Nucleic Acid Content and Nuclear Chromatin Structure of Human Bladder Cell Culture Lines as Studied by Flow Cyt fluorometry

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SUMMARY

Two human bladder cell lines, T-24 and HCV-29, are studied with flow cyt fluorometry and acidine orange staining to determine relative DNA and RNA content per cell and to measure resistance to thermal denaturation of DNA in situ. The RNA/DNA ratio for HCV-29 is over twofold higher than that for T-24, a difference that is consistent with the differences in cytological morphology and staining characteristics of these two cell lines and is sufficient to distinguish them completely, although measurements of DNA or RNA alone may not. In addition, the two cell lines show differences in DNA "melting" curves that indicate structural or conformational differences in nuclear chromatin. It is evident that the features are related to nuclear and cellular morphology, and they may be of value as additional parameters for characterizing tissue culture cell lines.

INTRODUCTION

The cells of urinary bladder carcinoma differ from those of benign transitional epithelium primarily in nuclear chromatin staining (15, 20, 22, 28), probably reflecting both a qualitative difference in total DNA and qualitative changes in DNA conformation (12, 13, 16, 18). These nuclear abnormalities may vary from one tumor to another and are the basis for a system of grading that can be related to clinical prognosis (3). Techniques have been developed to study the nuclear chromatin in cells with flow cyt fluorometry to determine dye binding to DNA (and RNA) per cell (7, 9, 26) and to provide information on the conformation of DNA in situ by measuring resistance to denaturation (6, 8, 10, 11). We (6, 7, 26) and others (1, 2) have demonstrated differences in nucleic acid dye binding and differences in the stability of DNA in situ for a number of cell types, including some cancer and benign cells. In this paper, we compare the amount of AO3-stainable DNA and RNA per cell and the resistance of DNA in situ to heat denaturation as possible parameters for characterizing bladder epithelial cell lines with apparently different nuclear chromatin by light microscopy.

Rationale for DNA/RNA Staining and Nuclear Chromatin Analysis by AO Fluorescence. AO is a fluorescent metachromatic dye that can be made to stain differentially double- and single-stranded nucleic acids. Under proper conditions, the dye molecules intercalate within double helical nucleic acids, where they are maintained in monomeric form and fluoresce green (530 nm) in blue light. The interaction of AO with single-stranded nucleic acids involves dye-dye aggregation (stacking), and in this polymeric form AO fluoresces red (640 nm). Thus, the metachromatic properties of AO can be used to stain differentially (double-stranded) DNA versus (single-stranded) RNA (9). Portions of native RNA that are double stranded are selectively denatured by treatment with chelating agents prior to staining (9).

After extraction of RNA, AO also can be used to study the stability of DNA under conditions that cause its denaturation. Heat, acid, or alkaline treatment will destroy the hydrogen bonding between base pairs of double-stranded native DNA and result in separation of the 2 strands. This phenomenon is called DNA "denaturation," "melting," or "helix-coil transition." As noted, AO stains the native, double-stranded DNA by intercalation and fluoresces green (530 nm), while DNA that is denatured or single stranded will fluoresce red (640 nm) (4, 17). In the absence of RNA, the cell fluorescence intensities at 530 (green) and 640 nm (red) provide a relative measure of the amount of double- and single-stranded DNA, i.e., the extent of denaturation (6, 8, 10, 11). Preparations of free DNA denature under specific conditions of temperature, pH, and ionic strength that depend upon the primary structure, i.e., base composition, of the DNA. DNA in chromatin, whether extracted from the cell (19, 25, 27) or in situ in unbroken cells (6, 8, 10, 11), is locally stabilized to varying degrees via interactions with neighboring macromolecules. Histones (7, 8) and nonhistone proteins (11), for example, modulate the stability of DNA in situ. The extent and strength of local stabilizations of the DNA helix in chromatin are reflected in a multiphasic curve of DNA denaturation ("melting profile"), and an analysis of this curve can provide some insight into the molecular structure of the nuclear chromatin. By an appropriate choice of conditions, it is believed that cells of different types or cells in different functional states having differences in nuclear chromatin structure can be distinguished by differences in chromatin stability (6).

