[CANCER RESEARCH 43, 1361–1364, March 1983]
0008-5472/83/0043-0000$02.00

Communication

Aphidicolin-induced Endoreduplication in Chinese Hamster Cells[1]

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ABSTRACT

Aphidicolin, a specific inhibitor of DNA polymerase α, was found to induce high frequencies of endoreduplication in Chinese hamster V79 cells in a dose-dependent manner. The aphidicolin-induced endoreduplication was observed when cells were incubated at 37° but not at 41°. Since it is known that DNA polymerase β is more thermally labile than is DNA polymerase α, the data are consistent with the hypothesis that DNA polymerase β might be responsible for endoreduplication as was reported in mouse trophoblast cells. From the induced diplochromosomes, it was observed that the two unifilarly 5-bromodeoxyuridine-substituted chromatids are generally paired and located inside, whereas the two bifilarly 5-bromodeoxyuridine-substituted chromatids are flanking outside regardless of the presence of sister chromatid exchange or intradiplochromatid interchange.

INTRODUCTION

Endoreduplication is a variant process of cell replication which involves 2 successive rounds of DNA replication without intervening cytokinesis. The observable characteristics of endoreduplicated cells are the presence of 4-stranded chromosomes (diplochromosomes) and the doubling of DNA content compared to diploid cells at metaphase (3). As a consequence, each of the resulting daughter cells contains twice as many chromosomes as its parental cell. The phenomenon occurs spontaneously in normal (2, 45) and tumor tissues (6, 19, 22) or in tissues from other clinical syndromes (1, 27) at relatively low frequency. Various treatments have been shown to increase the frequency of endoreduplication. These include physical (4, 16) and chemical mutagens (42, 44), sulfhydryl compounds (23–25, 38, 43), base analogues (29, 34), and metabolic or mitotic inhibitors (33). The highest frequencies of endoreduplication ever achieved by most of these treatments, however, have not exceeded 10%. The mechanism for its production remains unclear.

Aphidicolin is a specific inhibitor of DNA polymerase α and blocks DNA synthesis *in vivo* (21, 30–32, 47). In some studies, the compound has been implicated in the inhibition of repair replication (5, 10, 12, 17, 28, 40). Recently, in our study of the induction of SCEs[4] by aphidicolin in Chinese hamster V79 cells, we discovered that aphidicolin induced a high frequency of endoreduplication. Our initial observation was done in cells treated with both aphidicolin and BrdUrd (20). To clarify whether endoreduplication can be induced by aphidicolin alone and to determine whether the induction is dose dependent, a detailed study involving various doses of aphidicolin in the presence and absence of BrdUrd was carried out.

MATERIALS AND METHODS

Cell Culture. Chinese hamster V79 cells were grown in modified Eagle's medium (15) (Earle's balanced salt solution with a 50% increase in all vitamins and essential amino acids except glutamine), supplemented with nonessential amino acids (100% increase), 1 mM sodium pyruvate, and 5% fetal calf serum. Cells were grown in a humidified air atmosphere with 5% CO_2 at 37°.

Cell Treatments and Chromosome Preparation. Chinese hamster V79 cells (1.2×10^6) were inoculated into each of two 25-sq cm flasks containing 10 ml of modified Eagle's medium. After 4-hr incubation, the cells were exposed to various concentrations of aphidicolin (supplied by the Developmental Therapeutics Program, National Cancer Institute) in the presence or absence of BrdUrd (15 μM) for various durations (0 and 0.05 μM, 26 hr; 0.1 μM, 32 hr; 0.2 μM, 36 hr; 0.3 μM, 42 hr; and 0.4 μM, 48 hr) at 37° to allow for 2 rounds of DNA synthesis. The cells were then treated with Colcemid (0.04 μg/ml) for 3 hr and harvested by trypsinization. Chromosome preparations were made by dropping cells, which have been treated with hypotonic solution (0.075 M KCl) for 6 min and fixed with methanol/acetic acid (3:1, v/v), on ice-cold slides on a slide warmer (60°). Differential chromatid staining was achieved by soaking slides in Hoechst 33258 (5 μg/ml in Sorensen's buffer, pH 6.8) for 15 min and exposure of slides, which were mounted with coverslips using Sorensen's buffer (pH 8.0), on a slide warmer (40°) to high-intensity UV for 15 min. Slides were then washed twice with Sorensen's buffer (pH 8.0) and once in double-distilled water and stained with 5% Giemsa (Gurr's R66 prepared in Sorensen's buffer, pH 6.8) for 7 min. Metaphases were examined and photographed at ×1000 magnification under oil. More than 100 metaphases were examined per treatment (Table 1).

