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Mechanical and structural properties of rat and human lymphocytes after the exposure of the whole blood to X-rays *in vitro*

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Abstract

Objective. By the means of atomic force microscopy to determine the changes in the parameters of the structural and mechanical properties of peripheral blood lymphocytes induced by the irradiation of whole blood by X-rays and identifying the possibility of assessing a state and radiation-induced lymphocyte death programs by analyzing a set of such parameters.

Materials and methods. Whole blood of rats and humans was irradiated with X-rays (1–100 Gy) *in vitro.* Lymphocytes were isolated from the blood after a day of storage, placed on glass slides, fixed with glutaraldehyde and dried. The study of structural and mechanical properties was carried out with the help of atomic force microscope Bruker Bioscope Resolve in Peak Force QNM mode in air. For the sets of AFM parameters, which included elastic modulus, adhesion force, cell surface roughness and cell sizes, a k-mean clustering of data was carried out for the studied experimental groups.

Results. The X-ray irradiation of the blood caused changes in the structural and mechanical properties of lymphocytes measured by AFM at the nanoscale. Clustering analysis of the sets of AFM parameters revealed clusters with similar structure in each experimental group (humans, 6- and 16-month rats). The studied four clusters were associated with cell states and cell death programs: non-activated cells, activated cells with increased stiffness, apoptotic cells with reduced stiffness, and cells dying via programs other than apoptotic ones with increased stiffness. Each cluster (cell type) with a specific set of AFM parameters was represented differently in the blood lymphocyte population, depending on the dose of X-rays.

Conclusion. The set of ACM parameters of lymphocytes including elastic modulus, adhesion force, roughness, and cell sizes, can be helpful for automatically determining the state and death program of lymphocytes after the local irradiation of humans with the involvement of peripheral blood (for example, after radio-therapeutic causes).

Keywords: *X-rays, rat, human, blood, lymphocytes, mechanical properties, atomic force microscopy*

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Механические и структурные свойства лимфоцитов крысы и человека после воздействия рентгеновского излучения на цельную кровь *in vitro*

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Резюме

Цель исследования. Выявить с помощью атомной микроскопии изменения параметров структурных и механических свойств лимфоцитов периферической крови, вызванные облучением цельной крови рентгеновским

излучением, и определить возможность оценки состояния и радиационно-индуцированные программы гибели лимфоцитов с использованием анализа набора таких параметров.

Материалы и методы. Цельную кровь крыс и человека облучали рентгеновским излучением (1–100 Гр) *in vitro*. Лимфоциты выделяли из крови после суток хранения, помещали на стеклянные пластины, фиксировали глутаровым альдегидом и высушивали. Изучение структурных и механических свойств проводили с помощью атомно-силового микроскопа (АСМ) Bruker Bioscope Resolve в режиме PeakForce QNM на воздухе. Для наборов АСМ-параметров, в которые были включены модуль упругости, сила адгезии, шероховатость клеточной поверхности и размеры клеток, для разных экспериментальных выборок была проведена кластеризация данных методом k-средних.

Результаты. Облучение крови рентгеновским излучением вызвало изменение параметров структурных и механических свойств лимфоцитов, измеренных с помощью АСМ на наномасштабе. Кластеризация наборов АСМ-параметров выявила кластеры с подобной структурой в каждой экспериментальной группе (человек; крысы 6 и 16 месяцев). Изученные четыре кластера ассоциированы с разными состояниями клеток и программами их гибели: неактивные клетки, активированные клетки с повышенной жесткостью, апоптотические клетки со сниженной жесткостью и клетки, гибнущие по другим, отличным от апоптоза программам гибели с повышенной жесткостью. Каждый кластер (тип клеток) с определенным набором АСМ-параметров был разным образом представлен в популяции лимфоцитов крови в зависимости от дозы рентгеновского излучения.

Заключение. Комплекс АСМ-параметров лимфоцитов, включающий модуль упругости, силу адгезии, шероховатость и размеры клеток, может быть полезным при автоматическом определении состояния лимфоцитов и программы их гибели при локальном облучении организма с вовлечением периферической крови, например при радиотерапии.