MATERIALS AND METHODS

Cells and Staining. Two tissue culture cell lines, T-24 and HCV-29 (kindly supplied by Dr. J. Fogh, Sloan-Kettering Institute for Cancer Research, New York, N. Y. 10021) were used. The cytosol was removed from the tissue culture flask and the cells were treated with trypsin to disperse them into a single-layer suspension. The cells were then washed and resuspended in a medium containing 0.1 M NaCl, 0.01 M phosphate buffer, pH 7.4, and 0.01% sodium azide. After an additional wash, the cells were resuspended in media containing 0.1 M NaCl, 0.01 M phosphate buffer, pH 7.4, 0.01% sodium azide, and 0.01% bovine serum albumin. The cells were incubated in this media for 30 minutes at 37°C before they were stained with AO.

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2 To whom requests for reprints should be addressed, at Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, N. Y. 10021.
3 The abbreviation used is: AO, acidine orange.
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Institute, New York, N. Y.), were chosen for study, both derived from human bladder epithelium. The T-24 cell line was derived from bladder carcinoma by Bubenick et al. (5), and HCV-29 originated from presumed benign transitional epithelium of a patient previously treated for bladder tumor by irradiation. The cells of T-24 are specifically destroyed by lymphocytes from patients with bladder cancer and are believed to contain tumor antigen, while the cells of HCV-29 do not (24). Both cell lines grow as epithelial monolayers. A slow growth pattern, contact inhibition, and uniform cytology of HCV-29 (Fig. 1A) are all consistent with the presumed benign classification of these cells, whereas T-24 is a more rapidly growing cell line with nuclear hyperchromasia and the cytological morphology of a malignant tumor (Fig. 1B).

The cells were grown in BME medium (based on Earle's salt solution) supplemented with 15% fetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and 2.5 x 10⁻² M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer. When ready for harvesting, the cells were detached by incubation with trypsin-EDTA solution, washed twice in buffered saline (0.15 M NaCl, 0.015 M NaH₂PO₄-Na₂HPO₄, pH 7.0), and fixed in alcohol/acetone (1/1, v/v) as described before (8, 10).

After fixation, the cells were either stained directly with AO (1.5 x 10⁻⁸ M AO in 0.15 M NaCl, 5 x 10⁻³ M EDTA, 0.1 M phosphate-0.05 M citrate buffer, pH 6.0) to measure nucleic acid dye binding or treated with RNase or DNase prior to staining (8, 10). (Chromatographically purified AO was kindly supplied by Polysciences, Inc., Warrington, Pa.) RNase-treated cells also were suspended in a solution containing 10⁻⁴ M EDTA, 40% ethanol, (v/v), and 10⁻³ M cadoxylate buffer, final pH 6.0, for heating prior to staining to measure DNA resistance to denaturation (8, 10).

DNA Denaturation. Tubes with 0.25-ml aliquots of cell suspensions (approximately 2.5 x 10⁶ cells) were heated for 5 min in a water bath at appropriate temperatures. The tubes were then transferred to an ice-cold bath, and 1 ml of ice-cold AO solution dissolved in a solution of 0.25 M sucrose and 5 x 10⁻³ M MgCl₂ buffered with 2 x 10⁻³ M Tris-HCl to pH 7.4 was added to each tube to give a final AO concentration of 2.13 x 10⁻⁸ M. This concentration of AO gives optimal resolution in differential staining of double-versus single-stranded DNA in cells (8). Taking into account AO concentration and quantity of cells per tube, the ratio of AO molecules per DNA phosphate is approximately 5/1. The tubes are equilibrated to room temperature for measurements, the measurements always being completed within 2 to 5 min after heating. Control tests have indicated that 5 min of heating are adequate to induce maximal DNA denaturation at a given temperature and that no measurable renaturation occurs for up to 2 hr after cell cooling in the presence of AO (8).

