CHANGES IN THE STRUCTURE OF CHROMATIN IN HEPATOCYTE NUCLEI OF RATS ADAPTED TO HYPOXIA

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UDC 612.35.014.24-06:612.273.2.017.2

The structure of chromatin in the nuclei of isolated surviving hepatocytes and of isolated hepatocyte nuclei was studied by fluorochroming with Acridine Orange and microfluorometry of the luminescence of chromatin-bound dye at 530 and 590 nm in intact rats and rats adapted to hypoxia in a pressure chamber for 60 days. Hepatocyte nuclei of intact rats were shown to be distributed on the basis of their fluorescence at 530 nm into three classes, with a ratio between intensities of 1:2:4, whereas hepatocyte nuclei of rats adapted to hypoxia formed only one class, corresponding to the second class in the control. The ratio between the intensities of luminescence at 590 nm and 530 nm (the coefficient α) forms a normal distribution in intact rats, but in adapted rats it formed a bimodal distribution with a shaft of the maxima toward both sides of the control. During hypoxia repression of some genes and depression of others is considered to take place in the chromatin of liver nuclei.

KEY WORDS: *hypoxia; liver; structure of hepatocyte chromatin; Acridine Orange; microfluorometry.*

During the adaptation of animals to hypoxia many investigators have found, in addition to systemic reactions, definite reorganizations of intracellular metabolism in various organs, connected with intensification of the synthesis of RNA and some key enzyme proteins [2, 5, 6]. Adaptation to hypoxia is thus achieved at the cellular level evidently on account of changes in the operation of the genetic apparatus [5]. However, no direct evidence of structural changes in the nuclear chromatin of animals adapted to hypoxia has yet been reported in the literature.

The object of this investigation was to look for such structural changes. For this purpose, the character of interaction between the fluorescent dye Acridine Orange and the nuclear chromatin of intact cells of isolated nuclei was studied. Measurement of fluorescence of Acridine Orange, bound with chromatin, in the green (530 nm) and orange-red (590-615 nm) regions of the spectrum is known to be a sensitive test for the detection of functional changes in chromatin [4, 8, 10]. Interaction between Acridine Orange and nuclear chromatin can be investigated both in fixed [4, 8, 10] and in living (surviving) cells [7, 9, 10].

EXPERIMENTAL METHOD

Experiments were carried out on adult noninbred male albino rats. The animals were adapted to hyposia in a pressure chamber by successive elevation to an "altitude" of between 2500 and 7600 m, by Barbashova's method [1]. The experimental animals were tested on the 60th day of adaptation, when its level was maximal. Experimental and control animals were killed by decapitation and the liver was then immediately perfused in order to obtain

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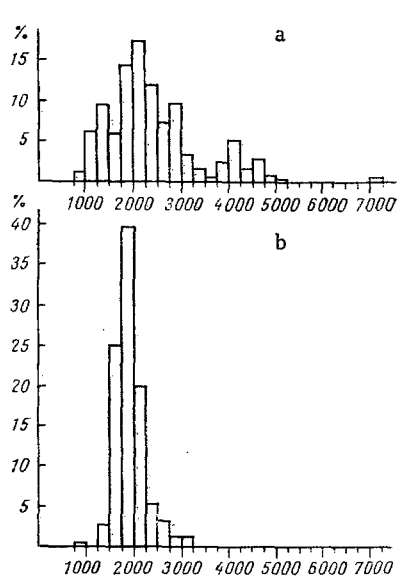

