GLUCOCORTICOID-MEDIATED DNA DEGRADATION IN THE THYMOCYTES OF RATS WITH TRANSPLANTED ZAJDELA'S ASCITES HEPATOMA

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Stress arising in response to exposure to unfavorable factors is accompanied by activation of the pituitary-adrenal system, one result of which is involution of the thymus. The writers showed previously that the same response (involution of the thymus and DNA degradation to nucleosomes) develops in animals with growing tumors, and that if tumor transplantation is preceded by bilateral adrenalectomy, this response is completely prevented [8]. Other investigators recorded fragmentation of thymocyte DNA after irradiation of animals [1, 2, 6. 9]. The elucidation of the details of DNA degradation in the thymocytes of tumor-bearing animals is of great interest for two reasons: first, it may shed light on theoretically and practically important aspects of the distant action of a tumor on remote tissues of the body, and second, it may help to explain the molecular mechanisms of programmed cell death — an extremely widespread general biological phenomenon [5].

The aim of this investigation was to compare degradation products of total thymocyte DNA in animals with transplanted Zajdela ascites hepatoma, on the one hand, with some of the specific sequences composing it (genes of histones, ribosomes, and heat shock hsp70), on the other hand, The aim was to discover whether heterogeneity of the degradation products of total DNA is due to superposition of a set of homogeneous distributions specific for individual genes or whether, on the contrary, breakdown products of individual genes are just as heterogeneous as total DNA. This would point to disordered degradation.

EXPERIMENTAL METHOD

Experiments were carried out on noninbred male albino rats weighing 140-180 g. ZAH cells were inoculated intraperitoneally (0.2 ml of ascites fluid per animal). The rats were used on the 5th day of tumor development. Animals of the control group underwent bilateral adrenalectomy 4 days before transplantation of the tumor. Healthy intact male rats of the same weight served as an additional control. In some experiments healthy animals were given an injection of hydrocortisone hemisuccinate (3 mg/100 g body weight 6 h before sacrifice) or of dexamethasone (200 µg/100 g body weight 90 min before sacrifice). DNA was extracted from isolated thymus nuclei by deproteinization with phenol in the presence of 1% sodium dodecylsulfate, and with phenol and chloroform, followed by treatment with RNase (50 µg/ml, 37°C, 30 min) and pronase (50 µg/ml, 37°C, 1 h), followed by repeated reprecipitation with ethanol. Nuclei isolated from rat liver were purified by centrifugation through 2.2 M sucrose, suspended in buffer containing 5 mM $MgCl_2$, 0.25 M sucrose, and Tris-HCl (pH 7.8), and incubated for various times (from 1 to 20 min at 0°C) to induce DNA degradation by endogenous nucleases, after which DNA was isolated as described above. After electrophoresis in 0.8% agarose in Tris-phosphate buffer (0.08 M Tris-phosphate, 0.008 M EDTA, pH 7.8) for 2-3 h at 5-7 V/cm the DNA was transferred to nitrocellulose filters and hybridized with cloned DNAs in accordance with previous recommendations [4]. Plasmids ph22 with insertion of a cluster of histone genes (7000 base pairs — bp) of the sea urchin *Ps. milliaris* in the Hind III site of the pUC8 vector (constructed in M. Brinstiel's laboratory, Switzerland); pDP8 with insertion of cDNA (1750 bp) of human heat shock gene hsp70 in the EcoRI — SalI site of

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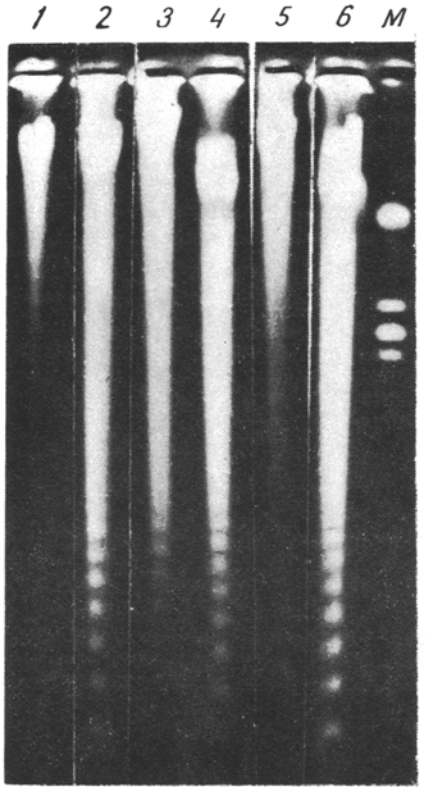

