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COLCHICINE-LIKE EFFECT OF DIETHYLSTILBESTROL (DES) ON MAMMALIAN CELLS IN VITRO *

MINORU SAWADA and MOTOI ISHIDATE Jr.

Department of Chemical Pathology, National Institute of Hygienic Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158 (Japan)

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Summary

Diethylstilbestrol (DES), a synthetic estrogen, showed colchicine-like effects in vitro on cells of the cell lines such as Chinese hamster fibroblast of thymus origin (CHT), rat liver (DL), rat erythroblastic leukemia (EDEN-1/TC) and HeLa-S3. Metaphase arrest was induced 3 h after treatment with 15 μg/ml of DES and polyploid or polynucleated cells were prominently observed more than 24 h after treatment. The arrest, however, was reversible when the agent was removed from the medium. Tetraploid karyotypes induced by DES in CHT cells consisted of all double sets of diploid chromosome constitution except one chromosome marker.

By clonal selection, several hypotetraploid sublines were successfully isolated from a CHT cell population after the treatment with 15 μg/ml of DES for 48 h. Some comparative studies of cytological effects of DES with those induced by colcemid indicated that the DES effect was also a mitotic inhibition similar to colchicine.

Introduction

DES has been currently used as a food additive for cattle and as a postcoital contraceptive for women. In 1971, however, Herbst et al. reported that the latent appearance of genital tract tumors in young women whose mothers had been given DES during gestation [8]. This delayed side-effect of DES has been further confirmed clinically [6,7,9,19,21] as well as in experimental animals

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Abbreviations: CHT, Chinese hamster fibroblast of thymus origin; DES, diethylstilbestrol; DES-dp, DES diphosphate.

[18]. DES has been also used in hormonal therapy for patients with prostatic carcinoma [5] or advanced breast cancer [14].

Apart from these pathological findings, some evidence has been reported on the cytological effects of DES on mammalian cells in vivo or in vitro. Kuchler and Grauer [15] found that DES inhibited selectively cellular metabolism, particularly DNA and protein synthesis in cultured human fibroblasts. Rao and Engelberg [20] exposed HeLa cells to DES and found that it induced a significant incidence of mitotic chromosome nondisjunction. Chrisman and Lasley checked the cytotoxicity of DES diphosphate (DES-dp) on bovine lymphocyte culture and found that there was a significant difference in the mitotic indices between concentrations [3]. Aneuploid cells were also detected in 8-day embryos and in bone marrow cells when DES-dp was administered in vivo to pregnant and male mice, respectively [1,2].

During the process of our screening for chemical mutagens or carcinogens [12], we found that carcinogenic DES induced a marked polyploidization in Chinese hamster cells in vitro. The present report deals with a further detailed survey of the cytological effects of DES on mammalian cells in culture.

Materials and methods

Cell lines used

A fibroblast cell line, CHT, was established in our laboratory from the thymus of a young male Chinese hamster. The cells have been maintained by 5-day passages in Eagle's MEM with 20% heat-inactivated fetal calf serum (Gibco). During serial passages, the cells retained their karyotype of normal diploid and the doubling time of about 12 h.

For comparison, three other cell lines, DL, EDEN-1/TC and HeLa-S3 cells were used. The DL, a rat-liver cell line, was established by Dr. M. Umeda, Yokohama City Med. Sch., from the liver of a Japanese Donryu rat. The EDEN-1/TC, a rat leukemia cell line, was established in our laboratory from a transplantable erythroblastic leukemia induced by *N*-ethyl-*N*-nitrosourea in a female Donryu rat. These cell lines were maintained in the same medium as used for CHT cells. HeLa-S3 was maintained in the MEM with 10% calf serum (Gibco). The modal number of chromosomes of DL, EDEN-1/TC and HeLa-S3 were 41—42, 42—43 and 62—63, respectively.

Treatment with DES

DES was kindly supplied by Dr. M. Nakadate, Dept. of Synth. Chem. of our Institute. It was dissolved in absolute ethanol which was adjusted to a final concentration of 1.0%. Cells growing exponentially were treated with DES at a final concentration of 7.5 or 15.0 μg/ml and incubated at 37°C in the presence of 5% CO_2. Cultures treated with only solvent served as controls. For a comparative study 0.2 μg/ml of colcemid was used.

