Reduction of Phase Thickness Is a Characteristic Reaction of the Nucleoli to Toxicity as Shown by Coherent Phase Microscopy

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Optical parameters of human cell nucleoli (HCT116 colorectal cancer cells) in depolymerization of microtubules and depletion of intracellular ATP pool were studied by coherent phase microscopy. These influences were associated with a rapid (recorded within the first minutes) reduction of the phase thickness of the nucleoli. These changes are similar to the nucleolar response to direct inhibitors of transcription. Hence, quantitative parameters of coherent phase microscopy describe common reaction of the nucleolus to stress; reduction of optical thickness of the nucleolus is a component of this reaction.

Key Words: *coherent phase microscopy; electron microscopy; transcription; adenosine triphosphate; microtubule*

The method of coherent phase microscopy (CPM) is based on measurement of the refraction value, depending on the morphology and function of the object [2,4,10]. We previously demonstrated the possibility of quantitative analysis of the structure and metabolism of an extensive class of microobjects, from chloroplasts and spores to isolated organelles and eukaryotic cells [1,3,8-10]. The possibility of using CPM for studies of the "nucleolar stress" (a sum of structural and functional changes in the nucleolus during cell treatment with pharmacological compounds directly inhibiting gene transcription, such as actinomycin D and olivomycin [4,5]) seemed to be particularly important. The CPM method records changes in optical parameters of the nucleoli within the very first minutes of exposure to these inhibitors. Spatial redistribution of phase thickness of the nucleoli and index of refraction can be a sign of segregation of nucleolar components in suppressed transcription.

The nucleolus is a self-regulated dynamic system reacting to agents whose intracellular targets are located outside the nucleolus, and therefore the structural and functional organization of the nucleoli is modified in response to various factors, including those not directly modulating the transcription system.

The aim of this study was to clear out whether reduction of the nucleolar phase thickness is the main modification under the effect of transcription inhibitors, characteristic of disorders in the cytoskeleton dynamics, cell de-energization, and DNA synthesis blocking. In order to answer these questions, we studied the changes in the optical characteristics of the nucleoli under the effects of oligomycin and a combination of rotenone with SF6847,

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reducing the intracellular pool of ATP, DNA synthesis blocker aphidicolin, and vincristine, inducing depolymerization of microtubules.

MATERIALS AND METHODS

Colorectal cancer HCT116 cells were cultured in DMEM with 5% FCS (BioWhittaker), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C, and 5% CO_2 in humid atmosphere. Cells in the phase of logarithmic growth were inoculated onto slides so that their density on the day of experiments was about 50%. Rotenone (inhibitor of mitochondrial respiratory chain I complex; 50 μM) in combination with 10 μM SF6847 uncoupling respiration and oxidative phosphorylation, vincristine (tubulin polymerization blocker; 1 μM), and aphidicolin (10 μM; Sigma) were added to cultures for a period of 5-60 min, after which the slides were removed and directly examined by CPM. Individual cells were observed in the CPM optic channel, then their topograms and phase thickness profiles were measured. Up to 20 native cells selected at random in different visual fields were analyzed in each sample. The measurements were carried out under an Airiscan microscope with a He-Ne laser (λ=633 nm) as the source, LI-620 dissector for registration of interference signal, and an electron block for computer presentation of phase images. The velocity of the image input was 1 msec/pixel. The size of image field for Olympus objective (20×/0.4) was 12 μ. Transverse parameters of the nucleolus were evaluated at the level of half-height at an accuracy of up to 100 nm, optical thickness (OT) Δh at an accuracy of up to 3 nm.

For studies of the nucleolar ultrastructure, the cells (intact and treated with drugs) were washed in Hanks solution, harvested, precipitated by short centrifugation, and fixed in 2.5% glutaraldehyde solution. Transmission electron microscopy was carried out by the standard method. The preparations were examined under a JEM-1200 EX-II microscope.

RESULTS

Coherent phase microscopy shows the object image as a two-dimensional distribution of optical path-length difference (OT) $h(x,y)$, where x, y are coordinates in the object plane. The maximum OT Δh and cross-section size (diameter) of structural element d are the main values measured in the phase thickness profile. The $\Delta h=d\times\Delta n$ relation is true for an object of presumable spherical or cylindrical shape, when the physical (geometrical thickness of the object $H\approx d$ is equal to the transverse size. Here $\Delta n=n_{x,y,z}-n_0$, where n_0 is refractoriness equal to the difference in the mean (by volume) value of the object refraction and environment n_0.