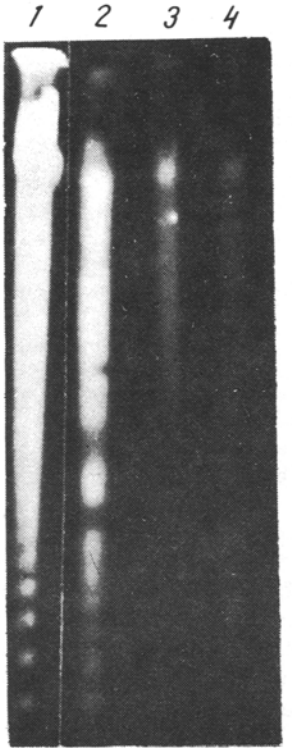

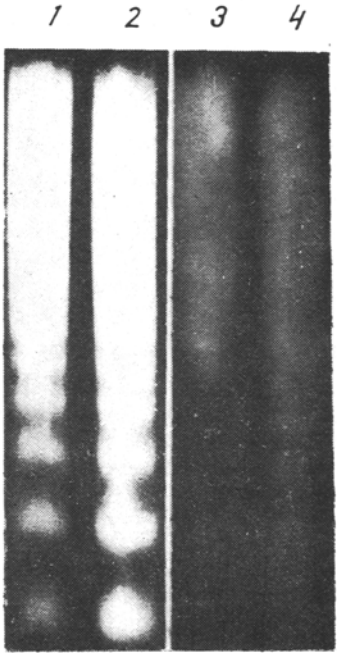

Fig. 1 Fig. 2 Fig. 3

Fig. 1. Electrophoresis of thymocyte DNA (stained with ethidium bromide) after various procedures. 1) Thymus of intact animals; 2) injection of exogenous glucocorticoids; 3) tumor development in animals (5th day after inoculation); 4) combined action of exogenous glucocorticoids and tumor growth; 5) bilateral adrenalectomy on tumor-bearing animals; 6) injection of glucocorticoids into tumor-bearing animals after adrenalectomy. M) Markers (fragments of DNA of phage λ measuring 22,000, 7600, 5700, and 4850 bp).

Fig. 2. Electrophoresis and blot hybridization of thymus DNA fragments from tumor-bearing animals with specific probes. 1) Distribution of total DNA (stained with ethidium bromide); 2, 3, 4) autoradiographs of DNA fragment hybridized with specific DNA sequences — with histone genes, ribosomal genes, and heat shock gene hsp70 respectively.

Fig. 3. Autolytic degradation of DNA from isolated rat liver nuclei. Incubation for: 1) 10 min; 2) 20 min. Stained with ethidium bromide; 3, 4) autoradiographs of DNA fragments hybridized with specific DNA sequence (fragments of tyrosine aminotransferase gene).

plasmid vector pUC13 (G. P. Thomas, England); pAβE with insertion of a fragment of the gene of human 28S rRNA in plasmid pBR322; and a fragment of the gene of rat liver tyrosine aminotransferase in Charon 4A vector (clone 28), also containing unidentified repeat sequences, were used as molecular probes. Procedures connected with nick-translation of the DNA probes, DNA hybridization, and development of the autoradiographic signal were carried out in accordance with known recommendations [4].

EXPERIMENTAL RESULTS

The results of electrophoresis of thymocyte DNA of intact rats and rats exposed to experimental procedures are shown in Fig. 1. DNA in intact animals (lane 1) was mainly of high molecular weight, and the visible tail of low-molecular-weight components probably reflects hydrodynamic fragmentation of the DNA during its isolation. Under the influence of excess of exogenous glucocorticoids (lane 2) or of glucocorticoids produced in the body as a result of development of the tumor [7] (lane 3), or by the action of both these factors together (lane

4), the thymocyte DNA is degraded in vivo with the formation of a typical nucleosomal "string of beads", evidence of activation of an endogenous mechanism (evidently Ca^{++}, Mg^{++}-activated nuclease). The action of the tumor on the thymus is evidently mediated through the adrenal cortex, for bilateral adrenalectomy completely prevented degradation of thymocyte DNA in tumor-bearing animals (lane 5), whereas injection of exogenous hydrocortisone into such animals restored the typical nucleosomal distribution (lane 6).

These results are evidence that double-stranded breaks develop in linker DNA of thymocytes dying under the influence of glucocorticoids, leading to excision of the nucleosomes. This is evidently not all, and in addition to double-stranded breaks, single-stranded breaks also are formed in the nucleosomal DNA itself, as is shown by disappearance of the nucleosomal "string of beads" on electrophoresis of the same DNA preparations as in Fig. 1, but under denaturing conditions (not shown here). This observation is in agreement with data in the literature [3].

