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Is Estradiol a Genotoxic Mutagenic Carcinogen?*

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ABSTRACT

The natural hormone 17β-estradiol (E_2) induces tumors in various organs of rats, mice, and hamsters. In humans, slightly elevated circulating estrogen levels caused either by increased endogenous hormone production or by therapeutic doses of estrogen medications increase breast or uterine cancer risk. Several epigenetic mechanisms of tumor induction by this hormone have been proposed based on its lack of mutagenic activity in bacterial and mammalian cell test systems. More recent evidence supports a dual role of estrogen in carcinogenesis as a hormone stimulating cell proliferation and as a procarcinogen inducing genetic damage. Tumors may be initiated by metabolic conversion of E_2 to 4-hydroxyestradiol catalyzed by a specific 4-hydroxylase (CYP1B1) and by further activation of this catechol to reactive semiquinone/quinone intermediates. Several types of direct and indirect free radical-mediated DNA damage are induced by E_2, 4-hydroxyestradiol, or its corresponding quinone in cell-free systems, in cells in culture, and/or *in vivo*. E_2 also induces various chromosomal and genetic lesions including aneuploidy, chromosomal aberrations, gene amplification, and microsatellite instability in cells in culture and/or *in vivo* and gene mutations in several cell test systems. These data suggest that E_2 is a weak carcinogen and weak mutagen capable of inducing genetic lesions with low frequency. Tumors may develop by hormone receptor-mediated proliferation of such damaged cells. (*Endocrine Reviews* **21:** 40–54, 2000)

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I. Carcinogenicity of E_2

THE INDUCTION of tumors by E_2 and its esters was described in the late 1930s by Lipschutz and Vargas in guinea pigs and by Gardner in the early 1940s in mice [reviewed by the International Agency for Research on Cancer (IARC) (1, 2)]. Since that time, many more reports of tumor induction by estrogens have been published, and many rodent tumor models have been introduced (1, 2). In contrast, the potential carcinogenic activity of estrogen-containing medications in humans has not been recognized for many years. Estrogens have generally been considered beneficial, based on a variety of hormonal effects. However, in the past 15–20 yr, epidemiological studies have increasingly pointed to an increased breast or uterine tumor risk associated with estrogens. This text cannot provide a detailed review of the animal and human carcinogenicity data [which may be found elsewhere (1, 2)], but can only highlight key reports.

A. Carcinogenicity of E_2 in animals

The evidence for the carcinogenic activity of 17β-estradiol (E_2) in animals has been deemed sufficient by the IARC to consider this hormone a carcinogen (1, 2). This conclusion is based on numerous tests of E_2 administered to rodents by oral or subcutaneous administration. For instance, the administration of E_2 to mice increased the incidence of mammary, pituitary, uterine, cervical, vaginal, testicular, lymphoid, and bone tumors (3–6). In rats, E_2 or estrone (E_1) increased the incidence of mammary and/or pituitary tumors (7–9). In hamsters, a high incidence of malignant kidney tumors occurred in intact and castrated males (10–13) and in ovariectomized females, but not in intact females (10). In guinea pigs, diffuse fibromyomatous uterine and abdominal lesions were observed (14). E_2 also induced tumors when administered orally in the drinking water or in rodent chow (4, 5, 15, 16). All these tumor models have been developed using pharmacological doses of E_2 with the aim of examining

the tumorigenic activity of this hormone in a relatively short period of time.

The purpose of all these studies was the development of useful and practical animal models for the investigation of mechanistic aspects of hormone-induced tumorigenesis. No animal models have been developed in which tumors are induced by very low doses of E_2, presumably because of the cost of maintenance of a large number of animals for such a model and the difficulty of dosing in view of the varying levels of endogenous estrogen in cycling females. The same considerations, however, are also true for almost all other carcinogens known to man, which have been established as carcinogens at high doses in small groups of animals over a short period of time. Although the predictive value of carcinogenicity testing at high doses has been questioned (17, 18), estrogens are nevertheless considered to be carcinogens, based mainly on two types of evidence (1, 2): various tumor types are induced in animals in many organ sites under a variety of treatment conditions as discussed above. Moreover, a consensus is developing that estrogens impart a defined carcinogenic risk to human populations exposed to the low concentrations of estrogens used for medication purposes as discussed below.

