Changes in Nitric Oxide and Superoxide Levels in Human Endotheliocytes and Carcinoma Cells after Exposure to Low-Dose Ionizing Radiation

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The exposure of HeLa G63 and ECV-304 cells to γ-rays of ^{137}Cs as well as β-particles of $^{3}H_2O$ and ^{3}H-thymidine induced changes in redox status of not only irradiated cells, but also their progeny. Increased intracellular levels of nitric oxide (NO) were observed only in HeLa G63 cells and persisted over three cell generations; β-particles from $^{3}H_2O$ were most efficient. Intracellular superoxide (O_2^-) level had similar dynamics in both cell lines. Intracellular O_2^- level decreased immediately after irradiation, but then increased and significantly surpassed the control level. These changes in the intracellular level of O_2^- were accompanied by decondensation of nuclear chromatin. Increased level of free radicals in the progeny of irradiated cells and changes in chromatin conformation and the absence of correlation between radiation-induced structural damage to chromosomes and intracellular level of free radicals suggest participation of epigenetic mechanisms of inheritance.

Key Words: *nitric oxide; superoxide; ionizing radiation; cytometry; HeLa G63; ECV-304*

Sources of β and γ radiation are used in radiotherapy of tumors and endovascular radiotherapy for prevention of neointimal hyperplasia after angioplasty [9]. Free radicals formed during water radiolysis ($H^{\bullet}$, $OH^{\bullet}$ and e^-_{hyd}) are the main pathogenic factor of ionizing radiation. At the same time, NO and O_2^- enzymatically produced in cells are also involved in the maintenance of free radical balance. Increased NO production is due to radiation-induced expression of inducible NO-synthase (iNOS) gene. Increased NO levels were also recorded after cell treatment with H_2O_2. It was assumed that H_2O_2 produced in irradiated cells triggers NO production [15]. It is also believed that DNA double-strand breaks (DSB) play an important role in stimulation of NO production [14]. Significantly reduced rate of DSB repair after cell irradiation in low doses (10 mGy) in comparison with doses >1 Gy was also reported [10,13]. Cells pretreatment with 10 mM H_2O_2 eliminated these differences, though it produced only DNA single-strand breaks (SSB) and base damage via generation of free radicals. It can be hypothesized that oxidative stress contributes to activation of the repair system [10]. We have previously shown that low-dose of β-particles emitted by ^{3}H-thymidine incorporated into DNA of human endothelial cells (in contrast to β-particles of $^{3}H_2O$) induced high levels of unstable chromosomal aberrations. It was hypothesized that the high genotoxicity of ionizing radiation depends on the position of the radiation source relative to the sensitive target, DNA [3,4].

To clarify the relationship between the damage to cell structures that contain genetic information and changes in the intracellular level of free radicals, we compared the efficacy of NO and O_2^- induction by incorporated and external sources of ionizing radiation in two strains of human cells, expressing different NO-synthase isoforms.

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MATERIALS AND METHODS

The study was carried out on ECV-304 human endotheliocytes and HeLa-G63 human carcinoma cells. Cells were cultured in modified Eagle's medium with 10% fetal serum (Biolot) and gentamicin. O_2^- level was measured with redox-sensitive probe dihydroethidine and intracellular NO level was assayed using 4,5-diaminofluorescein diacetate (DAF-2-DA). The techniques were described previously in detail [2,7]. Evaluation of chromatin conformation during cell incubation of with ethidium bromide in "non-saturating" concentrations (10^{-6} M) was described previously [5]. Cytofluorometric analysis was performed on a flow cytometer developed at the Group of Radiobiology and Medicine. The cells were irradiated with ^{137}Cs γ-rays in a dose poser of 1 Gy/min and dose range 1-6 Gy on a MIDI device. Tritiated water (^3H_2O, Izotop) and [methyl-^{3}H]-thymidine were used as the sources of β-radiation. The dose of incorporated ^{3}H-thymidine was evaluated from the average β-particle energy E_{av} of 5.7 keV (9.12×10^{-16} J), path length of β-particle with such energy in the aquatic environment 0.5-0.7 μ, cell diameter, and the time of contact with ^{3}H [3,4].

