Effect of Estradiol and Ethynylestradiol on Microtubule Distribution in Chinese Hamster V79 Cells

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The effects of estradiol (E_2) and ethynylestradiol (EE_2) on the chromosome number and cellular microtubule architecture of Chinese hamster V79 cells were studied using fluorescent anti-tubulin antibody. Treatment with 20 μM E_2 for 48 h induced only a small amount of tetraploid cells, but the normal microtubule network was disrupted completely by only 3 h of treatment.

This data reveals that E_2 has higher microtubule-disruptive activity than diethylstilbestrol in V79 cells.

Keywords estradiol; ethynylestradiol; microtubule distribution; aneuploidy; immunofluorescence; chromosome aberration

A causal link between estrogens and a variety of human cancers has been known for a long time,[1] and some estrogens are considered to affect cellular differentiation.[2] There is also a growing awareness of the carcinogenic potential of both natural and synthetic estrogens.[3]

We reported previously that diethylstilbestrol (DES; Chart 1), and a variety of its analogues, inhibit the *in vitro* polymerization of microtubule proteins isolated from porcine brain,[4-6] and that estradiol (E_2; Chart 1) does not inhibit *in vitro* microtubule assembly in the same assay system.[4] E_2 induces morphological transformation and aneuploidy,[7] and a 100% incidence of bilateral and multiple renal tumors in castrated male hamsters treated for 9.0 months.[8] On the other hand, treatment with ethynylestradiol (EE_2; Chart 1), a hemi-synthetic estrogen with very strong estrogenicity, produces only a 20% incidence of renal tumors in the same assay system,[8] and we have found that EE_2 has lower inhibitory activity against *in vitro* microtubule assembly than does DES (unpublished data).

In the present study, we investigated the effects of E_2 and EE_2 on the induction of aneuploidy and distribution of cytoplasmic microtubules in Chinese hamster V79 cells in comparison with those of DES.[9]

Experimental

Materials Estradiol was obtained from Seikagaku Corporation (Tokyo). Ethynylestradiol was obtained from Sigma Chemical Corporation (St. Louis, MO, U.S.A.). DES was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo). Dimethyl sulfoxide (DMSO) was purchased from Pierce Chemical Co. (Rockfield, IL) and colcemide from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Gibco after lot-checking.

Cell Culture Chinese hamster V79 cells were grown in monolayer culture in Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co., Ltd. (Tokyo)) with 10% heat-inactivated FBS. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

Relative Plating Efficiency The relative plating efficiencies in the presence of different concentrations of drugs were determined as the ratio of the number of colonies at a given drug concentration to that obtained in the control culture in the absence of any drug. Two hundred cells were seeded on 60/15-mm Petri dishes (Falcon; Becton Dickinson & Company (Lincoln Park, NJ)) in 4 ml of MEM supplemented with 10% FBS. At 24 h after seeding, E_2, EE_2 and DES dissolved in DMSO were added to the culture for 48 h, respectively, and in the control culture, 4 μl of DMSO was added. Then the medium was replenished, and the culture was continued for 48 h without drugs. The dishes were then fixed with methanol and stained with 7% Giemsa solution. The numbers of colonies (>50 cells/colony) were counted under a dissecting microscope. Groups of 4 replicate dishes were used to assess the effects of drugs on plating efficiency.

Determination of Chromosome Number and Chromosome Aberrations Cells (0.5—2×10^5, determined by a relative plating efficiency experiment), were seeded in 100-mm Petri dishes in 10 ml of MEM with 10% FBS. After overnight incubation, E_2, EE_2 and DES dissolved in DMSO (10 μl) were added to the culture, and in the control culture 10 μl of DMSO was added. After each incubation, the medium was changed, colcemid (0.2 μg/ml) was added, and the cells were treated for 2 h. After trypsinization, the cells were treated with 0.075 M potassium chloride at room temperature for 25 min, fixed with Carnoy's solution (methanol–acetic acid, 3:1), spread on glass slides and air-dried. The specimens were stained with 2% Giemsa solution in 1/15 M Sorensen phosphate buffer (pH 6.8) for 5 min. Over 100 metaphases were counted for examination of the number of chromosomes and chromosome aberrations.

