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Original Article

Evolution of Mechanical Stress (*In-vitro*) on Properties of Granulocytes in Acute Lymphoblastic Leukemia

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Abstract

Acute lymphoid leukemia (ALL) affects the lymph cells or lymphocytes that make up the lymph tissue and prevents the proper maturation of the bone marrow cells. The processes through which cells convert mechanical stimuli into biochemical signals are called mechanical transitions and result in the sensation of specific cellular responses. In the present study, the functional properties of granulocytes of the patients with ALL were investigated using the *in vitro* mechanical stress model. The experimental part of the work was executed using blood from patients with ALL (n=30) being treated in the Hematological Department of Belgorod Region Hospital, Belgorod, Russia. The patients were in the age range of 18-45 years. Sample blood was obtained from all the patients who underwent a standard course of chemotherapy. Blood sampling was performed using a venepuncture and collected into the vacuum tubes Vacuette K3E. Blood samples from each experimental group were divided into two groups of control and experiment. The injection model of mechanical stress was used for the experiment group *in vitro*. Subsequently, the adenosine triphosphate (ATP) concentration increased by 1.8 times in this group, compared with the controls. Young's module, which numerically characterizes the rigidity of the granulocytes' plasmalemma, decreased by 54.4% ($P<0.05$) under the influence of mechanical stress. The surface potential of plasmalemma was not significantly different between samples in the group of control and experiment in patients with ALL. However, the adhesive force between erythrocyte and granulocyte increased by 30.7% ($P<0.05$). The osmotic load test showed an increase in the cell's volume during incubation. The use of membrane reserve by granulocytes increased by 47% ($P<0.05$) at the initial seconds of incubation. The obtained results pointed to the regulatory role of ATP molecules in intercellular signaling and add to the present literature regarding the mechanisms of intercellular interaction in the microvasculature on the development of leukemia. Moreover, the obtained results can be taken into account for the development of new pharmacological immune correctors.

Keywords: Adhesive properties of biomembranes, Atomic force microscopy, Granulocytes, Potential surface, Young's module

1. Introduction

Lymphoblastic Leukemia is one of the most common childhood malignancies affecting about 40 in every one million children under the age of 15. Acute lymphoblastic leukemia (ALL) accounts for about 75% of leukemia cases. Due to the prevalence of leukemia in children, faster diagnosis of the disease and identification of early clinical and laboratory symptoms is of great importance. ALL is divided into T or B cell categories. The peak incidence of ALL is observed between the age of 2 and 5 in children and is higher in boys than girls. It is worth mentioning that, T cell type ALL is more prevalent among males. Signs and symptoms of ALL are associated with infiltration of leukemia cells into normal tissues which can result in bone marrow failure or specific tissue infiltration

(lymph nodes, liver, spleen, bone marrow, skin, or testes) (1).

Extracellular messenger purine signaling is mediated by purine nucleotides and nucleosides, such as adenosine and ATP. Purine receptors are activated in the cell and/or in adjacent cells to regulate cell functions. The ATP acts as a short-term signaling molecule in neurotransmission, neuromodulation, and neurosecretion (2). The purinergic signaling system acts as a paracrine mechanism for the regulation of intercellular communication and has a spectrum of biological effects on the functions of both normal and tumor cells (3). In particular, some studies have demonstrated the role of extracellular nucleotides (ATP, ADP, UTP, UDP) in regulating cell proliferation, migration, and death, based on the expression of the purinergic receptor subtype and concentration of nucleotides in the extracellular environment (4). Purines are involved in neurotransmission and neuromodulation since their effects are mediated by the purine and pyrimidine receptor subfamilies P1, P2X, and P2Y. The researchers showed that purinergic mechanisms and subtypes of particular receptors are involved in various pathological conditions, including brain injury and ischemia; neurodegenerative diseases, including neurological immune and inflammatory neurological reactions; as well as neuropsychiatric disorders, including depression and schizophrenia (5).

In response to the mechanical "stress", the erythrocytes and endothelium cells excrete ATP molecules into the intercellular space which are key participants in the intercellular interactions and act as regulators of the immune activity of leucocytes due to activation of purinergic receptors localized on the cell membranes (6). The main suppliers of extracellular ATP are erythrocytes, which release ATP in response to mechanical stress (7). A particular P2X7 has an essential role in the organization of inflammation (8) and the functioning of tumor cells (9).

