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Induction of apoptosis and autophagy in T-lymphocytes of patients with Systemic Lupus Erythematosus

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Abstract

Aim. To analyze the expression of key apoptosis (Bcl-2, caspase-3) and autophagy (Beclin 1, Vps34, p62 and LC3) proteins regulators in peripheral blood T-lymphocytes of patients with systemic lupus erythematosus.

Methods. The object of the study was peripheral blood T-lymphocytes of healthy donors and patients with systemic lupus erythematosus. To obtain T cells, we used the immunomagnetic separation method. Protein expression was analyzed using the Western blot method. Statistically analyzing the results was performed using the R software environment. The data was represented using boxplots. Groups were compared using the Mann–Whitney test.

Results. According to the results of the study of the apoptotic proteins, we found an increased content of caspase-3 and the absence of significant changes in the content of the anti-apoptotic protein Bcl-2 in patients with lupus, which indicates active apoptotic activity. A comparative analysis of Beclin 1 and Vps34 showed their increased content in the cells of patients, which indicates the activation of autophagy. The analysis of two isoforms of LC3 protein revealed their low content in the group of patients. Since the scatter of indicators was very different from the average value, we analyzed these indicators depending on the severity of the disease. In the acute course group, high content of protein LC3-I was detected, the content of form II was lower. In the group with the subacute course, the number of both isoforms is lower than in the other groups. In the group with a chronic course, significant increases of protein LC3-II and a decrease in the ratio of LC3-I/LC3-II were found.

Conclusion. The study showed that depending on the severity of systemic lupus erythematosus, the content of protein LC3 isoforms changes, which can be used for differential diagnosis of disease forms.

Keywords: Bcl-2, caspase-3, Beclin 1, Vps34, LC3, apoptosis, autophagy, systemic lupus erythematosus.

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Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease of unknown etiology. Clinical manifestations visible in most organs are initiated by autoantibody synthesis to nuclear antigens, serum proteins, and cell surface proteins [1–3]. One of the most common pathogenetic mechanisms in SLE is a T-lymphocyte homeostasis disorder [4, 5]. Apoptosis regulates and supports peripheral lymphocyte homeostasis. Therefore, immunity disorders, such as immunodeficiency and autoimmunity, arise because of improper regulation of lymphocyte apoptosis. Aberrant apoptosis in SLE, especially increased lymphocyte apoptosis, has been proven in several experiments [2, 6, 7]. In addition, chronic lymphopenia [2] and the compartmentalized release of autoantigen [8] in SLE may result in increased apoptosis of circulating T cells.

In addition to apoptosis, recent studies have shown the role of autophagy in maintaining lymphocyte homeostasis [9–11]. Autophagy is a catabolic process mediated by lysosomes, participating both in the basal circulation of cellular components and in response to stressful conditions. During autophagy, a part of the cytoplasm with damaged proteins or organelles is isolated using double-membrane vesicles (autophagosomes), which degrade after fusion with lysosomes for sub-

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sequent recycling [9–12]. Adverse environmental conditions, such as nutrient depletion and oxidative stress, can cause autophagy by activating Atg proteins, such as microtubule protein 1 LC3, a mammalian homolog of Atg8 yeast [13–16].

Autophagy dysregulation has been detected in several diseases, including autoimmune disorders [17, 18]. Genetic studies have revealed mutations in regulators of autophagy [12, 19], as well as in T cells of mice prone to lupus, and in patients with abnormal autophagy in SLE [18, 20].

However, the exact role of autophagy in the life of cells remains controversial, partly because of the complex functional and molecular intersection of autophagy and apoptosis. The interaction of molecular pathways between autophagy and apoptosis is a complex process, and regulators of apoptosis are able to activate autophagy. For example, in the cytoplasm, the high-mobility box 1 (HMGB1) protein interacts with a complex of two proteins, B-cell lymphoma 2 (Bcl-2)–Beclin 1. HMGB1 binds to the Bcl-2 protein, and the complex disintegrates. This is due to the intramolecular disulfide bridge between positions 23 and 45 (C23 and C45). After that, Bcl-2 blocks apoptosis and Beclin 1 triggers autophagy [21–23]. However, the way these interactions affect cells remains controversial, and the role of autophagy and apoptosis in T-lymphocytes in patients with SLE needs to be determined.

