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ANALYSIS OF THE RELIEF OF MCF-7 TUMOR CELLS DURING APOPTOSIS ON THE BASIS OF PHASE-CONTRAST IMAGES OBTAINED BY LASER INTERFERENCE MICROSCOPY

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Data on the surface topography of the cell makes it possible to predict its behavior and is an indicator to assess whether it is in a state of apoptosis, that is, in a state of death due to the activation of intracellular reactions; this state of a cell occurs both during normal development and as a result of a pathological process. A universal method for recording the state of the relief of the cell and its other mechanobiological parameters has not been developed, and therefore laser interference microscopy is promising for this purpose. This work aims to evaluate the state of the surface of tumor cells during the apoptotic changes in breast cancer cells by analyzing the phase contrast (phase) images obtained by laser interference microscopy. The object of the study is the cells of the MCF-7 line (epithelial-like adenocarcinoma of the human mammary gland) in a native state and in a state of induced apoptosis under the action of doxorubicin. The analysis of the cell surface involves the interpretation of data on the optical thickness of cells and their morphology through the construction of isolines. Based on the analysis of phase images of cells, their general morphometric parameters were determined. Visualization of optical data and their interpretation was performed using the ImageJ–Fiji software. According to the Wilcoxon–Mann–Whitney test, the samples under study are significantly different in maximum diameter, perimeter, and sphericity coefficient. Based on the analysis of isolinear images, a diagram of the phase state of cells in a native state and in a state of apoptosis was constructed. According to the diagram, the cross-sectional area of apoptotic cells is generally much smaller than the cross-sectional area of native cells at the same height levels. The characteristics of cells within the framework of the proposed phase diagram are consistent with the morphological cell apoptosis signs, which are recorded using classical microscopic methods. The construction of the diagrams of the state of cells according to the parameters of their relief holds promise for assessing the cellular behavior, predicting it, and analyzing the effect of drugs and therapeutic methods on pathological cells.

Key words: artificial ground freezing, frost heave, numerical simulation, fiber-optic sensor

1. Introduction

A cell surface is a mechanobiological object that includes the plasma membrane and the cytoskeleton, and its state, in particular the relief, depends on external physical influences and biochemical processes both inside the cell and in its environment. It is known [1, 2] that extracellular biophysical signals have a significant effect on cellular behavior, including cell growth, cell motility, differentiation, apoptosis, gene expression, adhesion, and signaling ability. The importance of the laws of physics and the need to incorporate them into biological models was emphasized in the classic work by the founder of mathematical biology D'Arcy Thompson (On Growth and Form, 1917).

In later works [3–5] devoted to the mechanobiology of cells, the idea is expressed that directed changes in the mechanical properties of intracellular structures, such as the cytoskeleton (a structure of microtubules and proteins providing the cell with shape, strength, and motility) and cytoplasm (the contents of the cell, limited by its membrane, with the exception of the nucleus), will allow to control intracellular signals and thus initiate or suppress both physiological and pathological cellular processes. It has been proven [6] that changes in intracellular structures caused by external forces, interaction with other cells or materials can change the phenotype (set of individual characteristics) of a cell, and cells of different phenotypes have different morphology and surface conditions [7]. Knowledge of the physical and mechanical background for a phenotype change is essential in understanding the genesis of the most dangerous diseases, including cancer, central nervous system

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diseases, fibroid diseases, and myocardial infarction. In this regard, the identification of pathological cells through assessing their morphology and relief appears to be a promising method for research and clinical practice.

The fundamental physical and mechanical process for understanding cell interaction with the environment is adhesion. Adhesion between cells maintains the mechanical integrity of multicellular tissues and bacterial layers and, as stated in [8], acts like mechanical transducers, converting mechanical signals into biochemical ones. As a result of this transformation, as well as the mechanical effect on the cells, the membrane tension changes, which brings a change in the mechanical properties of the components of the cell cytoskeletal apparatus, and in the aggregate of these factors leads to a change in its shape, surface relief, and, consequently, its functions.

According to research in the field of cell mechanobiology, it can be concluded that the morphology and parameters of the surface relief act as indicators of the cell state and can be used to predict the behavior of individual cells and their aggregates.