This study aimed to examine the granulocytes' properties (mechanical, adhesive, and electrical) in patients with ALL during the stimulation of elements of purinergic signaling pathways on the model of mechanical stress *in vitro.* The lymphoid tissue of adaptive immunity is damaged in the blood system in ALL, which is a natural model of the tumor process. The granulocytes are full-fledged cells that support the implementation of immune responses in the microvasculature and are directly involved in intercellular communications, both with healthy and tumor lymphoblasts.

2. Material and Methods

The experimental part of the present study was executed using blood samples from patients with ALL (n=30) being treated in the Hematological Department of Belgorod Region Hospital, Belgorod, Russia. The patients were in the age range f 18-45 years. Blood sampling was performed on all patients who underwent a standard course of chemotherapy. There were no blast forms of lymphocytes in the blood samples. The study was carried out in compliance with the requirement of the Helsinki Declaration and written informed consent was obtained from the participants following the provision of required recommendations.

The special medical personal performed the blood sampling using venipunctures. Blood was collected into the vacuum tubes Vacuette K3E. Blood samples from participants were divided into two groups of control and experiment. The condition of mechanical stress was modeled in the experimental group. The injection model of mechanical stress *in vitro* as described in the work of Oonishi, Sakashita (10) was implemented on the samples in the group of the experiment. However, no intervention was performed on the samples in the group of control, and they remained intact.

The ATP concentration in the experiment and control samples was measured using a photometer at a wavelength of 670 nm against physiological saline. The ATP was measured three times for each sample.

The properties of granulocytes were studied by a method of atomic force microscopy (AFM). Elastic properties of the granulocytes' plasmalemma were estimated through the numerical data of Young's

module. The modified AFM-probes were prepared according to tipless and polymer hemispheres (with a curvature radius of 5 mkm) (11). It should be noted that 20 cells from each sample were scanned.

Electrical properties of granulocyte's membrane were evaluated through the measurement of surface potential in the mode of Kelvin probe on an AFM using cantilevers with a conductive titanium coating of the NSG03/TiN (Nanoworld, USA). The preparation of the cell suspension for measuring surface potential was carried out according to the method described in the study performed by Sladkova and Skorkina (12). About 20 granulocytes were scanned in each sample. The obtained scans were processed using the Nova program (NT-MDT, Russia). The value of surface potential was calculated based on the obtained scans that reflect the distribution of potential on the leukocyte's surface in 10 points of each cell by "Point Instrument" software in the Nova program (NT-MDT).

The adhesive forces were measured in the AFM mode of force spectroscopy. The biosensor chip was constructed according to the native erythrocyte and Tipless AFM Probes (CSG11, USA) following the pathway set out in the study conducted by Skorkina, Shamray (13). The adhesive force in the "erythrocyte-granulocyte" system was measured by the registration of the force curves from the surface of 20 cells. The adhesive force was calculated using the software Nova (NT-MDT, Russia).

The model of the hypoosmotic load was applied to the granulocytes after the mechanical stress. According to the results of some previous studies, the granulocytes' membranes have mechanical osmotic sensors, as TRPV 4 channels (14) which transform physical irritants to the Ca^{2+}-dependent ATP releasing receptor (15). Leucocytes from whole blood were separated by centrifuging at 1500 rpm for 5 min. Leukocyte suspension in the control and experimental samples were divided into two parts. An equal amount of the autologous plasma was added and to the first part, and sodium chloride 0.45% was supplemented to the second part. The suspension samples were formed and the images of native leukocyte suspension were recorded after every 30 sec using scanning confocal microscope Nikon (Tokyo Byoke, 2011) and Nis-Elements Documentation software (Version 2.32). The duration of the hypoosmotic load was 5 min. The diameter of 50 cells from each sample was measured based on the obtained images, and the square and volume of cells were calculated as well. The relative membrane reserve was calculated according to the following formula:

$$MRrel = \frac{Shyp - Sp}{Vp}, \quad (1)$$

In this formula,

MR_{rel}: relative membrane reserve of cells (mkm^{-1});

S_{hyp}: square of cell's surface in the hypotonic medium (mkm^2);

Sp: square of cell's surface in the autologous plasma (mkm^2);

V_p: cell's volume in the autologous plasma (mkm^3).

