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RELATIONSHIP OF APOPTOSIS AND SECONDARY NECROSIS OF LEUCOCYTES WITH ACTIVITY OF DISEASE AND CLINICAL MANIFESTATION IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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Abstract

The presence of several different autoantibodies (auto-AT) at the same time is a specific peculiarity of the "autoantibody profile" of SLE (systemic lupus erythematosus). It is known that the induction of auto-AT formation involves both nonspecific and antigen-specific immunoregulatory disorders. In apoptosis, the primary changes in the cell membrane composition or/and the excretion of intracellular compounds into the intercellular milieu lead to an inflammatory reaction.

The purpose of the study was to highlight the connection between apoptosis and secondary necrosis of granulocytes and mononuclear (lymphocytes and monocytes) with inflammation activity in patients with SLE to improve diagnosis and basic therapy efficacy. In patients with SLE, secondary necrosis of granulocytes was 3.4 times higher compared to healthy control.

Moreover, the level of apoptotic monocytes was 1.87 times higher, and secondary necrosis of monocytes was 5.58 times higher than healthy control. The secondary necrosis of lymphocytes was higher 9.0 times than in the case of healthy control. The usage of Apolect technology in patients with SLE allows differentiating various cell types of immunological inflammation with the analysis of the degree of apoptosis and secondary necrosis of immunocompetent cells (granulocytes, monocytes, lymphocytes) to determine the aggressiveness of cytostatic therapy and predict the development of complications of this disease.

Keywords: Systemic lupus erythematosus, Apoptosis, Secondary necrosis, Monocytes, Lymphocytes, Granulocytes.

Зв'язок апоптозу та вторинного некрозу лейкоцитів із активністю хвороби та клінічною маніфестацією у пацієнтів з на СЧВ

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Одночасна наявність декількох різних типів аутоантитіл (ауто-АТ) є специфічною ознакою «профілю аутоантитіл» СЧВ (системного червоного вовчака). Відомо, що в індукції утворення аутоАТ беруть участь як неспецифічні, так і антигенспецифічні порушення імунорегуляції. При апоптозі спостерігаються первинні зміни складу клітинної мембрани та/або викид внутрішньоклітинного вмісту у міжклітинне середовище, що призводить до розвитку запальної реакції.

Мета дослідження — оцінка зв'язку апоптозу та вторинного некрозу гранулоцитів і мононуклеарів (лімфоцитів і моноцитів) із активністю запалення у хворих на СЧВ для покращення діагностики та ефективності базисної терапії. У хворих на СЧВ вторинний некроз гранулоцитів був у 3,4 рази вищий порівняно зі здоровим контролем. Крім того, рівень апоптичних моноцитів був вищим у 1,87 рази, а вторинний некроз моноцитів – у 5,58 разів порівняно зі здоровим контролем. Вторинний некроз лімфоцитів був вищим у 9,0 разів, ніж у здорового контролю. Застосування технології Arolect у хворих на СЧВ дозволяє диференціювати різні типи клітин та імунологічне запалення з ступенем апоптозу та вторинного некрозу імунокомпетентних клітин (гранулоцитів, моноцитів, лімфоцитів) для визначення агресивності цитостатичної терапії та прогнозування розвитку ускладнення цього захворювання.

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**Дозвіл комісії з біоетики щодо про-
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Introduction. Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder with plenty of autoantibodies in the bloodstream and abnormal innate and adaptive immune reactions [1]. Changes in cell death pathways, including apoptosis and NETosis, a specific form of death implemented by neutrophils, represent a crucial fount of autoantigens. These changed autoantigens are presented to autoreactive B cells by follicular dendritic cells in germinal centers of secondary lymphoid organs [2, 3]. The production of autoantibodies to nuclear antigens (including NETs) leads to continued inflammation and tissue lesion in patients with SLE. Alterations in apoptosis regulation are linked to the pathogenesis of SLE [4]. Otherwise, cumulated apoptotic cells may cause inflammation or immune reactions, resulting in immune resistance and autoimmune disorder [3, 4]. The implications of incomplete clearance of apoptotic cells and NETs in the progression of clinical features in SLE are discussed [3]. Apoptosis, primary necrosis, and particular necrotic changes may occur in the same tissues. In the absence of complete clearance, secondary necrosis occurs [3, 5]. Secondary necrosis is characterized by the release of damage-associated molecular patterns (DAMPs) and consequently causes autoimmune diseases. Autoantibodies induce the taking up of secondarily necrotic cell-derived debris by phagocytes, accompanied by inflammatory cytokines. Circulating immune complexes (ICs) that included nucleic acid excreted by necrotic and late apoptotic cells induced secretion of IFN- α in plasmacytoid dendritic cells (pDCs). In SLE, DCs produced IFN- α [4, 6].

