Microfluorometric Comparisons of Heat-induced Nuclear Acridine Orange Metachromasia between Normal Cells and Neoplastic Cells from Primary Tumors of Diverse Origin

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SUMMARY

Nuclear fluorescence metachromasia of heated fixed cells subsequently stained with acridine orange was compared in smears and isolated nuclei of various types of primary tumors and normal cells from the tissues that gave rise to the tumors. The ratios of fluorescence emission at 590 and 530 nm reflect the thermal stability of chromatin in situ. The results show that the mean thermal stability of the chromatin in neoplastic cells was lower than the stability of their normal counterparts in all cases. This was found in both spontaneous and chemically induced tumors as divergent in type as a dog vaginal tumor and murine lymphocytic leukemia. These data, together with our previous observations in other neoplastic systems, indicate that reduced chromatin thermal stability may be a general characteristic of cells that have undergone neoplastic transformation and is not confined to rapidly growing tumors. The present investigation identifies the sources of variability encountered in measuring fluorescence metachromasia in slide preparations, and methods of minimizing this variability for potential cytodiagnostic application are discussed.

INTRODUCTION

Studies using a number of different cell systems have shown that the thermal stability of nuclear chromatin decreases sharply immediately prior to the activation of cellular proliferation or growth (2-4, 6, 9, 10, 14, 15). This physicochemical change is detectable by measuring the metachromatic shift in the fluorescence of nuclei stained with AO after thermal denaturation of the chromatin (13). The physicochemical basis of AO chromatin metachromasia in situ has been extensively studied (11, 12). While it is not the purpose of this communication to elaborate on these studies, the basis may be essentially summarized as follows. AO is thought to bind to double-stranded DNA by intercalation of the dye molecule between base pairs in the interior of the helix. The DNA:AO complex, when excited, fluoresces green with a maximum emission at 530 nm. On the other hand, AO binds to single-stranded DNA by an aggregation phenomenon where the dye molecules stack between phosphates as a result of dye-dye interaction. This latter DNA:AO complex, when excited, fluoresces red at an emission maximum of around 590 nm. Thus, if the chromatin in a fixed cell is denatured by heat, i.e., DNA strand separation is induced, and the cell is subsequently stained with AO, the color of the nuclear fluorescence is indicative of the degree of denaturation achieved. This may be semiquantitated, as in the present study, by measuring the ratio of fluorescence emission at 590 and 530 nm, the numerical value of the ratio being directly proportional to the degree of denaturation.

The author has recently demonstrated a difference in the thermal stability of chromatin between normal and neoplastic cells using AO metachromasia and proposed that this parameter might prove useful in the cytological detection of cancer (1). However, this previous study used murine tumors that had undergone a long series of transplantations and, therefore, probably represented a rather atypical system, far removed from the clinical condition. Furthermore, the normal cells used for the comparison were from tissue types other than those from which the tumors originally arose. Thus, the questions remained as to whether the differences that were observed were due to (a) long-term changes in the tumors arising from repeated transplantation, (b) the fact that the normal cells were not compared to their neoplastic derivatives, or (c) if, in fact, there is a difference in chromatin thermal stability between normal and neoplastic cells in general.

This paper reports microfluorometric measurements of chromatin thermal stability in which cells of widely divergent types of primary tumors were compared with cells of normal tissues of the type from which the tumors were derived. In addition, certain technical aspects of the methodology pertinent to its use as a cytodiagnostic tool are discussed.

MATERIALS AND METHODS

Tumors. Four different types of primary tumors were used for the investigation: (a) unclassified vaginal tumors of unknown etiology that arose spontaneously in a mongrel dog housed in the animal quarters; (b) several spontaneous mammary adenocarcinomas of different sizes and location in C3H female mice; (c) lymphocytes from leukemic female...
mice of the AKD2F/J strain (The Jackson Laboratory, Bar Harbor, Maine); and (d) mammary adenocarcinomas induced in female Wistar rats by intubation with N-methyl-N-nitrosourea at a dose of 70 mg/kg body weight (7). The above tumors were selected for study because of their diverse tissues of origin and etiology and because cells from these types of tumors are easily obtainable in large quantities.

