Cell Morphology of a Human Diploid Cell Strain (WI-38) after Treatment with Arabinosylcytosine

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Summary

Cell morphology was studied in a human diploid cell strain (WI-38) treated with various concentrations of arabinosylcytosine for different periods. Mitotic inhibition followed most of these treatments except for the short treatments with the lowest concentration, which allowed mitosis in the posttreatment growth period. Paired acentric fragments were seen in anaphases after these treatments.

The treated cells exhibited certain morphologic characteristics, such as clustering of cells, appearance of oval, elongated nuclei and cells, increased cytoplasmic granularity, increased size of nuclei and nucleoli, fusion and vacuolation of nucleoli which became darker, and organization of new nucleoli and decreased stainability of the reticulum. The changes in the appearance of the nucleoli may indicate increased metabolic activities. Many of these morphologic characteristics resemble changes seen in transformed cells.

Introduction

Arabinosylcytosine (1-β-d-arabinofuranosylcytosine, ara-C) is an analog of deoxycytidine and cytidine and has been observed to interfere with DNA synthesis and cell reproduction by many authors (3, 5-7, 13, 14). It has also been demonstrated that ara-C causes breakage of chromosomes in 2 tissue culture systems, viz., human leukocytes and a human cell line (2, 14, 19), as well as in vivo (1). These breaks resembled those occurring after virus infection (18, 20-22).

Nichols and Heneen (19) reported initial comparative studies between long-term effects of ara-C on cell cultures and cell transformation following virus infection (9, 16, 23, 26). The present study extends these initial observations to more detailed cell morphology and pattern of growth in a human diploid cell strain (WI-38) treated with ara-C and subjected to a differential stain technique.

Materials and Methods

A diploid cell strain (WI-38) originally derived from a female human embryo lung was used. These cells were grown in Hank's Eagle's medium with 30% newborn calf serum in Petri dishes containing coverslips. The different treatments were applied on cells growing on coverslips when in Passages 23-32.

Arabinosylcytosine in final concentrations of $10^{-4}$ M, $10^{-5}$ M, and $10^{-7}$ M was used. The treatments were started 1-2 days after transfer before the formation of a dense sheet of cells on the coverslip. In one series, ara-C treatments were continuous for 1, 3, 24, and 48 hr before fixation. In another, the analog was removed after such treatments and fresh medium was added. In this series, fixations were made at intervals of 2 days for a period of 8 days, after removing the analog. During the posttreatment period the cells were refed every 2nd day. Controls for all treatments were fixed or refed at similar times or intervals.

Before fixation, the coverslips were dipped for a few sec in NaCl solution (0.85%) to take away remnant medium. Kahle's modified fixative [formula given by Smith (25)], composed of 95% ethyl alcohol-formalin-glacial acetic acid (15:6:1), was used for 10 min. The coverslips were then passed in an alcohol series before hydrolysis for 10 min in 1 N HCl at 60°C and differentially stained for chromatin and nucleoli with Feulgen's procedure and light green (Östergren, Koopmans, and Reitalu; see Ref. 24).

Results

Frequency of Mitosis

The frequency of mitosis after continuous treatment with ara-C and during the period of posttreatment growth in ara-C-free medium is presented in Table 1. Mitosis in frequencies less than the control level were observed after a short treatment of 1 or 3 hr with all the concentrations ($10^{-4}$, $10^{-5}$, or $10^{-7}$ M) of ara-C used except for the 1-hr $10^{-4}$ M treatment, which had about the same mitotic index as in the control. After 24- and 48-hr treatments, no mitoses were seen except for a low frequency after the 24-hr treatment at the lowest concentration ($10^{-7}$ M). Dead cells were of frequent occurrence after the 24- and 48-hr treatments, and the appearance of their chromatin material indicated death while in division.

During the posttreatment period (4 analyses at 2-day intervals) in ara-C-free medium, no mitosis was observed after the $10^{-4}$ M treatment. This concentration of ara-C, even if applied only for 1 hr, caused complete inhibition of mitosis in the subsequent period of observation. The $10^{-4}$ M concentration caused strong inhibition of mitosis, but in this case a few mitoses occurred in the first 2-4 days after a short treatment of 1 or 3 hr. On the 2nd day, after the $10^{-7}$ M concentration was applied for
Cell Morphology after Arabinosylcytosine Treatment

Table 1
Frequency of Mitosis* in WI-38 Cells after Treatment with Arabinosylcytosine and during the Period of Posttreatment Growth

<table>
<thead>
<tr>
<th>ara-C molar concentration</th>
<th>Duration of treatment (hr)</th>
<th>Posttreatment period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>10^-6</td>
<td>1.55</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>10^-6</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>10^-7</td>
<td>2.20</td>
</tr>
<tr>
<td>Control</td>
<td>2.25</td>
<td>2.25</td>
</tr>
</tbody>
</table>

* Mitotic indices based on counts of 2000-6000 cells in each treatment; dashes (—) indicate no mitosis.

