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INVOLVEMENT OF GENOTOXIC EFFECTS IN THE INITIATION OF ESTROGEN-INDUCED CELLULAR TRANSFORMATION: STUDIES USING SYRIAN HAMSTER EMBRYO CELLS TREATED WITH 17β-ESTRADIOL AND EIGHT OF ITS METABOLITES

Takeki TSUTSUI[1], Yukiko TAMURA[1], Eiichi YAGI[1] and J. Carl BARRETT[2]*

[1]*Department of Pharmacology, The Nippon Dental University, Tokyo, Japan*
[2]*Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA*

To examine a direct involvement of genotoxic effects of estrogens in the initiation of hormonal carcinogenesis, the abilities of 17β-estradiol (E_2) and 8 of its metabolites to induce cellular transformation and genetic effects were studied using the Syrian hamster embryo (SHE) cell model. Treatment with E_2, estrone (E_1), 2-hydroxyestrone (2-OHE_1), 4-hydroxyestrone (4-OHE_1), 2-methoxyestrone (2-$MeOE_1$), 16α-hydroxyestrone (16α-OHE_1), 2-hydroxyestradiol (2-OHE_2), 4-hydroxyestradiol (4-OHE_2) or estriol (E_3) for 1 to 3 days inhibited SHE cell growth in a concentration-dependent manner. Concentration-dependent increases in the frequency of morphological transformation in SHE cells were exhibited by treatment for 48 hr with each of all estrogens examined, except for E_3. The transforming activities of the estrogens, determined by the induced transformation frequencies, were ranked as follows: 4-OHE_1 > 2-OHE_1 > 4-OHE_2 > 2-OHE_2 ≥ E_2 or E_1 > 2-$MeOE_1$ or 16α-OHE_1 > E_3. Somatic mutations in SHE cells at the Na^+/K^+ATPase and /or *hprt* loci were induced only when the cells were treated with 4-OHE_1, 2-$MeOE_1$ or 4-OHE_2 for 48 hr. Some estrogen metabolites induced chromosome aberrations in SHE cells following treatment for 24 hr. The rank order of the clastogenic activities of the estrogens that induced chromosome aberrations was 4-OHE_1 > 2-OHE_1 or 4-OHE_2 > 2-OHE_2 > E_1. Significant increases in the percentage of aneuploid cells in the near diploid range were exhibited in SHE cells treated for 48 hr or 72 hr with each of the estrogens, except for 4-OHE_1 and E_3. Our results indicate that the transforming activities of all estrogens tested correspond to at least one of the genotoxic effects by each estrogen, *i.e.,* chromosome aberrations, aneuploidy or gene mutations, suggesting the possible involvement of genotoxicity in the initiation of estrogen-induced carcinogenesis. *Int. J. Cancer* 86:8–14, 2000.
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Estrogens are carcinogenic in humans and rodents (IARC, 1979), but the mechanisms by which these hormones induce cancer are not fully elucidated. 17β-estradiol (E_2) and its metabolites are carcinogenic in animals (IARC, 1979). E_2 undergoes oxidative metabolism at C-17 to yield estrone (E_1), which can be hydroxylated to yield 16α-hydroxyestrone (16α-OHE_1) and catechol estrogens [2-hydroxyestrone (2-OHE_1) and 4-hydroxyestrone (4-OHE_1)]. 16α-OHE_1 is metabolized to estriol (E_3) by 17β-hydroxysteroid dehydrogenase. E_2 also can be hydroxylated to 2-hydroxyestradiol (2-OHE_2) or 4-hydroxyestradiol (4-OHE_2). These catechol estrogens are mainly inactivated by *O*-methylation catalyzed by catechol-*O*-methyltransferase (reviewed by Zhu and Conney, 1998*a*), but conjugative metabolism by glucuronidation and/or sulfonation also plays a role in conversion of the catechol estrogens to hormonally inactive metabolites (reviewed by Zhu and Conney, 1998*a*).

Recently endogenous estrogens have been implicated as a possible etiological factor in the causation of certain types of human cancers such as breast, endometrium, ovary, prostate and, possibly, brain cancers (reviewed by Zhu and Conney, 1998*a*). In particular, 4-hydroxy catechol estrogens (4-OHE_1 and 4-OHE_2) are considered critical intermediates in estrogen-induced cancers because they are oxidized by metabolic redox cycling to catechol estrogen-3,4-quinones (CE-3,4-Q) that covalently bind to DNA and form depurinating adducts. The resultant apurinic sites in critical genes may generate mutations that initiate cancer (Cavalieri *et al.,* 1997).

The cytochrome P450 enzymes (CYP) play a major role in estrogen metabolism. Estrogen 4-hydroxylation is catalyzed primarily by CYP1B1, which is found in several tissues including mammary, ovary and adrenal (reviewed by Zhu and Conney, 1998*a*). *CYP1B1* gene expression is induced by xenobiotics such as polycyclic aromatic hydrocarbons and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (reviewed by Zhu and Conney, 1998*a*). In human breast epithelial cell lines and human breast tumor cell lines, the *CYP1B1* gene expression is stimulated by TCDD, and E_2 4-hydroxylase activities in the cell lines accompany *CYP1B1* expression (reviewed by Zhu and Conney, 1998*a*). If the catechol estrogen metabolism is increased by CYP1B1 induction, and/or its metabolic clearance is decreased by inhibition of catechol estrogen *O*-methylation, 4-hydroxy catechol estrogens may accumulate in target cells. The presence of increased amounts of 4-hydroxy catechol estrogens can subsequently lead to increased formation of CE-3,4-Q.