In testing for the effect of temperature on aphidicolin-induced endoreduplication (Table 2), the protocols of aphidicolin treatment and chromosome preparation were the same as those described above, except cells were incubated from cell plating to harvesting at 2 different temperatures. All cultures for the experiment presented in Table 2 contained 15 μM BrdUrd.

RESULTS AND DISCUSSION

The results presented in Table 1 clearly showed that endoreduplication can be induced by aphidicolin alone in a linear dose-responsive manner. The frequencies were not significantly modified by BrdUrd. At 0.4 μM, the aphidicolin induced endoreduplication in more than 30% of the treated cells. This concentration has been shown to reduce survival to 1% for Chinese hamster V79 cells if the drug was present for the entire period of colony development (9). The survival can be

[1] Supported by grants from the National Cancer Institute (CA 21104 and CA 26803).

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[4] The abbreviations used are: SCE, sister chromatid exchange; BrdUrd, 5-bromodeoxyuridine.

Received August 16, 1982; accepted December 7, 1982.

Table 1

Percentage of cells with endoreduplicated chromosomes after various aphidicolin and BrdUrd treatments

The detailed procedures for cell treatments and chromosome preparation are described in "Materials and Methods."

Presence of BrdUrd (15 μM)	% of cells at following concentration of aphidicolin					
	0 μM	0.05 μM	0.1 μM	0.2 μM	0.3 μM	0.4 μM
+	1	1	3	13	22	39
−	0	0	2	10	20	32

Table 2

Effect of temperature on frequencies of aphidicolin-induced endoreduplication in Chinese hamster V79 cells

The values shown are percentages of cells with endoreduplicated chromosomes based on analysis of 100 metaphases per treatment. The procedures of cell treatments and chromosome preparation are described in "Materials and Methods."

Temperature	Frequency of endoreduplication (%) at following concentration of aphidicolin		
	0 μM	0.2 μM	0.4 μM
37°	1	7	27
41	0	0	1

increased to 10 to 60% if the treatment is only 1 to 2 days as was used in the present experiment. Double endoreduplicated cells with quadruplochromosomes were also found in cultures treated with aphidicolin but at much lower frequency.

It seems ironic that a compound like aphidicolin can be simultaneously an inhibitor of DNA replication and an inducer of an extra cycle of DNA synthesis. Therefore, we speculated that endoreduplication might be the function of another type of DNA polymerase not sensitive to aphidicolin (*i.e.*, β- or γ-polymerase). Furthermore, it has been demonstrated that DNA polymerase β from a variety of mammalian cells (13, 14), including Chinese hamster cells (41), is more rapidly inactivated *in vitro* by elevated temperature than is DNA polymerase α. Consequently, we conducted an experiment to investigate the effect of high temperature on endoreduplication. Chinese hamster V79 cells grow and form colonies normally at either 37° or 41°. However, the same aphidicolin treatment that significantly enhanced the frequency of endoreduplication at 37° failed to elicit an effect at 41° (Table 2). The results are consistent with the prediction that endoreduplication might be the function of β-polymerase, when the α-polymerase is inhibited. If β-polymerase is responsible for endoreduplication, the inactivation of the enzyme at high temperature should eliminate endoreduplication as was found in our experiment. These results by themselves do not provide direct evidence for the hypothesis, since the temperature increase could have inactivated other substances which would have been responsible for the endoreduplication. However, after our observations were made, Siegel and Kalf (39) reported that DNA polymerase β seemed to be responsible for endoreduplication in rat giant trophoblast cells.