Indirect Immunofluorescence Chinese hamster V79 cells (800 cells/40 μl) were placed on one of several 8-well multitest slides (Flow Laboratories Ltd., Irvine, Scotland), incubated for 24 h at 37 °C in a wet box, grown to approximately 40% confluence, and then treated with drugs. At the time of the treatment, 20 μl medium per well were removed and 20 μl of a drug medium which contained twice the concentration of E_2, EE_2 and DES dissolved in DMSO (final conc. of DMSO was 0.1%) were added; in the control culture the same volume of DMSO was added.

The cell fixing and staining methods were performed according to the previous report.[9] Cells on slides were fixed for 1 min with 3% formaldehyde in PBS, permeabilized with cold methanol (-20 °C)[10] for 5 min, and air-dried. Cells were treated for 20 min with 1% Triton X-100 in PBS, preincubated for 30 min with 2% dry milk in PBS at room temperature, and then incubated for 1 h with 20 μl per well of mouse monoclonal anti-β-tubulin antibody (10—20 μg/ml; Amersham Laboratories, Buckinghamshire, England) at 37 °C. Following a 35-min rinse in PBS containing 2% dry milk, the cells were reincubated for 1 h with 2 μl per well FITC-conjugated sheep anti-mouse immunoglobulin G_1 (IgG_1) (125 μg/ml; Binding Site Ltd., Birmingham, England) at 37 °C. Following a 15-min rinse in PBS containing 2% dry milk, and then in PBS, the wet slides were mounted in FA mounting fluid at pH 9 (Difco Laboratories, Detroit, MI). The slides were examined and photographed with Fujicolor Super HG 400 (ASA 400) film under a 100 × Olympus DApo UV lens, using an Olympus BHS-RFK fluorescence microscope with an Olympus PM-10ADS autophotography system.
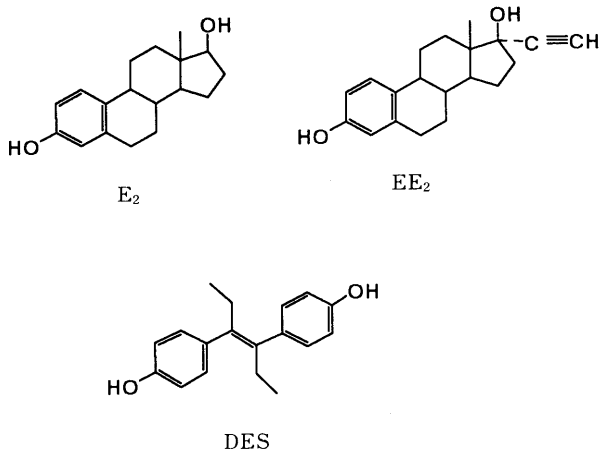

Chart 1

Results and Discussion

Cytotoxicities of E_2 and EE_2 in V79 Cells The cytotoxicity of E_2 and EE_2 in V79 cells was measured first by determining relative plating efficiency when the cells were treated with various concentrations of the drugs for 48 h.[9] As shown in Fig. 1, the IC_{50} (the concentration required for 50% inhibition of colony formation) was 11 μM for E_2, 8 μM for EE_2 and 13 μM for DES.

Dose Effects of E_2 and EE_2 on Aneuploidy and Chromosome Aberration The effects of E_2 and EE_2 on chromosome number in V79 cells were studied. V79 cells were incubated with various concentrations of E_2 and EE_2 for 48 h, then treated with colcemid for 2 h.[9] As summarized in Table I, both E_2 and EE_2 induced aneuploidy through chromosome loss and gain, where untreated V79 cells had 22 or 21 chromosomes, but these activities were lower than that of DES.

When the cells were treated with 20 μM DES, they had chromosomes close to the octaploid range (chromosome number > 80). However, when treated with 20 μM E_2, only 29% of the cells had chromosomes near the diploid range (chromosome number 18—26), and when treated with 30 μM E_2, 58% of cells had chromosomes near the tetraploid range (chromosome number 36—46) but none
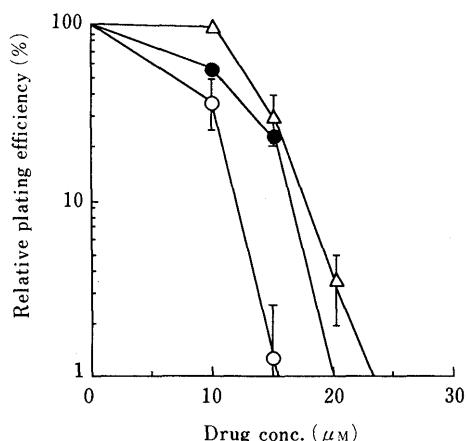

Fig. 1. Relative Plating Efficiencies of Chinese Hamster V79 Cells

Treated with E_2, EE_2 and DES for 48 h at various concentrations. ○, EE_2; ●, E_2; △, DES

near the octaploid range. When the cells were treated with 20 μM EE_2, 43% had chromosomes near the tetraploid range and 5% near the octaploid range.