There is strong evidence that estrogens are epigenetic carcinogens, acting via a promoting effect related to cellular proliferation, mediated through the estrogen receptor (Yager and Yager, 1980). However, it has been shown that estrogenic activity is not sufficient to explain the carcinogenic activity *in vivo* and *in vitro* under certain experimental conditions because not all estrogens are carcinogenic (reviewed by Tsutsui and Barrett, 1997). Another mechanism, related to genetic alterations, has been suggested in studies of estrogen-induced carcinogenesis (reviewed by Tsutsui and Barrett, 1997). The application of cell cultures to study carcinogenic mechanisms of chemical/physical carcinogens can provide insights into the cellular and molecular mechanisms of carcinogenesis, which is difficult in whole-animal or human systems. We have used Syrian hamster embryo (SHE) fibroblast cell cultures as a model system to study the ability of estrogens to transform cells directly (reviewed by Tsutsui and Barrett, 1997). An advantage of the SHE cell model for studies of carcinogenesis is that cellular transformation and genetic effects can be measured in the same target cells (Barrett *et al.,* 1978). SHE cells do not express measurable levels of estrogen receptor, and estrogen treatment is not mitogenic to the cells (reviewed by Tsutsui and Barrett, 1997). Thus, estrogenic stimulation of cell proliferation can be excluded as the mechanism of action in this *in vitro* assay. The cells do, however, have the ability to metabolize estrogens (reviewed by Tsutsui and Barrett, 1997).

We have shown that treatment of SHE cells with diethylstilbestrol (DES), a synthetic estrogen, or E_2 induces cell transformation without measurable gene mutations, unscheduled DNA synthesis (UDS) or structural chromosome aberrations (reviewed by Tsutsui

*Correspondence to: Laboratory of Molecular Carcinogenesis, NIEHS, MD C2-15, Research Triangle Park, NC 27709, USA. Fax: +1 919 541-7784. E-mail: barrett@niehs.nih.gov

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and Barrett, 1997). Under the same conditions, both estrogens induce a specific type of genetic change, *i.e.*, aneuploidy. Chromosome losses and gains are induced (reviewed by Tsutsui and Barrett, 1997), suggesting a nondisjunctional mechanism involved in the transforming activity. DES and E_2 bind and disrupt polymerization of microtubules in cultured mammalian cells (reviewed by Tsutsui and Barrett, 1997). However, estrogens and their metabolites induce DNA adduct formation in SHE cells, corresponding to the induction of cellular transformation (reviewed by Tsutsui and Barrett, 1997). In addition, treatment of SHE cells with DES in the presence of an exogenous metabolic activation system enhances the frequency of morphological transformation of the cells. This treatment elicits DNA damage (determined by UDS) and gene mutations in the cells at the Na^+/K^+ATPase locus (reviewed by Tsutsui and Barrett, 1997). Thus, we have proposed 2 potential mechanisms for estrogen-induced cellular transformation; in one the target of the estrogen is not DNA but rather microtubule disruption and the other is associated with DNA damage (reviewed by Tsutsui and Barrett, 1997). Although it has not been clear which cytogenetic endpoints are more correlated on a causal basis with the estrogen-induced transformation, our studies suggest that estrogens have the ability to transform cells directly and that multiple effects of estrogens act together to cause multiple types of genetic alterations leading to cellular transformation.

In the present study, we examined a direct involvement of genotoxic effects of estrogens in the initiation of carcinogenesis. The abilities of E_2 and 8 of its metabolites to induce cellular transformation and genetic effects were studied simultaneously using the SHE cell model. The results obtained indicate that the transforming activities of all estrogens tested correspond to at least one of the genotoxic effects by each estrogen, *i.e.*, chromosome aberrations, aneuploidy or gene mutations, suggesting the involvement of genotoxicity in the initiation of estrogen-induced carcinogenesis.

MATERIAL AND METHODS

Cells and chemicals

SHE cell cultures were established from 13-day-gestation hamster fetuses and grown as previously described (Barrett *et al.*, 1978). E_2, estrone (E_1), 2-hydroxyestrone (2-OHE_1), 4-hydroxyestrone (4-OHE_1), 2-methoxyestrone (2-$MeOE_1$), 16α-hydroxyestrone (16α-OHE_1), 2-hydroxyestradiol (2-OHE_2), 4-hydroxyestradiol (4-OHE_2) and estriol (E_3) were purchased from Sigma (St. Louis, MO) and dissolved with DMSO at 3 mg/ml. DMSO was added to control cultures at a final concentration of 0.33%. 6-Thioguanine (TG) (Sigma), ouabain (Oua) (Sigma) and benzo[α]pyrene (B[α]P) (Aldrich, Milwaukee, WI) were obtained from the indicated sources.

Growth curve

Cells (3×10^4) in logarithmic growth phase were plated on 35 mm dishes (Falcon, Oxnard, CA). After overnight incubation, the cells were treated with test estrogens at various concentrations for 1 to 3 days. The number of cells per 35-mm dish was determined after trypsinization. Cell counts are presented as mean ± SD from 4 dishes per counting point (1, 2 and 3 days after start of treatment).

Cellular transformation and somatic mutations

Cells (2.5×10^5) were plated into 75-cm^2 flasks (Falcon), incubated overnight and treated with test estrogens for 48 hr. After trypsinization, a part of the cell suspension was assayed for morphological transformation, and the remaining cells were subcultured at the density of 4.0×10^5 cells/75 cm^2 flask for mutation experiments. For morphological transformation, 2,000 cells were replated on 100-mm dishes (20 dishes for each group) and incubated for 7 days to form colonies. The cells were fixed with absolute methanol and stained with a 10% aqueous Giemsa solution. The number of surviving colonies with > 50 cells and morphologically transformed colonies were scored using previously established criteria (Barrett *et al.*, 1978). For mutation experiments, the cells were grown for an expression time of 4 days, then 10^5 cells were plated on 100-mm dishes (10 dishes for each group) with medium containing 18 μM TG or 1.1 mM Oua and incubated 7 days for colony formation (Barrett *et al.*, 1978). The mutation frequency was calculated as described previously (Barrett *et al.*, 1978).