**Cell Preparations.** Cells were scraped from the surfaces of the dog vaginal tumors with a metal spatula and immediately smeared uniformly on acid-cleaned glass slides. Scrapes were also taken from other areas of the vaginal epithelium that were free from tumor masses. The smears were fixed immediately, before drying, in ice-cold acetone: ethanol (1:1, v/v) and stored in the fixative at 4°C until used.

Cells were also obtained by scrapes from the mammary tumors, both spontaneous and chemically induced. The animals were killed by etherization and the tumors were excised. Obvious necrotic areas were removed and the remaining tumor was bisected. The scrapes were taken with a metal spatula from the internal cut surfaces, smeared on slides, and fixed and stored as above.

Normal mammary cells for comparison were taken from nonpregnant, nonlactating female animals. Patches of skin, approximately 4 sq mm, including the nipple, were excised after removal of the hair with a depilatory. The patches were incubated at 37°C for 30 min in 0.25% trypsin freshly prepared in Sorensen's buffer at pH 7.2. Following incubation, the dermal surfaces were gently scraped with a metal spatula and smears were prepared as above.

Twelve groups of mice were used in the leukemia experiments. Each group consisted of 1 leukemic AKD2F/J, 1 nonleukemic animal of the same strain, and 1 animal of the nonleukemic BALB/c strain. The presence of leukemia was verified at autopsy and by differential leukocyte counts of peripheral blood. Postmortem verification was based on the finding of enlarged thymuses and spleens and invaded livers. While all leukemic mice did not exhibit all 3 signs, enlarged thymuses and elevated lymphocyte counts were found in all mice used as leukemic.

The animals were etherized lightly and given i.p. injections of sufficient Ringer's solution at 37°C to distend the peritoneal cavity. The abdomen was massaged vigorously for several min and the animals were killed subsequently by etherization. The body wall was opened quickly and the Ringer's solution, containing suspended lymphocytes, was aspirated with a transfer pipet and placed in conical glass centrifuge tubes. The suspended lymphocytes were packed by centrifugation; the supernatant was discarded and replaced with fixative (acetone:ethanol, 1:1, v/v). The pellets were fixed for 30 min at room temperature, and the cells were suspended subsequently in fixative equal to 10-times the volume of the pellet. Aliquots were placed on slides and air dried. The volumes of the aliquots were adjusted so that the number of cells per unit area of slide was approximately the same on the 3 slides prepared from each group. Each set of 3 slides (representing 1 group) was processed for staining on separate days. The slides within a set were processed simultaneously. This procedure allowed an evaluation of interslide variability within and between sets.

**Nuclear Isolation.** Measurements of AO metachromasia were also done on nuclei isolated from the spontaneous and chemically induced mammary tumors. The purpose of this procedure was to evaluate the effect of cytoplasmic fluorescence on the nuclear fluorescence in intact cells obtained from scrapes.

Nuclei were isolated by a modification of the procedure of Hymer and Kuff (5), which has been published previously (1). Drops of the nuclear suspensions were placed on acid-cleaned glass slides and the nuclei were allowed to settle by gravity onto the surface. The number of nuclei per unit area of slide was adjusted by varying the settling time so that the density was nearly uniform on all slides. The preparations were fixed in the same way as the smears of whole cells and stored in the fixative at 4°C until use.

**Chromatin Thermal Denaturation.** Slides containing air-dried whole-cell smears or isolated nuclei were immersed for 30 min in vessels containing 100 ml of 0.15 M NaCl and 0.015 M sodium citrate dissolved in 10% formalin adjusted to pH 7.0. The temperature of the solution was maintained at 90 ± 0.5°C. This temperature was chosen on the basis of our previous experiments with transplanted tumors (1), which showed that the greatest differences in AO metachromasia between normal and neoplastic cells occurred at this temperature. The slides were quenched within 1 sec in ice-cold NaCl:sodium citrate dissolved in formalin for 5 min (11), rinsed in distilled water at room temperature for 5 min, and dehydrated in a graded ethanol series.