From this work and other studies reported in the literature, it seems clear that endoreduplication might be brought about by different mechanisms. Based on the positive effect of membrane and cytoskeleton binding compounds, such as phytohemagglutinin and colchicine, Sutou and Arai (43) have suggested that endoreduplication is a consequence of abnormal alterations in the cell membrane and/or the cytoskeleton. They realized that inducers such as base analogues and metabolic inhibitors are difficult to reconcile with the hypothesis. Our observation clearly suggests a different major cause of endoreduplication, *i.e.*, the arrest of normal DNA replication. Therefore, compounds or treatments that are known to stop normal DNA synthesis, such as DNA-damaging agents (4, 16, 42, 44) (*e.g.*, X-rays, 4-nitroquinoline 1-oxide), inhibitors of polymerase α (*e.g.*, aphidicolin), 1-β-D-arabinofuranosylcytosine (43), base analogues (29, 34) (*e.g.*, 8-azaguanine, 6-mercaptopurine), metabolic inhibitors (43) (*e.g.*, actinomycin, puromycin), and low temperature (8), are also capable of inducing endoreduplication.

From the induced diplochromosomes, we were able to look at the effect of SCE and intradiplochromatid interchanges on the position of unifilarly and bifilarly BrdUrd-substituted chromatids. In general, we confirmed previous observations that the polynucleotide strands of the DNA segregate into sister chromatids as though the newly synthesized strands were laid on the outside of the replicating double helix (18, 35, 37, 46, 48). Therefore, the 2 outer chromatids, which are bifilarly substituted, are faintly stained, whereas the 2 inner chromatids, which are unifilarly substituted, are darkly stained. Earlier reports, however, found that this rule can be violated in case of the presence of SCE or intradiplochromatid interchanges (35, 48). In contrast, we observed that the position of unifilarly and bifilarly BrdUrd-substituted chromatids remains the same even in the presence of SCE or intradiplochromatid interchanges (Chart 1; Fig. 1). Only a few exceptions were found in exchanges involving a small segment of chromatids (see Fig. 1, No. 6) or in late metaphase when paired chromosomes have started to separate. The discrepancy between our observation and other reports, we believe, is due to the different stages of metaphases being observed. If observations are made at early metaphase, the chromatid pairings are faithfully preserved, and one will find that chromatid positions are not affected by SCE or intradiplochromatid interchanges, except for exchanges that involve a very small segment of chromatids. When paired chromosomes started to separate, the original positions are inevitably disturbed. Our observation that chromatid exchanges essentially do not alter chromatid positions may be explained by the presence of pairing affinity at metaphase between parental as well as daughter-sister chromatids. The force that maintains the chromatid positions cannot be overcome by chromatid exchange except in a small exchange that is terminally located where pairing affinity may be relatively weaker.

The importance of chromosomal aberrations in the etiology of cancers was postulated by Boveri (7) as early as 1914. Since that time, specific types of chromosomal aberrations have been described in many hereditary as well as nonhereditary tumors. Furthermore, most malignant tumors of adulthood are characterized by abnormal karyotypes. The consequence of these changes remains unknown. Knudson (26) postulated that karyotype changes may be a third step following a second mutation in the development of cancer in some hereditary cancers such as colon carcinomas in Gardner's syndrome. Interestingly, the skin fibroblasts derived from this syndrome have been shown to exhibit high frequency of endoreduplication and tetraploidy (11). Since aneuploidy may also be generated by partial endoreduplication (19) and by multipolar division of endoreduplicated cells (36), a variety of

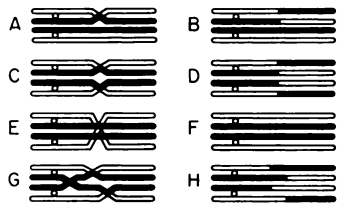

Chart 1. Within a diplochromosome, pairing affinity holds parental or daughter-sister chromatids together regardless of SCE or intradiplochromatid interchange. Therefore, configurations are seen as (*a*) *A* instead of *B* for single SCE (see Fig. 1, 1 and 2); (*b*) *C* instead of *D* for twin SCE (see Fig. 1, *3*); (*c*) *E* instead of *F* for intradiplochromatid interchange (see Fig. 1, *4*); and (*d*) *G* instead of *H* for 2 single SCEs and one intradiplochromatid interchange (see Fig. 1, *5*).

karyotypic aberrations characterizing tumor cells may be created by endoreduplication following carcinogen exposure. The origin of endoreduplication, therefore, may be related to mechanisms of tumorigenesis and evolution.