For subsequent hybridization analysis DNA isolated from thymocytes of tumor-bearing animals was used (Fig. 1, lane 3), and cloned DNAs of histone, ribosomal, and heat shock hsp70 genes were used as probes. These molecular probes were chosen because they are structurally and functionally different classes of eukaryotic DNA sequences: ribosomal and histone genes form large families; moreover, the former are constantly active in transcription, whereas the latter are evidently only weakly active — because of the low mitotic activity of thymus cells. As regards the heat shock genes, and, in particular, hsp70, they are represented in the genome by a much smaller number of copies than the previous genes, and their ability to be activated virtually instantaneously during stress situations places them in a special position and suggests the existence of unusual mechanisms of regulation [10].

Electrophoresis of total thymus DNA from tumor-bearing rats (lane 1, stained with ethidium bromide) and autoradiographs obtained on hybridization of this same DNA with DNA of ph22, pDP8, and $pA_{\beta E}$ (lanes 2, 3, and 4, respectively) are shown in Fig. 2. On comparison of degradation products of total DNA, on the one hand, and the sequences of histone, ribosomal, and stress genes contained in it, on the other hand, features of both similarity and differences will be noted. A heterogeneous distribution is common to them all, evidence in support of disordered degradation. In fact, the discovery of any of the sequences studied in the composition of both high-molecular-weight (over 20,000 bp) and low-molecular-weight (under 1000 bp) fragments indicates that degradation of a particular DNA sequence, once begun, takes place disorderly. Since we are concerned with an advanced process (flattened out on a plateau) with considerable involution of the thymus (5th day of tumor growth — the near-terminal stage), the heterogeneous distribution of fragments is difficult to explain by the unsynchronized nature of DNA degradation in different thymocytes, which is more likely to take place in the early stages of involution. Nevertheless, this hypothesis cannot be completely ruled out at this stage and it is being tested experimentally.

The features of difference in degradation of individual genes consist, first, of differences in the clarity of the nucleosomal "beads" — they are clearly visible in total DNA and in histone sequences, but they are very indistinct in ribosomal genes, possible evidence of their initially high transcription activity, whereas they are quite invisible in sequence of the hsp70 gene (in this last case, admittedly, the hybridization signal was relatively weak). Second, degradation of histone genes is accompanied, it may be seen, by the formation not only of oligonucleosomes, but also of fairly discrete blocks with a much higher molecular weight (from 2000 to 20,000 bp or more, approximately). The nature of these blocks, found after degradation of other genes also, will be a topic for future research.

If we turn to the obvious similarity of the degradation products of total DNA and of individual genes, it can be postulated that DNA degradation in thymocytes dying in vivo is just as disordered as during the well studied autolysis of isolated liver nuclei in vitro. In fact, the kinetics of excision of nucleosomes depending on the duration of incubation was studied in a model of autolysis of isolated rat liver nuclei (Fig. 3, lanes 1 and 2), and similarity of the nucleosomal distribution of total DNA and of the specific sequence — in this case, a fragment of the tyrosine aminotransferase gene, was established (Fig. 3, lane 3). To sum up the data described above it can be concluded that a tumor growing in the body acts as a chronic stress factor and induces a series of consecutive events: activation of the pituitary—adrenal system, release of an excess of glucocorticoids into the blood stream, and programmed death of thymocytes, the chief manifestation of which is disordered DNA degradation, effected by a system of endogenous nucleases, with the formation of double-stranded breaks in linker DNA and single-stranded breaks in nucleosomal DNA. DNA degradation in thymo-

cytes in vivo and in isolated liver nuclei in vitro is evidently effected by the same enzyme system, although it responds differently in different cells to glucocorticoids.

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CRITERIA OF SENSITIVITY OF ZAJDELA AND MORRIS HEPATOMA CELLS TO GLUCOCORTICOIDS

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Liver and hepatoma cells, in which glucocorticoids induce tyrosine aminotransferase (TAT) synthesis, are the classical model with which to study the molecular mechanism of action of steroid hormones on a target cell.

Morris hepatoma 7777 and Zajdela hepatoma cells were studied in the investigation described below. Glucocorticoids induce TAT synthesis in the former, whereas the latter are resistant to the hormone in this respect. The writers showed previously that cells of both hepatomas contain glucocorticoid receptors and form hormone-receptor complexes which can interact with nuclear structures [1, 2]. Analysis of interaction of glucocorticoids with isolated plasma membranes, and also with whole cells revealed, however, differences between the cells of the above hepatomas [3]. It was therefore decided to compare the response of the two hepatomas to this hormone with respect to several criteria.

EXPERIMENTAL METHOD

Cultures of exponentially growing cells of Morris and Zajdela hepatomas were used. The former were grown in Leibovitz L-15 medium (Flow Laboratories, England) with 10% embryonic serum, the latter in Eagle's medium with 10% bovine serum. The cells were subcultured twice a week, removed mechanically, washed 3 times with Hanks' solution (1000 rpm, 5 min, 4°C), and used in a concentration of $(0.5\text{-}1.0)\cdot 10^6$/ml.

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