**AO Staining.** The AO staining procedure used was essentially that previously described (1). In all cases, the times in each solution, the temperatures, and the ratios of number of slides per unit volume of solution were held constant. Microfluorometric measurements were performed immediately after staining.

**Microfluorometry.** The instrument and procedure used for the microfluorometric measurement of nuclear metachromasia have been described previously (9). In some cases, where whole cells were used, the determination of nuclear fluorescence emission at 590 and 530 nm was done using a 100 X oil-immersion objective. The image of the cytoplasm was excluded by interposing a variable diaphragm between the object plane and the ocular. Thus, the fluorescent image impinging on the cathode of the phototube was limited to the nucleus. Measurements of the ratio of fluorescence emission intensity at 590 and 530 nm (F900/ F800) of isolated nuclei were made using a 40 X objective.

In order to prevent photodecomposition that results from illumination of the fluorochromed object with high-intensity, short-wavelength light, the nuclei were aligned and focused under phase contrast using low-intensity tungsten illumination. Thus, the stained objects were exposed to the high-intensity exciting light for less than 5 sec at the time that the actual fluorescence intensity measurements were made.

**RESULTS**

**Dog Vaginal Tumors.** Two morphologically distinguish-
able cell types were seen in smears of whole cells scraped from the surface of the tumors. The most frequent type appeared nearly circular with a bright red fluorescent nucleus surrounded by a thin rim of cytoplasm of orange fluorescence. The chromatin appeared dispersed, with few visible chromocenters. The 2nd, less common type of cell visible in the smears resembled normal squamous epithelium, having a large, light green fluorescent cytoplasmic area containing a relatively small, dense, centrally placed nucleus that fluoresced light orange. The scrapes taken from vaginal surfaces free of obvious tumor masses contained from the surface of the tumors. The most frequent type appeared nearly circular with a bright red fluorescent nucleus that fluoresced light orange. The scraped nuclei from the surface of the tumors and the "normal" vaginal epithelial cells. The latter 2 gave nearly identical mean values. In view of this chemical and morphological similarity, it is concluded that the epithelium-like cells were probably surface contaminants on the tumors in the form of exfoliated epithelial cells. Thus, these data indicate that the chromatin thermal stability of the atypical cells was lower than the stability of the typical epithelial cells and that the 2 cell types are distinguishable from each other on this basis.

Spontaneous Mammary Tumor Cells versus Normal. In order to compare neoplastic mammary gland cells with those from the normal organ, it was necessary to establish a base-line value for normal cells. However, since AO metachromasia induced by chromatin denaturation is related to the functional state of the cell, it was evident that the values obtained in individual cases might reflect the functional state of the gland itself. Therefore, only females that were neither pregnant nor lactating were used as normal controls. These were animals that showed no signs of mammary tumor development on the basis of external examination.

Tumors from 10 C3H females were used in this phase of the study. Two slides were prepared from each tumor and stained as a set with 2 slides from a respective normal control. Thus, each of the 10 sets of 4 slides represented 2 animals, 1 with a tumor and 1 with normal mammary glands. Each set was processed for chromatin denaturation, AO staining, and microfluorometric measurement on separate days in order to evaluate the variability generated by the procedure. The metachromatic ratio of the nucleus was measured under oil immersion for 50 cells/slide.

The results are summarized in Table 2. The numerical values of $F_{590}/F_{530}$ varied among sets for both tumors and controls. However, in all cases the mean metachromatic ratios of the tumors were higher than the ratios of the normal mammary cells. These results are interpreted as meaning that the mean thermal stability of the chromatin from the population of tumor cells was lower than the stability of the normal mammary gland cells. The magnitude of the difference in means was not consistent between sets. The variation in S.D. is larger between sets than within sets with 1 notable exception (Table 2, Footnote b). This inconsistency appears to be generated by interslide variation and by the fact that the sets were processed separately. Nevertheless, in spite of the variation observed, the mean metachromatic ratios of nuclear fluorescence were found to be consistently higher in neoplastic cells in all cases.