Cytological observation

Chromosome preparations were made by the conventional air-drying method after colcemid treatment for 2 h. The incidence of polyploid cells was calculated from 100 metaphases on preparations from each of three dishes. G-band

technique was also applied to the chromosome preparations as follows: Slides were treated with 0.02% trypsin at room temperature for 30 sec and stained with 1.0% Giemsa (Merck)'s solution (phosphate buffer at pH 6.8) for 10 min. Squashed preparations stained by 2% acetic orcein were used for counting the mitotic index and incidences of each mitotic phase and polynucleated cells.

Observation under a phase-contrast microscope was also carried out as follows: Small pieces of a cover glass were placed on a 25 × 50 mm^2 cover glass and mounted on a slide to make a very thin chamber, in which a small number of CHT cells were seeded. Different shapes of the glass pieces help to identify the localization of each individual cell. After the treatment with DES, the individual cell was marked and periodically sketched showing the morphological changes under the microscope. The temperature was controlled at 37° C during this observation.

Experimental results

Polyploid incidences among different cell lines

The incidence of polyploid cells among different cell lines after treatment with 7.5 or 15.0 μg/ml of DES for 48 h is shown in Table 1. Approximately 10% of metaphases were polyploid after the treatment with 7.5 μg/ml. The incidence, however, increased markedly with 15.0 μg/ml and showed more than 90% in both CHT and EDEN-1/TC or 65.5% in HeLa-S3. The 15.0 μg/ml was very toxic to DL cells, so that no mitotic cells were detected at 48 h after treatment.

TABLE 1

INCIDENCES OF POLYPLOID CELLS IN DIFFERENT CELL LINES AFTER EXPOSURE TO DES FOR 48 h

Cell line	DES dose (μg/ml)	Incidence of polyploid cells (%)		
		$\leq 4n$	$>4n$	Total
CHT	Control	2.8 ± 0.5	0	2.8 ± 0.5
	7.5	8.6 ± 2.9	0	8.6 ± 2.9
	15.0	90.2 ± 2.7	2.7 ± 0.4	92.9 ± 2.4
HeLa-S3	Control	0.8 ± 0.3	0	0.8 ± 0.3
	7.5	9.3 ± 1.4	0.2 ± 0.3	9.5 ± 1.7
	15.0	65.2 ± 6.4	0.3 ± 0.6	65.5 ± 6.9
DL	Control	4.3 ± 1.2	0	4.3 ± 1.2
	7.5	12.8 ± 1.8	1.8 ± 0.3	14.7 ± 2.0
	15.0	—[a]	—[a]	—[a]
EDEN-1/TC	Control	0.8 ± 0.6	0	0.8 ± 0.6
	7.5	11.8 ± 1.9	0.2 ± 0.3	12.0 ± 2.2
	15.0	92.5 ± 1.3	3.0 ± 1.3	95.5 ± 2.3

[a] Cytotoxic (very few mitosis).

Mean ± S.D. for 3 plates.

Mitotic arrest and formation of polyploid or polynucleated cells by DES in comparison to those by colcemid

CHT cells were exposed to 15.0 μg/ml of DES or 0.2 μg/ml of colcemid for 3, 6, 12, 24, 36 and 48 h. The mitotic index and the incidence of polynucleated cells were calculated from 500 cells on each of 3 plates by the squash technique. The incidence of tetraploid cells was calculated from 40—50 metaphases on each plate. As shown in Table 2, the mitotic ratio is maximum at 12 h and shows 13.3% in DES or 29.3% in colcemid. The incidence of polynucleated cells increased gradually from 6 h after treatment in both cases and reached to 51.5% in DES or 78.9% in colcemid. Tetraploid cells also increased from 24 h in both cases and reached to 72.4% or 81.3%, respectively. Metaphase arrest was observed as early as 3 h after treatment and percentages of metaphases among 150 mitotic cells increased from 49.3% to 91.3% with DES, or from 52.0% to 96.0% with colcemid. This arrest was retained throughout 48 h after treatment. These results indicate that DES and colcemid both inhibit mitosis and produce polyploid in a similar pattern but at different dose levels.