The $n_{x,y,z}$ in the physical model [5] is the object refraction coefficient, H is physical (geometrical) thickness, and n_0 is the environment refraction coefficient. Optical path-length difference $h(x,y)$ in the object image and its maximum Δh value depend on the physical thickness and difference in the object ($n_{x,y,z}$) and environment (n_0) refraction coefficients $\Delta h=H\times[n_{x,y,z}-n_0]$. Phase image of the cell (three-dimensional topogram; Fig. 1) shows the nucleus and optically more dense nucleoli against this background. The nucleolus refractoriness $\Delta n=\Delta h_{\text{nucleolus}}/d_{\text{nucleolus}}$, determining its contrast in the phase image, depends on Δh excess over its OT above the nucleus. The actual value of the nucleolus refraction n can be determined as the sum $n=n_0+n_{\text{nucleus}}+\Delta n$, where n_{nucleus} is the indicator of the nucleus refraction, equal to $\Delta h_{\text{nucleus}}/d_{\text{nucleus}}$ ($\Delta h_{\text{nucleus}}$ is its OT and d_{nucleus} is its diameter).

Intracellular ATP was reduced by a combination of rotenone with SF6847 and oligomycin. A rapid drop of the nuclear OT and impairment of the nucleolar OT profile were recorded within 60 min in an HCT116 cell after addition of 50 μM rotenone and 10 μM SF6847. Two characteristic profiles are presented: of an intact cell and of a cell 60 min after addition of inhibitors (Fig. 2, *a*).The nucleolar and nuclear OT reduced. Intact culture (Fig. 2, *b*) was characterized by approximately equal ratio of cell counts with the mean nuclear OT values equal to 150, 250, and 420 nm. Cells with the mean OT of about 100 nm predominated on the histogram of de-energized cell nuclei OT (Fig. 2, *c*). The histo-

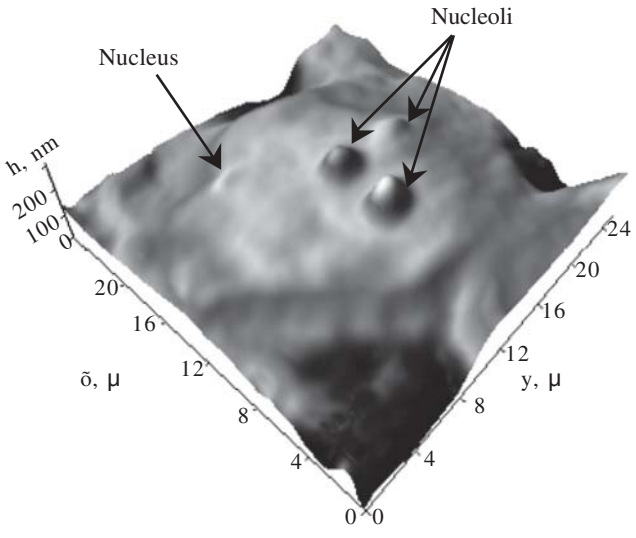

Fig. 1. 3D phase image of an HCT116 cell.

