EFFECT OF SINGLE-STRANDED BREAKS ON ULTRASTRUCTURAL ORGANIZATION AND CYTOCHEMISTRY OF TUMOR CELL CHROMATIN

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The superhelical conformation of DNA, which is particularly well marked in tumor cells [7], plays an important role in the regulation of chromatin function [1]. Data in the literature on the presence of torsional stress of superhelicity of transcriptionally active DNA are contradictory [8, 10, 12, 13].

The problem is beset by technical difficulties. In eukaryotes only about 5% of the DNA is transcriptionally active. The degree of superhelicity of DNA, determined in vitro on nucleoids, is largely generated by the actual procedure of preparation of dehistonized nuclei. The method of determination of torsionally stressed DNA in vivo, based on the kinetics of cross-linkage with psoralen, which gave negative results on HeLa and *Drosophila* cells, is perhaps insufficiently sensitive [13]. Indirect evidence in support of the presence of torsional stresses in vivo is given by the results of differential calorimetry of HeLa cells after short treatment with nuclease [14].

Our approach to the problem was to study the structural consequences of infliction of single-stranded breaks on DNA of living cells by a sensitive method of ultrastructural analysis in situ. It was assumed that the appearance of changes in the chromatin under these circumstances, leading to loosening of its structure, is evidence in support of torsional stresses.

To increase the reliability of the results two agents were used. The first, dimethyl sulfoxide (DMSO), through a chain of cellular reactions, activates the relaxing enzyme topoisomerase I [11], which is linked in vivo with transcriptionally-active regions of chromatin [6]; the second, bleomycin, unlike the first, has a primary DNase-like mechanism of action, and directly induces single-stranded breaks, primarily in transcriptionally active DNA [9]. To avoid accumulation of double-stranded breaks, short-term treatment with bleomycin was used.

EXPERIMENTAL METHOD

Experiments were carried out on cells of a Zajdela ascites hepatoma (ZAH) and a 1- to 3-day culture of transformed Djungarian hamster fibroblasts of diploid line 4/21. The cells were treated with 1% DMSO in culture for 1-2 h or with 100 μg/ml of bleomycin for 5-15 min after removal of the cells from the blast. The cells, washed with medium, were then tested for viability by blocking of intravital staining, and parallel films were prepared and sections cut for electron microscopy (2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, postfixation with 1% osmic acid), followed by embedding in Epon. Films fixed with ethanol and acetone were subjected to hydrolysis (5 N HCl, 60 min, 21°C) and stained for DNA with Schiff's reagent; the concentration of DNA-fuchsin was determined cytophotometrically on 100-120 nuclei per film. Fixed films also were stained with methylene, toluidine, and alcian blue, pH 4.1. Ultrathin sections were stained and studied in the JEM-100B electron microscope.

Single-stranded breaks were identified by comparing the results of neutral and alkaline elution of DNA from cells labeled for 24 h in culture with ^{3}H-thymidine (40 kBq/ml) [3].

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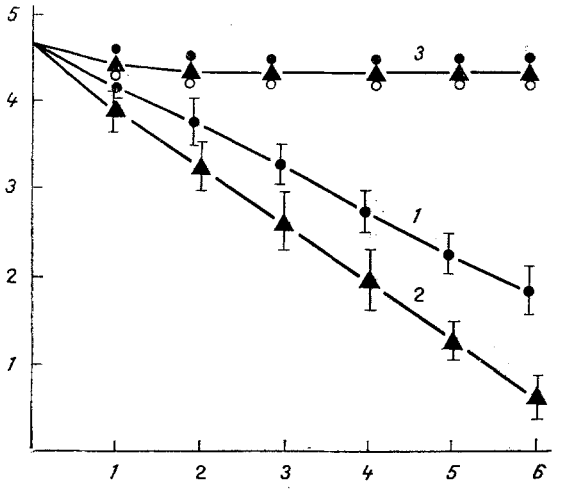

Fig. 1. Curves of alkaline (1, 2) and neutral (3) elution of DNA from cells of line 4/21. Filled circles indicates control cells, triangles — cells treated for 1 h with 1% DMSO, empty circles — 24 h after treatment with DMSO. Abscissa, Nos. of fractions; ordinate, natural logarithm of fraction of DNA remaining on filter. Fractions of alkaline elution collected every 30 min, neutral — every 90 min.