The differences in mean metachromatic ratios between slide sets as shown in Table 2 were attributed to differences in the number of cells per unit area of the slide. To test this, cells were isolated from the spleen of a normal BALB/c mouse and fixed. Dilutions of 0.1 and 0.01 were then made from the cells suspended in the fixative. Aliquots of the undiluted and diluted suspensions were placed on slides, air dried, and processed for denaturation and AO staining. The results of the measurements of the metachromatic ratios are shown in Chart 1, where a marked decrease in the $F_{590}/F_{530}$ ratio is evident with decreasing cell density on the slide.

The possible effect of tumor size on nuclear fluorescence metachromasia was also tested. Tumors from 10 different C3H females were used to prepare whole cell smears as before. The tumors were from various regions of the mammary area and ranged in size from $10 \times 15$ to $30 \times 35$.
Tumor dimensions (nm) along the smallest and largest axes. The results are shown in Table 3.

As before, there was considerable variation in the mean metachromatic ratios among the different tumors. However, the values did not show a positive correlation with tumor size. As may be seen, for example, Tumor 1 (10 x 15 mm) gave a mean $F_{590}/F_{580}$ ratio of 0.50 ± 0.09, while Tumor 8 (25 x 30 mm) gave a value of 0.50 ± 0.04. Thus, it was concluded that the age of the tumor was not a significant factor in determining the level of metachromasia and that the observable variations among tumors resulted primarily from differences in cell density on the slides as well as other unknown technical parameters.

**Spontaneous Mammary Tumor Cell Isolated Nuclei versus Normal.** One such unknown parameter that was investigated was the contribution made by the cytoplasm to the total fluorescence emission intensity of the cell. Even when the nucleus in an intact cell was framed by the diaphragm, thus eliminating a very large fraction of the cytoplasmic fluorescence, there was some cytoplasm overlying and underlying the nucleus. In cells poor in cytoplasmic RNA the cytoplasm fluoresced bright green. Thus, the ratio $F_{590}/F_{580}$ would be decreased by increasing the value of the numerator.

The effect of this factor was tested by using isolated nuclei from the mammary tumors for comparison with isolated nuclei from normal cells. While the ideal would have been to compare isolated tumor nuclei with those of normal mammary tissue, we found it impossible to obtain a sufficiently high yield from the latter to make the comparison statistically meaningful. Therefore, nuclei from other normal organs were used.

Nuclei were isolated from 5 mammary tumors from separate C3H females and from normal liver, kidney, and spleen of 5 separate normal mice of comparable age, sex, and weight. The number of nuclei per slide was adjusted so that it was approximately the same among slides. Sets of 4 slides consisting of 1 each of tumor, liver, kidney, and spleen were processed for denaturation, staining, and measuring on separate consecutive days. In spite of all precautions, however, preliminary scanning of the slides showed that the distribution of nuclei over the slide surface was not uniform. Thus, the following criterion was used for selecting the nuclei to be measured. The slides were scanned under fluorescence using low magnification. Areas showing the highest levels of metachromasia, i.e., the reddest areas, usually in the center of the smears, and with approximately equal densities, were measured. The results are shown in Table 4.

As may be seen by comparing Tables 3 and 4, variation in mean metachromasia among tumors remained when isolated nuclei were used. It was surmised that this variability might be due to localized differences in nuclear density among slides and to the subjective choice of areas to be measured. These factors apparently exert a measurable influence, in spite of the fact that a relatively large number of nuclei was measured per slide (n = 50/slide).

Again, the most significant aspect of these data is that, while it was technically impossible to eliminate interslide variability with the methods used, the mean metachromatic ratios of the tumors were in all cases significantly and consistently higher than the ratios from normal cell nuclei. This again implies a substantial difference between the thermal stabilities of the chromatin of normal and neoplastic cells.