Chromosome aberrations and chromosome number

SHE cells were plated into 75-cm^2 flasks at 1.3×10^5 cells/flask for the 72-hr group, 2.5×10^5 cells/flask for the 48-hr treatment group and 5.0×10^5 cells/flask for the 24-hr treatment group. The 24-hr treatment group was used for scoring chromosome aberrations, and the 48-hr and 72-hr treatment groups were used for scoring chromosome number. After overnight incubation, the cells were treated with test estrogens for 24 to 72 hr. Three hours before the end of the treatment time, Colcemid (GIBCO, Grand Island, NY) was administered at 0.2 μg/ml, and metaphase chromosomes were prepared. After trypsinization, cells were treated with 0.9% sodium citrate at room temperature for 13 min, fixed in Carnoy's solution (methanol:acetic acid, 3:1) and spread on glass slides using the air-drying method. Specimens were stained with a 3% Giemsa solution in 0.07 M phosphate buffer (pH 6.8) for 7 min. For determination of both chromosome aberrations (gaps, breaks, exchanges, dicentric chromosomes, ring chromosomes and fragmentations) and chromosome number, 100 metaphases per experimental group were scored. Achromatic lesions greater than the width of the chromatid were scored as gaps unless there was displacement of the broken piece of chromatid. If there was displacement, these were recorded as breaks.

RESULTS

The effects on cellular growth of treatment with varying concentrations of E_2 and 8 of its metabolites for 1 to 3 days were examined. No growth-stimulating activities were observed in SHE cells treated with the estrogens tested. Treatment of SHE cells with E_2 at 1 or 3 μg/ml did not affect cell growth. However, growth was markedly reduced by treatment with E_2 at 10 μg/ml (Fig. 1*a*). Treatment of cells with E_1 at ≤ 3 μg/ml also had little effect on the cellular growth. A slight decrease in cellular growth was observed in cells treated with E_1 at 10 μg/ml (Fig. 1*b*). On the other hand, treatment with 2-OHE_1 at 1 μg/ml caused a decrease in cellular growth, and the number of cells were markedly decreased by treatment with 2-OHE_1 at 3 μg/ml. Similar results were observed in cells treated with 4-OHE_1 (Fig. 1*c*,*d*). When SHE cells were treated with 2-$MeOE_1$, a slight decrease in the cellular growth was induced by treatment at 1 or 3 μg/ml. In cultures treated with 10 μg/ml 2-$MeOE_1$, the number of cells were maintained at similar levels to those at the beginning of the treatment for the first 2 days but subsequently decreased (Fig. 1*e*). Treatment of cells with 16α-OHE_1 at 1–10 μg/ml had little effect on cell growth (Fig. 1*f*). When SHE cells were treated with 2-OHE_2 or 4-OHE_2 at 1–10 μg/ml, cell growth was reduced in a concentration-related fashion, and the effect of 2-OHE_2 was stronger than that of 4-OHE_2 (Fig. 1*g*,*h*). Little effect on cell growth was induced in SHE cells treated with E_3 at 1–10 μg/ml (Fig. 1*i*). The rank order of the cytotoxic effect of each estrogen, determined by the growth curve, was 2- or 4-OHE_1 or 2-OHE_2 $>$ 4-OHE_2 $>$ 2-$MeOE_1$ $\geq$ E_2 $>$ E_1, 16α-OHE_1 or E_3.

Colony-forming efficiencies (CFEs) and frequencies of morphological transformation of SHE cells following treatment with test estrogens for 48 hr are shown in Table I. Treatment of SHE cells with 4-OHE_1 at 0.3 μg/ml and 2-$MeOE_1$ at 1 or 3 μg/ml increased the CFEs of the cells over the control level. However, exposure to E_2, 2-OHE_1, 2-$MeOE_1$ or 2-OHE_2 at the highest concentrations examined decreased the CFEs to 18% to 36% relative to control cells.