RESULTS

We present the results of one from the five experiments to assess the intracellular NO level after exposure of HeLa cells to γ-rays and β-particles (Fig. 1). In the nutrient medium, ^{3}H-thymidine and ^3H_2O were dissolved in concentrations from 0.066 mBq (0.46 Gy) to 0.132 mBq (0.92 Gy). The cells were incubated with the isotope for 20 h. Some cells were analyzed immediately after incubation others were further incubated in a fresh isotope free medium. The levels of NO and O_2^- were measured at different terms after isotope removal from the medium. β-Particles emitted by ^3H_2O and γ-rays dose-dependently increased fluorescence intensity of cells incubated with DAF-2 DA (Fig. 1, *b*, *d*). This is consistent with the results of other researchers evaluating this effect by other methods [11]. Comparison of the efficiency of NO induction by external γ-irradiation and β-particles incorporated and not incorporated into DNA showed that β-particles emitted by ^3H_2O were most effective. Isoeffective doses were 6 Gy for γ-rays of ^{137}Cs, 0.92 Gy for β-particles of ^{3}H-thymidine (Fig. 1, *c*), and 0.46 Gy for β-particles of ^3H_2O (Fig. 1, *a*, *b*). These doses induced an equal increase in fluorescence intensity evaluated by the position of fluorescence peak: the peak was shifted by about 28 channels from channel 64 in untreated control to channel 92 after incubation with the isotope or after γ-irradiation. This corresponds to a 1.5-fold increase in NO level. It should be noted that cytometric evaluation of intracellular levels of free radicals was semiquantitative, but it allows tracing the dynamics of changes in their intracellular levels induced by different agents. Increased NO was also reported in HeLa cells 48 h after irradiation (Fig. 1, *e*). Thus, radiation efficiency depended on the position of radiation source relative to DNA. However, in contrast to induction of chromosomal aberrations where a correlation was observed between the efficiency of radiation and the proximity of sensitive target, in this case an inverse relationship was observed. Taking into account the fact that the above concentrations of ^3H_2O did not induce chromosomal aberrations [4], we can assume that DSB in DNA do not play a role in stimulation of NO production and DNA is not the target responsible for this effect. No changes in intracellular NO after exposure of ECV-304 cells both to γ-rays (Fig. 1, *f*) and β-particles were registered [7]. It is noteworthy that the levels of radiation-induced chromosomal damage did not differ significantly in these cell lines [1]. This also confirms that structural damage to DNA is not essential in regulating the free radicals production. Radiation-induced changes in intracellular O_2^- levels were found in both HeLa G63 and ECV-304 cells. The results of one from the five experiments are presented in Figure 2. We monitored the changes in intracellular levels of this radical during 72 h (3 cycles of replication). The effect depended both on the dose (Fig. 2, *a*) and type of radiation: β-particles were more effective than γ-radiation because β-particles emitted by ^{3}H-thymidine in a dose of 0.92 Gy were more efficient than γ-rays in a dose of 6 Gy (Fig. 2, *e*). With increasing the time interval between irradiation and measurement of intracellular O_2^-, the effect changed to the opposite. Immediately after irradiation it was below the control levels (Fig. 2, *a*, *e*), but after incubation in a pure medium it significantly surpassed the control (Fig. 2, *c*). It should be noted that intracellular O_2^- dynamics depended not on the type of radiation (Fig. 2, I, *a*, *c*, *d*; II, *a*), but on its dose (Fig. 2, I, *b*; II, *b*). The cells irradiated at a lower dose more quickly reached and exceeded control values. Increased intracellular O_2^- was recorded in 3 generations irrespective of the type of radiation and cell type. Free radicals imbalance in the cell is obviously a delayed consequence of exposure to low-dose ionizing radiation, which either induce (β-particles emitted by ^{3}H-thymidine and γ-rays of ^{137}Cs) or not induce (β-particles emitted by ^3H_2O) damage to genetic structures in the cells. Altered redox status in the progeny of cells exposed to β-particles of ^3H_2O in doses not inducing DSB in DNA suggests that this change is inherited epigenetically. Our assumption is confirmed by other researchers demonstrating that elevated intracellular content of both O_2^- and NO_2 contributes to changes in

