Microfluorometric Comparisons of Chromatin Thermal Stability in Situ between Normal and Neoplastic Cells

Marvin R. Alvarez

Department of Biology, Cell Biology-Physiology Section, University of South Florida, Tampa, Florida 33620

SUMMARY

Microfluorometric comparisons of chromatin thermal stability in situ were made among nuclei isolated from various types of normal and neoplastic cells of the mouse. Normal tissue cell nuclei were isolated from liver, kidney, and spleen. Neoplastic cell nuclei were derived from transplanted murine lymphoma, sarcoma, mammary adenocarcinoma, and Ehrlich ascites tumor.

The isolated nuclei were seeded on glass slides in equal densities, fixed, and heated to various temperatures in 0.15 M NaCl-0.015 M sodium citrate containing formaldehyde in order to induce denaturation of the chromatin. The nuclei were subsequently stained with acridine orange. Chromatin thermal stability was compared by measuring the fluorescence emission ratios of the chromatin-ligand complex at 530 and 590 nm for each temperature.

The fluorescence emission profiles obtained were characteristic of each tissue type. However, the curves from all normal tissue cells showed a gradual shift in the emission ratio with temperature while those of the neoplastic cells, in general, showed a rapid shift in the fluorescence emission ratio and achieved a consistently higher maximum ratio. The data indicate that the chromatin of these neoplastic cells is more thermostable than that of normal tissue cells and suggest the possible application of the method to cytodiagnosis.

INTRODUCTION

Since genetic regulation in eukaryotic cells may be related to configurational changes in nuclear chromatin (8), it would appear profitable to compare the physicochemical state of chromatin in normal and neoplastic cells. This is particularly true if the comparison could be done at the level of the light microscope because it could serve as a means of distinguishing incipient neoplastic cells from normal cells, which may resemble morphologically.

Acridine orange, a fluorescent nucleic acid ligand, has been used as a probe to measure changes in the structural order of chromatin in situ (4, 5, 10, 12, 16, 20). The application of this microfluorometric procedure permits one to monitor subtle changes in the structural order of nuclear chromatin by measuring changes in the fluorescence emission ratio at 590 nm (F590) and 530 nm (F530) after having heated to various temperatures cells attached to glass slides.

This paper reports on microfluorometric comparisons of chromatin thermal stability between nuclei isolated from 3 normal tissues and 4 transplanted murine neoplasms. The purpose of the work was to attempt to distinguish tumor cells objectively on the basis of their acridine orange staining properties after thermal denaturation of chromatin.

MATERIALS AND METHODS

Nuclei of normal liver, spleen, and kidney cells were obtained from adult BALB/c mice of either sex. The animals were maintained in an air-conditioned room and provided with food and water ad libitum. Ehrlich ascites tumor was maintained by weekly transplant into BALB/c mice. Methylcholanthrene-induced sarcomas (MC-8) were obtained from C3H male mice after 174 passages, and lymphomas (M5183) were obtained from BALB/c females after 213 passages. Mammary adenocarcinomas (M4833) were taken from DBA/2 females after 157 passages. Mice bearing the latter 3 types of tumors were obtained from Dr. W. Dunning at the Panico-Caou Cancer Research Institute and were sacrificed immediately upon arrival at our laboratory.

Animals were killed by etherization, and the entire organ or tumor was quickly removed. In the cases of the solid transplanted tumors, any obvious necrotic peripheral tissue was dissected away upon excision of the tumors. The tissues were washed briefly in ice-cold 0.25 M sucrose containing 2 mM MgCl₂ (9). The minced pieces were gently homogenized in 9 volumes of the same solution for 2 to 4 min with a hand glass homogenizer. The homogenates were filtered through a single layer of cheesecloth into conical glass tubes and centrifuged at 900 × g for 15 min at 4°C. The supernatants were discarded, and the pellets were resuspended in a solution of 0.5% (w/v) Triton X-100, 0.25 M sucrose, and 1 mM MgCl₂ by repeated expulsion through a transfer pipet. The suspensions were centrifuged again at 900 × g for 15 min. This step was repeated until there was no evidence of erythrocytes in the suspensions. The final pellets were suspended in 0.25 M sucrose containing 1 mM MgCl₂. The isolation procedure was carried out at 4°C.

Nuclei were isolated from Ehrlich ascites tumor cells in the following manner. Intact cells suspended in ascites fluid were aspirated from the peritoneal cavities of mice killed by etherization between 6 and 8 days after inoculation. The cells in ascites fluid were packed by centrifugation, the supernatant was decanted, and the pellet was resuspended in 0.9% NaCl solution and packed again by centrifugation. At least 2 such washes were repeated to remove the ascites fluid. The cells, suspended in a solution of 0.5% Triton X-100, 0.25 M sucrose,
The homogenate was filtered through a single layer of cheesecloth into glass centrifuge tubes and centrifuged at 900 X g for 15 min. The supernatant was discarded, and the final pellet was resuspended in 0.25 M sucrose containing 1 mM MgCl₂.