Indication of the cell state and its surface relief by morphometric parameters, in particular, the registration of apoptotic changes, is necessary to identify the mechanism of development of pathological processes and assess the therapeutic effect of agents that initiate the destruction of pathological (cancer) cells. There is no unified approach to identifying the mechanobiological cell parameters now; laser interference microscopy appears to be one of the most up-to-date and promising methods for in vivo registration and follow-up of morphological cell parameters.

The purpose of this study is to analyze the relief of tumor cells during apoptotic changes using phase-contrast (phase) images of breast cancer cells of the MCF-7 line using laser interference microscopy (LIM).

2. Materials and methods

The object of the study is the MCF-7 cell line (epithelium-like adenocarcinoma of the human mammary gland), isolated at the National Medical Research Center of Oncology, named after N.N. Blokhin of the Ministry of Healthcare of the Russian Federation (Moscow). The MCF-7 cells were incubated in the DMEM medium (Dulbecco's Modified Eagle's Medium), manufactured by PanEco Research and Production Enterprise, Russia) with the addition of 10% fetal calf serum (produced by PAA Laboratories GmbH, Austria), 2 mmol of L- glutamine, and a mixture of 1% penicillin/streptomycin (1000 U/ml; 10 mg/ml) (manufactured by PanEco Research and Production Enterprise, Russia). The incubation was conducted in a CO2 incubator (Thermo Fisher Scientific, USA) at (+37.0±1.0) °C and (5.0 ± 0.5) % CO_2 in a humid atmosphere. We studied the native state of the cells and the state of induced apoptosis.

A synthetic anti-tumor drug doxorubicin hydrochloride (Mr 579.98) — C27H30ClNO11 (Tocris Bioscience, USA) was used as a pro-apoptotic agent. Doxorubicin hydrochloride was dissolved in 100% DMSO (dimethyl sulfoxide) and then diluted in the DMEM medium. The resulting solution was added to a monolayer of MCF-7 cells to a predetermined final concentration IC_{50}=0,3 μM. The IC_{50} concentration (the value of 50% inhibitory concentration) was determined using the cell metabolic activity assay (MTT assay) [9] based on dose-dependent curves and the GraphPad Prism 6.0 program.

The samples of the MCF-7 cells were prepared as follows. The previously cultured monolayer of the MCF-7 cells was detached from the surface of the culture flask using a mixture (1:4) of 0.25% trypsin solution (MP Biomedicals, USA) and Versene solution (0.2% EDTA in phosphate buffer (PanEco, Russia). The resulting cell suspension was centrifuged for 3 min at 1500 rpm, then the liquid phase remaining after the insoluble substances settled during centrifugation (supernatant) was removed, the cells were re-suspended (brought to suspended state again) in 3 ml of the DMEM medium. The number of cells was counted in a Goryaev chamber using a Meiji Techno TC5200 series optical microscope (Japan) at 200x magnification. Then, under aseptic conditions, 6 ml of the DMEM medium was added to a Petri dish 60 mm in diameter with a preliminarily introduced sterile glass 24×50 mm, and then the cell suspension was added to a final concentration of 3 × 105

cells/ml. The MCF-7 cells were incubated at (+37.0±1.0) °C in a humid atmosphere (5.0±0.5) % CO_2. After 24 h, doxorubicin solution was added to the MCF-7 cells in a Petri dish. The cells were incubated for an additional 18 hours under the above conditions. MCF-7 cells without doxorubicin exposure (native cells) were used as control samples.

Cell preparations for microscopy were prepared by applying a thin layer of silicone grease (spacer) along the perimeter of a non-conducting glass slide with mirror coating. Then the glass with the cells attached was transferred to the prepared spacer so that the cells were in the buffer zone in the nutrient medium. The obtained cell preparations were examined with the help of a laser interference microscope МИМ-340 (manufactured by Shvabe Holding, Russia) at the Research Equipment Sharing Center for Research of Materials and Substances at the Perm Federal Research Center of the Ural Branch of the Russian Academy of Sciences [11].