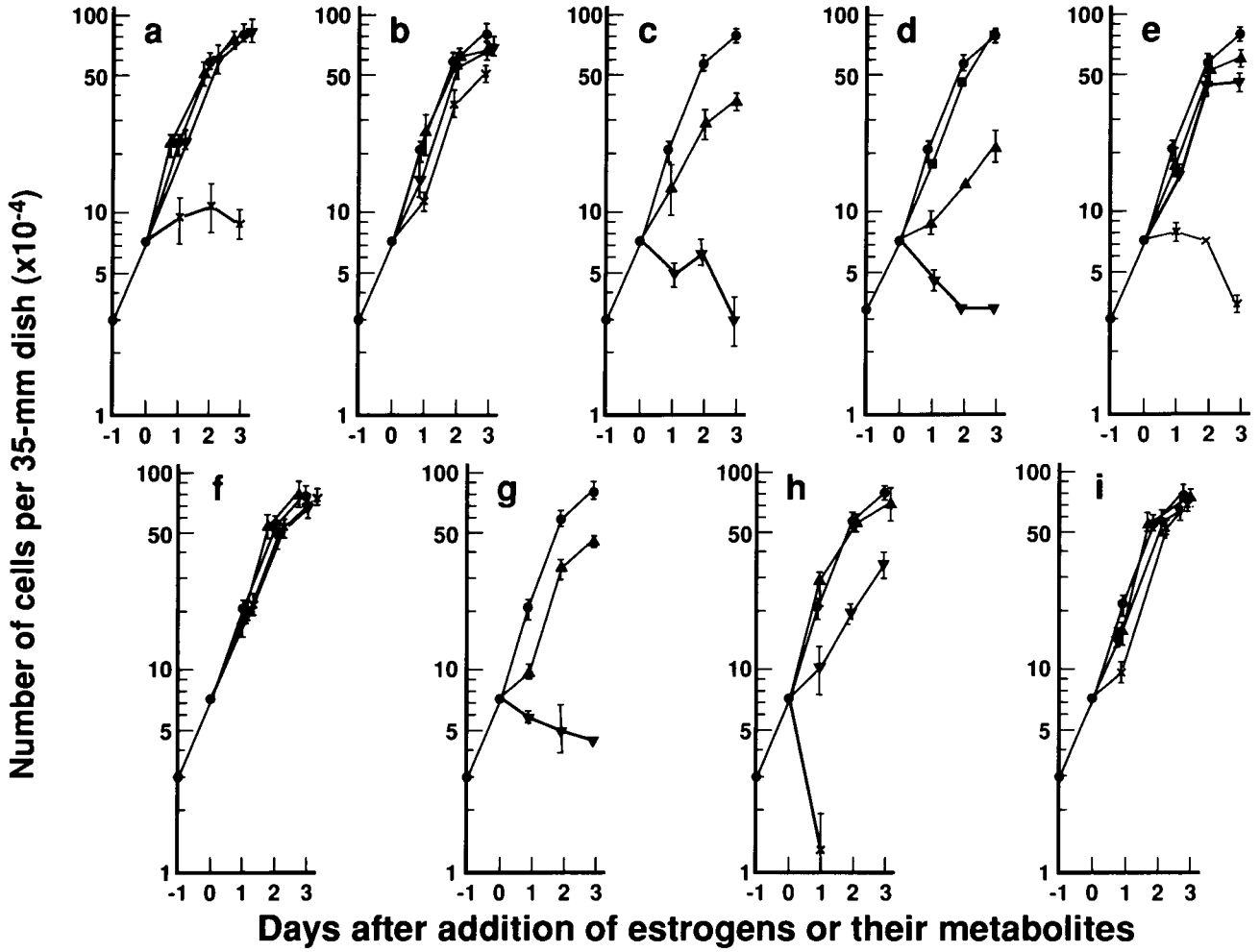

FIGURE 1 – Growth of SHE cells treated with E_2 or its metabolites. SHE cells were plated in quadruplicate on 35-mm dishes at a density of 3×10^4 cells/dish. After overnight incubation, cells were treated with (*a*) E_2, (*b*) E_1, (*c*) 2-OHE_1, (*d*) 4-OHE_1, (*e*) 2-$MeOE_1$, (*f*) 16α-OHE_1, (*g*) 2-OHE_2, (*h*) 4-OHE_2 or (*i*) E_3 at the concentrations of 0 (●), 0.3 (■), 1 (▲), 3 (▼) and 10 (×) μg/ml for 1, 2 and 3 days. Bars denote SD. When not indicated, SD are within symbols.

Morphological transformation in SHE cells was induced in a concentration-related manner by all estrogens examined, except for E_3. Morphologically transformed colonies induced by the estrogens were indistinguishable from those induced by B[α]P and other chemical carcinogens including DES (Barrett *et al.*, 1978; reviewed by Tsutsui and Barrett, 1997). The transforming activity (determined by the transformation frequencies) of E_1 was similar to that of E_2. Although 2-OHE_2 exhibited transforming activity similar or greater than E_1 or E_2, the other 3 catechol estrogens, 2-OHE_1, 4-OHE_1 and 4-OHE_2, showed activities higher than E_1 or E_2. The transforming activities of the 4 catechol estrogens tested were ranked as follows: 4-OHE_1 > 2-OHE_1 > 4-OHE_2 > 2-OHE_2. The transforming activities of 2-$MeOE_1$ and 16α-OHE_1 were lower than that of E_2.

Treatment of SHE cells with 4-OHE_1, 2-$MeOE_1$ or 4-OHE_2 induced statistically significant increases in the somatic mutation frequencies at the Na^+/K^+ATPase and/or *hprt* loci in a concentration-dependent manner. When SHE cells were treated with 2-OHE_2, only cells treated at 1 μg/ml exhibited a statistically significant increase in the mutation frequency at the Na^+/K^+ATPase locus. No significant increases in the mutation frequencies were observed in cells treated with any of the other estrogens examined (Table II).

To examine the ability of the estrogens to induce chromosomal abnormalities, SHE cells treated with the estrogens were analyzed for structural and numerical abnormalities of chromosomes. In control cultures, 1% of metaphase contained chromatid gaps. Although treatment for 24 hr of SHE cells with E_2, 2-$MeOE_1$, 16α-OHE_1 or E_3 at the concentrations examined did not induce a significant increase in the frequencies of chromosome aberrations, exposure to E_1, 2-OHE_1, 4-OHE_1, 2-OHE_2 or 4-OHE_2 at the highest concentrations tested elicited chromosome aberrations of the cells significantly (Table III). The rank of the inducibilities was as follows: 4-OHE_1 > 2-OHE_1 or 4-OHE_2 > 2-OHE_2 > E_1.

The effects of the estrogens on the induction of aneuploidy was measured by determining the chromosome number in metaphase cells 48 or 72 hr after the start of treatment. As shown in Table IV, the majority (92% or 93%) of metaphases from control cells had a diploid ($2n = 44$) number of chromosomes, and 6% or 7% was in the tetraploid and near tetraploid ranges (80–90 chromosomes). A small percentage (1%) of metaphases in the control cells had a near diploid number of chromosomes. Induction of aneuploidy in the near diploid range was elicited in cells treated with E_2 at 1 or 10 μg/ml for 48 hr. Statistically significant increases in the percentage of aneuploid cells in the near diploid range were induced by all estrogens examined, except for 4-OHE_1 and E_3. The percentage of

TABLE I – MORPHOLOGICAL TRANSFORMATION OF SHE CELLS TREATED WITH 17β-ESTRADIOL OR ITS METABOLITES

Chemical	Dose (μg/ml)	Relative cell survival (%)	Number of transformed colonies per number of colonies scored	% transformation
Control (DMSO)	0	100	0/9891[1]	0
Estradiol (E_2)	1	104	15/10,292[1]	0.15[3]
	3	90	22/8,894[1]	0.25[3]
	10	36	17/3,547[1]	0.48[3]
Estrone (E_1)	1	103	12/10,148[1]	0.12[3]
	3	108	32/10,684[1]	0.30[3]
	10	102	33/10,067[1]	0.33[3]
2-Hydroxyestrone (2-OHE_1)	0.3	107	13/10,551[1]	0.12[3]
	1	95	30/9,399[1]	0.32[3]
	3	29	19/2,844[1]	0.67[3]
4-Hydroxyestrone (4-OHE_1)	0.3	91	14/5,670[1]	0.25[3]
	1	65	37/4,039[1]	0.92[3]
	3	43	38/2,673[1]	1.42[3]
2-Methoxyestrone (2-$MeOE_1$)	1	79	7/7,771[1]	0.09[3]
	3	85	10/8,380[1]	0.12[3]
	10	8	2/791[1]	0.25[3]
16α-Hydroxyestrone (16α-OHE_1)	1	104	5/5,210	0.10
	3	113	14/11,145[1]	0.13[3]
	10	89	22/8,792[1]	0.25[3]
2-Hydroxyestradiol (2-OHE_2)	0.3	101	7/4,914	0.14[2]
	1	77	7/3,751	0.19[3]
	3	26	4/1,255	0.32[3]
4-Hydroxyestradiol (4-OHE_2)	0.3	103	10/5,025	0.20[3]
	1	95	13/4,606	0.28[3]
	3	94	16/4,585	0.35[3]
Estriol (E_3)	1	88	2/4,306	0.05
	3	98	0/4,785	0
	10	96	2/4,701	0.04