Aim

The study aimed to analyze the expression of key regulatory proteins of apoptosis and autophagy in peripheral blood T-lymphocytes of SLE patients. These results provide a new understanding of the pathogenic role of autophagy in the dysregulation of T-lymphocyte homeostasis in SLE.

Materials and methods

T-lymphocytes of peripheral blood of healthy donors and SLE patients were used as the study object. The peripheral blood samples of SLE patients were taken from 10 patients who received immunosuppressive therapy with chronic, acute, and subacute courses of the disease. The study enrolled two men and eight women aged 22 to 55 years (mean age 42 ± 12.41 years). The disease duration ranged from 1 to 30 years (an average of 9.14 ± 10.9 years). SLE activity was minimal in one (10%) patient, moderate in four (40%), and high in five (50%). All patients were diagnosed with SLE following the criteria of the American Rheumatological Association (1997). Clinical data, including age, gender, disease duration, and immunological analysis results, were obtained by interviewing patients and studying medical documentation.

The control group consisted of 10 practically healthy people: three (30%) men and seven (70%) women. In the group of volunteers, the minimum and maximum ages were 22 and 58 years.

Exclusion criteria were complaints and objective signs of acute diseases, chronic diseases in the acute phase, and rheumatological and autoimmune diseases in the family history.

The study was approved by the local ethics committee of Kazan State Medical University. Informed consent was obtained from the subjects to participate in the study.

Results

T-lymphocytes were isolated according to the standard method on a density gradient of Ficoll–Urographin (p = 1.077). The immunomagnetic separation method (Dynabeads Untouched Human T cells, Dynal, Invitrogen) was used to obtain a T-lymphocyte population. Cell counting was performed in a Goryaev chamber, stained with 0.1% trypan blue solution.

Western blot analysis of proteins was conducted. Radioimmunoprescipitation assay buffer supplemented with protease and phosphatase inhibitors (50 μL; Thermo Scientific) was added to the cell pellet. The cell lysates obtained were frozen, stored at –20°C, and thawed immediately before electrophoresis.

Protein concentration in the samples was determined using a bicinchoninic acid protein assay kit (Thermo Scientific). Before application to the gel, 0.5 μL of 1% bromophenol blue was added to the samples. Lysate samples containing ~20 μg of protein were separated in a 4–12% SDS-PAAG gradient. After electrophoresis, the gel was placed in a transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH = 8.3), and the proteins were transferred to the polyvinylidene fluoride membrane (Merck Millipore) for 1 h at a voltage of 350 V.

After transfer, the membrane was incubated sequentially with primary and secondary antibodies and visualized using an enhanced chemiluminescent kit based on a chemiluminescent substrate (Bio-Rad). Primary antibodies such as mouse antibodies to Bcl-2 (Cell Signaling Technology), rabbit antibodies to LC3 (Cell Signaling Technology), rabbit antibodies to caspase-3 (Invitrogen), antibodies to p62 (Invitrogen), rabbit antibodies to VPS34, mouse antibodies to Beclin 1 (Invitrogen), and antibodies to glyceraldehyde 3-phosphate dehydrogenase (Invitrogen) were used. Peroxidase conjugated antibodies (Life Technologies) were used as secondary antibodies. MagicMark XP (Invitrogen, Molecular Probes) was used as a protein marker.