Drops of the nuclear suspensions were placed on chemically clean thin glass slides and allowed to settle onto the surface. The densities were adjusted so that the average number of nuclei per unit area was the same for each cell type. The preparations were immediately fixed in 10% formalin, pH 7.0, at 26° for 30 min. The fixed, isolated nuclei attached to glass slides were immersed for 1 hr in vessels containing 100 ml of 0.15 M NaCl and 0.015 M sodium citrate made in 10% formalin adjusted to pH 7.0. The temperatures of the various baths ranged from 25° to 90°. All slides used to construct a given denaturation profile were treated simultaneously. After denaturation, the slides were quenched in ice-cold NaCl-sodium citrate containing formaldehyde for 5 min, dehydrated in a graded ethanol series, and processed for staining without storage (15).

The 3 phases of the acridine orange procedure, i.e., acetylation, staining, and diffusion, were carried out at 25 ± 0.5° (15). Reactions were timed precisely so that treatment of slide sets was as uniform as possible. Replicates were made of all runs and microfluorometric measurements in all cases were made immediately after staining.

Slide preparations were acetylated for 15 min in 40% acetic anhydride in pyridine following a 5-min equilibration in water-free pyridine. The nuclei were subsequently rinsed in 100% ethanol and brought to water through a graded ethanol series. Slides were equilibrated for 5 min in NaHPO₄-citrate buffer (pH 4.1, Γ/2 0.6) and stained for 15 min in 10⁻⁴ M acridine orange prepared in the buffer at pH 4.1. Excess dye was removed by diffusion in two 2.5-min changes of buffer, and coverslips were mounted over buffer and sealed with clear fingernail polish.

The fluorescence of the ligand bound to chromatin in individual nuclei was measured at 530 nm (F₅₃₀) and at 590 nm (F₅₉₀) with a previously described microfluorometer (14). Exciting radiation was generated by a xenon arc (XBO-150W) operating from a time-stabilized power supply. The exciting wavelength (λ = 400 nm) was selected with a BG-12 filter and focused on the specimen through a 0.90-N.A. field condenser with transillumination. Emitted and exciting light were collected by a Phaco 40 X, 0.65-N.A. objective and separated by a 510 nm cutoff filter. The image of the single nucleus being measured was delimited by a variable circular diaphragm and focused by an optical train onto the entrance slit of a Bausch and Lomb monochromator and projected to fill the grating. The monochromator was set up with a dispersion of 6.4 nm/mm with exit and entrance slits of 1.50 and 2.68 mm, respectively, thus giving a bandwidth of 9.6 nm. The photodetector was a RCA 1P21 photomultiplier tube positioned over the exit slit of the monochromator. The tube was operated at an input of 940 V from a solid-state zener-regulated power supply. The output of the phototube, which is proportional to the fluorescence intensity of the stained object, was amplified through the vertical amplifier of an oscilloscope and read as a vertical deflection on the screen in V/cm. For avoidance of photodecomposition which results from prolonged exposure to high-intensity exciting light, the nuclei were aligned and focused in low-intensity light effected by partial closure of the substage diaphragm.

RESULTS

Chromatin thermal denaturation profiles of normal spleen cell nuclei are shown in Chart 1. The curves obtained with this tissue are typical of those obtained with other normal tissue cells, and these data are presented to show the levels and sources of variation encountered with the procedure in the 3 normal tissues examined. The degree of denaturation at a given temperature was determined by the ratio of fluorescence emission of the dye-chromatin complex at 590 and 530 nm. Each point represents the mean ± S.E. from at least 10 nuclei selected from different locations on the slides. The 3 curves shown in this illustration were derived from 3 different animals of approximately the same age and weight. The curves were run at different times, but the slides within a given run were treated simultaneously.

Interuclear variation within a slide heated to a given temperature was consistently small, although the standard errors of the means generally increased with temperature. Although the curves differed somewhat from run to run in spite of the fact that controllable parameters of the procedure

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**Figure 1.** In situ thermal denaturation profiles of chromatin of normal isolated mouse spleen cell nuclei. Each curve was from a different animal and was run independently. Note small intracurve variation in contrast with variation between curves.
were held constant, the profiles resemble each other and are distinguishable from that of other normal tissues.

Three chromatin thermal denaturation profiles of Ehrlich ascites tumor cell nuclei are shown in Chart 2. Again, the 3 curves were derived from tumors from 3 different animals and each curve was run independently of the others. As in the case of the spleen, intraslide variability was minimal, but there was a notable difference between runs.

However, the fluorescence emission ratio of the tumor cells is higher than that of the spleen at 25° by a factor of nearly 2. Note also the steepness of the asci tes tumor curves compared to those of the spleen, indicating that deoxyribonucleoprotein denaturation occurs more rapidly in the ascites tumor cell nuclei than in the spleen cell nuclei.