The intensive of using the relative membrane reserve by cells was estimated by calculating the percent of relative membrane reserve used by the cells from potential membrane reserve, accepted as 100%.

The results of experimental studies were processed by methods of variation statistics. The significance of differences between control and experimental samples was determined using Student's t-test in the case of a normal distribution of traits. The Mann-Whitney U-test was used for the analysis of nonparametric data. A p-value less than 0.05 ($P<0.05$) was considered statistically significant. The mean values (M) and the statistical error of the mean (m) are measured in this study as well.

3. Results

The application of the mechanical stress model *in vitro* on the blood sample of patients with ALL increased the ATP concentration by 1.8 times,

compared with the controls. The Young's module, which numerically characterizes the rigidity of the granulocytes' plasmalemma, decreased by 54.4% under the influence of mechanical stress (P<0.05), compared with the group of control (Table 1).

No difference was observed between the blood samples of patients with ALL in terms of the surface potential of plasmalemma in the groups of control and experiment. However, the adhesive force between erythrocyte and granulocyte increased by 30.7% (P<0.05) in the group of experiment, compared with the group of control (Table 1).

The results of the osmotic load test showed that the cell's volume increased during the all-time incubation in the blood samples of the experiment group, compared with the control samples. At the initial stage of incubation, the use of membrane reserve by granulocytes increased up to 47% (P<0.05) under the influence of mechanical stress, compared with controls. The phase of regulatory volume decrease was observed in the next 90 sec of incubation in the experiment group. However, the use of membrane reserve by granulocytes was increased, compared with controls (i.e., on the 30 sec incubation by 33.7% (P<0.05), on the 60 sec by 30.3% (P<0.05), on the 90 sec by 38.3% (P<0.05; Figure 1).

Table1. Blood parameters in patients with ALL under the influence of mechanical stress

Parameters	Control	Experiment
ATP concentration in the whole blood (mkM)	0.0064 ±0.0005	0.0116 ± 0.008*
Young's module of granulocytes (mkPa)	2.38 ± 0.01	1.54 ± 0.01*
Adhesive force in the system "erythrocyte – granulocyte"(nN)	57.1 ± 0.3	82.5 ± 0.6*
Surface potential of granulocytes (mV)	-21.08 ± 1.7	-24.82 ± 1.8

Note: * – statistically significant differences between the indicators of experimental and control groups obtained by the Mann-Whitney U-test (P<0.05).

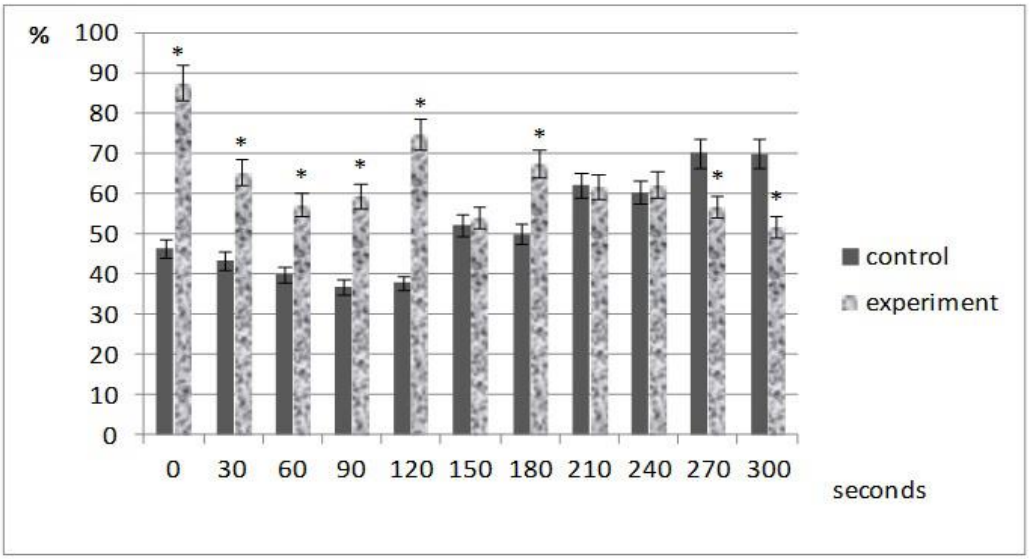

Figure 1. The intensity of the use of the membrane reserve by granulocytes in the hypoosmotic test (*in vitro*)*:* mechanical stress *in vitro* was applied in the group of experiment, while blood samples in the control remained intact.
*statistically reliable differences in the study, compared with the controls determined by the Student's *t*-criterion at p<0.05.

