Neoplastic Transformation of Cultured Mammalian Cells by Estrogens and Estrogenlike Chemicals
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Estrogens are clearly carcinogenic in humans and rodents but the mechanisms by which these hormones induce cancer are only partially understood. Stimulation of cell proliferation and gene expression by binding to the estrogen receptor is one important mechanism in hormonal carcinogenesis; however, estrogenicity is not sufficient to explain the carcinogenic activity of all estrogens because some estrogens are not carcinogenic. Estrogens are nonmutagenic in many assays but exhibit specific types of genotoxic activity under certain conditions. We have studied extensively the mechanisms by which estrogens induce neoplastic transformation in a model in vitro system and our findings are summarized in this review. 17β-Estradiol (E2) and diethylstilbestrol (DES) and their metabolites induce morphological and neoplastic transformation of Syrian hamster embryo (SHE) cells that express no measurable levels of estrogen receptor. Treatment of the cells with E2 or DES fails to induce DNA damage, chromosome aberrations and gene mutations in SHE cells but results in numerical chromosome aberrations (aneuploidy) that could arise from microtubule disruption or disfunction of mitotic apparatus. Estrogen-induced genotoxicity is detected in cells following treatment with estrogen metabolites or following exogenous metabolic activation of estrogens. The estrogens induce DNA adduct formation that is detected by 32P-postlabeling. Both aneuploidy induction and DNA damage caused by DNA adduct formation correlate with the estrogen-induced cell transformation and may be important in hormonal carcinogenesis. We propose that multiple effects of estrogens acting together cause genetic alterations leading to cell transformation. — Environ Health Perspect 105(Suppl 3):619-624 (1997)

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Estrogen use has been associated with an increased risk for breast cancer and endometrial cancers in women (1-4). Various natural and synthetic estrogens also induce mammary, pituitary, cervical, uterine, and renal tumors in rodents (4-5). Diethylstilbestrol (DES), a synthetic estrogen, is well known to be carcinogenic to humans (6). The cellular and molecular mechanisms whereby estrogen-induced neoplastic events occur have not been fully elucidated, but there is strong evidence that estrogens are epigenetic carcinogens, acting via a promoting effect related to cellular proliferation, mediated through the estrogen receptor (7-15). 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. Another mechanism, related to mutagenic changes, has been suggested in studies of estrogen-induced carcinogenesis (16-24).

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 directly transform cells.

Morphological and Neoplastic Transformation in Vitro by Estrogens and Estrogenlike Chemicals

Morphological and neoplastic transformation of SHE cells is induced by DES, 17β-estradiol (E2) and other estrogens. We observed that DES and E2 induce transformation of hamster cells that is indistinguishable from that induced by other chemical carcinogens such as benzo[a]pyrene (19,25). Sarcomas are also induced in Syrian hamsters in vivo following subcutaneous injection of DES (26). SHE cells do not express measurable levels of estrogen receptor and estrogen treatment is not mitogenic to the cells (27). Thus, estrogenic activity of the compounds can be excluded in this in vitro assay. The cells do, however, have the ability to metabolize estrogens (28,29), and the role of metabolic activation in the carcinogenic activity of estrogens in this model is under investigation as discussed later.

The role of mutagenesis in the neoplastic transformation of SHE cells by estrogens has been studied extensively. We have demonstrated that treatment of SHE cells with DES or E2 induces cell transformation without measurable gene mutations, unscheduled DNA synthesis (UDS) or structural chromosome aberrations (19,20,23). Under the same conditions, both estrogens induce a specific type of genetic change, i.e., aneuploidy. Chromosome losses and gains are induced (20,23), suggesting a nondisjunctional mechanism involved in the transforming activity. Structural analogues of DES have also been tested in this cell transformation system (25,30). Like DES, tetrafluorodiethylstilbestrol (TF-DES) and dimethylstilbestrol (DMS) induce morphological transformation of SHE cells. The transformation frequency of DMS is much less than that of DES and TF-DES. Hexestrol (HEX) and dimethoxydiethylstilbestrol (DM-DES) do not transform these cells. There is a good association between the metabolic conversion of DES analogues via a peroxidase-mediated