[1]Data compiled from 2 independent experiments.–[2]Statistically different from control ($p < 0.05$ by χ^2 test).–[3]Statistically different from control ($p < 0.01$ by χ^2 test).

aneuploid cells in the tetraploid and near tetraploid cells was significantly increased when SHE cells were treated with E_2, 2-OHE_1, 4-OHE_1, 2-$MeOE_1$, 2-OHE_2 or 4-OHE_2 for 48 or 72 hr (Table IV). The level of aneuploidy induction both in the near diploid range and in the tetraploid and near tetraploid ranges varied with each estrogen examined. In the near diploid range, high inducibility was observed in cells treated with E_2 or 2-$MeOE_1$, and intermediate or low inducibility was exhibited in cells treated with 2-OHE_2 or in cells treated with E_1, 2-OHE_1, 16α-OHE_1 or 4-OHE_2, respectively. Although not statistically significant, a concentration-dependent increase in the aneuploid cells in the near diploid range was observed in cells treated with 4-OHE_1. In the tetraploid and near tetraploid ranges, high inducibility was demonstrated in cells treated with 2-OHE_1 or 2-$MeOE_1$, and intermediate or low inducibility was observed in cells treated with 4-OHE_1 or in cells treated with E_2, 2-OHE_2 or 4-OHE_2, respectively.

DISCUSSION

In the present study, we examined the abilities of E_2 and 8 of its metabolites to induce cellular transformation and genetic effects using the SHE cell model to investigate a direct involvement of genotoxic effects of the estrogens in the initiation of hormonal carcinogenesis. No growth-stimulating activities were observed in SHE cells treated with any of the estrogens tested, which is consistent with the findings that estrogen treatment is not mitogenic to SHE cells lacking estrogen-receptor expression (reviewed by Tsutsui and Barrett, 1997). Morphological transformation was induced in a concentration-dependent manner in cells treated with all estrogens examined, except for E_3. The morphological transformation induced by these estrogens was stable because the cells were treated and the chemicals removed for one week before the transformed colonies were scored. In addition, the correlation between morphological and neoplastic transformations is well demonstrated in SHE cells induced by chemical carcinogens including DES (Barrett *et al.,* 1978; reviewed by Tsutsui and Barrett, 1997). These findings indicate that the estrogens tested have the ability to exert directly a heritable change in the cellular phenotype that initiates the cells to lead to neoplastic transformation.

The transforming activities induced by the estrogen and its metabolites varied with the following rank order: 4-OHE_1 > 2-OHE_1 > 4-OHE_2 > 2-OHE_2 ≥ E_2 or E_1 > 2-$MeOE_1$ or 16α-OHE_1 > E_3. This indicates that catechol estrogens, except for 2-OHE_2, have higher transforming activities than E_2 or other E_2 metabolites in this model. In particular, the transforming activities of catechol estrogens of E_1 were higher than the catechol derivatives of E_2. Moreover, 4-OHE_1 had high transforming activity compared with 2-OHE_1. The same difference was observed between 4-OHE_2 and 2-OHE_2. Because the differences in the transforming activities corresponded to the abilities of these catechol estrogens to induce chromosome aberrations and gene mutations in SHE cells (Table V), it is suggested that genetic damage at the gene and/or chromosome levels acts as one of the causal mechanisms of catechol estrogen-induced cellular transformation in SHE cells. However, it is not clear whether the difference in the transforming activities and the genetic effects of the catechol estrogens is attributed to the difference in the intrinsic feature or the metabolic fate of the chemicals. In the hamster kidney tumor model, 4-hydroxy catechol estrogens are carcinogenic, whereas 2-hydroxy catechol estrogens are not (reviewed by Roy and Liehr, 1999). This may come from the differences in the hormonal activity and/or the metabolic fate of both hydroxy catechol estrogens (reviewed by Roy and Liehr, 1999). Liehr and his collaborators demonstrated that hamster kidney contains high estrogen 4-hydroxylase activity (reviewed by Roy and Liehr, 1999) and that the methylation of 4-OHE_2 by catechol-*O*-methyltransferase is inhibited by 2-OHE_2 *in vitro* (reviewed by Roy and Liehr, 1999). This may facilitate accumulation of 4-OHE_2 in the hamster kidney. However, the lack of carcinogenic activity of 2-OHE_2 can also be due to rapid methylation and rapid metabolic clearance of 2-OHE_2 itself (reviewed by Roy and Liehr, 1999). Our results show that direct treatment of SHE cells with 2-hydroxy catechol estrogens (2-OHE_1 and 2-OHE_2) induces morphological transformation in the cells, suggesting an intrinsic carcinogenic and mutagenic ac-

