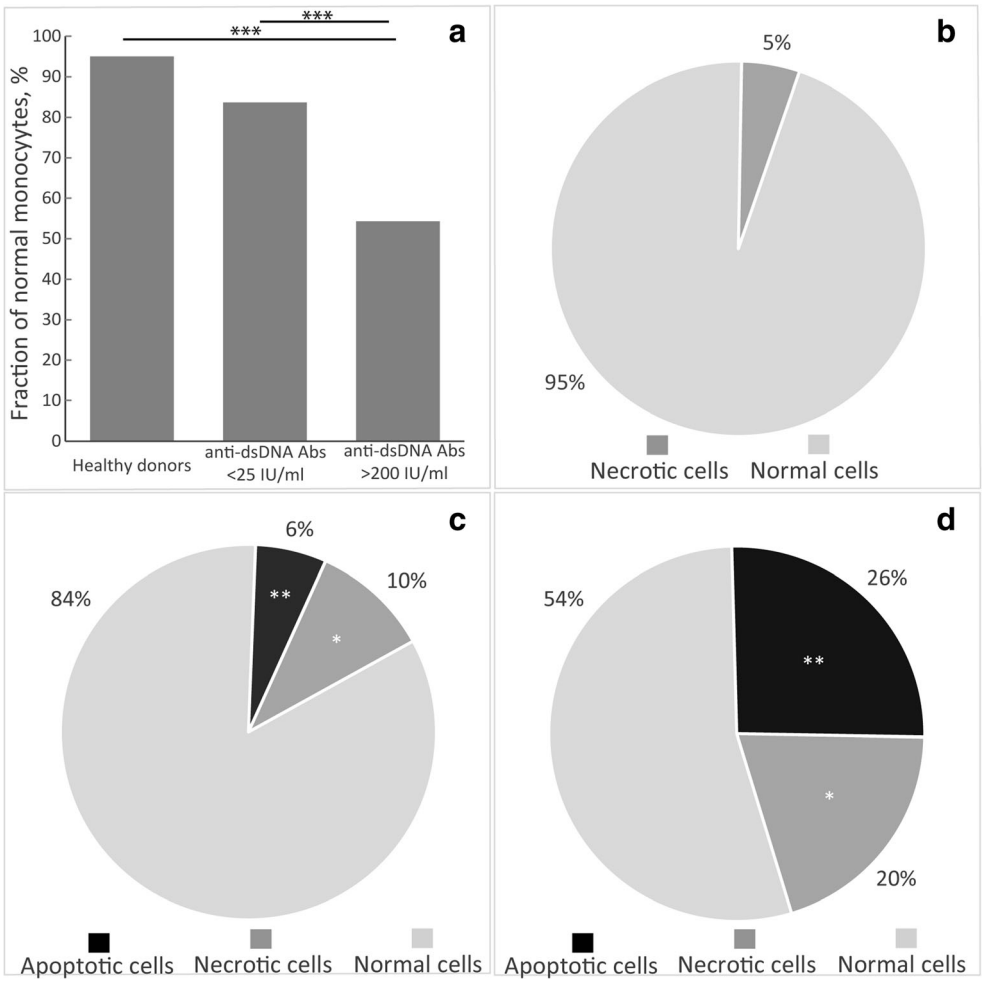

Fig. 2 **a** Percent of monocytes with normal ultrastructure isolated from the blood of healthy donors ($n = 22$), SLE patients with a low level of anti-dsDNA Abs ($n = 49$) and SLE patients with a high level of anti-dsDNA Abs ($n = 105$). **b**–**d** Fractions of apoptotic, necrotic and normal monocytes in healthy subjects (**b**), SLE patients with a low (**c**) or high (**d**) level of anti-dsDNA Abs. Statistical analysis was performed using a χ^2 test; $^*p < 0.5$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared the patients with a high and low level of anti-dsDNA Abs for **d**; n = number of cells analyzed

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monocytes have been shown to contribute to thrombotic events by expressing tissue factor as well as by initiating redox signaling in endothelial cells [11].

4 Conclusions

The systemic inflammation in SLE involves monocytes that can undergo dramatic morphological and functional changes leading to cellular apoptosis and/or necrosis. The incidence of these changes correlates directly with production of anti-dsDNA Abs, suggesting an immune injury of monocytes that can aggravate the course of SLE, including possible prothrombotic effects.

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