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Abbreviations used: DES, diethylstilbestrol; α-diestrol, trans-trans-diestrol; β-diestrol, cis,cis-diestrol; DMS, dimethylstilbestrol; DM-DES, dimethoxydiethylstilbestrol; E1, estrone; E2, 17β-estradiol; E3, estriol; DMS, hexestrol; MN, microucle; β-OH-E2, β-hydroxy-2-deoxyguanosine; 16α-CH3-E2, 16β-hydroxyestrone; 2-OH E2, 2-hydroxyestrone; 2-OH E3, 2-hydroxyestradiol; 4-OH E2, 4-hydroxyestradiol; PMS, postmitochondial supernatant; SHE, Syrian hamster embryo; TF-DES, tetrafluorodiethylstilbestrol; UDS, unscheduled DNA synthesis.
oxidative pathway and their ability to induce cell transformation. DES, DMS, and TF-DES can all be metabolized by peroxidase. In contrast, HEX and DM-DES are not metabolized via this pathway (25), suggesting that DES metabolism is important in its carcinogenicity. The peroxidative-mediated metabolism of DES that operates in SHE cells is also the major pathway of DES metabolism in the known DES target tissue [e.g., adult (31) or fetal uterus (32)] (25).

Treatment of SHE cells with DES in the presence of exogenous metabolic activation with rat liver postmitochondrial supernatant (PMS) enhances morphological transformation in a dose-dependent manner (33). Exposure of SHE cells to DES under the same conditions with exogenous metabolic activation induces DNA damage (determined by UDS) (21), and somatic mutation at the Na+/K+ -ATPase locus (33). SHE cells peroxidatively metabolize DES to cis,cis-dienestrol (β-dienestrol) (29), which does not induce UDS by itself (21). More β-dienestrol is formed in cells treated in the presence of rat liver PMS.

β-Dienestrol can potentially form a phenoxyl radical intermediate via a peroxidase-mediated pathway, and this intermediate may bind to DNA and induce UDS.

DES exhibits positive activity in other cell transformation systems as well. Fitzgerald et al. (34) demonstrated in the BALB/c 3T3 cell transformation system that DES displays transforming activity with no measurable induction of gene mutation at the Na+/K+ -ATPase locus (33). Transformation frequency of the cells is enhanced by DES when treated with DES in the presence of rat hepatocytes that are freshly prepared. Rinehart et al. (35) showed that chronic exposure to DES of human endometrial stromal cells with a temperature-sensitive SV40 large T antigen induces a dose-dependent increase in the immortalization of cells, which is determined by the ability to grow at the restrictive temperature. Moreover, the increase in cell proliferation at the restrictive temperature is concurrent with alterations in p53 in the cells. As immortalization is an important step in the carcinogenesis process, and immortalization of human cells may be analogous to initiation of rodent cells, DES could act as an initiator in the carcinogenic process of human cells (35).

We have studied the ability of estradiol (E2) and different metabolites of E2 to induce cell transformation. E2 metabolism is similar in rats and in humans. E2 is initially oxidized to estrone (E1), followed by hydroxylations at positions C-2 and 16α that are mutually exclusive (5). The principal products of 16α-hydroxylation are 16α-hydroxyestrone (16α-OH E1) and estradiol (E2). 2-Hydroxylation yields the catechol estrone 2-hydroxyestrone (2-OH E1) (36). P450 multisubstrate monooxygenases catalyze the oxidative metabolism of estrogens, and estrogen 2- and 16α-hydroxylases play a major role in the metabolism of these estrogens (37). E2 is also converted to catechol estrogen, postulated to arise from E2 via the 4-hydroxylation pathway, which is similar to the 2-hydroxylation pathway (38). The catechol estrogens e.g., 2-OH E1, 2-hydroxyestradiol (2-OH E2), and 4-hydroxyestradiol (4-OH E2) seem to be further oxidized to quinones that covalently bind to DNA (39).