The optical thickness of the cells was measured to assess the state of the cell surface and morphology; this index is calculated from the data on the local phase delay of the laser beam modulated by the cell. Then, the phase image of the cell was analyzed, which was formed based on its geometric parameters corresponding to the height, diameter (minimum and maximum), perimeter, area, and sphericity coefficient [10]. The laser interference microscope МИМ-340 used to measure the optical thickness of native and apoptotic cells had a semiconductor laser with a wavelength of 655 nm as a coherent radiation source and a lens with 10x magnification.

60 native and apoptotic MCF-7 cells were measured during the study, including 30 native cells and 30 cells treated with doxorubicin. The mathematical apparatus used to convert optical measurements into geometric characteristics is described in [12].

The cell surface analysis is based on the interpretation of isolines constructed from data on cells' optical thickness and morphology. The isolines formed a group of concentrically located figures corresponding to the cell sections, carried out at different heights parallel to the substrate (Fig. 1). The area of the figures corresponding to the sections decreased as the height relative to the substrate increased. The graphical dependence characterizing this change was taken in the study as a surface characteristic similar to the relief.

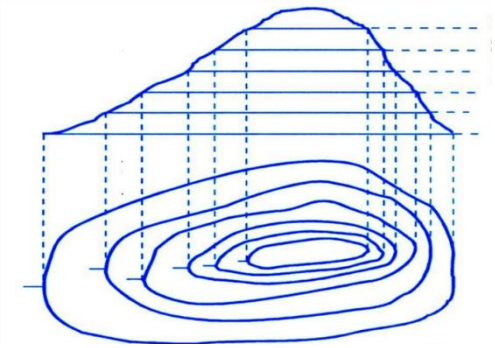

Fig. 1. Schematic view of the constructed isolines

Comparison of the established numerical parameters of control (native) and apoptotic (after exposure to doxorubicin) MCF-7 cells was conducted with the help of the nonparametric Wilcoxon–Mann–Whitney test [13]. The visualization of optical data and their interpretation through plotting isolines were conducted using the ImageJ-Fiji software.

3. Results and discussion

Figure 2 demonstrates the visualization of the phase image of the MCF-7 cells from the control group (native) (Fig. 2 *a* – *c*) and in a state of apoptosis after exposure to doxorubicin (Fig. 2 *d* – *f*). Based on these phase images, we generated three-dimensional visualizations (Fig. 3) and corresponding isoline patterns (Fig. 4).

In the obtained phase images of the cells, a contour was isolated, and the calculations of morphometric parameters, including the sphericity coefficient, were performed. Table 1 contains the main morphometric characteristics of phase images of the cells. The difference between the median values of height, maximum and minimum diameters, perimeter, area, and sphericity coefficient of the two groups on average is 2.5, 57, 11, 30, 47, and 14%, respectively. According to the Wilcoxon–Mann–Whitney test, the analyzed samples are significantly different in maximum diameter, perimeter, and sphericity coefficient (the relative error in determining the sphericity coefficient (p -value) is not higher than 0.01).

