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CELL BIOLOGY

Loosening of Condensed Chromatin in Human Blood Lymphocytes Exposed to Irradiation with a Low-Energy He-Ne Laser

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Abstract—It was shown that, 1 h after irradiation of human blood lymphocytes with a He-Ne laser at 56 J/m^2 (5.6 W/m^2, 10 sec), the relative optical density of condensed chromatin masses observed in ultrathin sections was decreased ($p < 0.01$); i.e., the condensed chromatin became less compact. Such transition of condensed chromatin to a more "open" state may improve its availability for regulatory proteins and transcriptional factors. The irradiation also results in dispersion of condensed chromatin clumps in the nucleoplasm and enhancement of their angularity, i.e., in extension of the clump surface. These shifts, correlating with the activation of transcription, may be due to decompaction of the chromatin fibers not only on the periphery of chromatin clusters in the center of the nucleus, but also within the masses of condensed chromatin.

Key words: He-Ne laser, low level radiation, human blood lymphocytes, condensed chromatin, loosening, electron microscopy.

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Low intensity laser radiation in the red to proximal infrared portion of the spectrum is widely used in the treatment of many pathological conditions, as an agent stimulating specific functions of different cells (Karu, 2007). The primary effect of low-intensity laser radiation on cells is explained by its influence on the mitochondrion. There is extensive proof that cytochrome C-oxidase, the terminal enzyme of the respiratory chain, absorbs light of certain wavelengths (Karu, 1999; Pastore et al., 2000). Irradiation with a He-Ne laser induces an increase in the mitochondrial membrane potential ($\Delta\psi_M$) and the proton gradient (ΔpH_M) (Passarella et al., 1984; Hu et al., 2007), along with intensification of ATP synthesis (Passarella et al., 1984; Karu et al., 1999). Such shifts initiate cascades that convey to the nucleus information about changes in the state of mitochondria. This mechanism is termed retrograde mitochondrial signaling (Butow, Avadhani, 2004). Assumedly, the retrograde mitochondrial signaling takes place in cells activated by exposure to light in the red to proximal infrared region of the spectrum (Karu, 2008). Signals that reach the nucleus trigger the activation of gene expression. The radiation-induced growing of $\Delta\psi_M$ stimulates expression of the *c-fos* gene (Greco et al., 2001). Irradiation of human blood lymphocytes with 632.8-nm or 670-nm light enhances the activity of the *c-myc* gene and the gene responsible for IFN-γ synthesis (Funk et al., 1993; Shliakova et al., 1996). Irradiation of murine spleen lymphocytes with a He-Ne laser boosts production of IL-2, IL-6, and IFN-γ (Novoselova et al., 2006). Analysis of the gene expression profiles in human fibroblasts irradiated with 628-nm light revealed activation of eleven genes, part of which were responsible for the formation of transcriptional factors (Zhang et al., 2003).

The exposure of human differentiated lymphocytes to He-Ne laser light with a wavelength of 632.8 nm at 56 J/m^2 (56 Wt/m^2, 10 sec) induced the following changes: (1) improvement of accessibility of the total nuclear chromatin to acridine orange (Fedoseyeva et al., 1988), which is attributed to multiplication of transcriptional sites (Baserga, 1979); (2) fusion of the major part of discrete mitochondria to form giant organelles (Manteifel et al., 1997; Bakeeva et al., 1999), which requires expression of the nuclear genes coding for mitofusins, the proteins of the mitochondrial fusion machinery in human cells (Legros et al., 2002); (3) a rise in the intracellular level of calcium (Karu, 1992), which participates in the regulation of gene expression as a secondary messenger (Greco et al., 2001); (4) an increase in the total cellular RNA level (Karu, 1992); (5) modification of the "silent" ring nucleoli, accompanied by enlargement of the structures that contain newly formed and partially processed pre-rRNA (Manteifel, Karu, 1992). Such changes in the nucleoli are attributed to derepression of ribosomal genes (Derenzini et al., 2006). On the whole, similar changes develop in human blood lymphocytes 1 h after

phytohemagglutinin (PHA) stimulation. However, unlike PHA, irradiation induces neither DNA replication nor blast transformation of lymphocytes (Karu, 1992; Wasik, 2007).