Table I also shows chromosome aberration induced by E_2 and EE_2. The frequency of chromosome aberration in the EE_2-treated cells increased dose-dependently, but it was lower than that in DES-treated cells. The frequency in the E_2-treated cells was ever lower, and 20 μM E_2 did not induce any chromosome aberration.

Effects of E_2 and EE_2 on the Cellular Microtubule Network Next, the effects of E_2 and EE_2 on cytoplasmic microtubules were investigated. Interphase cells in unsynchronized cultures were examined by the indirect immunofluorescence method employing anti-β-tubulin antibody.[9] Cells treated with a high concentration (20 μM) of E_2 and EE_2 for 3 h changed their morphology to a round shape with accompanying abnormal architecture of the cytoplasmic microtubules (Fig. 2). We treated cells for 1 h with various concentrations (0.1—50 μM) of E_2 and EE_2, and the effects were evaluated by measuring the percentage of cells with a normal microtubule network in comparison with total cells (> 100) observed.[9] As shown in Fig. 3, E_2 and EE_2 dose-dependently decreased the percentages of cells with a normal microtubule network more effectively than DES. In particular, a marked difference between E_2 and EE_2 and DES with regard to microtubule-disruptive activity was observed at medium concentrations (1—10 μM). The EC_{50} (the concentration required for 50% disruption of the microtubule network) was 3.3 μM for E_2, 2.7 μM for EE_2, and 25 μM for DES. In untreated control cultures the proportion of normal cells was 90.0%.

In the present investigation we studied the effect of E_2 and EE_2 on aneuploidy, chromosome aberration and the microtubule network in Chinese hamster V79 cells, in comparison with those of DES.[9] The data for aneuploidy and chromosome aberration was consistent with our previous results using in vitro results,[4] which indicated that E_2 has no ability to interact with microtubules or microtubule protein from porcine brain. However, the disruptive effect on the microtubule network was not consistent with the in vitro effects. In connection with this data, Ravindra reported that E_2 inhibits tubulin-induced guanosine triphosphate (GTP) hydrolysis activity in a
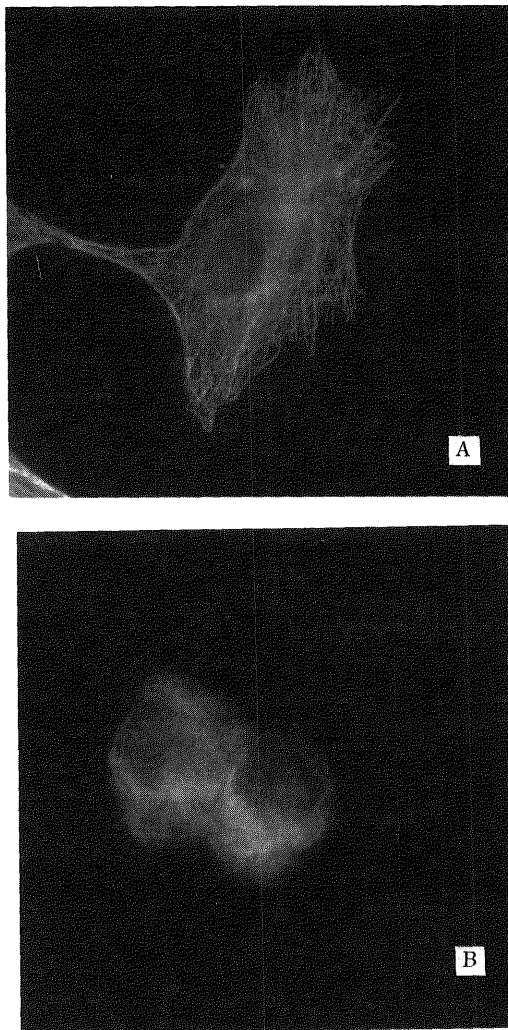
TABLE I. Chromosome Analysis of V79 Cells Treated with E_2 and EE_2