A damaged function of “professional phagocytes” - monocytes, macrophages, neutrophils, dendritic cells, etc. leads to disturbances of antigen presentation and the development of autoimmune disease [7, 8]. Generally, secondary necrosis and apoptosis are the first mechanisms that may be included in the pathogenesis and progression of SLE through secreting DAMPs and autoantigens [1]. Together, they enhance the inflammatory and immune responses, the release of cytokines, production of autoantigens, tissue damage in SLE, and play significant roles in SLE pathogenesis and development [1, 9]. The lack of clearance partakes in the breakdown of the

lenience to itself, promoting autoimmune reactions mainly directed against nuclear autoantigens. Deposits of antinuclear antibodies (ANA) and ICs in tissues can induce inflammation with complement and vascular damage [3].

Consequently, apoptosis is a non-inflammatory and predominant immune tolerance-inducing way of cell death. Augmented apoptosis encountered with incomplete clearance in SLE can lead to the mass production of apoptotic cells through secondary necrosis [1, 10]. Deprivation of cell membrane integrity and excretion of the cellular composition by secondary necrotic cells can cause autoimmune disorders and promote the progression of SLE [1, 11].

In the case of secondary necrosis, violation of the integrity of the plasma membrane at later stages of apoptotic death makes dying cells exhaust their energy reserves to maintain the integrity of plasma membrane. Over time, they will not be phagocytosed by defective phagocytosis. The first ones immediately release their components to the environment, and the second ones first undergo a component modification, including oxidation and caspase-dependent degradation, and then release them into extracellular space [12, 13].

Excessive production of extracellular neutrophilic traps with systemic inflammation can be fatal and have negative long-term consequences because an accumulation of oxidized histone complexes and DNA provokes the production of autoantibodies [10]. The defective clearance of dying cells can lead to the accumulation of apoptotic cell debris. Phagocytosis of apoptotic cells results in the absorption of complex autoantigens (nucleic acid etc.) and actively inhibits inflammation by diminishing the synthesis of pro-inflammatory cytokines and inducing the production of anti-inflammatory factors - TGF- β 1 and PGE2 [8,9].

As a multicomponent disease, SLE does not have the only diagnostic signs, and the routine diagnosis is based on clinical and laboratory rules described by the American College of Rheumatology (ACR). Therefore, ANA has the power to be used as a biomarker of several clinical signs and symptoms of SLE, and its elucidation is the main primary labo-

ratory test for SLE [3, 14]. The manifestation of various autoantibodies directed against cell compounds strongly argues that SLE's autoimmunity is initiated by defective clearance of dead and dying cells [3, 15]. With the understanding of the etiopathogenesis of SLE and its multisystem nature, the investigators can implement new diagnostic markers and therapeutic plans for treating this autoimmune disease [4].

The purpose of the study was to examine the relationship between apoptosis and secondary necrosis of granulocytes and mononuclear (lymphocytes and monocytes) cells and inflammation in patients with systemic lupus erythematosus to improve diagnosis and determine the efficacy of basic therapy.

Materials and methods.

Participants.

This study was performed following the Declaration of Helsinki and approved by the ethics committee of Danylo Halytsky Lviv National Medical University (approval protocol No. 5 of May 16, 2016). Written informed consent was obtained from all examined subjects. There were 17 patients (2 men and 15 women) treated in the Rheumatology department of Lviv Regional Clinical Hospital and consulted at the Department of Clinical Immunology and Allergology. The average age of patients was 34.6 ± 11.8 years with SLE duration from 3 months to 15 years. The diagnosis of SLE was presented by the commendations of the ACR (2010). The control group consisted of 20 healthy people. Clinical and laboratory methods included: 1) immunological, genetic, allergic, epidemiological anamnesis; 2) a general clinical examination; 3) general laboratory tests 4) evaluation of the clinical activity of SLE with SLEDAI and BILAG indices [7]. All research was conducted to refine the functional condition of organs and systems; determine the degree of activity and complications of the underlying disease and concomitant pathology.