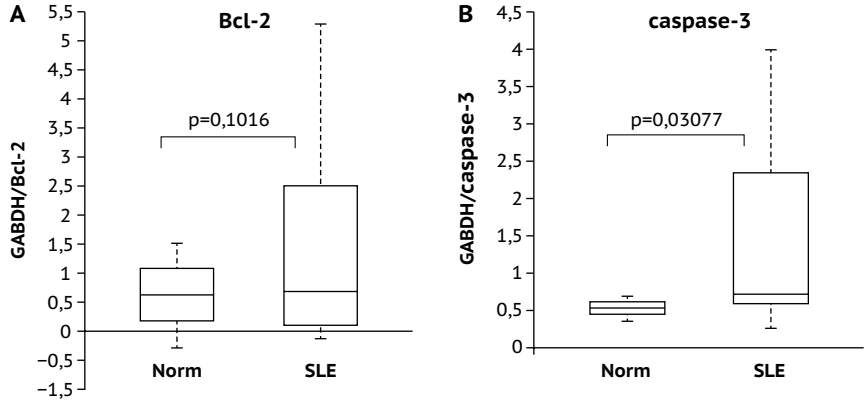

Fig. 1. Analysis of the content of (A) B-cell lymphoma 2 (Bcl-2) and (B) caspase-3 proteins in T-lymphocyte lysates obtained from healthy donors and patients with systemic lupus erythematosus (SLE).

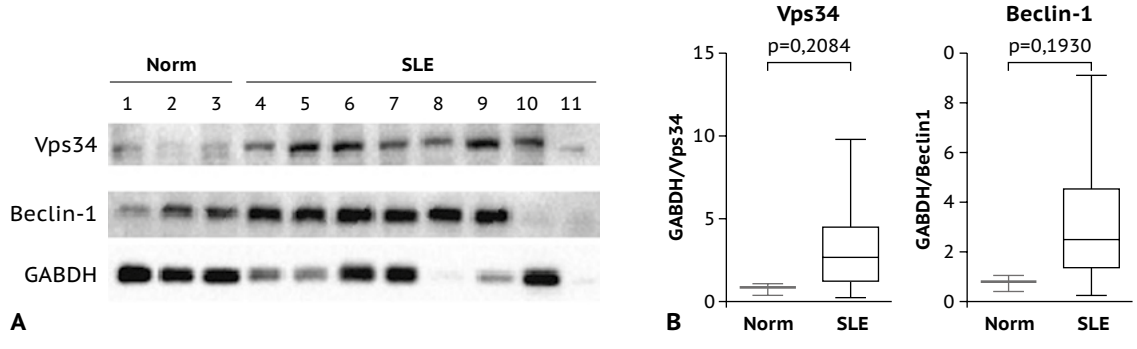

Fig. 2. Analysis of the content of Vps34 and Beclin 1 proteins in T-lymphocyte lysates. (A) Representative Western blot analysis of Vps34 and Beclin 1 protein content in T cell lysates. The immunograms show control cell lysates and samples of lysates obtained from cells of patients with systemic lupus erythematosus (SLE). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as a "boot" control. (B) Results of a statistical analysis of (A) Beclin 1 and (B) Vps34 protein expression data in T-lymphocytes obtained from healthy donors and SLE patients.

Mathematical analysis of the results was performed on a personal computer using the Excel software package (Microsoft Office 2007). Since the medians (Me) of the samples differed from their average values, this meant that the variation of individual indicators does not comply with the law of normal distribution. Therefore, the use of statistical characteristics such as mean and standard deviation is incorrect to characterize the sample. The results were processed in statistical medium R. Boxplots were used to represent the data. For comparison of the three groups, the Kruskal–Wallis test was used, followed by paired comparisons with Bonferroni correction and Benjamini–Hochberg correction. Two groups were compared using the Mann–Whitney test. The differences were considered statistically significant with the values of bilateral $p < 0.05$.