Drug	Conc. (μM)	% diploid cells	% heteroploid cells			Aberrant metaphases (%)	Type of aberration (%)								
			Near di-	Near tetra-	Near octa-		G	ICG	E	O	B	D	P	Ero	R
Control		95	5	0	0	0	0	0	0	0	0	0	0	0	0
E_2	10	94	5	1	0	0	0	0	0	0	0	0	0	0	0
	15	73	21	1	0	0	0	0	0	0	0	0	0	0	0
	20	69	29	2	0	0	0	0	0	0	0	0	0	0	0
	25	69	29	2	0	2	0	0	0	0	0	0	0	2	0
	30	11	31	58	0	28	2	2	0	0	0	10	8	9	1
EE_2	10	84	16	0	0	0	0	0	0	0	0	0	0	0	0
	15	37	54	9	0	6	2	2	0	0	0	2	0	2	0
	20	4	48	43	5	19	2	0	0	0	0	8	4	9	2
	25	9	29	54	8	30	3	2	0	2	3	10	8	9	1
DES	10	88	11	1	0	4	1	0	0	0	0	0	0	0	3
	15	59	26	15	0	9	2	0	0	0	0	1	2	0	5
	20	1	7	31	61	59	11	0	2	0	3	22	6	9	24

G, gap; ICG, isochromatid gap; E, exchange; O, O-ring; B, break; D, dicentric; P, pulverization; Ero, erosion; R, ruffling.

Fig. 2. Indirect Immunofluorescence Staining of Chinese Hamster V79 Cells with Anti-β-tubulin Antibody

A) Untreated cells in interphase with extensive microtubule network; cells treated for 3 h with (B) 20 μM E_2.
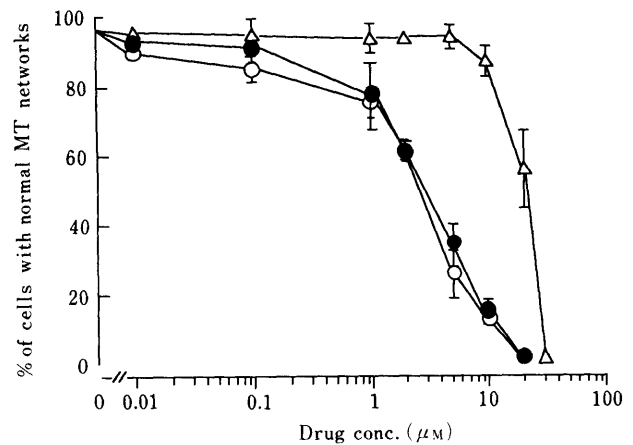

Fig. 3. Effects of E_2, EE_2 and DES on Microtubule Network in Chinese Hamster V79 Cells

Cells were treated with various concentrations of E_2 (●), EE_2 (○), or DES (△) for 1 h.

metabolize xenobiotics, the effects of metabolites would be neglected in this experiment. In the present study, however, 20 μM E_2 induced only a small amount of tetraploid cells after 48 h treatment, but completely disrupted the normal microtubule network after 3 h of treatment.

Although the relationship between microtubule function and carcinogenesis has not been clarified, the present results on microtubule-disruptive activity of naturally occuring estrogen E_2 would be important from the point of view of control of the cell cycle and also carcinogenesis.

dose-dependent manner, and also tubulin assembly *in vitro*.[11] This contradiction would be due to the difference in concentration of a GTP in a GTP-assembly buffer. Ravindra proved that at 25 μM GTP, E_2 inhibited tubulin assembly at 7—34 μM, but in our *in vitro* study, which used a higher concentration of GTP (1 mM), E_2 did not show an inhibitory activity for tubulin assembly. Although the detailed mechanism of GTP-promoted reversible self-assembly of tubulin into microtubules is not clarified, it is established that GTP is required for polymerization. Further, the concentration of guanine nucleotides in cells would make some contribution to their regulatory effect on microtubule formation. The regulatory effect of guanine nucleotides remains a major question although many studies have addressed it.[12] Inhibition of the microtubule assembly has been proposed as a possible inducer of aneuploidy.[13] However, these results, showing that E_2 had higher microtubule-disruptive activity but lower aneuploidy-inducing activity than DES, indicated that microtubule-disruptive activity could not easily be connected with aneuploidy. Recently, a metabolite of E_2 was shown to be extremely toxic regarding the division of MCF-7 and HeLa cells.[14] However, since V79 cells had no ability to

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