Blood sampling

Blood sampling for laboratory tests was carried out by venipuncture at the time of the patient's admission to the hospital (before treatment) and processed within 2 h of the collection.

Whole blood APOLECT assay

The assay was performed according to the manufacturer's instructions [16]. Briefly, 100 μ L of whole heparinized blood in a tube with 10 μ L of propidium iodide (Sigma-Aldrich, USA) was added. The mixture was incubated at 4 °C for 30 min. The sample was washed with PBS (150 mM sodium phosphate; 150 mM NaCl; pH 7.2 ± 0.1 (25 °C) (Sigma-Aldrich, USA). After centrifugation at 2504g for 10 min, the buffer was removed, and 100 μ L of BSA (bovine serum albumin) (Sigma-Aldrich, USA) was added. Then, solutions A, B, and C were added to the sample successively, and the mixture was vortexed every 15 s. Finally, 2 μ L of NPL-FITC was added and incubated at 4 °C for 30 min. After staining, the sample was analyzed by the FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). An excitation wavelength of 488-nm and 515-nm bandpass filter for fluorescence emission was used.

Statistical methods

Clinical results were added to digital databases in Microsoft Excel (Microsoft Office and STATISTICA 9.0). To describe the results, M was calculated. Since the distribution of data in all groups corresponded to the normal distribution and the study task involved a pairwise comparison of results, the Student's criterion was used to assess the probability of this statistical study. The Student's method was chosen because the distribution (dispersion) in the comparison groups was correct, and only two groups were compared. Given that the primary material was presented for analysis in the form of absolute values, we used a straight-line correlation coefficient based on the method of K. Pearson for the correlation analysis. The results of the t-criterion were compared with normal values. Results exceeding table value were interpreted as a reliable correlation coefficient with a probability of an error-free prediction of 95% ($p < 0.05$), 99% ($p < 0.01$) or 99.9% ($p < 0.001$).

Results

In this study, 17 SLE patients (2 men and 15 women) and 20 Healthy Control groups (HC) were enrolled. Table 1 presents demographic and medical history data of SLE patients. Study groups were matched by age and gender.

All patients were ANA seropositive (100%). All patients displayed high SLEDAI that ranged from 5 to 7 (mean 5.59 ± 0.28). One patient was diagnosed with mild normochromic anemia, leukocytosis was diagnosed in 16 (94.1%), neutrophilic leukocytopenia in 7 (41.2%), and thrombocytopenia in three (17.6%) patients. In 88% patients, CRP level was increased (average concentration is 19.62 mg/L). An elevated ANA was found in all (100%) patients (ranging from 1.3 to 4.0 IU/mL, mean 1.5 ± 1.3 IU/mL) and anti-dsDNA in 16 (94%) patients (47.2 ± 200 U/mL).

Question OK. Did the authors look into the possible correlation between clinical Dx with the status of apoptosis or necrosis and the type of treatment mentioned in Table 1 on a patient-by-patient basis?

Answer. Due to the small number of patients in the sample, the small number of patients receiving biological monoclonal antibodies (2) and nonsteroidal anti-inflammatory drugs (2) and methotrexate (7) were not studied.

The clinical picture of SLE is diverse, complicating early diagnosis. SLE was often accompanied by tissue self-destruction realized through apoptosis of cells. Apoptosis control is essential for diagnosing, predicting, and treating autoimmune inflammatory disease. Apoptotic and secondary-necrotic blood cells (granulocytes, monocytes, and lymphocytes) in patients with SLE and the healthy control group are described in Table 2.

The monocytes, neutrophils, and lymphocytes in peripheral blood were analyzed by two-color

Table 1.

Demographic and disease features of SLE patient group and healthy control group

	SLE patient group (n=17)	Healthy control group (n=20)	P-value
Age: median (min-max), years	32 (18-44)	38 (22-40)	> 0.05
Sex: ratio female/male	15/2	15/5	> 0.05
Disease duration: median (min-max), years	5 (2-11)	-	-
Swollen and painful joints	17(100)	-	-
C-reactive protein (%)	15 (88)	-	-
ANA-positive (%)	17 (100)	-	-
Anti-dsDNA-positive (%)	16 (94)	-	-
Current treatment with methotrexate (%)	7 (41,2)	-	-
Current treatment with DMARDs (%) 2 (9.5)	2 (11.7)	-	-
Current treatment with biologic agents (%)	2 (11.7)	-	-
Current treatment with glucocorticoids (%)	17 (100)	-	-

Table 2.