TABLE II – INDUCTION OF SOMATIC MUTATIONS IN SHE CELLS BY 17β-ESTRADIOL AND ITS METABOLITES

Chemical	Dose (μg/ml)	Number of mutant colonies/number of cells assayed		Specific locus mutation frequency	
		Oua^r	TG^r	Oua^r	TG^r
Control (DMSO)	0	$1/1.7 \times 10^7$	$4/1.7 \times 10^7$	3.3×10^{-7}	1.2×10^{-6}
Estradiol (E_2)	1	$0/3 \times 10^6$	$0/3 \times 10^6$	$<3.0 \times 10^{-6}$	$<3.0 \times 10^{-6}$
	3	$0/3 \times 10^6$	$0/3 \times 10^6$	$<3.0 \times 10^{-6}$	$<3.0 \times 10^{-6}$
	10	$0/3 \times 10^6$	$0/3 \times 10^6$	$<3.0 \times 10^{-6}$	$<3.0 \times 10^{-6}$
Estrone (E_1)	1	$0/3 \times 10^6$	$0/3 \times 10^6$	$<3.0 \times 10^{-6}$	$<3.0 \times 10^{-6}$
	3	$0/3 \times 10^6$	$1/3 \times 10^6$	$<3.0 \times 10^{-6}$	1.7×10^{-6}
	10	$1/3 \times 10^6$	$0/3 \times 10^6$	1.9×10^{-6}	$<3.0 \times 10^{-6}$
2-Hydroxyestrone (2-OHE_1)	0.3	$0/3 \times 10^6$	$2/3 \times 10^6$	$<3.0 \times 10^{-6}$	3.3×10^{-6}
	1	$0/3 \times 10^6$	$0/3 \times 10^6$	$<3.0 \times 10^{-6}$	$<3.0 \times 10^{-6}$
	3	$0/3 \times 10^6$	$0/3 \times 10^6$	$<3.0 \times 10^{-6}$	$<3.0 \times 10^{-6}$
4-Hydroxyestrone (4-OHE_1)	0.3	$0/2 \times 10^6$	$2/2 \times 10^6$	$<2.0 \times 10^{-6}$	5.0×10^{-6}
	1	$0/2 \times 10^6$	$10/2 \times 10^6$	$<2.0 \times 10^{-6}$	2.5×10^{-5}[2]
	3	$1/2 \times 10^6$	$10/2 \times 10^6$	2.8×10^{-6}[1]	2.5×10^{-5}[2]
2-Methoxyestrone (2-$MeOE_1$)	1	$3/3 \times 10^6$	$1/3 \times 10^6$	5.6×10^{-6}[2]	1.7×10^{-6}
	3	$0/4 \times 10^6$	$7/3 \times 10^6$	$<4.0 \times 10^{-6}$	1.2×10^{-5}[2]
	10	$11/3 \times 10^6$	$4/4 \times 10^6$	2.1×10^{-5}[2]	5.0×10^{-6}
16α-Hydroxyestrone (16α-OHE_1)	1	$1/3 \times 10^6$	$1/4 \times 10^6$	1.9×10^{-6}	1.3×10^{-6}
	3	$1/3 \times 10^6$	$3/4 \times 10^6$	1.9×10^{-6}	3.8×10^{-6}
	10	$1/3 \times 10^6$	$2/4 \times 10^6$	1.9×10^{-6}	2.5×10^{-6}
2-Hydroxyestradiol (2-OHE_2)	0.3	$1/3 \times 10^6$	$2/3 \times 10^6$	1.9×10^{-6}	3.3×10^{-6}
	1	$3/3 \times 10^6$	$0/4 \times 10^6$	5.6×10^{-6}[2]	$<4.0 \times 10^{-6}$
	3	$0/4 \times 10^6$	$0/4 \times 10^6$	$<4.0 \times 10^{-6}$	$<4.0 \times 10^{-6}$
4-Hydroxyestradiol (4-OHE_2)	0.3	$0/3 \times 10^6$	$2/3 \times 10^6$	$<3.0 \times 10^{-6}$	3.3×10^{-6}
	1	$2/3 \times 10^6$	$4/3 \times 10^6$	3.7×10^{-6}[2]	6.7×10^{-6}
	3	$8/3 \times 10^6$	$0/4 \times 10^6$	1.5×10^{-5}[2]	$<4.0 \times 10^{-6}$
Estriol (E_3)	1	$0/2 \times 10^6$	$0/2 \times 10^6$	$<2.0 \times 10^{-6}$	$<2.0 \times 10^{-6}$
	3	$0/2 \times 10^6$	$0/2 \times 10^6$	$<2.0 \times 10^{-6}$	$<2.0 \times 10^{-6}$
	10	$0/2 \times 10^6$	$0/2 \times 10^6$	$<2.0 \times 10^{-6}$	$<2.0 \times 10^{-6}$
Benzo[α]-pyrene (B[α]P)	1	$13/2 \times 10^6$	$11/2 \times 10^6$	3.6×10^{-5}[2]	2.8×10^{-5}[2]

[1]Statistically different from control ($p < 0.05$ by χ^2 test).–[2]Statistically different from control ($p < 0.01$ by χ^2 test).

TABLE III – CHROMOSOME ABERRATIONS IN SHE CELLS TREATED WITH 17β-ESTRADIOL OR ITS METABOLITES FOR 24 HR

Estrogen	Dose (μg/ml)	Type of aberrations[1] (%)						Aberrant metaphases (%)
		G	B	E	D	R	F	
Control (DMSO)	0	1	0	0	0	0	0	1.0
Estradiol (E_2)	1	2	0	0	0	0	0	2.0
	3	3	0	0	0	0	0	3.0
	10	1	0	0	0	0	0	1.0
Estrone (E_1)	1	1	0	0	0	0	0	1.0
	3	3	2	0	0	0	0	5.0
	10	3	5	2	0	0	0	10.0[2]
2-Hydroxyestrone (2-OHE_1)	0.3	2	0	0	0	0	0	2.0
	1	5	0	0	0	0	0	5.0
	3	10	14	1	0	0	0	22.0[3]
4-Hydroxyestrone (4-OHE_1)	0.3	1	3	0	0	0	0	4.0
	1	2	5	0	0	0	0	7.0
	3	17	26	7	4	0	0	36.0[3]
2-Methoxyestrone (2-$MeOE_1$)	1	2	0	0	0	0	0	2.0
	3	1	0	0	0	0	0	1.0
	10	1	1	0	0	0	0	2.0
16α-Hydroxyestrone (16α-OHE_1)	1	1	0	0	0	0	0	1.0
	3	1	0	0	0	0	0	1.0
	10	1	2	0	0	0	0	3.0
2-Hydroxyestradiol (2-OHE_2)	0.3	4	2	0	0	0	0	6.0
	1	4	3	0	0	0	0	7.0
	3	9	6	2	0	0	0	17.0[3]
4-Hydroxyestradiol (4-OHE_2)	0.3	1	1	0	0	0	0	2.0
	1	2	4	0	0	0	0	6.0
	3	10	12	1	0	0	0	21.0[3]
Estriol (E_3)	1	4	0	0	0	0	0	4.0
	3	1	0	0	0	0	0	1.0
	10	1	2	0	0	0	0	3.0