A secondary effect of the low-intensity He-Ne laser on differentiated lymphocytes, consisting in enhancement of nuclear gene expression, logically, is expected to be linked to changes in the chromatin structure. The present work is aimed at testing this hypothesis by comparing human blood lymphocytes exposed to the He-Ne laser in vitro with intact lymphocytes. Using ultrathin cell sections impregnated with heavy metals, we analyzed the quantitative parameters characterizing condensed chromatin (i.e., clusters of compact chromatin), because such preparations do not allow visualization of the dispersed chromatin. First of all, we measured the optical density of randomly chosen condensed chromatin regions to determine their degree of compaction. Then we performed a morphometric analysis of the condensed chromatin clusters, comparing the effect of irradiation with that of PHA which, after brief (1 h) contact with lymphocytes, activates expression of genes responsible for the entrance of lymphocytes into the cell cycle (Irving et al., 1989).

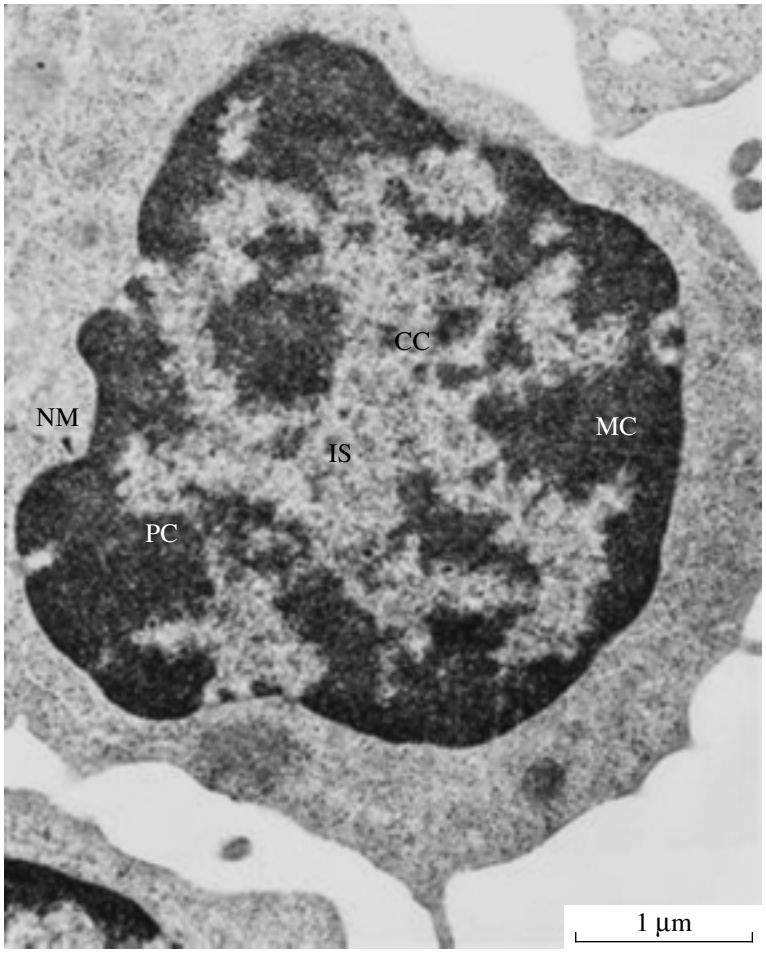
MATERIALS AND METHODS

Lymphocytes were obtained from blood of healthy male donors by centrifuging it in the Fikoll-Paque gradient (Pharmacia, Sweden). The bulk mass of cells thus obtained was formed by lymphocytes that were distinguished from other white blood cells by their ultrastructure. A suspension of lymphocytes (1×10^6) in medium 199 was placed into quartz cells and irradiated with a He-Ne laser. The optimum radiation dose (56 J/m^2 (5.6 W/m^2, 10 sec)) was determined based on analysis of the dose–effect correlation, by the ability of chromatin to bind acridine orange (Fedoseyeva et al., 1988). The maximum effect, as dependent on time, was detected one hour after irradiation. Lazed (at the optimal dose) and intact (control) lymphocytes were incubated for one hour in medium 199 at 37°C. Then the cell suspensions were centrifuged and the precipitated cells were fixed by a 25-% glutaraldehyde solution in 0.1 M phosphate buffer at pH 7.2–7.4. Subsequently, the cells were further fixed by a 1-% OsO_4 solution in the same buffer, dehydrated, and applied to Epon 812, according to the standard technique. Sections of lymphocytes, about 80-nm thick, were stained with heavy metals and examined with an electron microscope JEM 100CX (Japan). Images of lymphocyte profiles at a final 380000-fold magnification were processed with an electronic calculator fitted with a graphic input device. The relative profile area of condensed chromatin regions was measured using a previously described method (Manteifel et al., 1982). It is known that the total profile area of structures on arbitrarily chosen cell sections is proportional to their volume in the whole nucleus (Weibel, 1979). Additionally, angularity indices of condensed chromatin clumps and of the contour of membrane-associated chromatin projected into the nucleoplasm were calculated as, respectively, the ratio of the clump perimeter to its profile area and the ratio of the contour length to the length of the nuclear membrane bordering on the chromatin. This series of experiments compared the effects of lazing and PHA at a concentration of 2 μg/ml (Bacto phytohemagglutinin P, United States). After addition of PHA, the cells were incubated at 37°C for 1 h. Fifty lymphocytes were studied in each sample including the control; one section of each cell was analyzed. The packing density of the chromatin fibers composing the condensed chromatin was estimated using the same material. For this purpose, we measured the relative optical density of arbitrarily chosen islands of condensed chromatin on positive images of lymphocyte sections, magnified 100000-fold, using the application PhotoM (A. Chernigovskii, Institute of Cytology, Moscow). We did not measure the structural characteristics of chromatin located proximal to the interchromatin space, in order to eliminate the perichromatin region, as it may contain RNP fibers, along with chromatin fibers (Fakan, 2004). Regions lying close to nuclear pores were not measured either, because this kind of chromatin is characterized by an increased transcriptional activity (Misteli, 2004).