Fluorescence Measurements. A flow-through Cytofluorograf, Model 4801 (Bio/physics Systems, Inc., Mahopac, N. Y.), interfaced to a NOVA 1220 minicomputer (Data General, Southboro, Mass.), was used to measure the fluorescence and light scatter signals of individual cells, as previously described (21, 23). The green fluorescence (F₅₃₀, measured in a band 515 to 575 nm) and red fluorescence (F₆₀₀, measured in a band 600 to 650 nm) from each cell are separated optically, subtracted from background, and quantitated by separate photomultipliers. Sensitivity of the photomultipliers was standardized, as described before (8), to ensure that the recorded fluorescence at F₅₃₀ and F₆₀₀ remain in proportion to the extent of stainable double- and single-stranded DNA in cells, respectively. Based on the intensity of green fluorescence, which is sensitive to DNA, it is possible to estimate the relative content of stainable DNA per cell. Frequency distribution histograms of cells according to their F₅₃₀ values indicate the proportions of cells in G₁, S, and G₂ + M phases of the cell cycle. In the cultures growing exponentially, over 70% of HCV-29 and 60% of T-24 cells were in G₁ phase (not shown). For the sake of simplicity, only this main (G₁) subpopulation was analyzed for each cell type. The G₁ subpopulation was selected for analysis by setting appropriate thresholds in the computer to eliminate subpopulations with increased DNA content (S and G₂ + M). Control experiments on cells in a stationary phase of growth confirmed the position of the G₁ clusters; over 85% of HCV-29 and 90% of T-24 cells were in G₁ phase in stationary cultures. Since the mean and median values that are reported by us refer only to the G₁ subpopulations of cells in logarithmic growth and a total of 5 x 10⁶ cells from all phases of the cycle are measured per sample, the number in G₁ that are actually analyzed are over 70% of each sample for HCV-29 cells and 60% for T-24 cells. A more complex analysis of cells in all phases of the cycle will be presented at a later date.

The extent of DNA denaturation within the cells is expressed in an index, αₐ, indicating a ratio of F₆₀₀ to total cell fluorescence:

\[ \alpha_a = \frac{F_{600}}{F_{600} + F_{530}} \]

The mean values of αₐ for cells in G₁ subpopulations were calculated from the means of F₆₀₀ and F₅₃₀ of these subpopulations, obtained as described above. Provided that F₆₀₀ and F₅₃₀ represent AO interaction with denatured and native DNA, respectively, and that the respective fluorescence intensities are in proportion to the quantities of these 2 DNA species, the αₐ index is expected to represent the proportion of DNA denatured within the cell (8).

RESULTS

Stainability of DNA and RNA. Chart 1 illustrates the stainability of DNA (F₅₃₀, green fluorescence) and RNA (F₆₀₀, red fluorescence) per cell for the G₁ subpopulations of HCV-29 and T-24 cells. The intensity of green fluorescence per cell, indicating the amount of stainable DNA, makes it possible to establish the relative ploidy level for that cell, as well as its position in the cell cycle. However, F₅₃₀ does not represent staining of total nuclear DNA but rather of the fraction that is unmasked and available for the intercalating probes (7, 26).

A characteristic feature of HCV-29 compared to T-24 cells is their lower DNA stainability. The intensity of green fluorescence (F₅₃₀) for the main subpopulation (G₁ peak) of HCV-29 cells is 30% lower than for T-24 (Table 1). The possible
significance of this difference will be discussed later. On the other hand, red fluorescence is greater for HCV-29 cells than for T-24 (the mean values of F₉₆₀₀ for HCV-29 and T-24 are 46.8 and 35.1, respectively). Most of the F₉₆₀₀ disappears after treatment with RNase and is presumed to be due to RNA (Chart 1; Table 1). The ratio of RNase-sensitive F₉₆₀₀ to F₅₃₀ is 1.09 for HCV-29 and 0.52 for T-24. These ratios for stainable RNA to DNA differ more than either RNA or DNA alone and may be used as parameters for distinguishing and characterizing the 2 cell lines.

Forward light scatter measurements (1–19°), which were obtained for both cell populations, probably are related to cell size and refractive index and indicate no significant difference as measured in this instrument system (Table 1).

**Thermal Denaturation of DNA In Situ.** All experiments were performed on cells pretreated with RNase. Consequently, nearly all fluorescence could be attributed to interactions of AO with DNA. Fluorescence at F₅₃₀ represented reaction with double-stranded DNA, and that at F₉₆₀₀ represented reaction with single-stranded DNA. Heat denaturation experiments were carried out on: (a) HCV-29 cells alone, (b) T-24 cells alone, and (c) HCV-29 and T-24 cells mixed together. In the 3rd series of experiments, the cell samples were mixed in fixative prior to processing, to exclude any unnoticed variations in cell handling as a possible reason for differences between the 2 cell lines. Further, the mixed population provided a means of demonstrating whether the 2 cell lines could, in fact, be separated by measurements of AO fluorescence following heat denaturation of DNA.