[1]G: gaps; B: breaks; E: exchanges; D: dicentric chromosomes; R: ring chromosomes; F: fragmentations.–[2]Statistically different from control ($p < 0.05$ by χ^2 test).–[3]Statistically different from control ($p < 0.01$ by χ^2 test).

tivity of 2-hydroxy catechol estrogens. Because SHE cells have endogenous metabolizing enzymes that exhibit oxidative and peroxidative activities (reviewed by Tsutsui and Barrett, 1997), the catechol estrogens could undergo metabolic conversion in SHE cells to reactive intermediates such as quinone estrogens (CE-2,3-Q and CE-3,4-Q) that can form DNA adducts (Cavalieri *et al.*, 1997).

TABLE IV – CHROMOSOME NUMBER DISTRIBUTION OF SHE CELLS TREATED WITH 17β-ESTRADIOL OR ITS METABOLITES

Chemical	Treatment time (hr)	Dose (μg/ml)	Number of chromosomes per metaphase												% diploid cells	% aneuploid cells	
			37	38	39	40	41	42	43	44	45	46	47–55	80–90		Tetraploid and near tetraploid	Near diploid
Control (DMSO)	48	0							1	92				7	92.0	7.0	1.0
	72	0								93	1			6	93.0	6.0	1.0
Estradiol (E_2)	48	1							5	84	5			6	84.0	6.0	10.0[1]
		10			1		1	1	13	45	11	7	3	18	45.0	18.0[1]	37.0[3]
Estrone (E_1)	48	1							2	88	2			8	88.0	8.0	4.0
		3							1	91	1			7	91.0	7.0	2.0
		10							3	83	4			10	83.0	10.0	7.0
		30						2	4	79	1	1		13	79.0	13.0	8.0[1]
2-Hydroxyestrone (2-OHE_1)	48	0.3							1	89	1			9	89.0	9.0	2.0
		1							2	71	2			25	71.0	25.0[2]	4.0
		3	Few metaphases														
	72	0.3						1	1	72	3	1	1	21	72.0	21.0[2]	7.0
		1						1	4	67	3			25	67.0	25.0[3]	8.0[1]
		3	Few metaphases														
4-Hydroxyestrone (4-OHE_1)	48	0.3								87	3			10	87.0	10.0	3.0
		1							1	83	3			13	83.0	13.0	4.0
		3							1	74	1	2		22	74.0	22.0[2]	4.0
	72	0.3							1	82	5			12	82.0	12.0	6.0
		1							4	76	2			18	76.0	18.0[1]	6.0
		3	Few metaphases														
2-Methoxyestrone (2-$MeOE_1$)	48	1								94	2			4	94.0	4.0	2.0
		3								92	4			4	92.0	4.0	4.0
		10						2	2	45	16	3	4	28	45.0	28.0[3]	27.0[3]
16α-Hydroxyestrone (16α-OHE_1)	48	1							2	92				6	92.0	6.0	2.0
		3							1	91	2			6	91.0	6.0	3.0
		10							3	87	5			5	87.0	5.0	8.0[1]
2-Hydroxyestradiol (2-OHE_2)	48	0.3						1	1	93	1			4	93.0	4.0	3.0
		1							6	68	5	1		20	68.0	20.0[1]	12.0[2]
		3	Few metaphases														
4-Hydroxyestradiol (4-OHE_2)	48	0.3							1	91				8	91.0	8.0	1.0
		1			1		1	1	4	79	2	1		11	79.0	11.0	10.0[1]
		3	1						3	72	3	1	2	18	72.0	18.0[1]	10.0[1]
		10	Few metaphases														
Estriol (E_3)	48	1							2	87	2			9	87.0	9.0	4.0
		3								92				7	92.0	7.0	1.0
		10							3	88	2	1		6	88.0	6.0	6.0

[1]Statistically different from control ($p < 0.05$ by χ^2 test).–[2]Statistically different from control ($p < 0.01$ by χ^2 test).–[3]Statistically different from control ($p < 0.001$ by χ^2 test).

TABLE V – COMPARATIVE QUALITATIVE FINDINGS AND MULTIPLE ENDPOINT MEASUREMENTS OF SHE CELLS EXPOSED TO 17β-ESTRADIOL OR ITS METABOLITES

Genetic endpoint	E_2 and its metabolites								
	E_2	E_1	2-OHE_1	4-OHE_1	2-$MeOE_1$	16α-OHE_1	2-OHE_2	4-OHE_2	E_3
Morphological transformation	++[2]	++	++++	+++++	+	+	++	+++	−
Chromosome aberration	−	+	+++	++++	−	−	++	+++	−
Aneuploidy[1]	+++	+	+	−	+++	+	++	+	−
Gene mutation	−	−	−	+	+	−	±	+	−

[1]Numerical abnormality of chromosomes in the near diploid range.–[2]Estrogen-associated increases in measured SHE cell endpoints.

This possibility is supported by our previous findings that when detected by the ^{32}P-postlabeling assay, treatment of SHE cells with 2- or 4-OHE_2 induced covalent DNA adduct formation in the cells, corresponding to the induction of cellular transformation (reviewed by Tsutsui and Barrett, 1997). We analyzed the ability of estrogens to cause somatic mutations by detecting the phenotypic resistance to TG and/or Oua. Genotypic rather than phenotypic evidence might be necessary to fully demonstrate the mutagenic activity of the various estrogens. There are several lines of evidence to support the concept that estrogens induce gene mutations. The DNA repair enzyme DNA polymerase β mRNA of hamster kidney tumors induced by DES contains several mutations in the catalytic domain compared with that of age-matched controls, and estrogen-induced kidney tumors and kidney of hamsters treated with E_2 are altered in repeat sequences of microsatellites (reviewed by Roy and Liehr, 1999).