At stage 1 of the study, the analysis of two main apoptosis-regulating proteins (Bcl-2 and caspase-3) was performed. The proteolytic cleavage of procaspase-3 with the formation of effector caspase-3 is a biochemical marker of the activation of apoptosis in cells. Enzyme activity results in the degradation of chromosomal deoxyribonucleic acid (DNA), causing chromatin condensation [24]. A comparative analysis between the groups revealed a significant increase in protein content in the group of SLE patients ($p = 0.03077$), which indicates apoptosis activation and is consistent with the results of other studies (Fig. 1).

Protein Bcl-2 inhibits multiple forms of apoptosis and plays a key role in its regulation in various types of cells, including T-lymphocytes. The expression of Bcl-2 is strictly regulated in monocytes, neutrophils, and T cells, which indicates the critical role of this protein in cell death. In our study, we found that the Bcl-2 content in healthy donors and SLE patients does not have a significant difference ($p = 0.1016$; Fig. 1A).

At the next stage of the study, the content analysis of key autophagy proteins (Beclin 1, Vps34, p62, and LC3) in the T-lymphocytes of SLE patients and healthy donors was performed (Fig. 2).

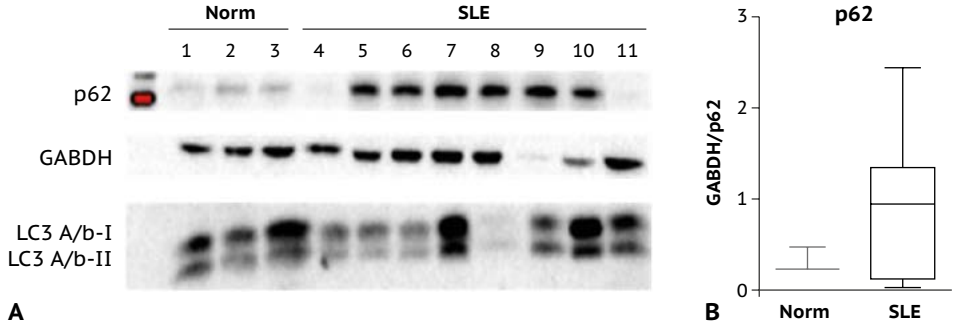

Fig. 3. Analysis of the content of p62 protein in T-lymphocyte lysates. (A) Representative Western blot analysis of p62 protein content in T cell lysates. The immunograms show control cell lysates and samples of lysates obtained from cells of patients with systemic lupus erythematosus (SLE). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as a "boot" control. (B) Results of a statistical analysis of p62 expression data in T-lymphocytes of healthy donors and SLE patients.

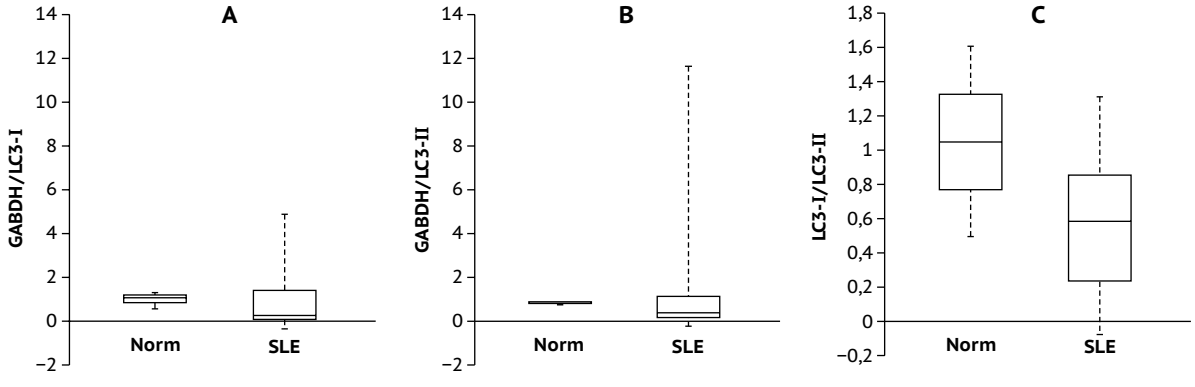

Fig. 4. Analysis of the content of LC3 protein isoforms (forms I and II) and their ratio in T-lymphocyte lysates of healthy donors and systemic lupus erythematosus (SLE) patients. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