Ключевые слова: *рентгеновское излучение, крыса, человек, кровь, лимфоциты, механические свойства, атомно-силовая микроскопия*

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Introduction

The cell's responses to ionizing radiation include changes in its biomechanics [1]. The irradiation of the whole blood with ionizing radiation causes changes in the properties of all blood-formed elements (erythrocytes, leukocytes, and platelets). It has long been known that lymphocytes are the most radiosensitive cells of the hematopoietic system [3-7]. Ionizing radiation induces various signaling pathways, leading to cell death. The cell's mechanical properties depend on the cell's state and cell death program [8].

There are many methods to measure the parameters of cell mechanical properties, among which atomic force microscopy (AFM) occupies a key position [2]. AFM provides not only three-dimensional imaging of the surface of single cells or small areas of their surface with nanoscale resolution, but it also maps the mechanical properties (elastic and adhesive) of the cell's surface. Using a Quantitative Nanomechanical Mapping AFM mode (PeakForce QNM), several maps of different cell's surface properties can be simultaneously recorded. Each small cell's surface area can be characterized by a set of parameters averaged over the scanned area: the elastic modulus, or Young's modulus, E; the adhesive force, F_a; the roughness of the cell topography, $R_{q\ topo}$; or any other recorded property map, for example, an adhesion map, $R_{q\ Fa}$. This set of cell parameters describes cell's mechanical phenotype, defining cell type and cell state. AFM is a powerful method of automatic analysis of cellular mechanical phenotypes based on the processing of large volumes of multidimensional data (big data).

Our work is the first attempt to develop theoretical and experimental bases for the application of ACM for automatic analysis of lymphocyte properties and states after blood irradiation by ionizing radiation. To obtain general and objective results, we conducted experiments using different species (humans and rats) and ages (6 and 16-month-old rats). We have clustered ACM parameter sets for each experimental group to identify the sets of ACM parameters characterizing different cell states and death programs. At present, we have no information published in the available literature about any ACM

studies of radiation-induced changes in the mechanical properties of lymphocytes.

Objective

The study aims at determining by atomic force microscopy the changes in the parameters of the structural and mechanical properties of peripheral blood lymphocytes induced by the irradiation of whole blood by X-rays and identifying the possibility of assessing a state and radiation-induced lymphocyte death programs by analyzing a set of such parameters.

Materials and methods

Blood samples

All animal experiments were approved by the Animal Care and Ethic Committee of the Gomel State Medical University. The ethical considerations of the use of the rats were in accordance with the international recommendations of the "European Convention for the Protection of Vertebrate Animals used for Experimental or other Scientific Purposes". All experimental procedures used in the present study were conducted according to the rules of Directive 2010 / 63 / EU of the European Parliament and the Council of the European Union on the protection of animals used for scientific purposes. Male Wistar rats (16 months) were housed under standard vivarium conditions with ad libitum access to food and water. The rat blood was taken from the portal vein of the liver under deep anesthesia. A human blood sample was taken from the ulnar vein of a male volunteer (24 years old) after receiving the informed consent. The volunteer had no history of malignancy, immune deficiencies, autoimmune disorders, hepatitis, or HIV infection.

Blood irradiation

Whole blood was irradiated using a X-RAD 320 Biological Irradiator (Precision X-Ray, USA) by X-rays (320 kV, 12.5 A, 50 cm, filter 1.5 mm Al, 0.25 mm Cu, 0.75 mm Sn). The absorbed doses of 1, 25, 50 and 100 Gy were used. Lymphocytes were isolated after one day of irradiated blood storage at 4° C in a density gradient (ROTI®Sep 1077, Carl Roth), immobilized on adhesive-coated glass slides, fixed with 1% glutaraldehyde and washed with phosphate-salt buffer and distilled water.