EXPERIMENTAL RESULTS

After exposure to 1% DMSO for 1 h, single-stranded breaks were formed in DNA which did not change with time into double-stranded breaks, to judge from the acceleration of alkaline, but not of neutral, elution of DNA (Fig. 1). The DNA content likewise was not reduced in Feulgen preparations, confirming the absence of its depolymerization. Since internal irradiation of cells labeled for biochemical monitoring may itself induce DNA breaks, in morphological experiments the Feulgen data were used for guidance. According to this criterion, the concentration and minimal essential time of treatment with bleomycin (5 min) were chosen at which the DNA concentration was not reduced, but clear ultrastructural changes were present in the chromatin. For comparison, prolongation of the treatment to 15 min caused extraction of 25% of the DNA and definite removal of diffuse chromatin.

Under the conditions chosen, the cells preserved their ability to block intravital staining.

Both agents had a similar morphological action on the two tumor objects (Fig. 2). In the control, compact chromatin of ZAH cells and of a 1-day culture of line 4/21 consisted of 25- to 30-nm supranucleosomal globules, whereas the diffuse chromatin, as was described previously for rat liver [2], consisted mainly of chains of looser globules of about the same size. After both treatments the substructure of the compact chromatin remained unchanged, although it was somewhat condensed. Enlargement of zones occupied by diffuse chromatin was observed, together with loosening of its structure. In these regions a finely fibrillar structure of the chromatin became predominant, with single fibrils about 2.5 nm in thickness.

Under the influence of both agents, abasophilia of the chromatin was intensified after staining with basic dyes and contrast stains (quantitative analysis was not undertaken because of accompanying staining of the cytoplasm).

The similarity of the ultrastructural changes in the chromatin under the influence of these two agents is evidence that they share a common mechanism of action, and it suggests that the cause of the changes discovered is the presence of single-stranded breaks in DNA.

The essence of the changes taking place in diffuse (active) chromatin of these cells is loss of the discrete globular structure and thinning of the chromatin fibril. Besides increased accessibility of the phosphoric acid groups of the DNA for basic dyes under these circumstances, these changes also indicate loss of nucleosomal organization and dissociation of histones from DNA. The same phenomenon — cooperative shedding of proteins — has also been found by other workers during relaxation of torsionally stressed DNA [4, 8].

The results can therefore be interpreted as supporting the presence of torsional stresses in active chromatin of tumor cells in vivo. In that case the loose globular subunit of diffuse