The Beclin 1 gene (Atg6) plays a key role in the process of autophagy. The protein encoded by this gene is actively involved in autophagy initiation, namely, it gives rise to the formation of autophagosomes by joining the Vps34-PI3K-CIII complex. It also interacts closely with other autophagy proteins, such as Ambra1, ULK1, and Vps15. We showed that the content of Beclin 1 was significantly higher in the T cells of SLE patients compared with that of the healthy donors ($p = 0.1930$; Fig. 2B).

Representative Western blot analysis of Vps34 and Beclin 1 protein content in T cell lysates. The immunograms show control cell lysates and samples of lysates obtained from cells of patients with systemic lupus erythematosus (SLE). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as a "boot" control. (B) Results of a statistical analysis of (A) Beclin 1 and (B) Vps34 protein expression data in T-lymphocytes obtained from healthy donors and SLE patients.

At the next stage, the task was to assess the Vps34 protein content in both groups under study. The results showed that the content of this protein, as well as Beclin 1, was higher in the SLE patient group ($p = 0.2084$; Fig. 2).

Protein p62 serves as a central marker of autophagic degradation and plays a role in autophagy induction. The loss of Atg or factors necessary for the fusion of autophagosomes with lysosomes leads to a significant increase in p62-positive aggregates. Our study showed that p62 expression in SLE patients is higher than in the control group ($p = 0.0097$; Fig. 3). Since the protein content during the normal course of the process should decrease, this may indicate a blockage of downstream members of the autophagy protein family. Thus, at the final stage of the study, an analysis of the content of protein LC3, its isoforms, was performed.

Western blot analysis of p62 protein content in T cell lysates. The immunograms show control cell lysates and samples of lysates obtained from cells of patients with systemic lupus erythematosus (SLE). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as a "boot" control. (B) Results

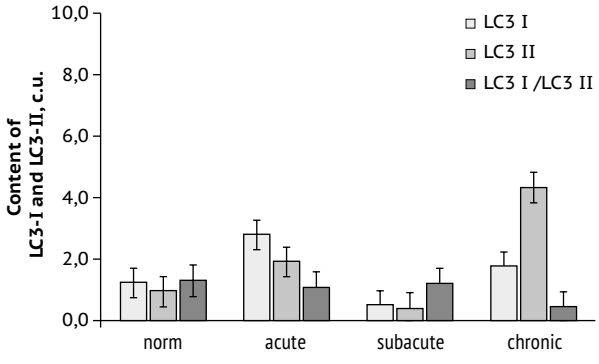

Fig. 5. Analysis of the content of LC3 protein isoforms (forms I and II) and their ratio in T-lymphocytes of healthy donors and patients with systemic lupus erythematosus with acute, subacute, and chronic courses of the disease.

of a statistical analysis of p62 expression data in T-lymphocytes of healthy donors and SLE patients.

The LC3 protein is present in the cytosol of most cell types. When autophagy starts, it is decomposed proteolytically to form LC3-I. The released LC3-I protein is activated and transferred to the Atg3 protein until it is conjugated to phosphatidylethanolamine, and LC3-II is formed. The LC3-II protein is involved in the growing phagophore and is located on the inner and outer membranes of the autophagosome; therefore, its number correlates well with the number of autophagosomes. This LC3 conversion characteristic can be used to monitor autophagy.

In our study, we found that the content of LC3 protein isoforms, as well as their ratio, is lower in the group of SLE patients than in the control group (Fig. 4). However, in the group of patients, a significant deviation from the average value is noticeable. For this reason, we analyzed these indicators, depending on the nature of the disease course (acute, subacute, and chronic).