**Induced Mammary Tumors versus Normal.** In the previously described experiments, the tumors that were used were spontaneous in origin. We also wished to determine whether decreased chromatin thermal stability of tumor cells was a characteristic of induced neoplasias. For this purpose, mammary tumors induced by intubation of female Wistar
From our studies it appears that 2 factors contribute to the absolute values of the metachromatic ratios in a given preparation. These are the degree of chromatin denaturation in each cell and the diffusion of the dye bound in aggregate form in the final rinse solutions. The former is biological in origin and is indeed the parameter we wished to measure. The latter factor is technical and is influenced by the following conditions: (a) the number of cells per unit area of slide; (b) the duration of the final rinse; (c) the number of slides per unit volume of rinse bath; and (d) the gravity inevitably results in uneven distribution. Perhaps the solution to this technical problem lies in applying the cells to a centrifuge, such as the instrument described by Leif et al. (8). However, if this is done, precautions must be taken to ensure that excessive centrifugal force does not rupture the nucleus or mechanically alter the physical state of the chromatin.

**DISCUSSION**

As expected, the mean nuclear metachromatic ratios of the neoplastic cells were significantly higher than the ratios of normal cells, regardless of whether whole cells or isolated nuclei were used. This finding is of particular interest because the tumors were excised at a very early stage in their development. Thus, the heat-induced nuclear metachromasia procedure was able to detect the abnormality at a fairly early stage in the development of the tumor.

**Murine Leukemia.** In view of the interest in murine leukemias as model systems in oncology, we decided to compare the heat-induced nuclear metachromasia of normal and neoplastic lymphocytes.

Six sets of 6 slides were used, each consisting of lymphocytes from a leukemic mouse of the AKD2F1/J strain, a nonleukemic animal of the same strain, and a healthy animal of the BALB/c strain. Three slides from each set were processed daily. The metachromatic ratios of 100 cells/slide were determined. The results are shown in Table 6.

The metachromatic ratios of lymphocytes from normal AKD2F1/J and BALB/c animals were not significantly different within a given run. Interrun (interanimal) differences, again, are probably attributable to technical parameters. However, as in the case of all the other comparisons made in primary and transplanted tumors, the mean metachromatic ratios of the neoplastic cells were significantly and consistently higher than the ratios of their normal counterparts.

**Table 4**

Heat-induced chromatin fluorescence metachromasia of isolated nuclei from spontaneous murine mammary tumors and normal liver, kidney, and spleen

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Tumors</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.60 ± 0.07</td>
<td>0.33 ± 0.05</td>
<td>0.46 ± 0.04</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>0.78 ± 0.06</td>
<td>0.57 ± 0.06</td>
<td>0.55 ± 0.04</td>
<td>0.56 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>0.83 ± 0.06</td>
<td>0.64 ± 0.06</td>
<td>0.60 ± 0.06</td>
<td>0.61 ± 0.05</td>
</tr>
<tr>
<td>4</td>
<td>0.71 ± 0.06</td>
<td>0.63 ± 0.06</td>
<td>0.57 ± 0.04</td>
<td>0.56 ± 0.08</td>
</tr>
<tr>
<td>5</td>
<td>0.82 ± 0.24</td>
<td>0.57 ± 0.07</td>
<td>0.59 ± 0.07</td>
<td>0.47 ± 0.09</td>
</tr>
</tbody>
</table>

* Mean ± S.D.; n = 50 for each mean.

**Table 5**

Chromatin fluorescence metachromasia in cells and isolated nuclei of N-methyl-N-nitrosourea-isolated mammary tumors, normal mammary glands, liver, kidney, and spleen of the rat

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>Normal mammary cells</th>
<th>Tumor nuclei</th>
<th>Liver nuclei</th>
<th>Kidney nuclei</th>
<th>Spleen nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.46 ± 0.06</td>
<td>0.32 ± 0.04</td>
<td>0.55 ± 0.04</td>
<td>0.36 ± 0.04</td>
<td>0.34 ± 0.03</td>
<td>0.39 ± 0.04</td>
</tr>
</tbody>
</table>

* Mean ± S.D.; n = 50 for each mean.