We examined the transforming activity of these estrogens using the SHE cell assay system. Treatment of SHE cells with E1, E2, 16α-OH E1, 2-OH E1, or 2-OH E2 induces morphological transformation of cells in a dose-related manner. Exposure to E2 fails to elicit SHE cell transformation (unpublished data). Higher transforming activity is observed in cells treated with 16α-OH E1 or 2-OH E1, when compared to other estrogens. 16α-OH E1 could be capable of inducing UDS and anchorage-independent growth in mouse mammary epithelial cells (40). Additionally, 16α-OH E1 binds covalently not only to nucleohistones in vitro (41), but also to nuclear regulatory proteins, specifically the estrogen receptor, in estrogen target cells (42). This may disturb normal gene functions, possibly participating in the transformation process (43). Elevated levels of 16α-hydroxylation are detected in breast tissue from women with breast cancer (43) and in mouse strains with a high incidence of mammary tumor formation (44). However, the high frequency of morphological transformation of cells induced by 2-OH E1 could be due to genotoxicity of 2-OH E1 or its metabolites converted in SHE cells, because treatment with 2-OH E1 induces chromosome aberrations in SHE cells (unpublished data). E2 induces morphological transformation of BALB/c 3T3 cells, a mouse fibroblast cell line having 2- and 4-hydroxylase activity. The transformation efficiency does not increase with increasing hormonal potency of the estrogens examined, but correlates well with the relative rates of catechol estrogen formation (45).

Tamoxifen, toremifene, and ICI 164,384 are positive in the SHE cell transformation assay well (46). Tamoxifen, a triphenylethylene nonsteroidal antiestrogen, is a structural analogue of DES and exerts mixed or partial agonist/antagonist effects with estrogens. Toremifene is a new triphenylethylene nonsteroidal antiestrogen; its molecular structure closely resembles that of tamoxifen. Toremifene differs from tamoxifen by the presence of a chlorine atom at the end of the ethyl side chain. ICI 164,384 is the 7α-alkylamide analogue of E2, and a new steroidal antiestrogen with complete pure antagonistic properties (47). The results confirm that hormonal effects are not implicated in cell transformation. Rather, a role for estrogen metabolism seems to be important in estrogen-induced cell transformation or carcinogenesis. No reports on the cell transforming activity and carcinogenicity of other estrogen blockers, e.g., EM 800 and ICI 182,780, are available.

**Mechanisms of Cell Transformation by Estrogens**

**Aneuploidy Induction**

DES induces numerical chromosome changes (aneuploidy) in SHE cells (20). The aneuploidy induction occurs at nontoxic doses and correlates with the ability to induce cell transformation with parallel dose–response curves. Treatment of synchronized cultures with DES results in a cell cycle-dependent induction of aneuploid cells that parallels the induction of cell transformation, with the greatest level observed following treatment during mitosis. Parallel dose–response curves for cell transformation and aneuploidy induction by DES are observed when the synchronized cultures are treated during the mitotic phase of the cell cycle (20). A nonrandom chromosome gain accompanies DES-induced immortalization and tumorigenic conversion of SHE cells (48). These suggest that DES-induced aneuploidy is mechanistically involved in estrogen-induced cell transformation and possibly in carcinogenesis (7,22). E2 also induces a dose-dependent increase in the frequencies of aneuploid cells, corresponding to the inducibility of morphological transformation (23).

E2 and DES bind and disrupt polymerization of microtubules in cultured mamalian cells (49–53). DES inhibits in vitro assembly of microtubules purified from porcine brain (54), and induces a decrease in the number of spindles and cytoplasmic microtubule fibers in SHE cells (49) and Chinese hamster V79 cells (50). E2 has no ability to interact with microtubules or...
microtubule protein in vitro (54), but the quinone metabolites of both E2 and DES bind covalently to the C-terminal regions of β-tubulin, which are important in regulation of microtubule assembly and disruption (55). E2 exhibits microtubule-disrupting activity both in estrogen receptor-positive and receptor-negative human breast cancer cell lines (51). The disrupting activity is demonstrated also in V79 cells (51,52), which have little capability of metabolizing xenobiotics (56). These findings suggest that E2 itself induces microtubule disruption independent of its binding to estrogen receptor. Therefore, E2-induced microtubule disruption in living cells seems to be due to a more complex involvement with factors regulating microtubule assembly, such as Ca²⁺, microtubule accessory proteins, the calcium regulatory protein calmodulin, adenylate cyclase, or the protein kinases activated by the cyclic nucleotides (57–59). Microtubule-disrupting activities of E2 and its metabolites in living cells vary with their chemical structures. Aizu-Yokota et al. (53) have examined the activity in V79 cells by the indirect immunofluorescence method using anti-β-tubulin antibody and determined the rank-order of the potencies as follows: E₂ = 2-OH E₂ > 4-OH E₂ > 16α-OH E₁ > 2-OH E₁ = E₃. 16α-OH E₁ and 2-OH E₁ exhibit activities about one-fourth to one-fifth that of E₂. The disruptive activities of E₃ and E₂ are negligible when compared to that of E₂. Functional or conformational change in microtubule organization could lead to chromosomal nondisjunction, aneuploidy induction, and cell transformation. Tamoxifen, toremifene, andICI 164,384 induce aneuploidy in SHE cells with no increases in the frequency of chromosome aberrations (46). Tamoxifen binds to calmodulin and acts as a calmodulin antagonist (60). Sargent et al. (61) reported that both unipolar spindles and incompletely elongated spindles were observed in cultured hepatocytes from rats treated with tamoxifen, as well as in calmodulin-defective mutants in yeast (62). Some calmodulin is associated with the spindle pole body and plays an important part in the proper function of mitotic spindles (62). Tamoxifen-induced aneuploidy may be due to the inhibitory effect of calmodulin by tamoxifen.