Fig. 1

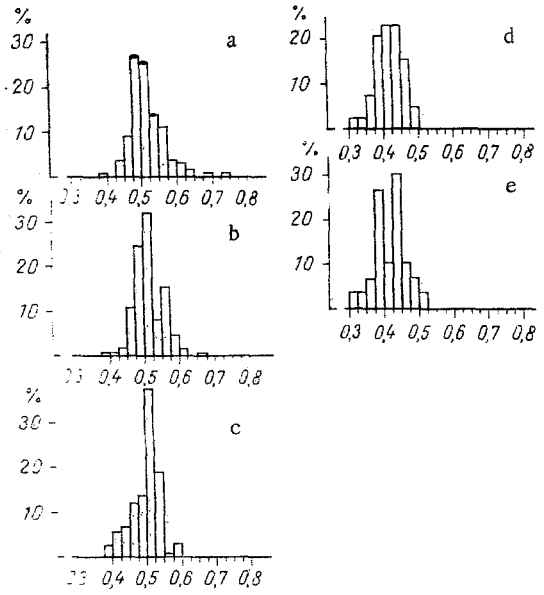

Fig. 2

Fig. 1. Distribution of hepatocyte nuclei from intact rats (a) and rats adapted to hypoxia (b) based on intensity of fluorescence at 530 nm. Abscissa, intensity of fluorescence (in working units); ordinate, percentage of nuclei with the given intensity of fluorescence. Step of histogram 250 working units.

Fig. 2. Distribution of hepatocyte nuclei by coefficient α from intact rats (a) and rats adapted to hypoxia (b, c) and distribution of isolated nuclei by coefficient α in intact rats (d) and rats adapted to hypoxia (e). Abscissa, value of coefficient α (dimensionless); ordinate, percentage of nuclei with the given value of α. Step of histogram 0.025 working unit.

living isolated cells, as described by the writers previously [2]. A suspension of isolated cells was sedimented by centrifugation and the cells were resuspended in $1 \cdot 10^{-4}$ M Acridine Orange solution in 0.9% NaCl, pH 7.0. Staining was carried out for 20 min at room (18-20°C) temperature. The excess of fluorochrome was then washed off by two repeated resuspensions in 0.9% NaCl solution followed by centrifugation, after which the suspension of fluorochromed cells was placed on a quartz slide and covered with another quartz coverslip, which was surrounded by a ring of paraffin wax to prevent the specimen from drying. Examination and fluorometry of the preparation were carried out on the MLD microscope equipped with an EMEL-1A photometric attachment. The source of light was a KIM-75 iodine-cycle lamp, powered by a stabilized dc supply. An FSI = I + C3C7 excitation filter and ZhS18 + ZhS19 suppression filter were used. Green and red fluorescence of chromatin-bound Acridine Orangewere isolated by means of interference filters with transmission maxima at 530 and 590 nm. Fluorometry was carried out with the MSHCH-L 40 × 0.65 objective, and the diameter of the fluorometric probe in the plane of the preparation was 9 μ. By moving the preparation around, the nuclei of the isolated hepatocytes were placed successively in the photometric probe and the luminescence of each of them was measured at 530 and 590 nm. Fluorescence of the cytoplasm of the hepatocytes, of the isolated hepatocyte nuclei constantly present in the preparations, and also of the background was measured in the same way. The coefficient α was calculated:

$$\alpha = \frac{\text{Fluorescence of nucleus at 590 nm} - \text{fluorescence of background at 590 nm}}{\text{Fluorescence of nucleus at 530 nm} - \text{fluorescence of background at 530 nm}}$$

Fluorescence of 225 nuclei of isolated hepatocytes and of 40 isolated nuclei obtained from 5 animals was measured in the experimental series and fluorescence of 289 hepatocyte nuclei and of 39 isolated nuclei from 6 rats in the control series. The results of the measurements were subjected to statistical analysis on the "Nairi-3" computer; mean values, confidence limits for a 95% level of significance, and characteristics of the distribution were calculated. Histograms also were plotted.