Further experiments were done in which cells were washed after the treatment with DES or colcemid for 12 h. As shown in Table 3, after washing both the mitotic index and incidence of metaphase decrease, while the incidence of anaphase plus telophase increases within the first 30 min, indicating that the mitotic arrest induced by DES could be reduced by washing. By colcemid, however, such recovery effects seemed to be slightly delayed, although there were no marked differences in their pattern of the recovery between these two agents.

Direct observation under a phase-contrast microscope

In CHT cells, the metaphase finished generally within 10 min and continued to the anaphase. After the treatment with DES, however, chromosomes at metaphase did not move into two poles and remained in the metaphase plate for several hours. Such metaphase cells began to bud and to form a gourd-like

TABLE 2

MITOTIC INDEX AND INCIDENCES OF POLYNUCLEATED AND TETRAPLOID CELLS AFTER THE EXPOSURE TO 15 μg/ml OF DES OR 0.2 μg/ml OF COLCEMID

Exposure time (h)	Mitotic index [a]		Polynucleated (%) [b]		Tetraploid (%) [c]	
	DES	Colcemid	DES	Colcemid	DES	Colcemid
0	3.5 ± 0.1	3.2 ± 0.4	1.1 ± 0.2	1.1 ± 0.4	1.4 ± 2.4	0.0 ± 0.0
3	7.7 ± 1.1	12.6 ± 0.9	2.9 ± 1.4	0.9 ± 0.8	0.0 ± 0.0	0.0 ± 0.0
6	8.5 ± 0.9	22.3 ± 3.3	6.0 ± 1.2	3.1 ± 0.7	0.7 ± 1.2	0.0 ± 0.0
12	13.3 ± 2.4	29.3 ± 1.7	11.8 ± 3.1	18.2 ± 1.2	0.7 ± 1.2	0.7 ± 1.2
24	6.5 ± 0.7	10.0 ± 1.4	35.7 ± 3.4	71.9 ± 5.7	13.8 ± 4.8	7.7 ± 4.3
36	5.6 ± 1.4	6.7 ± 0.5	51.5 ± 2.6	78.9 ± 1.7	38.3 ± 4.7	77.7 ± 6.9
48	6.3 ± 0.9	10.0 ± 0.1	46.5 ± 6.0	81.4 ± 1.7	72.4 ± 8.0	81.3 ± 8.7

[a] Mitotic cells/500 cells in each of 3 plates.
[b] Polynucleated cells/500 cells in each of 3 plates.
[c] Tetraploid cells/40~50 metaphases in each of 3 plates.
Mean ± S.D.

TABLE 3

RECOVERY TESTS ON CHT CELLS AFTER TREATMENT FOR 12 h WITH 15 μg/ml OF DES OR 0.2 μg/ml OF COLCEMID

Time after washing (min)	Mitotic index [a]		Metaphase (%) [b]		Anaphase plus telophase (%) [c]	
	DES	Colcemid	DES	Colcemid	DES	Colcemid
0	16.8 ± 0.6	25.3 ± 2.9	91.3 ± 4.0	97.3 ± 3.1	0.0 ± 0.0	0.0 ± 0.0
15	15.4 ± 1.4	—[d]	44.3 ± 0.6	—	47.0 ± 3.0	—
30	4.8 ± 0.9	26.1 ± 2.5	34.0 ± 9.5	98.7 ± 0.6	32.3 ± 10.5	0.0 ± 0.0
60	2.7 ± 0.3	24.4 ± 3.0	55.3 ± 8.1	71.0 ± 1.7	11.3 ± 2.5	20.7 ± 2.1
90	—	9.7 ± 1.4	—	50.7 ± 10.4	—	33.4 ± 4.6
120	2.8 ± 1.2	5.5 ± 0.2	62.0 ± 3.0	57.3 ± 5.7	8.0 ± 3.0	19.0 ± 4.6

[a] Mitotic cells/500 cells in each of 3 plates.
[b] Metaphases/100 cells in each of 3 plates.
[c] Anaphases + telophases/100 mitotic cells in each of 3 plates.
[d] Not tested.
Mean ± S.D.

shape. Chromosomes were usually located at the opposite site of the bud. The area containing chromosomes then blistered to form several lobes. Nuclear membranes appeared around the chromosome clumps which had been located in different lobes. The cells formed then polynuclei and stretched on the glass surface without normal cytokinesis. The majority of these polynucleated cells became disintegrated thereafter in the presence of 15 μg/ml of DES. When DES was removed from the medium at 24 h after treatment, a few cells developed to the next cell cycle and formed metaphases with double sets of chromosome