**Table 6**

Chromatin fluorescence metachromasia in lymphocytes of leukemic AKD2F1/J, normal AKD2F1/J, and normal BALB/c mice

<table>
<thead>
<tr>
<th>Animal</th>
<th>Leukemic AKD2F1/J</th>
<th>Normal AKD2F1/J</th>
<th>Normal BALB/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.76 ± 0.10</td>
<td>0.61 ± 0.08</td>
<td>0.64 ± 0.09</td>
</tr>
<tr>
<td>2</td>
<td>0.79 ± 0.11</td>
<td>0.53 ± 0.05</td>
<td>0.51 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>0.72 ± 0.05</td>
<td>0.46 ± 0.05</td>
<td>0.39 ± 0.05</td>
</tr>
<tr>
<td>4</td>
<td>0.74 ± 0.11</td>
<td>0.56 ± 0.07</td>
<td>0.55 ± 0.07</td>
</tr>
<tr>
<td>5</td>
<td>0.72 ± 0.07</td>
<td>0.54 ± 0.06</td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td>6</td>
<td>0.63 ± 0.05</td>
<td>0.52 ± 0.06</td>
<td>0.52 ± 0.01</td>
</tr>
<tr>
<td>7</td>
<td>0.71 ± 0.05</td>
<td>0.54 ± 0.05</td>
<td>0.55 ± 0.05</td>
</tr>
<tr>
<td>8</td>
<td>0.67 ± 0.06</td>
<td>0.53 ± 0.05</td>
<td>0.53 ± 0.05</td>
</tr>
<tr>
<td>9</td>
<td>0.65 ± 0.05</td>
<td>0.54 ± 0.04</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>10</td>
<td>0.74 ± 0.07</td>
<td>0.56 ± 0.07</td>
<td>0.59 ± 0.06</td>
</tr>
<tr>
<td>11</td>
<td>0.63 ± 0.07</td>
<td>0.56 ± 0.06</td>
<td>0.50 ± 0.06</td>
</tr>
<tr>
<td>12</td>
<td>0.84 ± 0.09</td>
<td>0.61 ± 0.07</td>
<td>0.65 ± 0.06</td>
</tr>
</tbody>
</table>

* Mean ± S.D.; n = 100 for each mean.

With N-methyl-N-nitrosourea-isolated mammary tumors, normal mammary glands, liver, kidney, and spleen of the rat.
The following 3 conclusions or inferences may be drawn from the present data.

1. The thermal stability of chromatin in neoplastic cells, as judged from the level of AO metachromasia, is on the average lower than in normal cells in the case of primary tumors, both spontaneous and chemically induced. Thus, it is concluded that repeated transplantation of the tumors in our previous study (1) was not responsible for the differences in nuclear metachromasia that we found. Furthermore, the differences observed in the present study exist between neoplastic cells and cells from the same normal tissues that gave rise to the tumors.

2. The enhanced nuclear metachromasia resulting from thermal denaturation of chromatin appears to be characteristic of widely different types of neoplasias and may prove to be a characteristic of neoplastic cells in general. This inference is supported by the fact that most of the tumors examined were large and showed little mitotic activity. Indeed, in the case of 1 of the dog vaginal tumors, the mass actually appeared to be regressing. Also, the data show that nearly every neoplastic cell that was measured had reduced chromatin thermal stability, indicating that the cell need not be in the S phase of the cycle to exhibit the enhanced fluorescence metachromasia.

3. Although enhanced nuclear metachromasia has been found in all cases where neoplastic cells have been compared to normal cells, there is some interslide variability. Thus, using the present method, large numbers of cells must be measured in order to obtain statistically significant results if comparisons are made among several slides processed at different times. In other words, if only a relatively few cells are available, as is frequently the case with smears, the slides must be processed simultaneously for denaturation and staining in order to obtain valid comparisons. However, as in the case of the dog vaginal tumors, when normal and neoplastic cells exist side by side on the same slide, the neoplastic cells are distinguishable from the normal cells solely on the basis of the fluorescent color of the nucleus. In this connection, it would be of great interest to examine smears of human cervicovaginal epithelium to see whether carcinoma in situ could be detected. The procedure might prove to be particularly valuable in such an application because the neoplastic cells are not readily distinguishable from normal epithelium on morphological grounds alone.

REFERENCES

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