EXPERIMENTAL RESULTS

Ultrathin sections of lymphocytes isolated from human blood reveal electron-dense clusters of condensed chromatin (pattern) in the nucleus, whose total area makes up more than 50 percent of the nuclear section area (1). Nearly 80 percent of the total condensed chromatin area is occupied by a thick layer of chromatin contiguous to the membrane. The rest of the condensed chromatin is represented by clumps occurring in the central part of the nucleus. Such clumps may be connected with each other and with the membrane-associated chromatin. The interchromatin space, devoid of condensed chromatin, is characterized by a lowered electron density. It contains clusters of interchromatin RNP granules, single perichromatin RNP granules, other fibrillar and granular structures, and a nucleolus that is not seen in the section (figure). A detailed characteristic of the structural and functional organization of the components of interchromatin space has previously been given (Spector, 2003).

Results of the morphometric analysis of condensed chromatin showed that the profile area of membrane-associated condensed chromatin was similar in trial and control samples; no change in the nuclear volume was revealed. Neither did we detect any variation in the angularity index for the membrane-associated condensed chromatin surface oriented towards the nucleoplasm (it reached 1.95 ± 0.06 in trial and control samples). The cells subjected to irradiation showed a downward trend of the total clump profile area and an upward



Electron microscopy image of the nucleus in a peripheral human blood lymphocyte section. NM—nuclear membrane; MC—membrane-associated condensed chromatin; CC—condensed chromatin clumps; IS—interchromatin space; PC—perichromatin region.

trend of the clump surface angularity and the number of clumps (Table 1). The effect of PHA augments the angularity index, as compared with both the control cells ($p < 0.001$) and lazed cells ($p < 0.01$).

The compaction degree of chromatin was estimated by measuring the optical density of randomly chosen zones within clusters of condensed chromatin. In sections treated with heavy metals, the fibers composing clusters of condensed chromatin were characterized by increased electron density, whereas the space between the fibers had a low electron density. According to the definition, the higher the optical density, the greater the compaction degree of chromatin clusters, and vice versa.

Based on the facts about the repressed state of peripheral chromatin (Kosak, Groudin, 2004, and other works), we assumed that this chromatin was condensed to the limit. For this reason, we compared the optical density of the peripheral condensed chromatin immediately adjacent to the nuclear membrane (±150 nm in width) with the optical density of the rest of the condensed chromatin. Such analysis confirmed that the peripheral chromatin in control samples was characterized by a reliable increase in the optical density (by 13.2%), as compared with the rest of the condensed chromatin (Table 2). This property of the peripheral chromatin was used for a methodological purpose. To neutralize the variation in the optical density among different cells, which is linked to the difference in the impregnation degree of sections, we divided the optical density of the zones to be measured by the density of peripheral chromatin zones. Measurement of the relative optical density of condensed chromatin zones in lazed lymphocytes revealed a reliable 8.5-% drop in the compaction index of the membrane-associated chromatin, as compared to the control (Table 3). A decrease in the specified parameter for centrally localized chromatin was less pronounced (5.8%).