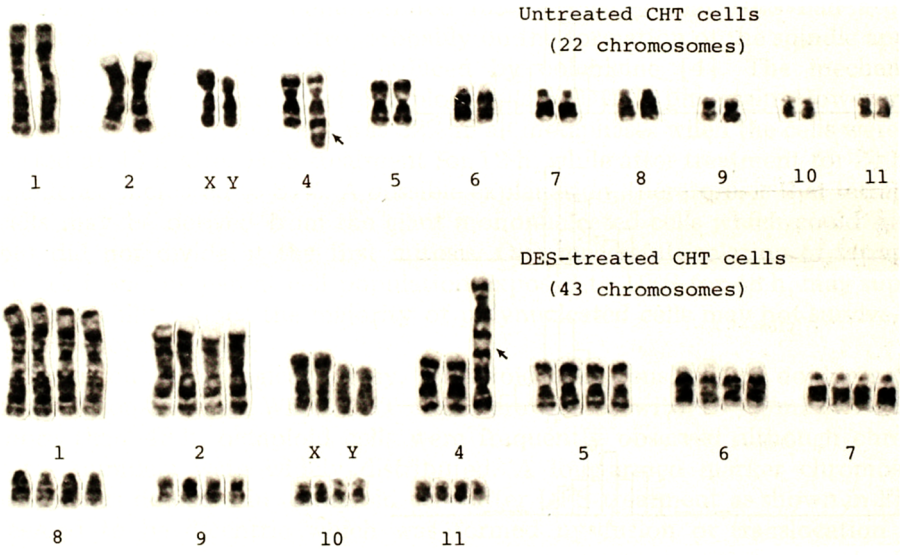

Fig. 1. Representative karyotypes of untreated and DES-treated CHT cells. The cells were treated with 15 μg/ml of DES for 48 h. Note a rearranged No. 4 chromosome (upper arrow) and a long marker chromosome (lower arrow).

constitution. During this process synchronous mitoses were observed among nuclei within a polynucleated cell. When only metaphases were collected and exposed to 15 μg/ml of DES in the chamber for 24 h, polynucleated cells rather than mononucleated giant cells were predominantly observed.

Karyotype of tetraploid CHT cells induced by DES

Chromosomes were arranged according to the report by Kato and Yoshida [13]. A shown in Fig. 1, untreated cells have 22 chromosomes with a rearranged chromosome at No. 4 which is formed by attachment of an unknown chromosome fragment on its distal end of the long arm (arrow). The cells treated with 15 μg/ml of DES for 48 h consisted of a wide range of distribution of chromosome numbers as shown in Fig. 2, but showed a modal number of 43 chromosomes with all duplicated sets of diploid. These tetraploid cells had one big marker chromosome which was possibly formed by a fusion of the two rearranged chromosomes detected in untreated cells (Fig. 1, arrow).

