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

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The effects of estradiol (E2) and ethynylestradiol (EE2) on the chromosome number and cellular microtubule architecture of Chinese hamster V79 cells were studied using fluorescent anti-tubulin antibody. Treatment with 20 μM E2 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 E2 has higher microtubule-disruptive activity than diethylystilbestrol in V79 cells.

Keywords estrogen; 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 (E2; Chart 1) does not inhibit in vitro microtubule assembly in the same assay system.4) E2 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 (EE2; Chart 1), a semi-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system.8) and we have found that EE2 has lower inhibitory activity against in vitro microtubule assembly than does DES (unpublished data).

In the present study, we investigated the effects of E2 and EE2 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 coelemid 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% CO2/95% 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, E2, EE2 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 (per 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 × 105, 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, E2, EE2 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, coelemid (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 Sörensen 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 multistest 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 E2, EE2 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.3) Cells on slides were fixed for 1 min with 3% formaldehyde in PBS, permeabilized with cold methanol (−20°C) 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 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, (IgG)(2) (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 x Olympus DAPo UV lens, using an Olympus BHS-RFK fluorescence microscope with an Olympus PM-10ADS autophotography system.

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Results and Discussion

Cytotoxicities of E₂ and EE₂ in V79 Cells  The cytotoxicity of E₂ and EE₂ 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. As shown in Fig. 1, the IC₅₀ (the concentration required for 50% inhibition of colony formation) was 11 μM for E₂, 8 μM for EE₂, and 13 μM for DES.

Dose Effects of E₂ and EE₂ on Aneuploidy and Chromosome Aberration  The effects of E₂ and EE₂ on chromosome number in V79 cells were studied. V79 cells were incubated with various concentrations of E₂ and EE₂ for 48 h, then treated with colcemid for 2 h. As summarized in Table I, both E₂ and EE₂ 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 octoploid range (chromosome number > 80). However, when treated with 20 μM E₂, only 29% of the cells had chromosomes near the diploid range (chromosome number 18—26), and when treated with 30 μM EE₂, 58% of cells had chromosomes near the tetraploid range (chromosome number 36—46) but none near the octoploid range. When the cells were treated with 20 μM EE₂, 43% had chromosomes near the tetraploid range and 5% near the octoploid range.

Table I also shows chromosome aberration induced by E₂ and EE₂. The frequency of chromosome aberration in the EE₂-treated cells increased dose-dependently, but it was lower than that in DES-treated cells. The frequency in the E₂-treated cells was ever lower, and 20 μM E₂ did not induce any chromosome aberration.

Effects of E₂ and EE₂ on the Cellular Microtubule Network  Next, the effects of E₂ and EE₂ on cytoplasmic microtubules were investigated. Interphase cells in unsynchronized cultures were examined by the indirect immunofluorescence method employing anti-β-tubulin antibody. Cells treated with a high concentration (20 μM) of E₂ and EE₂ 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 E₂ and EE₂, and the effects were evaluated by measuring the percentage of cells with a normal microtubule network in comparison with total cells (>100) observed. As shown in Fig. 3, E₂ and EE₂ dose-dependently decreased the percentages of cells with a normal microtubule network more effectively than DES. In particular, a marked difference between E₂ and EE₂ and DES with regard to microtubule-disruptive activity was observed at medium concentrations (1—10 μM). The EC₅₀ (the concentration required for 50% disruption of the microtubule network) was 3.3 μM for E₂, 2.7 μM for EE₂, 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 E₂ and EE₂ on aneuploidy, chromosome aberration and the microtubule network in Chinese hamster V79 cells, in comparison with those of DES. The data for aneuploidy and chromosome aberration was consistent with our previous results using in vitro results, which indicated that E₂ 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 E₂ inhibits tubulin-induced guanosine triphosphate (GTP) hydrolysis activity in a

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### Table I. Chromosome Analysis of V79 Cells Treated with E₂ and EE₂

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. (μM)</th>
<th>% diploid cells</th>
<th>% heteroploid cells</th>
<th>Aberrant metaphases (%)</th>
<th>Type of aberration (%)</th>
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G, gap; IC, isochromatid gap; E, exchange; O, O-ring; B, break; D, dicentric; P, pulverization; Ero, erosion; R, ruffling.

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Fig. 1. Relative Plating Efficiencies of Chinese Hamster V79 Cells
Treated with E₂, EE₂ and DES for 48 h at various concentrations. ○, EE₂; ●, E₂; △, DES
dose-dependent manner, and also tubulin assembly in vitro. 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, E2 inhibited tubulin assembly at 7—34 μM, but in our in vitro study, which used a higher concentration of GTP (1 mM), E2 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. Inhibition of the microtubule assembly has been proposed as a possible inducer of aneuploidy. However, these results, showing that E2 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 E2 was shown to be extremely toxic regarding the division of MCF-7 and HeLa cells. However, since V79 cells had no ability to metabolize xenobiotics, the effects of metabolites would be neglected in this experiment. In the present study, however, 20 μM E2 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 occurring estrogen E2 would be important from the point of view of control of the cell cycle and also carcinogenesis.

References