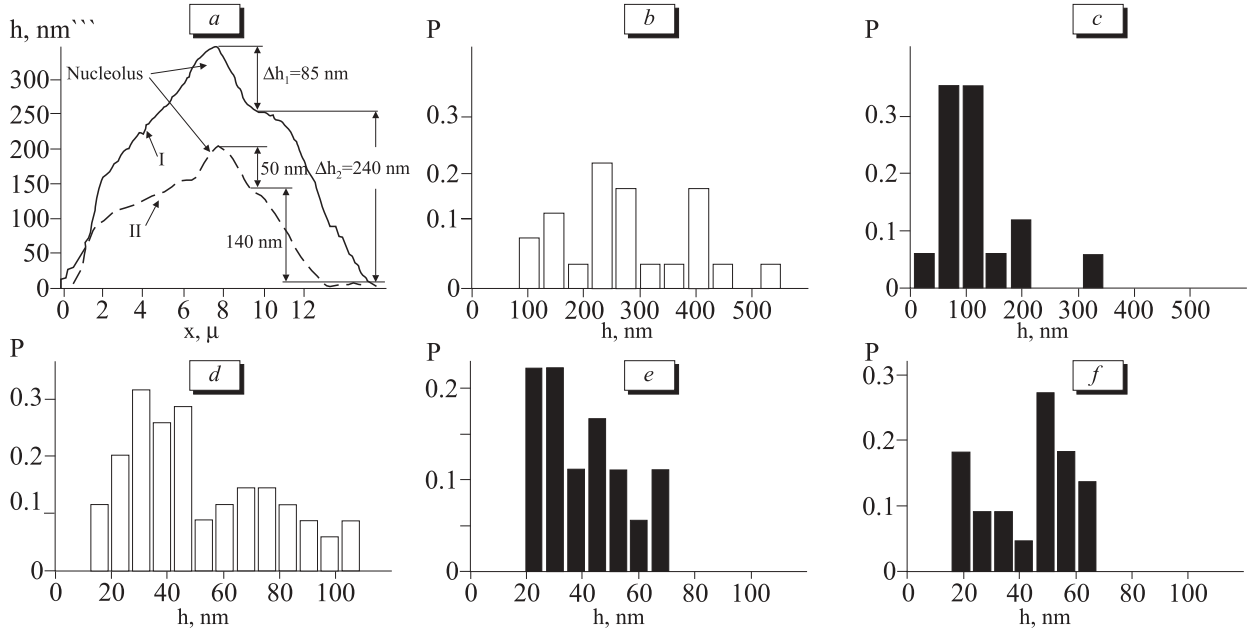

Fig. 2. OT after deenergization of HCT116 cells. *a*) changed OT profile after addition of rotenone and SF6847. Continuous line: intact cells; interrupted line: de-energized cells Δh_1: $\Delta h_{nucleolus}$, Δh_2: $\Delta h_{nucleus}$; *b*) histogram of nuclear OT in intact HCT116 cells; *c*) histogram of nuclear OT in HCT116 cells treated by rotenone and SF6847; *d*) histogram of intact nucleolar OT; *e*) histogram of nucleolar OT after addition of rotenone and SF6847; *f*) histogram of nucleolar OT after addition of oligomycin. *P*: probability of drop-out of the value in the interval.

gram of intact cell nucleoli shows two groups with OT of about 40 and 80 nm (Fig. 2, *d*). The distribution of nucleolar OT under the effects of rotenone and SF6847 (Fig. 2, *e*) indicated the absence of a group with the mean OT of about 80 nm. The group with OT of about 80 nm also disappeared after oligomycin treatment (Fig. 2, *f*). Hence, deenergization of cells led to reduction of the nuclear OT and in the number of "active" nucleoli with OT of about 80 nm.

The CPM data were compared with the ultrastructural changes. Treatment with rotenone and SF6847 led to reduction of chromatin density, densities of fibrillar centers and granular component of the nucleolus; dense granular zones were shifted (Fig. 3, *b*). Oligomycin appreciably reduced the electron density of the nucleoli in comparison with intact cells (Fig. 3, *c*), which is in good agreement with the nucleolar OT distribution after treatment with ATP synthesis inhibitors on histograms (Fig. 2, *d-f*). Ultrastructural disorders in the shape and ratio of components were observed in the majority of the examined nucleoli; however, little changed nucleoli were also seen. Presumably, the severity of changes depended on the cell cycle phase and metabolic activity of the nucleoli.

After aphidicolin treatment (10 μM), the mean nucleolar OT decreased to 20 nm (*vs.* the mean 40 and 80 nm OT values in the control, Table 1). The group with OT of 80 nm can be referred to "active" nucleoli [5,6]. Physical diameter of the nucleoli did not change. Differences in the nuclear and nucleolar response to stress can be caused by distribution of cells by cycle phases.

Vincristine (1 μM) significantly changed the nucleoli of both groups. The percentage of nucleoli with OT<40 nm increased, while the nucleoli with OT>70 nm disappeared. Histograms showed peaks at 25 and 45 nm. Hence, the effects of vincristine were directed to nucleoli with lesser and greater

TABLE 1. Time Course of the Mean Optical Parameters of "Active" Nucleoli after Drug Treatment

Parameter	HCT116 cells				
	control	rotenone+ SF6847	oligomycin	aphidicolin	vincristine
Mean OT (Δh), nm	80	45	50	20	40
Mean refractoriness (Δn)	0.040	0.025	0.025	0.020	0.015

OT. The OT values were evenly shifted towards the lower values, while the range of values decreased in both populations of the nucleoli. Two new contrast areas formed from one fibrillar component, presumably two fibrillar centers formed from one. This effect was detected in statistical processing of the data for several cells and analysis of one cell. The study of the dynamics of the nucleolar fibrillar center gave similar data [7]. The study of the nucleolar ultrastructure showed its degradation and loss of shape (Fig. 3, *d*), the density of granular and fibrillar components decreased. The nuclear reaction consists in reduction of chromatin density and absence of its accumulations (less pronounced at the periphery of the nucleus). Chromatin is evenly distributed and is rarely condensed near the nuclear membrane.