Charts 2 and 3 illustrate the change in AO-DNA fluorescence with increasing heat denaturation of DNA. In Chart 2 green fluorescence (F₅₃₀) is plotted against red fluorescence (F₉₆₀₀) for a mixed sample of the 2 cell lines at 24, 75, 90, and 100°. At 24° (unheated), T-24 has slightly greater green fluorescence (F₅₃₀) than HCV-29, but measurements of the 2 populations overlap. With denaturation by heating, there is distinct separation. In Chart 3 the ratio of αₐ (F₉₆₀₀/F₅₃₀), which is effectively the proportion of total stainable DNA that is single stranded or denatured, is plotted against the temperature to which the cells were heated. At 24° the cells fluoresce primarily in the green (F₅₃₀) with minimal red (F₉₆₀₀) component, T-24 having a slightly lower αₐ value due to greater F₅₃₀ (Charts 2A and 3). At increasing temperatures, there is a gradual increase in αₐ for both HCV-29 and T-24. Above 85° the rate of denaturation increases sharply.
DNA is due to a difference in masking by histones and other proteins (7, 26). Studies regarding the staining mechanism in which we are presently engaged support this view. The relatively high ratio of DNA to RNA fluorescence with AO staining of T-24 compared to HCV-29 is consistent with the high measured ratio of nuclear to cytoplasmic size for malignant compared to benign exfoliated bladder epithelial cells (14) and to the relative staining intensities of nucleus and cytoplasm of these 2 cell lines (Fig. 1).

The DNA melting curve in Chart 3 can be divided into a part representing the thermolabile DNA portion melting below 80-85° and a thermoresistant DNA portion denaturing above that temperature. This type of biphasic melting curve has been characteristic of all cells studied by us so far and may depend upon general structural features of chromatin within the intact nucleus (10). The greatest difference between HCV-29 and T-24 is in the thermoresistant fraction of DNA, which denatures at lower temperatures in T-24 than in HCV-29 cells. Findings by Alvarez (1, 2), who compared a number of tumor and benign cells using a different technique, led him to conclude that DNA is more labile in tumor cells than in their benign counterparts. However, the inverse is true at least in the case of some leukemias (6), and it seems probable that there will be considerable variation among other tumor types. As the mechanisms involved in DNA melting and in stainability of DNA and RNA become better understood, these measurements will yield information on the molecular structure of chromatin within intact cells and can be expected to contribute to our knowledge of the differences between benign and malignant cells. In the meantime, they have an empirical value in characterizing tissue culture cell lines, as we have done here, and perhaps in distinguishing benign and malignant epithelial cells in clinical cytology specimens.

DISCUSSION

The difference in cytological morphology of HCV-29 and T-24 cell lines, particularly with regard to nuclear chromatin structure and staining, is consistent with their presumed origins from benign and malignant bladder epithelium. These differences are reflected in different values of stainable DNA and RNA per cell with AO and also in a differing resistance to thermal denaturation of DNA in situ.

Stainable DNA per cell is greater for the G1 population of the 2 cell types at 24° based on green fluorescence (F530) of double-stranded DNA, the difference in resistance to heat denaturation makes separation quite easy when AO staining is carried out after heating to appropriate temperatures.

for both cell types and progresses more rapidly for T-24 than for HCV-29 (Charts 2, C and D, and 3). Thus, while it is not possible to distinguish G1 populations of the 2 cell types at 24° based on green fluorescence (F530) of double-stranded DNA, the difference in resistance to heat denaturation makes separation quite easy when AO staining is carried out after heating to appropriate temperatures.
REFERENCES


Fig. 1. The cytological morphology of HCV-29 (A) and T-24 (B) in tissue culture is compared. Note the nuclear hyperchromasia of T-24 compared with HCV-29 and the greater ratio of cytoplasmic to nuclear size for HCV-29. Papanicolaou stain. × 560.
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