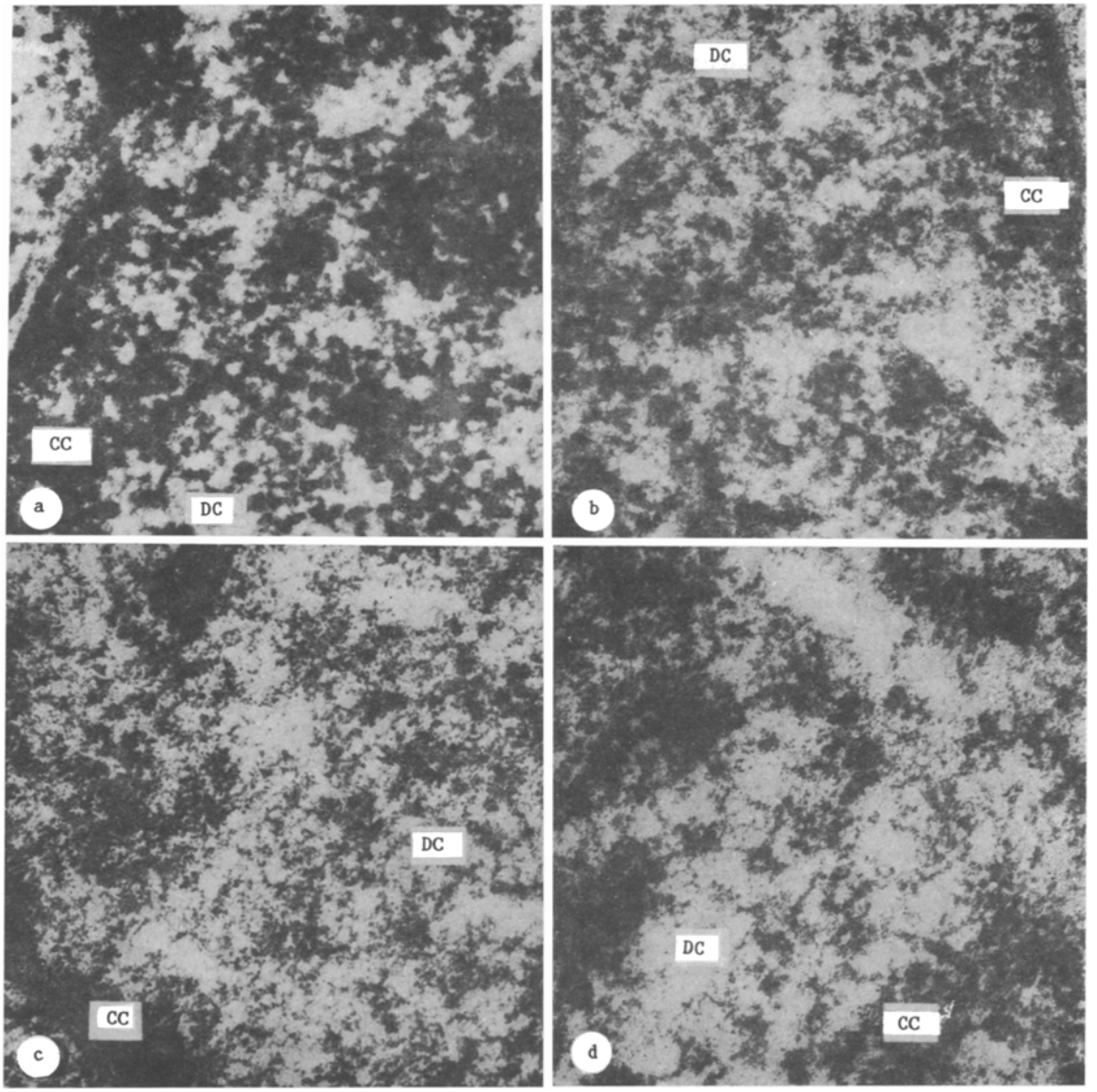

Fig. 2. Ultrastructural changes in nuclei on infliction of single-stranded breaks of DNA. a, b) Compact chromatin (CC) consists of 25-30 nm supranucleosomal globules, diffuse chromatin (DC) consists mainly of chains of looser globules of about the same size; c, d) treatment of loosely globular chromatin converts it into finely fibrillar chromatin; c) ZAH (1% DMSO, 1 h), d) 4/21 cells (bleomycin, 100 μg/ml, 5 min). 100,000 ×.

chromatin is a form of organization of torsionally stressed DNA. It evidently corresponds to the partially uncoiled nucleosome of functionally active chromatin [5].

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ACTION OF HYPOTHALAMIC HORMONE ON NUDE MICE WITH TRANSPLANTABLE STRAINS OF HUMAN TUMORS

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Melanostatin (the tripeptide propyl—leucyl—glycinamide, H—Pro—Leu—Gly—NH_2), a hypothalamic hormone which inhibits the melanocyte-stimulating hormone of the pituitary gland, has been synthesized at the All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR. The targeted synthesis of hypothalamic hormones became possible once their peptide nature had been established [6].

Considering the role of melanostatin in melanin formation, it was natural to suggest its action on melanomas. This paper discusses the results of a chemotherapeutic and cytological study of melanostatin on models of melanoma and other human tumors.

EXPERIMENTAL METHOD

Experiments were carried out on 6- to 8-week nude mice (based on line BALB/c) with subcutaneously inoculated (with a suspension of tumor cells in Hanks' solution) strains of human tumors. Melanostatin was dissolved in distilled water and injected into mice with melanoma (Mel-1) on the 10th day after transplantation (when the average volume of the tumors was 0.3 cm^3) in a dose of 70 mg/kg intraperitoneally, twice with an interval of 72 h between injections. Inhibition of tumor growth was calculated as the difference in their volume in the control and treated groups. The morphological composition of the peripheral blood was determined 3 days after the end of treatment: the functional state of the blood lymphocytes and tumor cells (in squash preparations of the tumors) was assessed cytochemically as succinate dehydrogenase (SDH) and α-glycerophosphate dehydrogenase (α-GPDH) activity, as described previously [1]. Fluorescein sodium (FlNa) was injected into retro-orbital sinus on the 10th day, 1 h before sacrifice of the mice with Mel-1; the method described in [2] was used for the investigation. Accumulation of FlNa was determined in squash preparations of the tumors and organs (liver, spleen, lungs, kidney) of the control and treated mice.

Accumulation of FlNa also was estimated after contact exposure of strains of melanoma (Mel-5), lung cancer (LC-1), and carcinoma of the liver (CLi), obtained from material removed at operation, to melanostatin in vitro. The strains were studied in the period of maximal tumor growth on the 14th-20th days after transplantation. Fragments of tumor (control and experiment) were incubated in Hanks' solution with FlNa for 5 min at 37°C, after which they were made.

The degree of fluorescence after injection of FlNa was determined under the "Lyumam I-3" microscope at $\lambda = 516 \pm 16$ nm, with FMÉL1-A photometric attachment and the Sh-4300 combined digital computer. The intensity of fluorescence of each squash preparation of tumors and organs was determined in ten fields of vision (U = 2v).

The results were subjected to statistical analysis by nonparametric tests [3].

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