Hence, our findings confirm the previous data [4,5] and indicate that CPM is an adequate method for online quantitative analysis of life-time structural and functional changes in eukaryotic cell nucleoli. Optical parameters were studied after treatment of cells with direct pharmacological blockers of transcription and after indirect modification of the nucleoli: in energy metabolism disorders, microtubule depolymerization (and disorders of the nuclear cytoplasmatic transport related to it). The results indicate that reduction of the nucleolar OT is a manifestation of the common cell reaction to various disorders in its metabolism. Hence, CPM as a noninvasive method detects an important relationship between physical parameters and morphofunctional status of microobjects: these parameters quantitatively describe the totality of processes constituting the "nucleolar stress".

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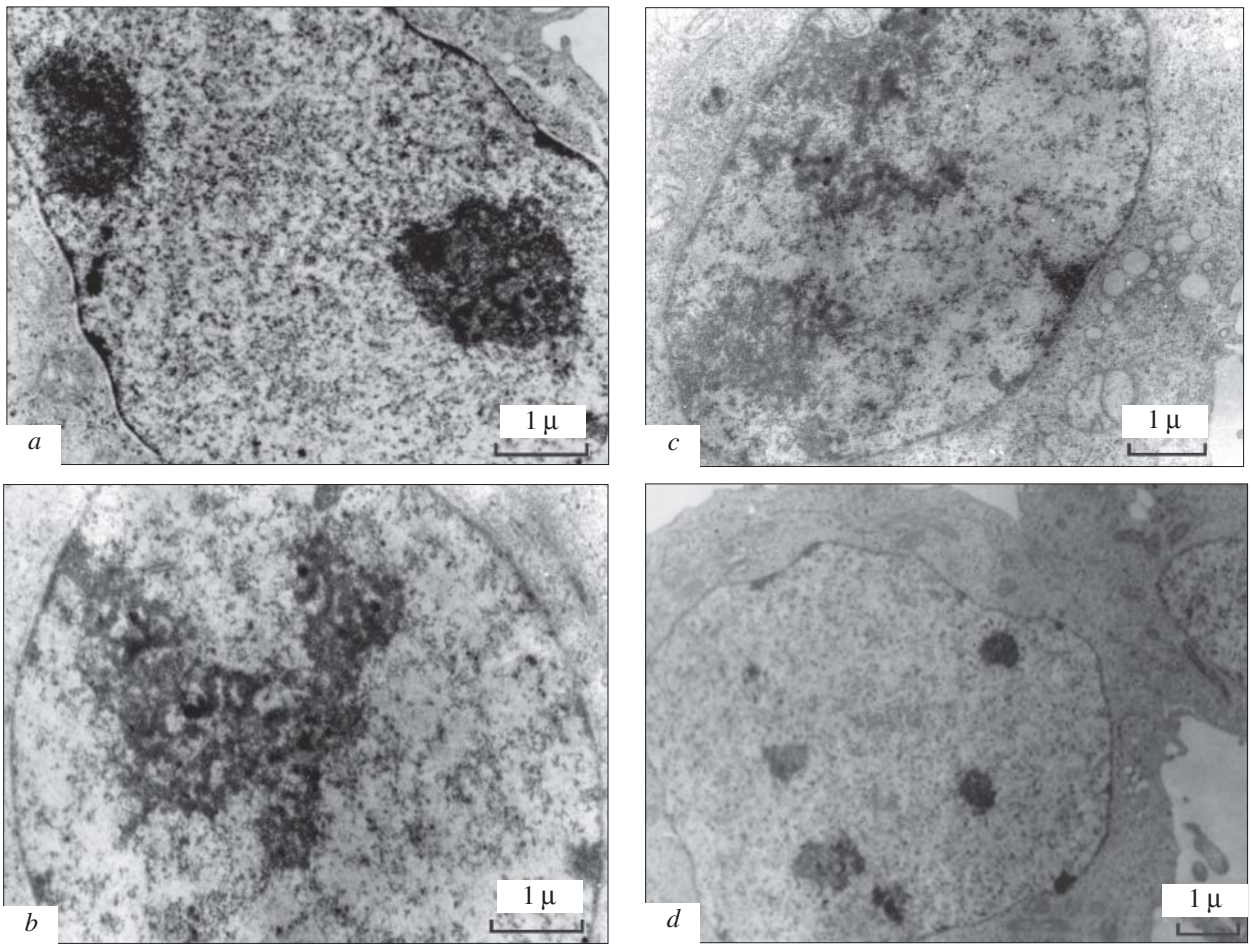

Fig. 3. Ultrastructure of HCT116 cell nucleoli. *a*) control (×12,000); *b*) rotenone and SF6847 (×12,000); *c*) oligomycin (×10,000); *d*) vincristine (×8000).

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