The differences in chromatin thermal stability observed between several normal and neoplastic cell types are summarized in Chart 3. This composite graph shows the mean profiles obtained from normal liver, kidney, and spleen and from Ehrlich ascites tumor, adenocarcinoma, lymphoma, and sarcoma cell nuclei. The curves from the neoplasms are shown in dashed lines and those of the normal tissues in dark, solid lines. In general, the curves of the neoplasms are steeper than those of the normal tissues. These data indicate that at 90° the degree of denaturation achieved is markedly and consistently greater in all of the neoplastic cells than in the normal cells. Thus, after heating at 90°, the neoplastic cells could be distinguished from normal cells solely on the objective basis of their fluorescence color.

**DISCUSSION**

Acridine orange binds to DNA in either of 2 modes (13, 14). The physical mode in which the dye binds appears to be determined by the degree of structural order of the polymer if other parameters such as dye concentration, pH, and ionic strength remain constant. Thus, double-stranded, helical DNA bound the dye by intercalation of acridine orange molecules between adjacent base pairs within the interior of the DNA helix. Single-stranded DNA regions bind the dye in an aggregate form through an electrostatic interaction between the negatively charged groups of the ligand molecule and the positively charged phosphate groups on the exterior of the DNA helix (13, 14). The DNA-dye complex fluoresces in the green region of the spectrum (530 nm peak) when the dye is bound in the intercalated form, but the emission peaks shift toward the red as single strandedness and thus electrostatic or aggregate dye binding increase (590 nm peak). Therefore, by measuring the ratio of emission at these 2 wavelengths, it is possible to obtain information about the degree of structural order of the DNA in situ (15).

The presence of polycationic histones decreases the amount of ligand bound by the DNA by neutralizing the phosphate groups on the exterior of the DNA helix and also stabilizes the helix against strand separation induced by heat, thus decreasing the binding of the ligand in aggregate form (7). It has been shown recently that acridine orange binds almost exclusively to diffuse chromatin in mammalian cells (8); this is also the phase that supports the bulk of RNA synthesis (7). The DNA-histone interaction in this diffuse chromatin phase is thought to be weaker than in the highly condensed chromatin (8).

The present results indicate that binding of the ligand shifts from the intercalated to aggregate mode at a lower temperature in neoplastic cells than in cells from normal tissues. This appears to be generally true of cells that are suddenly stimulated into increased RNA synthesis prior to extensive proliferation activity. Such changes have been observed during lymphocyte activation by phytohemagglutinin (10), in nucleated erythrocytes activated by cell hybridization (5), in activated lymphocytes in infectious mononucleosis (4), and in plant cells stimulated by kinetin (12). The present results indicate that proliferating neoplastic cells, rich in cytoplasmic RNA, are no exception and that this property may prove useful in recognizing malignant cells in the absence of mitotic figures.

While each of the transplantable tumors used in this investigation are composed of a homogenous cell population, the normal tissues with which they were compared contain several cell types. However, variations in the 590/530 ratios were small for each point throughout the thermal denaturation range in both cases. This unexpected observation results from the fact that only nuclei of 1 or 2 cell types for each tissue was measured. Thus, in the liver, only large round nuclei corresponding to hepatocytes were measured while smaller elongated nuclei corresponding to Kupffer cells or fibroblasts were ignored. The same was true in the kidney, in which parenchyma cell nuclei were selected in favor of fibroblast
nuclei, and in the spleen, in which lymphocyte and white pulp parenchyma predominate numerically.

The cytological recognition of malignant cells in a clinical situation is based primarily on nuclear characteristics such as variations in size, shape, structure, and hyperchromasia (11). However, the certainty of identification increases with the number of malignant characteristics recognized. The presence of 2 or more characteristics is necessary to make a safe diagnosis, and accuracy is increased when based on changes in many cells. For these reasons, additional characters, particularly objective ones, are most desirable in aiding cytdiagnosis.

Acridine orange has been used previously in clinical cytology to recognize malignant cells. The method of von Bertalanffy et al. (17) and its modifications are based on fluorescence metachromasia in cells resulting from the binding of the ligand to both DNA and RNA. Since malignant cells frequently exhibit more cytoplasmic RNA than normal cells, it is sometimes possible to distinguish them in smears on this basis. More recently, Wied et al. (18, 19) studied acridine orange binding in smears of glandular epithelium of uterine endometrium and endocervix in various pathological conditions. These authors measured microfluorometrically the amount of ligand bound in the intercalated mode by the cell nuclei. Although they found that statistically significant differences in fluorescence existed under several conditions of pathology, individual evaluations showed marked overlap.

A part of the problem in attempting to recognize malignant cells on the basis of the amount of fluorescent ligand bound by the nucleus lies in the fact that many, if not most, neoplastic cells are hyperdiploid. Since acridine orange binds semiquantitatively to DNA, the amount of dye bound is a reflection of the ploidy level and thus leads to scattered, overlapping values. The method described in this paper is based on differential thermal stability of the chromatin, which appears to be independent of ploidy (2). However, technical difficulties such as the effects of cell density (3) and the effects of mixing cell types (1) on chromatin-dye emission ratios must be further studied. If, however, the results of these preliminary investigations can be generalized to include many types of cancers, it appears possible that this procedure could be applied in cytodiagnosis to decrease subjectivity and might prove to be of value in the diagnosis of precancerous lesions.

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REFERENCES

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