Atomic force microscopy

In this study, we used Bruker's BioScope Resolve AFM. Scanning in air was performed using SCANASYST-AIR probes (Bruker, k=0.4 N/m, R=2 nm) in PeakForce QNM mode (NanoScope 9.4 software, Bruker). The surface of the whole cell was scanned using the following parameters: scan size of 10 μm × 10 μm, rate of 1 Hz, and resolution of 256 × 256 pixels. The small areas (three per each cell) of the cell surface were scanned using the following parameters: rate of 0.5 Hz, scan size of 250 nm × 250 nm, resolution of 256 × 256 pixels, force of 500 pN. Calibration of the probes was done before scanning by contact method according to the protocol of the microscope manufacturer (Bruker PeakForce QNM User Guide, #004-1036-000, 2011). The probe was calibrated at the frequency of 0.5 kHz; with a Ramp Size of 300 nm and a Ramp SetPoint of 0.3 V in air. Three types of AFM images were used in further analysis: topographic maps (Height), maps of elastic modulus (DMT modulus), and maps of adhesion force. To estimate the elastic modulus (Young's modulus), the linear segment of a retract curve recorded during scanning is fitted automatically using the Derjaguin-Muller-Toporov (DMT) model. The mechanical and structural parameters (the elastic modulus, E; the adhesion force, F_a, the roughness of topographic map, $R_{q\ topo}$, the roughness of adhesion map, $R_{q\ Fa}$) were assessed using them.