Based on the data in Figure 1, the phase of regulatory volume increased in the experiment group in the interval from 90 to 120 sec of incubation. The use of the membrane reserve by cells on the 120 sec increased by 49.5% ($P<0.05$), compared with controls. On the 150 sec, the cell volume was not different in both experiment and control groups; therefore, no significant change was observed in the use of membrane reserve. In the experiment group, the increase of membrane reserve by granulocytes has been fixed at 26% ($P<0.05$) within the 180 sec of incubation which was then replaced by the decrease in the use of membrane reserve by cells before the end of incubation (in the interval of 210 and 300 sec), compared with controls.

The phase of regulatory volume decrease in the control group occurred during the first 120 sec of incubation. The phase of regulatory volume increase was observed in the interval from 120 to 150 sec. The equilibrium phase has been established in the interval from sec 150 to sec 480, starting from sec 210, and the phase of regulatory volume decrease was registered at the end of incubation.

4. Discussion

The obtained results were consistent with the existing literature indicating that mechanical stress triggers a purinergic signal cascade in the blood cells by the release of ATP from erythrocytes (16) and neutrophils (17) when they are deformed in the microvasculature. Extracellular ATP is a powerful chemotaxis stimulus for granulocytes and triggers changes in the properties of plasmalemma which ultimately affects the implementation of inflammatory reactions in the body (18). ATP molecule activates the granulocytes via the opening receptor – ion channel P2X4 expressed in the surface of immune cells and having the high permeability for Ca^{2+} (19) that promotes the increase of cytosol Ca^{2+} concentration necessary for remodeling of the cytoskeleton (20). The decrease in stiffness of the cell surface of granulocytes in the study can be attributed to the rearrangement of cytoskeleton elements. The decrease of adhesive force in the system "erythrocyte-granulocyte" with the development of ALL confirms the important physiological role of erythrocytes in the adhesive function of leukocytes. It was proved that first-hand interaction between erythrocytes and leukocytes (flow around leukocytes by erythrocytes) was necessary for increasing the tangential force and torque that promote the rolling of leukocytes on the surface of the endothelium wall in the microvasculature (21).

The vesicular ATP release from granulocytes was indirectly proved in this study using the part of the membrane reserve and modeling the osmotic force to the cell after mechanical stress. Based on the obtained results, the relative membrane reserve involvement by granulocyte in their volume regulation was increased. According to the existing literature, the leukocytes is induced the TRPV4-cation channel as a sensor transforming the osmotic stimulus to the Ca^{2+}-depend ATP release due to vesicular transport involving pannexin-1 (22). The obtained data indicated that the membrane reserve of granulocytes was increased under the condition of osmotic stress.

5. Conclusion

Based on the obtained data, the mechanical deformation of blood cells (mechanical stress model *in vitro*) leads to the increase of ATP concentration in the blood, change in properties of granulocytes, decrease in the stiffness of cells surface, an increase of adhesive force in the "erythrocyte – granulocyte" system. The results confirm the regulatory role of the ATP molecule in intercellular signaling and can add to the existing literature about mechanisms of intercellular interaction in the microvasculature and the role it plays in the development of leukemia. Moreover, the presented results can be taken into account for the development of new pharmacological immune correctors that aim to maintain the functional activity of the healthy population of leukocytes involved in immune reactions

of patients with ALL.

Authors' Contribution

Study concept and design: Y. M. S.
Acquisition of data: A. S. T.
Analysis and interpretation of data: T. S. S.
Drafting of the manuscript: E. S. S.
Critical revision of the manuscript for important intellectual content: L. R. Z.
Statistical analysis: Y. M. S.
Administrative, technical, and material support: T. S. S.

Ethics

The study was carried out in compliance with the requirement of the Helsinki Declaration and written informed consent was obtained from the participants following the provision of required recommendations.

Conflict of Interest

The authors declare that they have no conflict of interest.

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