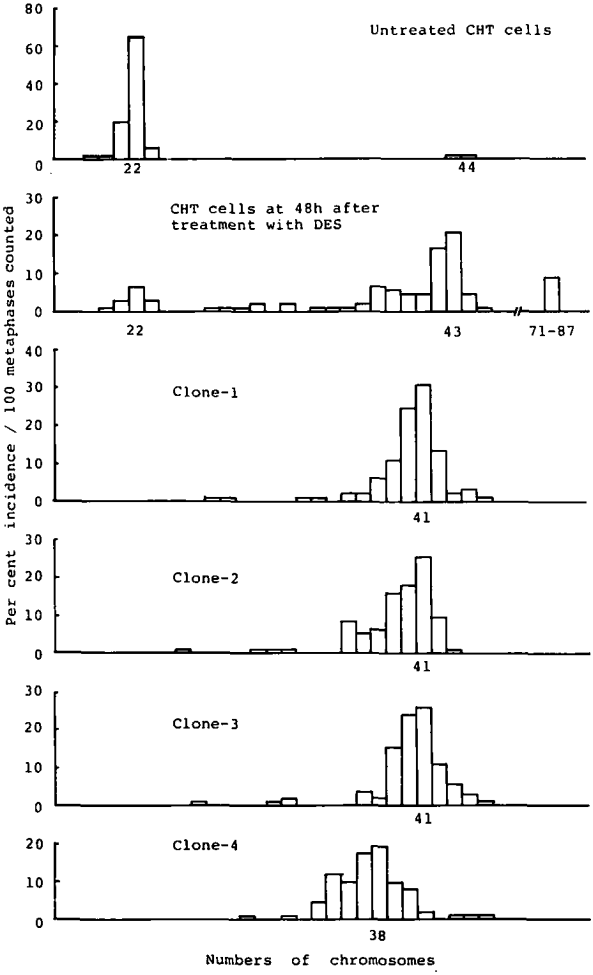

Fig. 2. Distribution of chromosome numbers of untreated CHT cells, those treated with 15 μg/ml of DES for 48 h, and 4 clones of the 7th passage which were isolated from the treated cells. Note changes in modal numbers of chromosomes of the DES-treated cells and hypotetraploidy in 4 different clones.

Isolation of tetraploid clones from DES-treated cells

Single-cell clones were isolated from a cell population treated with 15 μg/ml of DES for 48 h. Out of 13 clones 12 showed the karyotype of tetraploid range and one showed diploid range. The distribution of chromosome numbers of 4 out of 12 clones is shown in Fig. 2. They showed all hypotetraploidy throughout additional 14 passages, although the marker chromosome as demonstrated in Fig. 1 was not detected at their 3rd passage.

Discussion

DES is a potent synthetic estrogen widely used for estrogen replacement and as a growth stimulant in food-producing animals. Since 1971, evidence has accumulated that prenatal exposure to this agent may lead to pathological changes in the female genital tract and to induce clear cell adenocarcinoma of the vagina or cervix [8—10]. The problem, however, whether DES acts as a chemical carcinogen which may react to bind to cellular macromolecules, or as a hormone which may not involve such a covalent binding, is still unsolved. Chrisman [1] demonstrated significant increases of aneuploid cells in 8-day mouse embryos obtained from the mother which had been injected with DES-dp. It is also known that estrogens with hydroxyl units at positions 3 and 17 produce unusual mitoses in mammalian cells in vitro [15,20,17]. As to the question of the etiology of the aneuploid cells, there has been no clear-cut answer. Biochemical studies indicate that inhibitory effects on mammalian cells in vitro could be related to estrogen-cell membrane interaction resulting in physiological blockage of substrate transport [16]. Chrisman and Hinkle have suggested, however, that a more reasonable assumption would be that DES-dp has a direct influence on the spindle apparatus of mammalian cells [2].

Our present studies demonstrated further clearly that DES had a direct effect on cell mitosis in vitro, probably on the formation of the spindle apparatus, similarly to the effects induced by colchicine [4]. The mechanisms involved in the formation of polyploid cells by DES is uncertain. However, the incidence of polyploid cells was 22% in all metaphases when the cells were harvested at 48 h after DES treatment for 12 h, while after treatment for 24 h the incidence increased to 87%. A possible explanation, therefore, is that tetraploid cells may be derived from the giant mononucleated cells which could survive but did not divide at the first mitosis. Our successful isolation of tetraploid clones from the parent cell population exposed to DES for 48 h, may support this possibility, since the majority of polynucleated cells may not survive even after removal of DES.

From our karyological survey, tetraploid cells consisted of double sets of diploid constitution. When CHT cells were treated with 15 μg/ml of DES for more than 48 h, octaploid cells were frequently observed although chromosome numbers were widely distributed. A long-armed marker chromosome which was detected in tetraploid cells after DES treatment as shown in Fig. 1, seemed to be dicentric which was formed by fusion or translocation of 2 No. 4's of the diploid chromosome. This marker, however, may not be DES-specific, but specific to the rearranged chromosome found in the diploid CHT cells used in this experiment, since such markers were not detected in other cell lines after DES treatment.

The correlation between the effect of mitotic poison and the oncogenic property of DES is an important project in the future. There is a possibility that mitotic poisons could contribute as much to mutagenesis as true mutagens in mammalian cells [11], even though they could not be detected by mutation assays in bacteria, as indicated by our previous screening for chemical carcinogens [12].

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