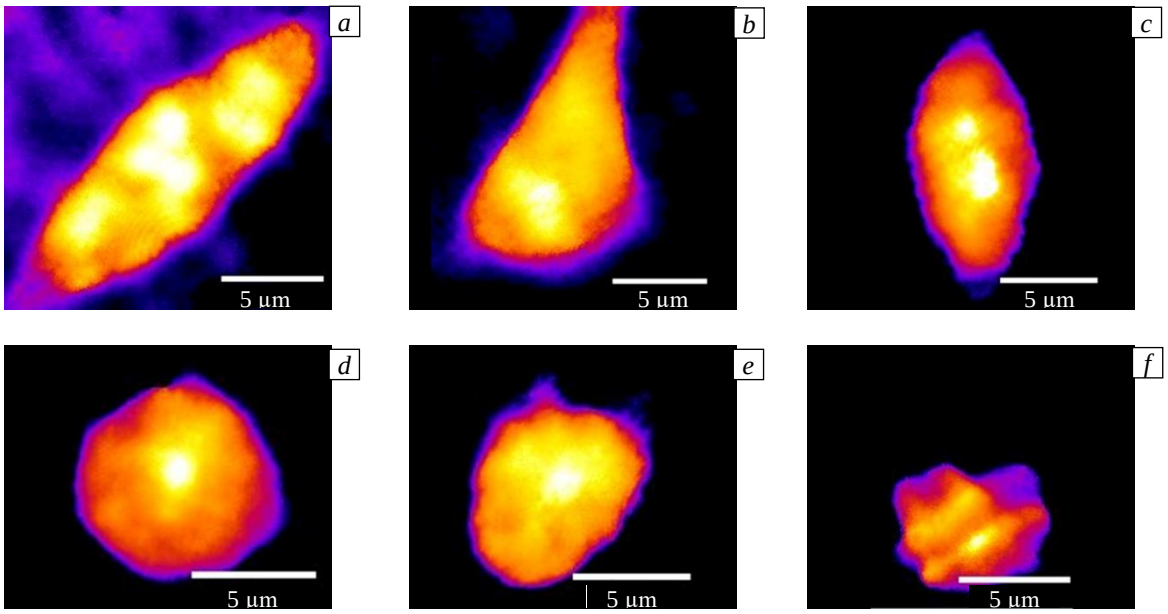

Fig. 2. The phase state of the MCF-7 cells: control group (*a* — *c*); after apoptosis induced by the synthetic anti-tumor drug doxorubicin hydrochloride (*d* — *f*).

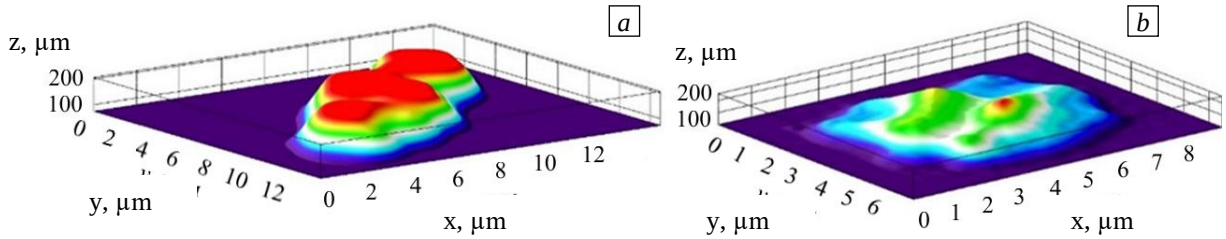

Fig. 3. The three-dimensional visualization of the MCF-7 cell relief: control group in the native state (a), after apoptosis induced by the synthetic anti-tumor drug doxorubicin hydrochloride (b)

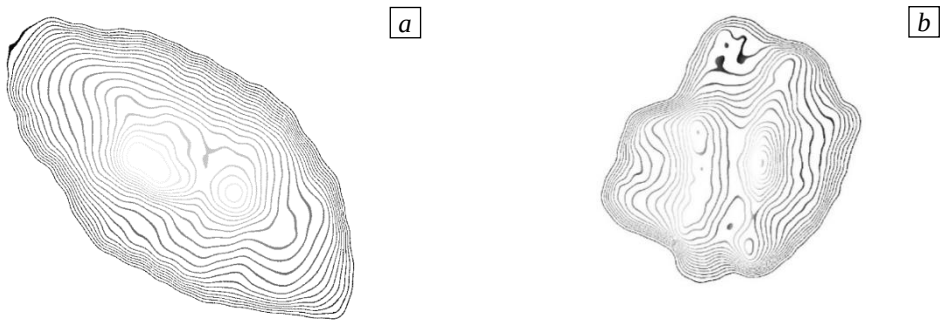

Fig. 4. The patterns of isolines characterizing the MCF-7 cell relief: control group in the native state (a), after apoptosis induced by the synthetic anti-tumor drug doxorubicin hydrochloride (b)

Table 1. Morphometric characteristics of the MCF-7 cells in the native state and after apoptosis induced by the synthetic anti-tumor drug doxorubicin hydrochloride

Cell state	Characteristic					
	Height, nm	Diameter D, μm		Perimeter P, μm	Area S, μm^2	Sphericity coefficient
		D_{max}	D_{min}			
Native	136.32 ±39.28	*14.74±2.56	7.68±1.42	**40.53±6.72	77.10±17.29	***0.60±0.08
Apoptosis	138.52±16.98	8.43±0.46	6.82±0.47	28.02±1.34	40.78±5.30	0.70±0.07

Notes: $*U = 35 < U_{кр} = 317$; $p = 0,004$; $**U = 25 < U_{кр} = 317$; $p = 0,009$; $***U = 310 < U_{кр} = 317$; $p = 0,01$,

where U is the Wilcoxon–Mann–Whitney test value for comparing data from the native state and the apoptosis groups $U_{кр}$ is the critical value of the Wilcoxon–Mann–Whitney test corresponding to the sample size against which the calculated test is assessed.