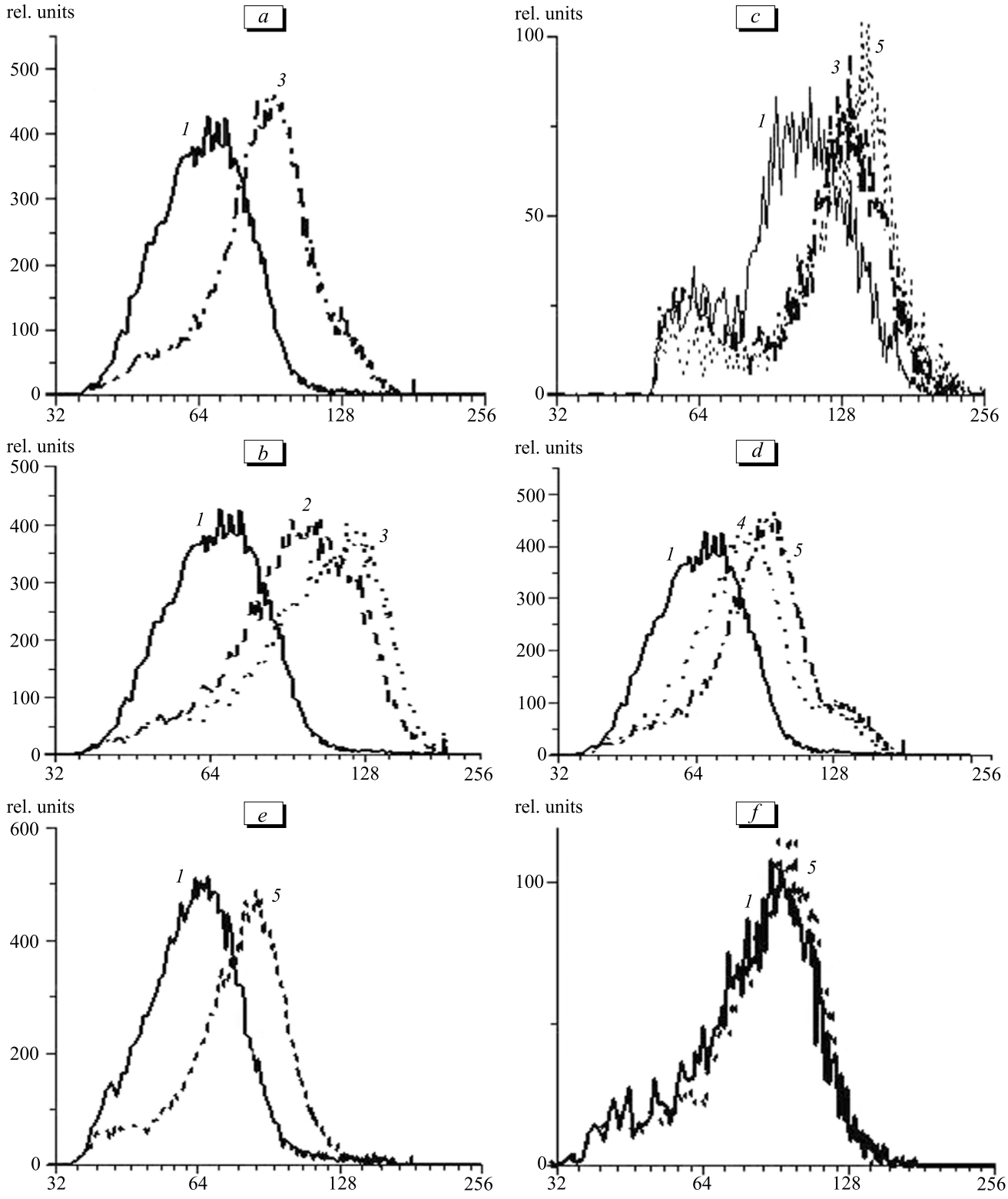

Fig. 1. The changes in intracellular NO levels after exposure of HeLa G63 cells to ionizing radiation: β-particles emitted by ^{3}H-thymidine (*a*), $^{3}H_2O$ (*b*); 0.92 Gy of β-particles emitted by ^{3}H-thymidine and 6 Gy of ^{137}Cs γ-rays immediately after irradiation (*c*); 6 Gy of ^{137}Cs γ-rays 4 h after irradiation (*d*); 6 Gy of ^{137}Cs γ-rays 48 h (*e*) and after irradiation of ECV-304 cells: 6 Gy of ^{137}Cs γ-rays (*f*). *1*) without irradiation and after irradiation in a dose of 0.46 Gy (*2*), 0.92 Gy (*3*), 3 Gy (*4*), and 6 Gy (*5*). Here and in Fig. 2, 3: Abscissa: fluorescence channel number ($\log_2$ scale); ordinate: number of cells.