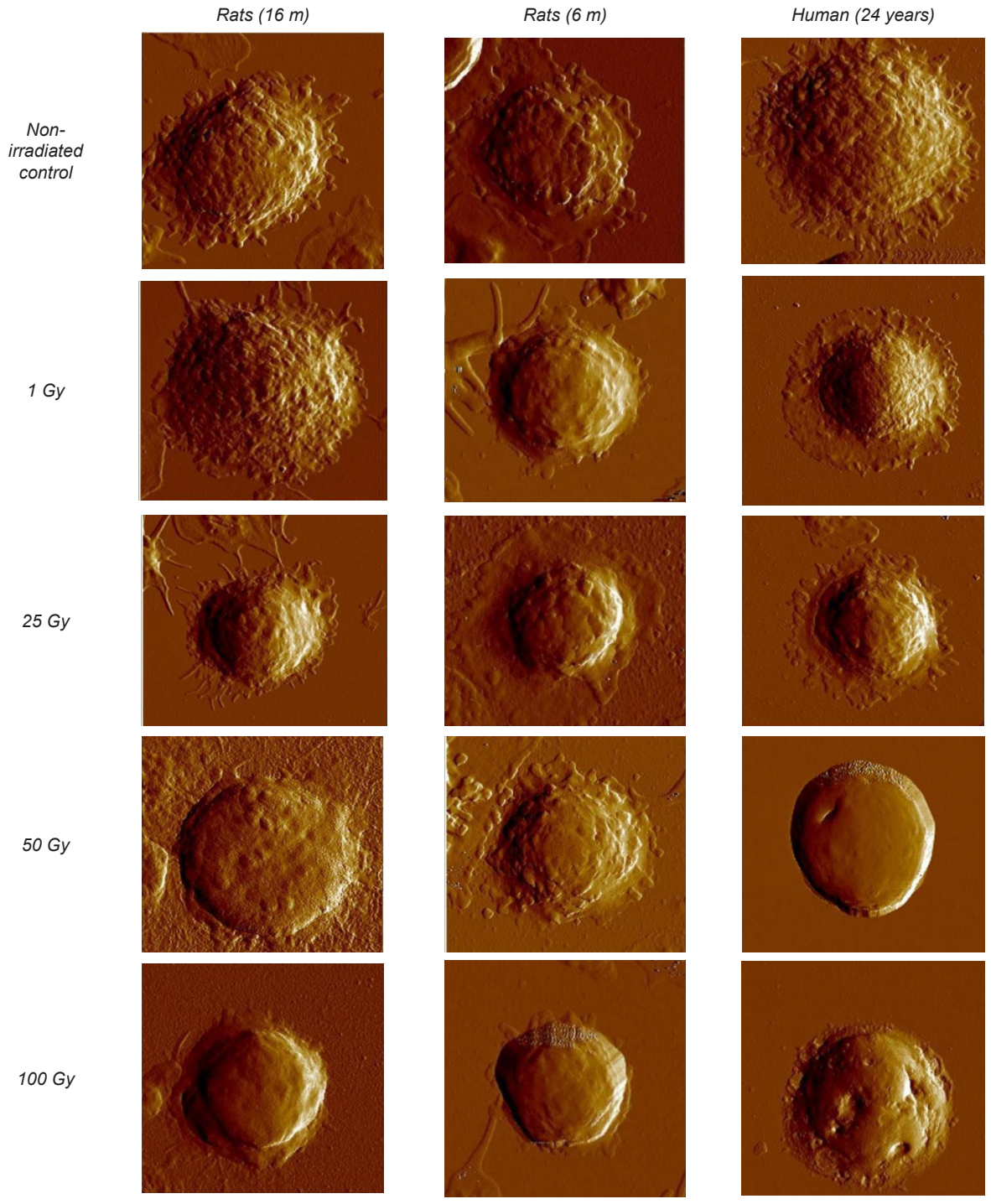
Data analysis

The data analysis was performed using NanoScope Analysis 1.9 software (Bruker). Statistical analysis of experimental data was carried out using R studio, MS Excel 2016, Statistica, and the Statistical Kingdom online calculator (https://www.statskingdom.com/). The raw AFM data from files generated by the Bruker instrument in the process of scanning the cell surface were transformed into ASCII files for further processing. Data analysis of ASCII files was performed using R (version 4.0.5). The data were checked for compliance with the normal distribution law using the Shapiro-Wilks test. The data are represented as the median and limits of the interquartile range (Me(LQ; UQ)), the mean and standard deviation (M±SD) or the 95% confidence interval (95%CI). Multiple comparison analyses were performed using the ANOVA Post-hoc test (Kruskal-Wallis test, Dunn's test with Bonferroni correction). Clustering parameter sets (E, Fa, $R_{q\ topo}$, $R_{q\ Fa}$, D, H) were performed using the classical method of cluster analysis, the k-means method implemented using Statistica software. The k-means method is a method of multivariate statistical analysis, the purpose of which is to divide m observations (from space R_n) into k clusters, with each observation belonging to the cluster to the center (centroid) of which it is closest. The number of clusters was 4. The effectiveness of clustering was analyzed using the Euclidean distances between clusters.

Results and discussion

The typical changes in lymphocyte morphology induced by whole blood X-ray irradiation are presented in Figure 1. The blood irradiation at doses of

1 and 25 Gy led to the smoothing of the lymphocyte surface and a decrease in the number of microvilli on the lymphocyte surface compared to the control non-irradiated blood samples for both species (rats and humans). At doses of 50 and 100 Gy, lymphocyte microvilli and lamellopodia were almost absent. Among the lymphocytes isolated from the blood irradiated with X-rays at 25-100 Gy, apoptotic lymphocytes were detected. In AFM images, apoptotic cells were characterized by the presence of globular structures, or apoptotic bodies, on the cell surface and the invaginations of the cell surface in the nuclear zone, corresponding to nucleus pyknosis. Pyknosis, or karyopyknosis, is the irreversible chromatin condensation in cell nuclei undergoing necrosis or apoptosis.



Scan size is 10 μm×10μm, resolution is 256×256 pixels

Figure 1. AFM images (PeakForce Channel data) of lymphocytes depending on the absorbed dose and species