1 or 3 hr, mitosis was close to the control level. This was followed by a decreasing frequency in the analyses made on Days 4, 6, and 8. Twenty-four- or 48-hr treatments with 10^-7 M ara-C completely inhibited mitosis in the posttreatment period.

As indicated in Table 1, mitosis in the control was frequent on Day 2 and decreased successively on Days 4, 6, and 8. These were periods corresponding to the days of growth in ara-C-free medium in the treated material. Some mitotic figures can be seen in the cells shown in Figs. 1-4, which represent the controls at various periods of growth.

The chromosomes in most of the metaphase plates seen in the treated material exhibited some degree of stickiness. This has been observed both directly after ara-C treatments and 2-8 days following these treatments. A metaphase figure from the control and an ara-C treatment are shown in Figs. 5 and 6, respectively. Persistent nucleolar material (arrows, Fig. 6) was of more common occurrence in the treated cells than in the control. For example, on Day 2 after a 3-hr 10^-7 M treatment, persistent nucleoli occurred in 46% of the metaphase plates and in 22% of the controls (67 and 151 metaphase analyses, respectively).

Besides stickiness and overcontraction of chromosomes at metaphase, aberrations at anaphase were also encountered. These were clearly demonstrable in the following 4 treatments of ara-C; 3 hr with 10^-4 M or 10^-3 M concentration, 24 hr with 10^-7 M concentration, and day 2 posttreatment analysis of the 10^-7 M 3-hr treatment. A total of 675 anaphases studied from these treatments showed aberrations in a frequency of 25-29%. In 492 anaphases analyzed in control material, the frequency of aberrations ranged from 3 to 11%. Besides the differences in frequency of anaphase aberrations, there was a difference in the type of aberration most frequently observed in the treated versus control material. The most common anaphase aberration after ara-C treatment was a pair of acentric fragments (Figs. 7, 8) which varied in size from dotlike to as large as whole chromosome arms. In the case of the control, most of the aberrant anaphases showed separation difficulties, giving rise to chromatid bridges (Fig. 9).

Pattern of Growth and Morphology of Cells

The treatment with ara-C caused a marked decrease in the number of cells that grew on the coverslip (Figs. 10-15). Control cells at various periods of growth are seen in Figs. 1-4. The intensity of cell loss increased with increasing concentrations and time of treatment. Proliferation of cells during the posttreatment period occurred only in the 1- and 3-hr treatments, with the lowest concentration, 10^-7 M, of ara-C.

A difference in growth pattern between cells treated with ara-C and the controls could sometimes also be seen. Treated cells tended to group together in clusters (Fig. 11). They also showed a more elongated appearance than the controls (Fig. 14). This often happened shortly before cells detached from the glass. A somewhat similar, but less marked, effect was seen in control cells as they became crowded on the glass.

During the posttreatment period, especially after long treatments with high concentrations of ara-C, the cytoplasm exhibited more granularity and stainability with light green (Figs. 12-15) and the cells often appeared to be degenerating.

Appearance of Nuclei

Compared with the control, there was a wider variation in nuclear sizes after treatment with ara-C. Small, shrunken nuclei were seen immediately after the short treatments (1 and 3 hr). After long treatments (24 and 48 hr) and during the posttreatment period, most of the nuclei were increased in size (Figs. 10-15). Some of these large nuclei were so much larger than the rest of the cells that they probably represented polyploid cells (Fig. 10).
A general increase in size of nuclei that was of a lesser degree than in these polypliod cells was also seen after treatment and may have represented either a real increase in nuclear material or a decreased compactness of the nucleus. Since there was a low staining density of these big nuclei, the latter was considered most likely. In degenerating cells, the faintly stained chromatin reticulum condensed into darkly stained granules distributed all over the nucleus (Figs. 14, 15). This was noticeable to a much lesser extent in the late periods of growth in the control.

**Appearance of Nucleoli**

In the control material (Figs. 1–4), nucleoli were small and varied in number and shape. From 2 to 4 nucleoli per nucleus were most frequently observed. In the treated cells, nucleoli were darker staining and appeared in a diffuse or branched condition (Figs. 10, 11). Attenuations of nucleoli appeared to be a condition prior to their fusion in later stages of growth in ara-C-free medium (Figs. 12–15). Vacuoles were frequently seen in large, fused nucleoli. The vacuoles were rounded, and varied in number from few to many, covering most of the nucleolar area (Fig. 16). Small vacuoles were seen much less frequently in nucleoli of the control. These effects were accentuated by increase in concentration and length of treatment.

In many of the treated cells, new, small nuclei appeared during the growth period in the ara-C-free medium (Fig. 17).

**Discussion**

The mitotic inhibition observed in WI-38 cells after arabinosylcytosine treatment was dependent on concentration and duration of treatment. The 1- and 3-hr treatments with $10^{-7}$ M were the only ones in which mitotic inhibition was not observed in the posttreatment period of growth. Karon et al. (13) and Buthala (3) reported similar observations in other systems, and the mitotic inhibitory action of ara-C has also been pointed out by other workers (6, 7, 11, 14, 19). The mitotic inhibitory effect of another analog, 2'-deoxy-5-fluorouridine (FUDR), counteracted by the addition of thymidine, was used as a method for synchronization and accumulation of mitoses (see Hsu et al. 12).