We confirmed our previous results that E_2 induces morphological transformation and aneuploidy in SHE cells, but fails to induce any detectable gene mutations and chromosome aberrations over the concentrations which induce cellular transformation (reviewed by Tsutsui and Barrett, 1997). Other studies have reported negative findings on E_2-induced mutagenicity (Drevon *et al.*, 1981) and chromosome aberrations (Abe and Sasaki, 1977). Conflicting findings of E_2-induced unscheduled DNA synthesis have been reported (Oshiro *et al.*, 1986). Because aneuploidy and cellular transformation in SHE cells are mechanistically related (reviewed by Tsutsui and Barrett, 1997), the ability of E_2 to induce aneuploidy could participate in SHE cell transformation by E_2.

E_1-induced cellular transformation, chromosome aberrations and aneuploidy in SHE cells (Tables III and IV). However, somatic mutations at the Na^+/K^+ATPase and *hprt* loci were not elicited in SHE cells by E_1 (Table II). Kochhar (1985) found no clastogenic activity of E_1 by using Chinese hamster ovary (CHO) cells. No mutagenic activity of E_1 was also noted by Drevon *et al.* (1981) in Chinese hamster V79 cells with a cell-mediated system using primary

hepatocytes from a rat. Clastogenic and/or aneugenic activities could play a role in the induction of SHE cell transformation by E_1.

The transforming activity of 16α-OHE_1 was lower than E_2, which is consistent with the observation that 16α-OHE_1 has low carcinogenic activity in hamster kidney relative to E_2 or 4-OHE_2 (Li *et al.*, 1995). 16α-OHE_1 forms covalent adducts with histones, particularly H1 histone, whereas E_1, E_2 and E_3 do not (Yu and Fishman, 1985). Binding of 16α-OHE_1 to histone could interfere with accurate reading of the DNA template during transcription or replication. In addition, 16α-OHE_1 binding could stimulate or block an essential site for biochemical modification of nucleohistones during changes in gene function (Yu and Fishman, 1985). Thus, this adduct formation is a possible mechanism for cellular transformation induced by 16α-OHE_1. 16α-OHE_1 induced aneuploidy in SHE cells (Table IV), and microtubule disruption is induced in V79 cells by 16α-OHE_1, but this latter effect is approximately one-fourth that of E_2 (Aizu-Yokota *et al.*, 1995). The interaction between estrogens and microtubules may mediate the induction of aneuploidy in mammalian cells (reviewed by Tsutsui and Barrett, 1997). As described above, aneuploidy is mechanistically related to SHE cell transformation, indicating a possible involvement of aneuploidy induction in SHE cell transformation by 16α-OHE_1. 16α-OHE_1 is mutagenic in the Ames test (his^+ revertants in the TA98 and TA1538 strains) (Arts *et al.*, 1990). DNA repair synthesis is induced by 16α-OHE_1 in mouse mammary epithelial cells in primary culture (Telang *et al.*, 1992). 16α-OHE_1 failed to elicit chromosome aberrations and gene mutations in SHE cells (Tables II and III). This estrone metabolite might exhibit clastogenicity and/or mutagenicity in SHE cells under an appropriate condition using a metabolic activation system.

Treatment of SHE cells with 2-$MeOE_1$ resulted in cellular transformation in SHE cells (Table I). 2-$MeOE_1$ failed to induce chromosome aberrations in SHE cells but induced somatic mutations at the Na^+/K^+ATPase locus partially in a concentration-dependent manner (Tables II and III). The estrone metabolite also induced aneuploidy in SHE cells at the efficiency similar to E_2 (Table IV). The results suggest that 2-$MeOE_1$ is genotoxic and potentially carcinogenic. The blood concentration of 2-$MeOE_1$ is very high in pregnant women (4 to 6 ng/ml) (Dunn, 1983). However, 2-$MeOE_1$ has a high affinity for globulin in blood compared with E_2 (Dunn, 1983). In addition, 2-$MeOE_1$ is postulated to be catalyzed by 17β-hydroxysteroid dehydrogenase and immediately converted to 2-methoxyestradiol (reviewed by Zhu and Conney, 1998*b*), which is known to be antitumorigenic (Fotsis *et al.*, 1994). These might contribute toward reducing the potential carcinogenic activity of 2-$MeOE_1$.

E_3 failed to induce morphological transformation in SHE cells. No genotoxic effects at 3 endpoints including chromosome aberrations, aneuploidy and somatic mutations were observed in SHE cells treated with E_3 (Tables I to IV). There are few other reports about the genotoxic effects of E_3. E_3 induces chromosome aberrations in CHO cells (Kochhar, 1985) but does not induce aneuploidy in cultured human lymphocytes (Hill and Wolff, 1983).

The results from 4 genetic endpoints measured in the present experiments are qualitatively summarized in Table V. Multiple E_2 metabolites, E_1 and 4 catechol estrogens (2- or 4-OHE_1 and 2- or 4-OHE_2), exhibited transforming activities similar or greater than E_2. The transforming activities of some E_2 metabolites, 2-$MeOE_1$ and 16α-OHE_1, were lower than that of E_2. Although E_2 displayed little activity at 2 genetic endpoints, chromosome aberrations and gene mutations, either or both of the genetic activities were positive when SHE cells were treated with E_1 or each of the 4 catechol estrogens. High levels of aneuploidy induction in the near diploid range were observed in SHE cells treated with E_2 or 2-$MeOE_1$, but the levels were decreased in cells treated with other E_2 metabolites.

Overall, the transforming activities of all estrogens tested corresponded to at least one of the genetic effects by each estrogen, *i.e.*, chromosome aberrations, aneuploidy or gene mutations, suggesting the involvement of the genetic effects in the initiation of estrogen-induced carcinogenesis. Our present results confirm our previous finding that multiple genetic effects of estrogens can act together to cause genetic alterations leading to cellular transformation (reviewed by Tsutsui and Barrett, 1997).

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