DISCUSSION OF RESULTS

Eukaryotic cells are characterized by a special arrangement of chromatin in a resting nucleus. This factor, along with molecular and supramolecular levels of chromatin organization, may control gene expres-

Table 1. Dynamics of quantitative characteristics of condensed chromatin (CC) profiles in lymphocytes 1 h after irradiation with a He–Ne laser or after addition of PHA, $\bar{X} \pm S_{\bar{X}}$

Parameter of CC cluster profiles	Control	Irradiation	PHA action
Total area of membrane-associated CC, % of a nuclear section area	47.75 ± 1.01	47.97 ± 1.24 $p > 0.89$	47.4 ± 1.29 $p > 0.71$
Total area of CC clumps, % of a nuclear section area	7.78 ± 0.55	6.64 ± 0.57 –14.7% $p > 0.17$	7.3 ± 0.5 –6.2% $p > 0.53$
The quantity of CC clumps	3.6 ± 0.31	4.04 ± 0.3 12% $p > 0.3$	3.19 ± 0.42 –11.4% $p > 0.15$
Angularity index of CC clumps	44.69 ± 3.46	50.61 ± 4.39 13% $p > 0.28$	68.49 ± 5.67 53.3% $p < 0.001$

Note: In all cases $n = 50$; percent deviations from the control are presented.

sion (Cremer T. et al., 2004). In situ application of fluorescent hybridization (FISH) that uses chromosome-specific dyes coupled with gene sensing showed that interphase chromosomes occupy certain regions in the three-dimensional volume of the nucleus. These regions consist of subchromosomal domains (Albeiz et al., 2006). In ultrathin cell sections, the chromosomal regions and subdomains may adjoin and overlap (Visser et al., 2000) and have the appearance of condensed chromatin clusters, made up of segments of compacted chromatin fibers (Manteifel et al., 1980; Fakan, 2004). Subchromosomal domains are separated by the interchromatin space with nonchromatin structures in it, the latter containing various regulatory proteins including transcriptional factors (Spector, 2003). It is suggested that channels of the interchromatin space do not enter between the fibers within subchromosomal domains (Cremer et al., 2006). According to this model, active genes are localized on the surface of domains, which puts them close to the transcriptional apparatus contained in the interchromatin space, whereas silent genes belong to the interior of domains (Fakan, 2004; Albeiz et al., 2006). This model suggests that the tight packing of fibers can prevent penetration of proteins inside the chromatin domains. However, this hypothesis was subjected to criticism based on experimental results confirming the penetration of high molecular weight proteins inside the condensed chromatin clusters (Verschure et al., 2003; Gorisch et al., 2005). Furthermore, within an in situ nucleus, a portion of the dispersed (transcriptionally active) chromatin was localized inside the condensed chromatin (Sadoni, Zink, 2004).

Table 2. Comparison of the optical densities of peripheral condensed chromatin (CC) and the rest of CC in the control, $\bar{X} \pm S_{\bar{X}}$

The optical density of peripheral CC	0.266 ± 0.006 $n = 80$
The optical density of CC in the remaining part of the nucleus	0.231 ± 0.005 $n = 113$
Reliable difference between the peripheral and the remaining CC	$p < 0.0001$

We showed that activation of human lymphocytes by low-intensity He-Ne irradiation brings about a decline in the relative optical density of the membrane-associated condensed chromatin that occupies roughly 80 percent of the total condensed chromatin in the section. This indicates a drop in the compaction degree, or loosening of the chromatin clusters. A less pronounced shift in the above parameter, as related to the condensed chromatin of the nucleoplasm, is attributable to the presence of chromosomal regions enriched with transcriptionally active genes in the central nuclear regions of human peripheral lymphocytes (Lukasova et al., 2002). Decompaction of condensed chromatin masses is not accompanied by changes in the relative area occupied by zones of condensed chromatin in a nuclear section (Table 1). This is consistent with the constancy of the volume of this chromatin in the whole nucleus (Weibel, 1979).