**Formation of Micronuclei**

Both DES and E₂ induce the formation of micronuclei (MN) in cultured mammalian cells (63–67). MN enclose acenitic chromosomal fragments or whole chromosomes that do not become incorporated into the main nuclei after cell division. MN are believed to arise from acenitic chromosomal fragments or from chromosomes lagging at anaphase resulting from mitotic disturbance (68). MN packing acenitic chromosomal fragments appears to be induced by clastogens, while MN enclosing whole chromosomes are induced by agents that affect the mitotic apparatus. The majority of DES-induced and E₂-induced MN contains whole chromosomes, which are demonstrated both with antikinetochore antibodies and with the centromere-specific DNA probe (67). DES may need peroxidative activation to produce metabolites that induce MN, because both DES oxidation and MN induction by DES are markedly decreased by indomethacin, an inhibitor of prostaglandin H synthase activity (66,69). E₂ may require metabolic activation for MN induction as well, as indicated by the following: a) 2-OH E₂, an E₂ metabolite converted by 2-hydroxylase, binds covalently to tubulin in vitro with or without peroxidative activation system (55), but E₂ itself does not; and b) E₂ exhibits comparable MN induction to DES in SHE cells (64), which have both oxidative (28) and peroxidative activities (29). Schnitzler et al. (67) have shown that the mechanism of DES-induced MN is different from that of E₂-induced MN using SHE cells and ovine seminal cells. DES-induced MN can arise through chromosome nondisjunction due to spindle disruption, whereas E₂ at the concentrations used in the MN assay exerts no detectable effect on the formation of the mitotic spindle, but causes chromosome dislocation, probably due to a functional loss of the mitotic apparatus.

**Genotoxicity**

Although DES is not genotoxic in many assays, in certain studies DES has been found to induce UDS (21,70–72), sister chromatid exchanges (73–75), chromosome aberrations (76,77) and gene mutations (33,78). The positive studies of DNA damage by DES use either cultured mammalian cells with exogenous metabolic activation or cells with possible endogenous activation capacity for DES. Therefore, we directly compared the cell transforming activity and genotoxicity of DES in the same cells with and without exogenous metabolic activation. When SHE cells are treated in the absence of a rat liver PMS-metabolic activation system, DES fails to induce DNA damage in SHE cells at doses that induce cell transformation (7,19,20,21,33). However, treatment of SHE cells with DES in the presence of an exogenous metabolic activation system enhances the frequency of morphological transformation of the cells. Furthermore, this treatment elicits UDS and gene mutations in the cells at the Na⁺/K⁺-ATPase locus (33). Thus, we have proposed two potential mechanisms for estrogen-induced cell transformation; in one the target of the estrogen is not DNA but rather microtubule disruption and the other is associated with DNA damage (33). Both pathways may involve active genotoxic metabolites of DES.