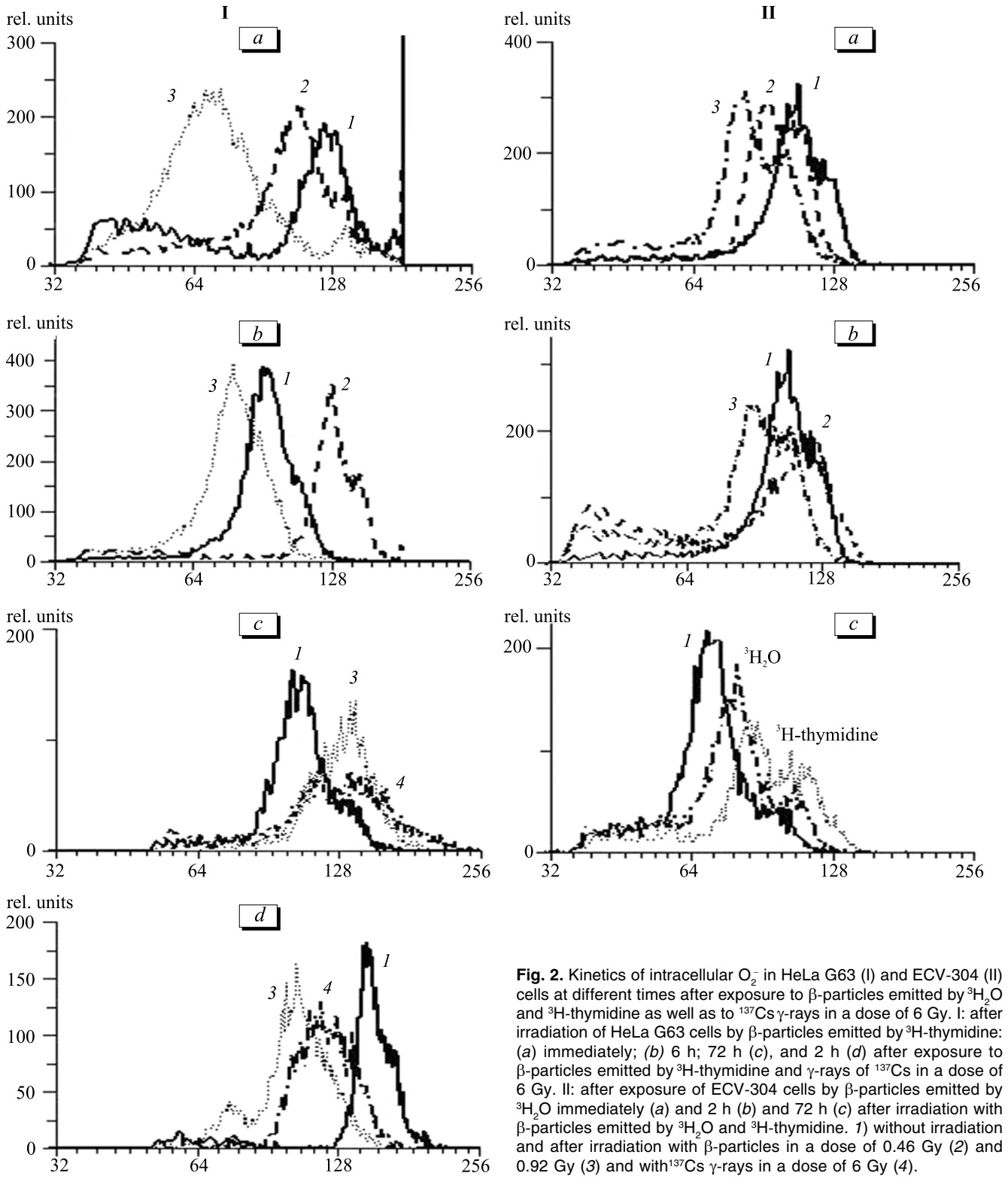

Fig. 2. Kinetics of intracellular O_2^- in HeLa G63 (I) and ECV-304 (II) cells at different times after exposure to β-particles emitted by ^3H_2O and ^{3}H-thymidine as well as to ^{137}Cs γ-rays in a dose of 6 Gy. I: after irradiation of HeLa G63 cells by β-particles emitted by ^{3}H-thymidine: (*a*) immediately; *(b)* 6 h; 72 h (*c*), and 2 h (*d*) after exposure to β-particles emitted by ^{3}H-thymidine and γ-rays of ^{137}Cs in a dose of 6 Gy. II: after exposure of ECV-304 cells by β-particles emitted by ^3H_2O immediately (*a*) and 2 h (*b*) and 72 h (*c*) after irradiation with β-particles emitted by ^3H_2O and ^{3}H-thymidine. *1*) without irradiation and after irradiation with β-particles in a dose of 0.46 Gy (*2*) and 0.92 Gy (*3*) and with ^{137}Cs γ-rays in a dose of 6 Gy (*4*).

DNA methylation status [8]. Changes in DNA methylation are underlain by epigenetic mechanisms and are usually associated with altered chromatin structure. Using the method of ethidium bromide staining in "non-saturating" concentrations [5], we evaluated chromatin conformational changes in ECV-cells induced by β-particles emitted both by ^{3}H-thymidine and ^3H_2O. β-Particles of ^3H_2O enhanced fluorescence intensity of cells in comparison with untreated control. This indicates facilitation of accessibility of dye