The analysis revealed the differences in the ratio of the LC3 protein isoforms depending on the course of SLE. In the group with an acute course, the value of the protein form I was the highest compared with that in the control. The content of form II was also higher compared with the control but lower in relation to that of form I. In the subacute group, the content of forms I and II of LC3 protein was almost the same, and it turned out to be lower in relation to the control. In the group with a chronic course, the change in the content of two forms of the LC3 protein was opposite to the group with the acute course of the disease, as form II content was significantly higher than form I. That is, the content of protein form II increases, which is localized on the membrane of autophagosomes. The ratio of LC3-I/LC3-II in the acute and subacute courses of the disease did not statistically differ from the control, and it was only lower than in healthy donors in the group with a chronic course (Fig. 5).

Discussion

Autophagy is a process that is responsible for the degradation of damaged proteins or those to be removed. If this process is ineffective, the proteins are accumulated. There is evidence confirming the relationship between clearance disorders of apoptotic proteins and an increase in the number of nuclear antigens presented to T cells [25], which is accompanied by autoimmune reactions that can lead to the development of SLE.

This work presents the analysis of the content of some key proteins of the apoptotic and autophagic pathways in peripheral blood T-lymphocytes of SLE patients. According to the results of the study of two main apoptotic proteins (Bcl-2 and caspase-3), we found an increased content of caspase-3 (an enzyme that induces chromatin condensation and DNA fragmentation), which indicates active apoptotic activity. In addition, this confirms the absence of a significant change in the content of anti-apoptotic protein Bcl-2 in patients compared with healthy donors. The result is consistent with previous data in the work of Rastin et al., where the content of this protein also remained at the control level [26]. Thus, we revealed that in the T-lymphocytes of SLE patients, apoptosis is more intense than in healthy donors.

There is a significant amount of evidence that impaired or delayed the clearance of apoptotic bodies in SLE, leading to the accumulation of apoptotic "debris," which correlates with the persistence of autoantigens [27]. Free apoptotic debris attached to dendritic cells, which represents antigenic signals to B cells, facilitates their differentiation into high-affinity plasma cells secreting antibodies. $Atg5^{-/-}$ embryos with autophagy deficiency have a more pronounced inflammation in tissues where the clearance of apoptotic cells is impaired [28]. This suggests that the lack of effective clearance of apoptotic cells can overcome tolerance to autoantigens and lead to SLE.

Based on the data obtained in our work, we can say that no abnormalities were found in the apoptotic process itself, and probably, the autoimmune burden was caused by insufficient activity of macrophages.

The relationship between apoptosis and autophagy has been demonstrated in a number of studies. It was established that in case of a disorder, in one of them, the other pathway can be activated, or both processes can occur in parallel in the same cell. For this reason, at the next stage of

our work, autophagy was evaluated by determining the content of its key proteins, namely, Beclin 1, Vps34, p62, and LC3. A comparative analysis of Beclin 1 and Vps34 showed an increased protein content in the T cells of SLE patients. The Beclin 1 (Atg6) gene plays a key role in mammalian autophagy. The protein encoded by this gene is actively involved in the initiation of autophagy, and as by joining the Vps34-PI3K-CIII complex, it induces the formation of autophagosomes. Since both of these proteins are part of the Beclin 1-Vps34-Atg14-PI3P complex, which is responsible for the elongation of preautophagosomes, it can be concluded that autophagy is activated in the T cells of SLE patients.

The p62 protein interacts with autophagic substrates and delivers them to autophagosomes for degradation. At that, the amount of p62 itself decreases, and when autophagy is induced, the level of p62 decreases consequently. On the contrary, analysis of the protein content in the groups revealed a significant increase in the number of p62 in SLE patients.