The number of viable, apoptotic, primary, and secondary necrotic granulocytes, monocytes, and lymphocytes in the peripheral blood

	Healthy Control n=20	SLE n=17
Granulocytes, viable %	97.06±0.41	92.05±2.18
Granulocytes, apoptotic %	0.45±0.17	0.58±0.18
Granulocytes, secondary necrotic %	0.42±0.06	1.44±0.47*
Granulocytes, primary necrotic %	2.14±0.31	5.92±1.88*
Monocytes, viable %	92.78±0.66	80.32±3.20
Monocytes, apoptotic %	0.93±0.13	1.74±0.56*
Monocytes, secondary necrotic %	1.80±0.33	10.04±3.07*
Monocytes, primary necrotic %	4.30±0.62	7.41±2.07
Lymphocytes, viable %	92.54±1.13	80.39±3.85
Lymphocytes, apoptotic %	2.67±0.94	3.40±0.51
Lymphocytes, secondary necrotic %	1.08±0.15	9.74±2.84*
Lymphocytes, primary necrotic %	2.98±0.91	7.21±2.45*

* P < 0.05 the difference compared to the healthy control group

flow cytofluorometric analysis, based on forward scatter (FSC) and side scatter (SSC) parameters. As shown in Table 2, there were no significant differences ($P = 0.05$) between the number of viable granulocytes ($97.06 \pm 0.41\%$ vs. $92.05 \pm 2.18\%$), monocytes ($92.78 \pm 0.6\%$ vs. $80.32 \pm 3.20\%$) and lymphocyte ($92.54 \pm 1.13\%$ vs. $80.39 \pm 3.85\%$) in control healthy and SLE groups, respectively. Next, we observed a significant increase in apoptotic peripheral blood monocytes in SLE patients compared to HC ($1.74 \pm 0.56\%$ vs. $0.93 \pm 0.13\%$, $P = 0.05$). There were no significant differences ($P = 0.05$) between apoptotic granulocytes ($0.58 \pm 0.18\%$ vs. $0.45 \pm 0.17\%$) and lymphocytes ($3.40 \pm 0.51\%$ vs. $2.67 \pm 0.94\%$) in patients with SLE compared with HC. Instead, we noticed a significant secondary necrotic change in the granulocytes, monocytes, and lymphocytes. Table 2 presented the number of secondary necrotic granulocytes significantly increased ($P = 0.05$) in SLE patients compared to the HC ($1.44 \pm 0.47\%$ vs. $0.42 \pm 0.06\%$). The percentage of secondary necrotic monocytes was significantly increased ($P = 0.05$) in SLE patients compared to the HC ($10.04 \pm 3.07\%$ vs. $1.80 \pm 0.33\%$). Also, the percentage of secondary necrotic lymphocytes was significantly increased ($P = 0.05$) in SLE patients compared to the HC ($9.74 \pm 2.84\%$ vs. $1.08 \pm 0.15\%$). After that, we analyzed the level of primary necrotic change in the peripheral blood. The number of primary necrotic granulocytes was significantly higher in SLE patients compared to HC ($5.92 \pm 1.88\%$ vs. $2.14 \pm 0.31\%$) as well as the number of primary necrotic lymphocytes ($7.21 \pm 2.45\%$ vs. $2.98 \pm 0.91\%$). Unlike primary necrotic monocytes, a significant difference was not observed ($7.41 \pm 2.07\%$ vs. $4.30 \pm 0.62\%$).

Discussion.