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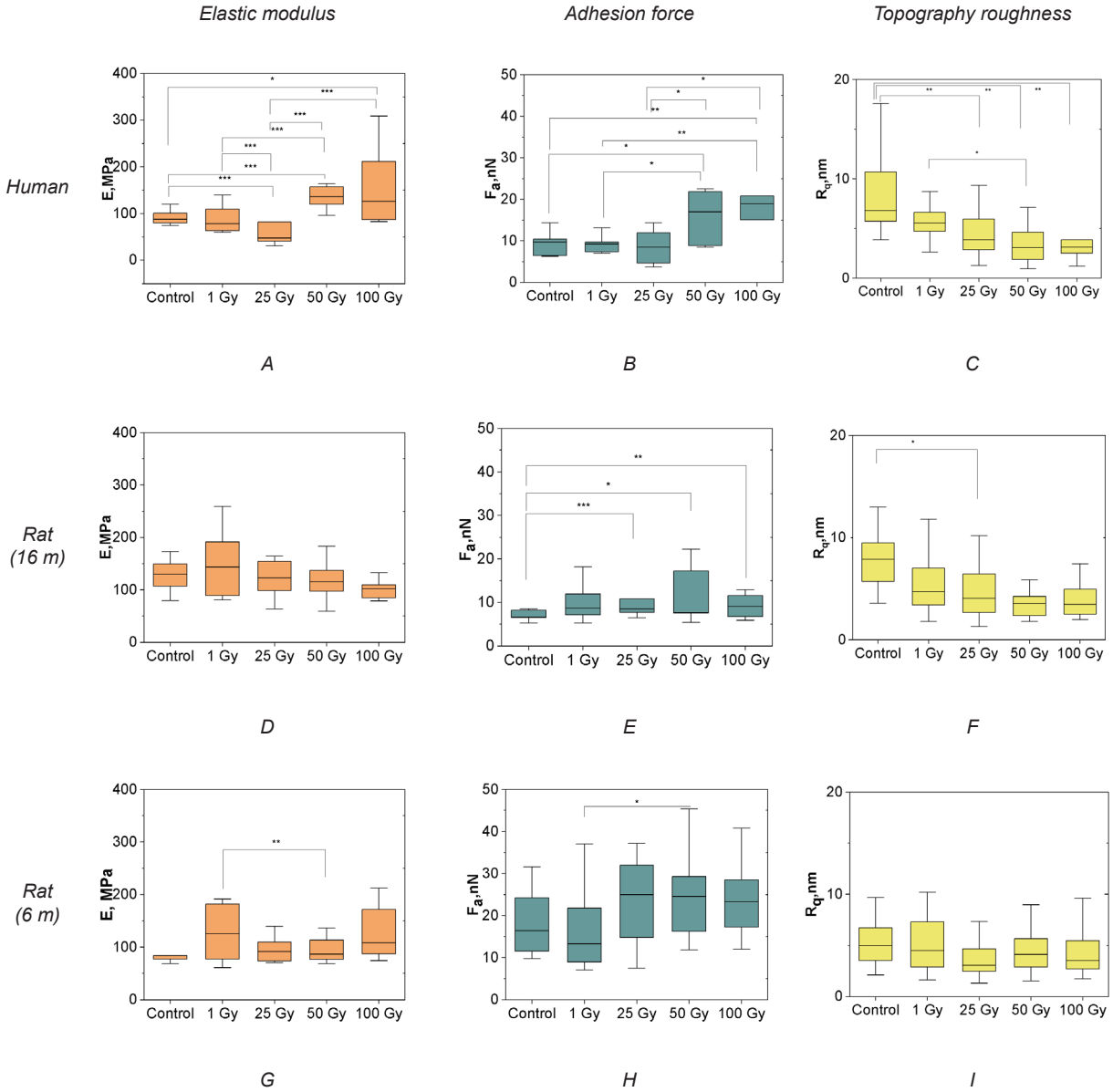
In Figure 2, several nanomechanical parameters (Young's modulus, E; adhesive force, F_a; roughness of cell topography, $R_{q\ topo}$) of the lymphocyte's surface depending on the absorption dose and species are presented. Comparing the characters of the revealed dose dependencies of the parameters for different species, we can observe the tendencies of the decrease in roughness, increase in adhesive force, and non-monotonical change of elastic modulus with the dose.

The dependency character significantly varied with experimental groups (species and age). The obtained data show a significant difference in the mechanical parameters caused by ionizing radiation (Figure 2, A, B, C, E, F, G, H), but their interpretation is difficult. The data obtained for the lymphocyte population isolated from the irradiated blood were heterogeneous for any experimental group. For example, the distribution of E for the control human blood lymphocytes is well described by the Gauss function with the mean elastic modulus of 102.62±30.26 MPa (95% CI), indicating a relative homogeneity in the elastic properties of the control cell surface. After the exposure of the whole blood to X-rays at doses of 1-100 Gy, the distribution of E became highly heterogeneous. The probability density curve is best described by a function representing the sum of the two Gaussian curves.