EXPERIMENTAL RESULTS

The suspension of isolated liver cells of control rats contained hepatocytes with nuclei that differed in size and brightness of fluorescence respectively. Measurement of luminescence of the hepatocyte nuclei in the 530 nm region showed the existence of 3 classes of nuclei, with intensities of luminescence relative to each other of 1:2:4 (Fig. 1a). Luminescence in the 530 nm region is known to be due to acridine, intercalated into the DNA double helix, and it reflects mainly the quantity of DNA in the nucleus [10]. On the other hand, it is well known from the results of cytophotometric determinations that the adult rat liver contains about 20% of diploid, 60% of tetraploid, and 10% of octaploid cells [2, 3]. The presence of 3 classes of nuclei with different intensities of luminescence at 530 nm in hepatocytes of normal liver thus reflects their heterogeneity as regards DNA content. The distribution of hepatocyte nuclei of rats adapted to hypoxia by the intensity of their luminescence at 530 nm differed sharply from the control and was close to the normal distribution (Fig. 1b). In this histogram the maximum coincided with the second maximum on the histogram for hepatocytes of the control rats, i.e., it evidently corresponded to cells containing the 4 n quantity of DNA in their nuclei.

Although normal liver cells are sharply heterogeneous for their DNA content per nucleus, their distribution on the basis of this coefficient was close to normal (Fig. 2a). The functional state of the chromatin in cells of different ploidy is evidently sufficiently similar. The distribution of hepatocyte nuclei from rats adapted to hypoxia, on the basis of their coefficient α, differed in character in both experimental groups from that in the control (Fig. 2b, c), although they also differed from one another. In the first experiment there was both an increase in the number of nuclei for which the coefficient α was below 0.525 (about 75% compared with 65% in the control) and a small increase in the number of nuclei for which the coefficient α was between 0.551 and 0.575 (about 15% compared with 11% in the control), as a result of which the distribution apparently became bimodal. It is interesting to note that a distribution of the same character for the value of α was found for isolated nuclei of the experimental rats of this group (Fig. 2e).

In the second experiment the histogram of rats adapted to hypoxia differed sharply from the normal distribution on account of an increase in the number of hepatocyte nuclei for which α was under 0.525 (more than 75%) and a sharp decrease in the number of nuclei for which α was between 0.551 and 0.575 (about 2%).

During measurement of luminescence of nuclei in isolated hepatocytes, areas of cytoplasm unavoidably were caught in the probe, and this was naturally reflected in the value of their coefficient α. For pure cytoplasm it averaged 0.707 in the control and 0.691 in the experimental. Meanwhile measurements on isolated nuclei showed that the character of their distribution by the value of α was generally the same in both the control and the experimental as for nuclei in hepatocytes (Fig. 2d, e) and the histograms were simply shifted to the left (i.e., toward a decrease in the coefficient α) on account of removal of the luminescence of the cytoplasm. The cytoplasm thus evidently is not an obstacle to the discovery of structural changes in chromatin in hepatocyte nuclei.

The study of changes in the coefficient α during differentiation and dedifferentiation and before the beginning of cell proliferation showed that an increase in α corresponds to activation of the genetic apparatus and a decrease to its repression [8]. In the light of these findings, changes discovered during adaptation to hypoxia in the first experiment can be interpreted as repression of one part of the genome of the hepatocyte nuclei and depression of the other. Changes in the second experiment point simply to repression of an appreciable part of the genome. It should be pointed out that the period of adaptation of 60 days adopted in these experiments corresponds to the maximum of adaptivity of the experimental animals: Usually continuation of the experiment led to overadaptation [1, 6]. Although all the physiological and biochemical indices of adaptation (including an increase in the content of particular enzyme proteins) reached a maximum, synthesis of m-RNA could possibly already have been slowed or, in some cases, even stopped, in good agreement with the state of the chromatin discovered in the hepatocyte nuclei in these experiments. Evidence was obtained that at the height of adaptation to hypoxia, changes of two types occurred in the nuclear chromatin of the hepatocytes: well-marked changes, pointing to possible repression of an appreciable part (about 10%) of the genome, and weakly defined, indicating derepression of another, smaller part of their genome.

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