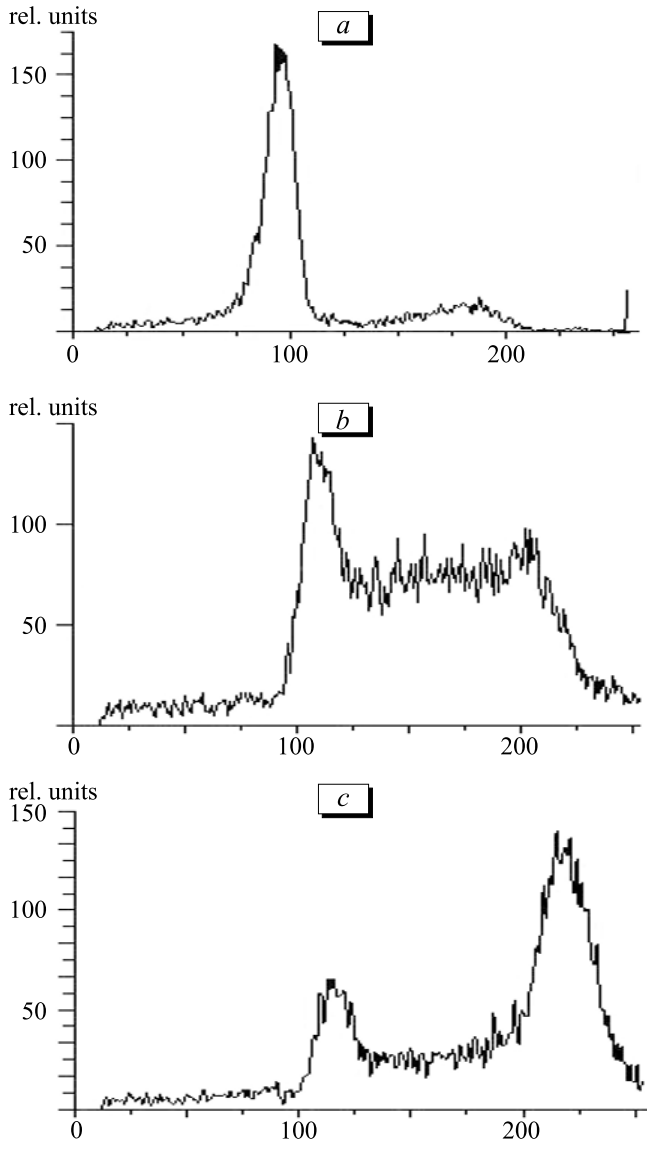

Fig. 3. Changes in chromatin conformational in ECV-304 cells after exposure to β-particles emitted by ^3H_2O (*b*) and ^{3}H-thymidine (*c*) in equal concentrations (0.066 mBq/ml) and untreated (*a*). Abscissa: linear scale.

binding sites or, in other words, chromatin decondensation. These results are consistent with our data on the assessment of radiation-induced chromatin decondensation in HeLa and V-79 cells *in vitro* as well as in hepatocytes of rats of different ages after exposure to different types of ionizing radiation *in vivo* [5,6]. Incubation of endothelial cells in the medium with ^{3}H-thymidine induced G2-phase cell cycle arrest accompanied accumulation of chromosomal aberrations [3]. Incubation of endotheliocytes in the medium with ^3H_2O at the same concentrations produced S-phase block without increase in the level of chromosomal aberrations [4]. Hence, changes in chromatin conformation and intracellular levels of free radicals apparently do not depend on structural chromosomal damage and hence on DSB of DNA. This hypothesis agrees with the results of our experiments demonstrating higher efficiency of γ-irradiation in induction of chromatin decondensation in comparison with neutrons, though the efficiency of neutrons in induction of chromosomal aberrations was almost 3 times higher than that of γ-rays [6]. Changes in chromatin conformation are known to modulate gene transcription. Changes in DNA methylation and chromatin conformation are indicators of triggering epigenetic mechanisms [12]. We previously studied the dynamics of intercellular NO and O_2^- after treatment with NO-synthase inhibitors. We detected decreased intracellular levels of both NO and O_2^- immediately after treating the cells with these inhibitors; 7 h after removal of inhibitors from the incubation medium, NO returned to the control value, but O_2^- level exceeded that not only within 7, 12, and 24 h, but also in three subsequent cell generations [2]. It should be noted that these changes were recorded only in HeLa cells. This can be explained either by selective action of NO-synthase inhibitors and different mechanisms underlying the regulation of redox status in endothelial and human carcinoma cells expressing different isoforms of NO-synthase. An important difference between the isoforms lies in the mechanisms regulating their activity in cells. Activity of constitutive forms depends on the concentration of intracellular calcium and calmodulin. Stimulation of mRNA synthesis increased activity of inducible form. This process is controlled by transcription factor NF-κB, which is activated by cytokines or factors stimulating generation of reactive oxygen species in cells.

The imbalance of free radicals obviously occurs during either activation or inhibition of free radical production in cells. Mild physical and/or chemical exposure is known to modulate cell functions. A possible mechanism underlying the switching between the cell functions is associated with modification of chromatin conformation and triggering epigenetic mechanisms of inheritance.

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