It is known that the level of apoptotic and secondary necrotic cells reflects the severity of inflammation. The usage of Apolect technology in the diagnostic of SLE allows differentiating various cell types of immunological inflammation with the analysis of the degree of apoptosis and secondary necrosis of immunocompetent cells (granulocytes, monocytes, lymphocytes). In the SLE, immune tolerance is defective, which may be due to 1) insufficient dead cell clearance; 2) anomalous presentation to T cells of autoantigens by

antigen-presenting cells (APCs). Impaired immune resistance and extended production of autoantibodies against nuclear autoantigens are two critical factors of SLE [1]. After the activation by their antigens, T cells promote auto reactivation of B cells, leading to the production of many autoantibodies. These autoantibodies bind to their antigens, forming ICs deposited in many organs, leading to damage to different tissues, organ inflammation, and dysfunction [4,5]. Our study revealed that in patients with SLE, secondary necrosis of granulocytes was 3.4 times higher than in healthy control. Moreover, the level of apoptotic monocytes was 1.87 times higher, and secondary necrosis of monocytes was 5.58 times higher than in healthy control. The secondary necrosis of lymphocytes was 9.0 times higher than in healthy control. While SLE clearance of apoptotic cells is defective, early apoptotic cells release anti-inflammatory cytokines (transforming growth factor β – (TGF- β), IL-10), realize “find me” signals (adenosine triphosphate (ATP), uridine triphosphate (UTP), dimer of ribosomal protein S19 (dRp S19)) and phagocytic molecules (phosphatidylserine (PS), uridine triphosphate (UTP) dimer of ribosomal protein S19 (dRp S19)), on the cell membrane, attracting phagocytes that migrate easily and express phagocytic molecules (phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE)), and “stay away” (Lactotransferrin – LTF)) signals to macrophages for induction to tolerance. Macrophages that have captured apoptotic cells release “tolerate me” cytokines (IL-10 and TGF- β), which create an anti-inflammatory environment [4]. This clearance stage begins when the apoptotic cell release “find-me” signals, such as sphingosine-1-phosphate (S1P), lysophosphatidylcholine (LPC), ATP, UTP, and fractalkine (CX3CL1), which promote leukocytes migration and activation towards dying cells. Phagocytes recognize directly apoptotic cells through phospholipid phosphatidylserines PS receptors, such as Tim-1, Bai-1, Tim-4, and Stabilin-2. So, the apoptotic cell clearance actively forms an anti-inflammatory environment [3,13].

Furthermore, it is known that impaired phagocytosis is also associated with decreased C-reactive protein levels and complements consumption in serum patients with SLE [4].

Redundant apoptosis has also been found in phagocytic cells, which are significant for the clearance of apoptotic cells. Increased monocyte/macrophage apoptosis is observed in patients with SLE and leads to the formation of autoantibodies and tissue damage [1,4]. Apoptotic glycoproteins in the plasma membrane of lymphocytes are localized and are essential because they determine the stage of apoptosis without compromising cell integrity. Domestic researchers Bilyy RO, Stoyka RS and a group of researchers led by M. Hermann described changes in surface glycans associated with apoptosis. Specific lectins carbohydrate-binding proteins were used to determine apoptotic changes in lymphocyte glycoproteins. It has been established that the increase in alpha-D-mannose and beta-D-galactose-enriched plasma membrane glycoproteins was characteristic of apoptotic cells. In experiments, it was determined that in the case of SLE, there is an increase in apoptotic lymphocytes, the number of which was approximately 20% higher than in virtually healthy individuals.

Apoptotic lymphocytes are actively taken away by tangible body macrophages (TBMs) from germinal centers. The number of TBMs found in some patients with SLE seems to be diminished. In patients with SLE, the autoimmune response shows signs of an antigen-driven T

cell-dependent immune response, which generally occurs in the germinal centers of secondary lymphoid organs. In this case, randomly generated autoreactive B cells leave the cell cycle and begin interacting with autoreactive follicular B helper T cells (TFH). This may provoke the emergence of autoreactive long-lived plasma cells and lead to autoimmunity initiation [3]. The number of apoptotic T cells was elevated in patients with SLE and displayed a positive correlation with the index of SLE disease activity [3]. In SLE patients, cutaneous lesions and redundant apoptosis of immune cells, including T lymphocytes, had been reported in PBMCs [4].

Conclusions

1. The development and use of Apolect technology allow differentiating various cell types of immunological inflammation. Using Apolect, we can analyze the degree of apoptosis and secondary necrosis of immunocompetent cells (granulocytes, monocytes, lymphocytes).
2. The results of studies indicated that in SLE, the number of secondary necrotic monocytes, lymphocytes, and neutrophils significantly are elevated compared to healthy control. Since apoptosis in SLE is defective, particles are released from secondary necrotic cells, and neoantigens are formed, which leads to increased autoimmunity.

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