Interestingly, p62 itself is considered optional for canonical autophagy. In contrast to almost all major Atg proteins, the loss of which in mice results in embryonic or neonatal mortality, and the only adverse outcome manifested in $p62^{-/-}$ mice is adiposity of mature age [29]. Several recent publications have shown that p62 is a multifunctional scaffold protein that interacts with different proteins to regulate various processes, including apoptosis and other cell death forms, such as necroptosis. P62 has been shown to interact with ERK1 to regulate adipogenesis and with protein kinase C zeta to regulate NF-kB signal transmission [30]. For this reason, along with determining the content of p62, another important marker of autophagy protein, LC3, has to be monitored.

The analysis of the content of two isoforms of the LC3 protein showed a low content of both isoforms and the ratio of LC3-I/LC3-II in the group with SLE. Since the scatter of indicators was very different from the average value, we decided to analyze these indicators depending on the nature of the disease course.

In the group with the acute course, a high content of protein isoform I was revealed. The content of form II was lower, but its value was higher compared with that in the control group. The ratio of protein forms remained at the control level. In the group with the subacute course, the content of both isoforms was lower compared with that in other groups under study, but the LC3-I/LC3-II ratio was at the control level. In the group with a chronic course, a significant increase in the protein LC3-II was established, compared with form I. And a significant decrease in the LC3-I/LC3-II ratio was registered. LC3-II is constantly present in the autophagosome and is considered the most reliable marker of autophagy. However, the determination of the content of the LC3-I isoform is no less significant. An increase in the LC3-I level indicates an increase in the activity of autophagy processes, and an increase in the LC3-I/LC3-II ratio may indicate a high level of autophagy initiation during the normal course of further stages of this process, such as vesicular transport, fusion with lysosomes, and proteolytic degradation of the contents of autolysosomes.

An increase in the LC3-II level, as well as a decrease in the LC3-I/LC3-II ratio, may indicate a disorder of the fusion of autophagosomes and lysosomes and, therefore, incomplete autophagy, leading to the accumulation of a large number of autophagosomes inside the cell [31, 32]. With the excessive accumulation of autophagosomes inside the cells, exosome formation processes are activated. As a result, protein aggregates with prion-like activity can be excreted into the intercellular space and captured by healthy cells, which contributes to the further spread of the pathological process and increases the rate of disease progression.

In our work, a tendency toward an increase in the LC3-II level and a decrease in the LC3-I/LC3-II rate was noted in patients with a chronic form. Based on the data obtained, we can state a high level of autophagy initiation in the group with the acute form of the disease. And in the group with an acute course, since the values of protein forms I and II were low, autophagy is probably low active.

The results of our study, for the first time, revealed that T-lymphocytes of patients with acute SLE tend to a higher level of autophagy activity than that of healthy people. In the group with a subacute form of the disease, on the contrary, the degree of autophagy activity was insignificant. In the group with a chronic form of the disease, despite the high level of LC3-II protein, there are probably disorders at the stage of fusion of autophagosomes with lysosomes. To confirm this hypothesis, further research is necessary.

Conclusion

The data obtained suggest the use of monitoring of the content of the LC3 protein isoforms for the differential diagnostics of SLE forms.

Author contribution. Yu.V.S. and Z.I.A. developed the study and were responsible for overall leadership and planning. R.R.I., B.R.I., and S.N.A. conducted enrollment of donors for inclusion in the study, prepared the

samples, and isolated T-lymphocytes from peripheral blood. Yu.V.S. and A.R.F. conducted experiments to determine the content of p62 and LC3 proteins by Western blot and performed statistical processing of the results. B.R.I. and S.N.A. conducted experiments to determine the content of Beclin 1 and Vps34 proteins by Western blot method and performed statistical processing of the results. E.M.B. and I.A.A. performed the experiments to determine the content of proteins Bcl-2, caspase-3 by Western blot method, and statistical processing of the results. Yu.V.S., A.N.M., and Z.I.A. contributed to the interpretation of the results. Yu.V. S. prepared a manuscript with the participation of all authors. All authors provided critical feedback and helped develop the study, analysis, and the manuscript.

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Conflict of interests. The authors declare no conflict of interest for the article.

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