The differences in frequency and type of aberrations found at anaphase between treated and untreated cells further confirm the chromosome-breaking effects of ara-C seen at metaphase (2, 14, 19).

A prominent effect of ara-C is that it interferes with or blocks DNA synthesis (3, 5–7, 14). The appearance of the treated cells thus reflects morphologic characteristics of cells that have been subjected to the interference of DNA synthesis by the analog, a condition which may be compared to the unbalanced growth induced in a thymine-less state in bacteria (8) and observed in HeLa S-3 cells after treatment with ara-C (15).

In short treatments with lower concentrations of ara-C, cells started to replicate again after the removal of the analog, whereas after long treatments with low or high concentrations, they remained in interphase.

The appearance of the nucleoli directly after the treatment and during the posttreatment period might give an indication of an increased metabolic activity. The nucleoli first appear in diffuse branched condition and then fuse together, forming big, darkly stained nucleoli. In the posttreatment period, new nucleoli are sometimes organized. The appearance of vacuoles in nucleoli may increase the area of the reactive surface (10). These changes do not necessarily indicate increased metabolic activity, and, indeed, in the protozoan Tetrahymena pyriformis, fusion of nucleoli takes place in aged cultures at the end of the log phase and during the stationary phase of growth (4). In this paper (4), the authors referred to works demonstrating the effect of heat shocks in causing nucleolar fusion. Also, vacuoles were seen in nucleoli of green monkey kidney cells after infection with Simian Virus 40 (17), and they may represent a stage of pyknosis in these cells.

Many of the features seen in the ara-C-treated cells presented here resemble those observed in transformed, or virus-treated, cells (e.g., 16, 17, 29). Included in these similarities are the increased cytoplasmic granularity, more conspicuous large nucleoli, enlargement of nuclei, and clustering of cells. Some of the observed morphologic characteristics are also seen in aged control cells. Attempts are in progress to follow up the cells treated with ara-C by repeated cloning and passing to detect cells that fulfill all the requirements of transformation.

**Acknowledgments**

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**References**


References

FIGS. 1-17. All figures are from material grown on coverslips, fixed in Kahle's modified fixative, and differentially stained with Feulgen's procedure and light green.

FIGS. 1-4. Human diploid cell strain (WI-38) control 1-2, 3-4, 5-6, and 7-8 days after passing, respectively. More mitoses in the earlier days of growth. In an older state, cells form a dense sheet of cells, nuclei are lighter in color, and nucleoli are fewer in number. × 560.

FIGS. 5-11. Human diploid cell strain (WI-38) treated with arabinosylecytosine (ara-C) (except cells in Figs. 5, 9).

Fig. 5. Metaphase or prometaphase in control. × 2000.

Fig. 6. A cell subjected to a 3-hr 10^{-7} M treatment and 2 days growth in ara-C-free medium showing stickiness of chromosomes and persistence of nucleolar material (arrows) in a metaphase or prometaphase. × 2600.

FIGS. 7, 8. Anaphases after 3-hr 10^{-6} M treatment exhibiting paired acentric fragments. × 3200.

Fig. 9. Anaphase with a chromatid bridge demonstrating the type of aberrations occasionally seen in control material. × 3200.

Fig. 10. Treatment with 10^{-7} M for 24 hr, exhibiting fewer cells, many of which seem to be polyploid. The diffuse or branched nature of nucleoli is also seen. × 560.

Fig. 11. Treatment with 10^{-3} M for 24 hr, demonstrating clustering of cells. × 560.

FIGS. 12-15. Human diploid cell strain (WI-38) treated with ara-C and grown in ara-C-free medium after treatment. × 560.

Fig. 12. Treatment with 10^{-4} M for 3 hr and 4 days' posttreatment period, exhibiting fused, rounded nucleoli.

Fig. 13. Treatment with 10^{-7} M for 3 hr and 8 days' posttreatment growth. Few dead cells with pyknotic chromatin material are seen.

Fig. 14. Treatment with 10^{-4} M for 3 hr and 8 days' posttreatment growth. Elongated nuclei in spindle-shaped fibroblasts and also granularity of the chromatin material in some degenerating nuclei are seen.

Fig. 15. Treatment with 10^{-7} M for 1 hr and 8 days' posttreatment growth, demonstrating degenerating nuclei.
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Fig. 16. Treatment with $10^{-4}$ M for 24 hr and 8 days' posttreatment growth in ara-C-free medium, showing fusion of nucleoli into 1 big nucleolus and presence of vacuoles. $\times$ 2600.

Fig. 17. Treatment with $10^{-4}$ M for 24 hr and 8 days' posttreatment growth in ara-C-free medium. Fusion of nucleoli into 1 big nucleolus and the organization of many small new nucleoli are seen. $\times$ 2600.
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