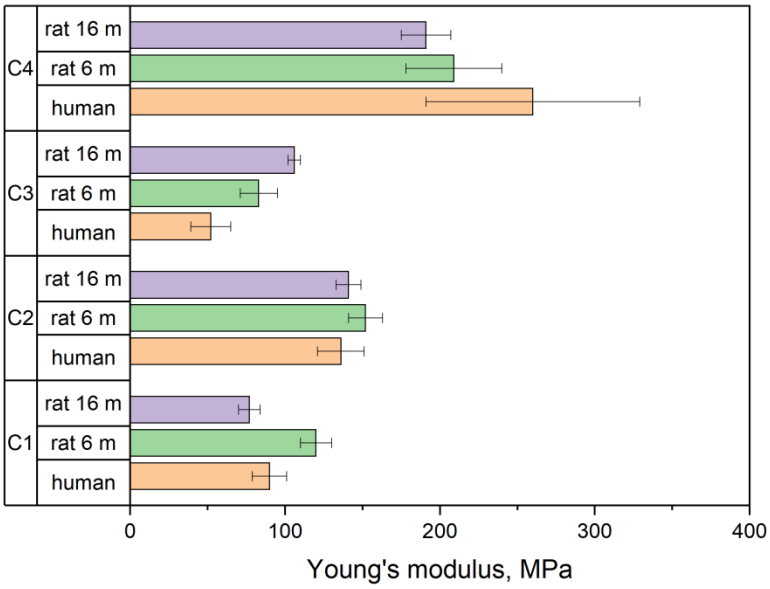


*Data are presented as the median, lower and upper quartiles, maximum and minimum. p<0.05, Kruskal-Wallis test, Dunn's test with Bonferroni correction.*p<0.05,**p<0.01,***p<0.001*

Figure 2. The influence of X-ray irradiation of the whole blood on Young's modulus, E (A, D, G), adhesion force, F_a (B, E, H), topographic roughness, R_q (C, F, I), of lymphocyte surface for different species

This fact may indicate either the presence of two types of cell surface areas at the nanoscale with different elastic properties (less or stiffer than the stiffness of control cells) or the heterogeneity of the cell population in cell elastic properties. The cell population heterogeneity of the studied properties can be related to the presence of cells with various activation states and at different stages of cell death programs. Even at a dose of 100 Gy in the blood lymphocyte population, it is possible to find the cells that do not die via the necrotic or apoptotic cell death program.

It becomes difficult to infer the general regularities of the influence of ionizing radiation in the studied doses on the mechanical properties of lymphocytes of different organisms and ages. These difficulties arise from small cell samples for ACM research and cell heterogeneity in response to ionizing radiation. The cells in the population will evolve in different states and ways after ionizing radiation. Therefore, we have attempted to classify (cluster) the lymphocytes of the studied samples based on the set of AFM parameters associated with the structural and biomechanical properties of their surface. The parameter set for clustering consists of the parameters of mechanical properties (E, F_a, and $R_{q\ Fa}$), parameters of morphology (diameter, D, and height, H, of cells), and the parameter of the surface nanoarchitecture ($R_{q\ topo}$). The data clustering was performed separately for human (24-year-old) and rat (6-month-old and 16-month-old) samples. At the beginning of the analysis, we assumed that the lymphocyte population may contain resting cells (C, a non-active control), activated cells (S), apoptotic cells (A), and necrotic cells (N). Therefore, we used four clusters for clustering analysis. A key parameter for clustering was the stiffness of the lymphocyte's surface. The average Young's modulii of lymphocytes of different clusters (C1, C2, C3, and C4) and experimental groups (human, rats 6 and 16 m) are presented in Figure 3.



C1, C2, C3, and C4 are cluster 1, cluster 2, cluster 3, and cluster 4, correspondingly. Data are presented as M±SD.
Figure 3. The average values of Young's modulus of lymphocytes belonged to different clusters for human and rat blood samples

Cluster 1 may correspond to control (C) lymphocytes without signs of apparent activation or death. Cluster 2 seems likely to link with a cell activation (S) state; there is an increase in elastic modulus. In an activated state, cells possess the developed structure of the cortical cytoskeleton, which leads to an increase in cell surface stiffness and smoothing of their surface at the nanoscale. Cluster 3 may correspond to an apoptotic (A) cell death program; the elastic modulus decreases. In apoptotic cells, the depolymerization of actin filaments of the cortical cytoskeleton leads to a reduction in cell stiffness and an increase in non-specific cell surface adhesion. Hu and co-authors [11], studying resting, activated and apoptotic lymphocytes with AFM, revealed that cell activation increased the cell stiffness and apoptosis reduced the cell stiffness compared to the stiffness of resting cells. The reduction in the elastic modu-

lus of apoptotic cells was proven by other authors [12, 13]. Cluster 4 may correspond to a cell death program other than an apoptotic one (necrotic cell death program (N)) as the elastic modulus increases. The cells have specific shapes, and typical cell edge structures such as microvilli and lamellopodia are absent.