**DNA Adduct Formation**

Cellular DNA damage induced by chemicals can be examined by detection of DNA adduct formation through a covalent modification of DNA. Liehr et al. (79) demonstrated the presence of covalent DNA adducts in premalignant kidneys of Syrian hamsters treated chronically with DES using a sensitive 32P-postlabeling assay. Because structurally diverse estrogens induced identical DNA adducts, they concluded that estrogens induce binding of the same unknown endogenous compounds to target tissue DNA. They also reported that a distinct pattern of DNA adducts was detected in the liver, kidney, uterus, and testes of Syrian hamsters following treatment with DES (80), and the major adducts found were similar to those produced by reaction of diethylstilbestrol-4’,4”-quinone with DNA (39). This suggests that DES acts as a genotoxic carcinogen via its metabolic activation to the electrophilic 4’,4”-quinone (39). There is another possible mechanism by which DES may cause DNA damage. Micromomediated redox cycling between DES or its catechol and the corresponding quinones generates superoxide radicals (O₂⁻•) and hydroxyl radicals (OH•) (81–84). Free radicals generated by the redox cycling of DES also oxidize 2’-deoxyguanosine to 8-hydroxy-2’-deoxyguanosine (8-OH-dG) in vitro (85) as well as in vivo (86). Small DNA adducts such as 8-OH-dG could be a causal basis for DES-induced DNA damage, which is detected as gene mutations and UDS in cultured SHE cells (21,33). Chronic exposure of Syrian hamsters to E₂ for 6 months induces renal tumors (6), and the treatment causes covalent DNA alterations (adduct formation) in the kidney (82). E₂ is metabolically oxidized to catechol estrogens (2-OH E₂ and 4-OH E₂), which are postulated to be capable of...
redox cycling (84). Free radical-mediated DNA damage might be involved in E2 carcinogenesis because 8-OH-dG levels increase in kidney DNA of male Syrian hamsters in chronic treatment with E2 (87).

To study the possible involvement of DNA damage in cell transformation induced by estrogens, we have examined whether DNA adduct formation is elicited in SHE cells treated with estrogens and their metabolites by means of the nuclease P1 enhancement version of 32P-postlabeling (30). DNA adduct formation is detected in SHE cells treated with DES, but not in SHE cells treated with trans, trans-dienestrol (α-dienestrol) or β-dienestrol. Similarly, morphological transformation of SHE cells is induced by DES, but not by α- or β-dienestrol. Treatment of SHE cells with DES in the presence of exogenous metabolic activation with rat liver PMS enhances morphological transformation in a dose-dependent manner. Exposure of SHE cells to DES under the same conditions with exogenous metabolic activation induces somatic mutations and UDS. However, following this treatment, DNA adduct formation is not detected in SHE cells. It is possible that DNA adducts may be formed but not detected because of the instability of the adducts (39). However, this is unlikely because DNA adduct formation is not detected even when SHE cells are treated with DES for 30 min (30). Exposure of SHE cells to E2 and its metabolites, 2-OH E2 and 4-OH E2, for 24 hr leads to covalent DNA adduct formation, corresponding to the induction of cell transformation (30). The results indicate that estrogens induce DNA adduct formation in cultured SHE cells, but the induction may not be the only mechanism relevant to the initiation of cell transformation. Because DES and E2 result in DNA adduct formation and aneuploidy in SHE cells, the possibility exists that DNA adduct formation is involved in nondisjunction leading to aneuploidy. Alternatively, DNA adduct formation may only correlate with other adducts in different critical macromolecules, such as tubulin (55,88,89), and may not be causally involved in either morphological transformation or aneuploidy induction.

E2 is metabolically oxidized to catechol estrogens that are also postulated to be capable of redox cycling, which would generate free radicals by redox cycling between 2- or 4-OH E2 and their corresponding quinones (82). Roy et al. (86) showed that 8-OH-dG levels increase in kidney and liver DNAs of male Syrian hamsters by chronic treatment with DES but not with E2. This suggests the involvement of different mechanisms in cell transformation induced by DES and E2.

In summary, estrogens (DES and E2) and their metabolites induce morphological transformation of SHE cells in a dose-related manner. DES and E2 do not cause significant increases in the chromosome aberrations in SHE cells, but induce numerical chromosome changes in the near diploid range, corresponding to the transforming activity. In addition, these estrogens result in DNA adduct formation in SHE cells. It has not been clear which cyogenetic endpoints are more correlated on a causal basis with the estrogen-induced cell transformation. Moreover, other effects by estrogens, e.g., covalent binding to proteins (55,88,89) and generation of reactive oxygen species (39,81,83,84,86) could participate into inducing transformed cells. Furthermore, we cannot rule out the possibility that multiple effects of estrogens act together to cause genetic alterations leading to cell transformation. Our studies do, however, suggest that estrogens have the ability to directly transform cells by multiple mutagenic mechanisms unrelated to estrogenicity. These estrogen-induced changes, in conjunction with epigenetic changes mediated through the estrogen receptor, may contribute to hormonal carcinogenesis.

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