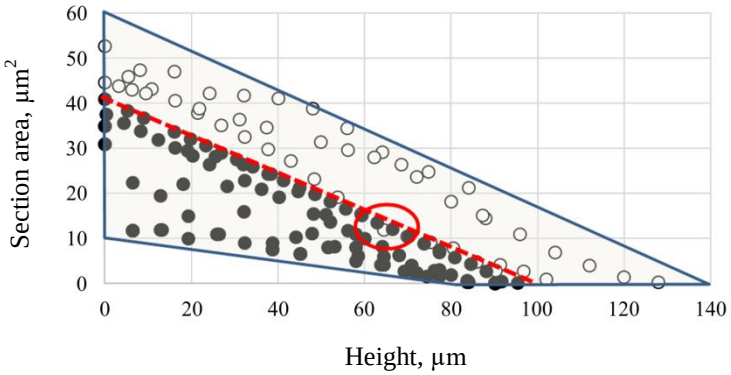

Fig. 5. The phase state diagram of the MCF-7 cells: ○ – native, ● – in the apoptosis

Figure 5 demonstrates the phase state diagram of all MCF-7 cells studied within the framework of the experiment, obtained as a result of processing and analysis of isolinear images. On the abscissa axis x are the values of the height, and on the ordinate axis y, are the values of the cross-sectional area of the cell, correlated with the height. The area inside the solid line is a phase area; that is, it generalizes the totality of all ratios of cross-sectional areas and cell heights. The "○" symbols denote the values for the cells of the control group; the "●" symbols denote the cells with induced apoptosis. The cells from both groups are arranged in an orderly manner, making it possible to divide the phase area into two zones, one characteristic of the cells in the native state, the other for the cells with apoptosis. The diagram demonstrates the area corresponding to the cross-sectional area of the cell 10–20 μm ^{2} at the heights of 50–70 nm, in which both groups lay over each other, and there is a conventional border (see the dashed line) between the areas of apoptosis and native state. This suggests that this parameter of the cell surface relief is the most stable indicator characteristic of the thermodynamic equilibrium and is present in both native cells and the cells subjected to apoptosis.

The phase diagram of the surface indicates that the relief of the cells, altered during apoptosis, is less uniform than that of the native cells; it demonstrates a more significant number of individual peaks at different heights, which form folds that are not characteristic of the native state. In general, the cross-sectional area of the apoptotic cells is considerably smaller than the cross-sectional area of the native cells at the exact coordinates of height. The characteristics of cells within the proposed phase diagram are consistent with the morphological features of the cell apoptosis process recorded with the help of the classical microscopic methods [14], as noted in the earlier works by some authors [12]. They reflect the change in the shape of the cell, consisting of the transition to a rounded shape and subsequent wrinkling with folds on the surface.

4. Conclusion

The authors analyzed the MCF-7 cell surface relief in the native state cells and in the presence of apoptotic changes on the basis of the phase-contrast images (phase images) obtained by laser interference microscopy (LIM). The study established the quantitative difference between the median values of the height, minimum and maximum diameters, perimeter, area, and sphericity coefficient of cells in these states. Compared with the native cells, certain features in the cell relief in the state of apoptosis were discovered, such as misaligned relief, allocation of multiple individual peaks at different heights, and a decrease in the cross-sectional area along with the height of the cells. Based on the differences discovered within the framework of the research, a phase diagram of the state of the MCF-7 cells was constructed, which allows distinguishing the native cells from the cells altered by apoptosis by morphological parameters of the surface relief, which is promising for

assessing cellular behavior, predicting it, as well as tracking the effectiveness of drugs and therapeutic methods of influencing pathological cells.

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