The results of AFM data clustering analysis, as a first approximation, satisfy the criterion of cell classification based on lymphocyte death programs and functional activity. The different cell clusters contribute differently to the population of the studied AFM samples for each absorbed dose and non-irradiated sample. In the control (non-irradiated) samples, cluster 1 was dominant: 83% (human), 63% (rat 6 m), and 80% (rat 16 m). The contribution of cluster 2 (activated lymphocytes) increased with the absorbed dose, with a maximum at 1 Gy (11% (human), 45% (rat 6 m), and 30% (rat 16 m). The percentage of the cells belonging to cluster 3 (apoptosis) increased with the absorbed dose, with a maximum at 25 Gy (83% (human), 20 (rat 6 m), and 40 % (rat 16 m).

We analyzed the effectiveness of cell clustering for each experimental group and all samples using the Euclidean distance between clusters. Four clusters with comparable parameters discriminate similarly for each experimental group (human, rat 6 m, and rat 16 m). The best discrimination was reached between resting (C) and necrotic (N) cells and between apoptotic (A) and necrotic (N) cells. The worst discrimination was obtained between resting (C) and activated (S) or apoptotic (A) cells.

Our findings have shown the heterogeneity of the cells in the blood lymphocyte population in terms of structural and mechanical properties after the irradiation of the whole blood with X-rays. The heterogeneity of cell states contributes significantly to the obtained values of the AFM parameters. All samples are characterized by the presence of non-activated and apoptotic cells with reduced stiffness, activated cells, and necrotic cells with enhanced stiffness. The results of data clustering analysis for non-irradiated blood lymphocytes indicate a predominance of non-activated forms of lymphocytes. As the dose of blood irradiation increases, the number of dying cells and activated cells changes. The radiation-induced apoptosis leads to a softening of the cell surface. Radiation-induced lymphocyte activation and necrosis can lead to stiffening of the cell surface. Depending on the percentage of cells in a certain state and the death program, the population of lymphocytes can be characterized by a smaller or bigger average value of elastic modulus.

Peripheral blood lymphocytes are a highly heterogeneous population of cell types: T-, B-, and natural killer lymphocytes, which in turn are divided into subclasses. Different lymphocyte types have different levels of radiosensitivity and radioresistance [12, 13]. The different lymphocyte subtypes show distinct radiosensitivity. Naive CD8+ effector T cells are more sensitive than memory T cells, while regulatory T cells are relatively resistant [14].

In the present work, we did not separate the different types of lymphocytes. The heterogeneity of the peripheral blood lymphocyte population in types can also cause the ambiguity of a classification (clustering) of lymphocytes by their structural and mechanical properties related to the cell state. To further develop the AFM-based method for automatic determination of the lymphocyte states after the exposure of the blood to ionizing radiation, it will be necessary to perform the experiments taking into account the lymphocyte types and involving a larger number of cells.

Conclusion

The work is the first AFM-based study of the nanostructural and nanomechanical properties of the surface of blood lymphocytes of rats and humans after whole blood irradiation with X-rays in vitro in the range of doses (1-100 Gy). In our work, we suggested characterizing the mechanical phenotype of lymphocytes and its radiation-induced change by a set of AFM parameters (elastic modulus, adhesion force, roughness of topography and adhesion map, cell diameter, and cell height). The obtained dose dependencies of separate AFM parameters change with the experimental group (different species and species ages) and the parameter type. The distributions of the parameters are heterogeneous. A set of used parameters characterizes the mechanical phenotype better than a single parameter. Clustering the sets of parameters for cells in each experimental group into four clusters showed the similarity between the corresponding clusters of different groups. Four clusters were suggested to relate to four cell states: non-active and activated cell states, apoptotic and necrotic death programs. The different clusters contribute differently, depending on the dose to the lymphocyte population after the blood irradiation. Apoptosis was accompanied by a decrease in the stiffness and roughness of the cell surface at the nanoscale (the number of cells undergoing apoptosis increases at doses of 1–25 Gy), and necrosis was accompanied by an increase in stiffness and adhesion, manifestation of karyopyknosis, and loss of functional activity of the actin cytoskeleton (the number of cells undergoing necrosis increases at doses of 50–100 Gy). Our findings demonstrate the possibility of applying the suggested set of AFM parameters for automatically determining the cell states and death programs after irradiation of the blood with ionizing radiation.

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