Loosening of condensed chromatin under the influence of laser radiation may promote the advancement

Table 3. The dynamics of the relative optical density of CC zones in lazed lymphocytes, $\overline{X} \pm S_{\overline{X}}$

Relative optical density of CC profiles localized in different parts of the nucleus	Control	Irradiation	The difference between trial and control samples, %
Near the membrane	0.858 ± 0.018 $n = 98$	0.785 ± 0.022 $n = 120$	–8.5 $p < 0.01$
In the central portion of the nucleus	0.838 ± 0.027 $n = 30$	0.780 ± 0.018 $n = 37$	–5.8 $p < 0.08$

of interchromatin space channels into condensed chromatin and the penetration of transcriptional factors and chromatin proteins to fibers comprising the chromatin clusters. Recently, it has been suggested that highly mobile proteins can penetrate inside the condensed chromatin, participating in the folding of chromatin fibers (Misteli, 2007).

Decompaction of the condensed chromatin in lazed lymphocytes is linked to gene activation (Funk et al., 1993; Shliakova et al., 1996; Novoselova et al., 2006). However, our data indicate that the optical density reflecting the degree of compaction of the membrane-associated condensed chromatin, in lazed lymphocytes, is reduced by 8.5% ($p < 0.01$). As the coding DNA makes up only about 10% of the genome in mammalian cells (Manuelidis, 1990), the loosening of the bulk condensed chromatin is too substantial to reflect the intensification of transcription proper. It may also be linked to changes in the chromatin conformation, which prepare it for the transcription (Studitskii, 2005).

Loosening of condensed chromatin under the influence of laser radiation is similar to chromatin changes in rat lymphocytes that develop after brief exposure to PHA (Manteifel et al., 1982). Ultrathin sections of the stimulated cells displayed increased relative distance between the fibers of condensed chromatin. Such chromatin alteration in guinea pig hepatocytes entering the G_1 phase was followed by a shift in the width distribution of chromatin fibers. This was accompanied by an increase in the number of thin chromatin fibers and the appearance of a peak corresponding to 10-nm fibers, whereas the 30-nm fiber peak became less pronounced (Manteifel et al., 1980; 1984). Unfolding of the highly condensed fibers in nucleo is attributable to activation of the genes responsible for proliferation (Irving et al., 1989). In the course of transcription, RNA-polymerase II is believed to be capable of traveling along a 10-nm fiber (Wegel, Shaw, 2005). The decompaction of condensed chromatin in lazed lymphocytes may also be induced by a partial unfolding of highly condensed 30-nm chromatin fibers.

A relevant trend in dynamics of the morphometric characteristics of chromatin clumps of the nucleoplasm is as follows (Table 1). In lymphocytes subjected to irradiation, the angularity index increases by 13% and the number of clumps, by 12%, which is equivalent to their dispersion. The variation of these parameters is not reliable; together they indicate enlargement of the clump surface. The increase in the angularity of clumps in human peripheral lymphocytes stimulated by PHA is much more pronounced. Lazed lymphocytes also exhibit a tendency towards reduction of the relative area occupied by the clumps (by 14.7 percent), as against the control. This may be due to a partial transmutation of condensed chromatin into transcriptionally competent dispersed chromatin on the periphery of clumps. Perichromatin RNP-fibers, containing newly formed and partially processed pre-mRNA, were found on the periphery of the condensed chromatin profiles (Fakan, 2004).

Thus, the experimental results have shown that the inducing influence of the low-intensity He-Ne laser radiation leads to chromatin alterations that may be caused by decompaction of fibers both inside the condensed chromatin clusters and on their surface. Induction of a more "open" structural conformation of condensed chromatin in differentiated lymphocytes by exposure to light with $\lambda = 632.8$ nm may reflect the transition to the transcriptional competence state and/or transcriptionally active state. Naturally, these hypothetical functional abilities of condensed chromatin require experimental proof. Nevertheless, there is some logic behind both existing alternative hypotheses suggesting that transcription sites are located inside the nucleus—at the interface (Cmarco et al., 1999) or within the condensed chromatin clusters (Sadoni, Zink, 2004)—in situ.

Thus, we have shown that low-intensity He-Ne lazing of lymphocytes brings about alterations in the ultrastructure of condensed chromatin and also induces the previously reported transformation of the "silent" nucleoli into transcriptionally active ones. These shifts reflect photosensitive activation of the genome that develops via the retrograde mitochondrial signaling (Karu, 2008). To improve our understanding of the

mechanism underlying the effect of low-intensity laser radiation, it is essential to continue electron-microscopic investigations introducing new criteria of the structural and functional characteristics of chromatin.

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