ACKNOWLEDGMENTS

We want to acknowledge P. K. Liu for his helpful discussions and Olivia Reid and Judy Copeman for their typing assistance.

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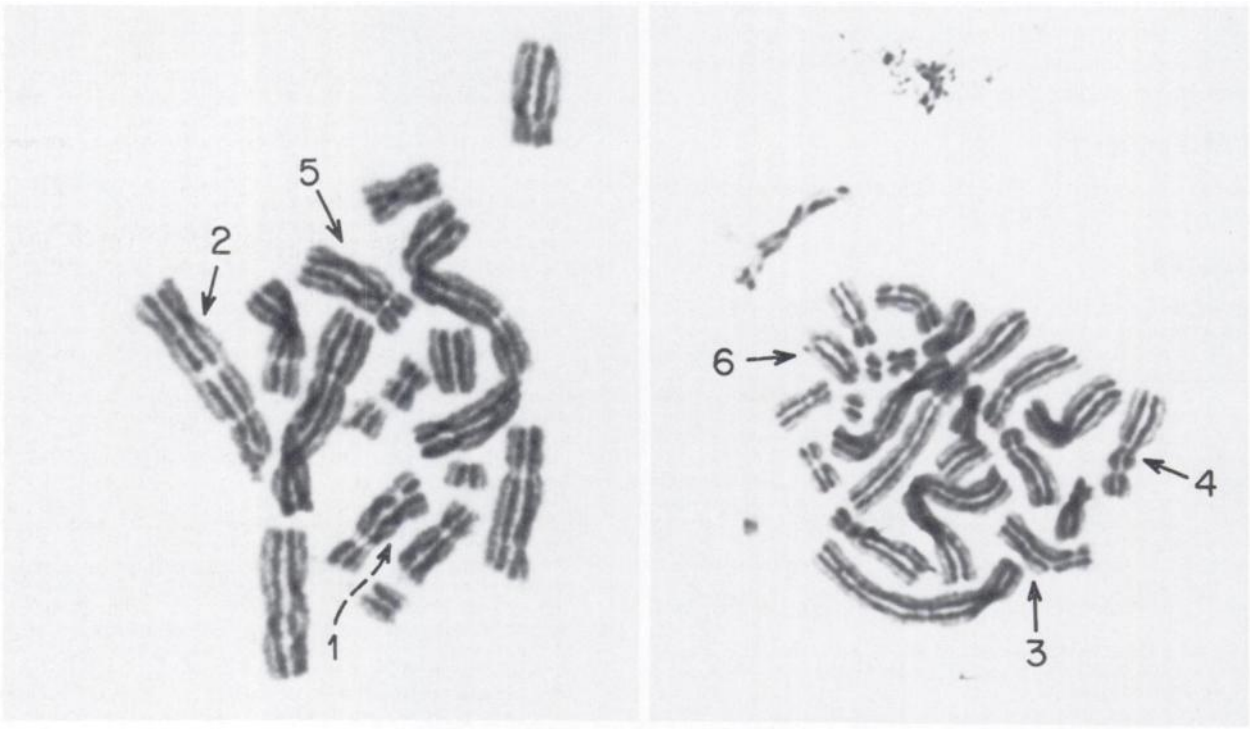

Fig. 1. Endoreduplication mitosis with differential chromatid staining induced by aphidicolin treatment (0.4 μM). Unifilarly BrdUrd-substituted chromatids are darkly stained. *Arrows*, chromosomes with SCE or intradiplochromatid interchange. See Chart 1 and text for related explanation.