B. Carcinogenicity of E_2 in humans

Estrogen administration is accepted by most epidemiologists as a risk factor of human endometrial adenocarcinoma (19, 20). Thus, estrogens unopposed by progestins increase the risk of uterine tumors. This risk increases with increasing doses of estrogen and with the length of treatment (21). Obesity also increases uterine tumor risk, most likely because the aromatase activity of adipose cells elevates tissue and circulating E_1 levels (21, 22).

Increasing evidence shows that slightly elevated levels of circulating estrogens are also a risk factor for breast cancer (23, 24). This role of endogenous estrogen in human breast carcinogenesis is supported by risk factors of breast cancer such as high serum or urine estrogen levels (25, 26), the early onset of menstruation, or late menopause (27). While early cohort studies failed to identify an association between serum hormone levels and breast cancer (28, 29) (presumably due to shortcomings of the assay methods), more recent cohort studies have demonstrated strong relationships between endogenous estrogen levels and breast cancer risk (25, 30–33). The role of endogenous E_2 as a risk factor in human breast cancer is reviewed in more detail in the epidemiological literature (Refs. 23, 24, and 27 and references cited therein).

Exogenous estrogens, alone or in combination with progestin, also elevate breast cancer risk (34–36). Progestin added to the estrogen medications does not inhibit mammary carcinogenesis (37) because the former hormone appears to be the primary mitogen of mammary ductal epithelial cells (38), whereas estrogen appears to function in this manner in the uterus. Pike *et al.* (39) summarized the population-based studies of oral contraceptive use and breast cancer among women under 45 yr of age that had been published through 1990 and derived a weighted average of approximately 3.1% increase in breast cancer risk per year of oral contraceptive use (relative risk estimate: 1.36). The weighted relative risk for young women who consumed oral contraceptives for 10 yr before their first full-term pregnancy was 1.45 compared with nonusers.

Pike *et al.* (39) also summarized the population-based epidemiological studies that had been published through 1990 and derived a weighted average of the relative breast cancer risk from use of hormone replacement therapy. Of the 10 studies reviewed, 9 showed a positive association and the results of 5 were statistically significant. Based on these studies, the average annual increase in breast cancer risk was 3.1% per year of estrogen replacement therapy use. For women with 10 yr of use, the risk of breast cancer was 1.36 times that of women who have never used these preparations. In a more recent meta-analysis of more than 50 studies, the relationship has been examined between breast cancer risk and estrogen replacement therapy during menopause (40). Although no randomized, controlled, double-blind studies have been conducted, the observational data available show an increased risk of breast cancer with the use of estrogen replacement therapy for more than 5–10 yr. The relative risk of breast cancer under these circumstances increases by about 30%. The absolute risk is small with about one additional breast cancer case/100 women of age 50 who have taken estrogen for at least 10 yr.

C. Conclusion: carcinogenicity of E_2

These biological studies in animals and epidemiological studies in humans all clearly identify E_2 as a carcinogen. Tumors are induced in small groups of animals with pharmacological doses of E_2 in a short period of time. In humans, slight elevations of circulating estrogen levels caused either by elevated endogenous production of hormone or by therapeutic doses of estrogen medications also increase breast or uterine cancer risk (1, 2, 39, 40). This carcinogenic activity of steroidal estrogens is recognized by the IARC, which classifies the evidence for the carcinogenicity of steroid estrogens to humans as sufficient (1, 2).

The human epidemiological data point to E_2 and other estrogens as only weak carcinogens. This conclusion is not contradicted by laboratory animal tests, which provide only qualitative results given the difficulties with appropriate dosing. Thus in animals, E_2 may well be only a weak carcinogen compared with other laboratory carcinogens such as benzo[a]pyrene or 7,12-dimethylbenzanthracene. However, only a weak carcinogenic activity is to be expected because E_1, E_2, and other steroidal estrogens are endogenous hormones at low picomolar levels and because a strong carcinogenicity would have provided an evolutionary disadvantage to humans and many other species.

II. Hormonal Contributions of E_2 to Carcinogenesis

There is widespread agreement among scientists that oncogenesis in hormone-responsive tissues such as in the mammary gland or the uterus is not possible without a contribution by receptor-mediated hormonal effects. E_2 regulates or, in conjunction with other hormones, participates in the regulation of the development of reproductive organs early

in life, in differentiation, and later in their proper functioning during reproduction (41, 42). The biological basis for this role of E_2 and of other steroid hormones is the differential control of gene expression and of the stimulation of proliferation of uterine or mammary epithelial cells or other responsive cells. The mechanism of E_2-induced cell proliferation is still under discussion and beyond the scope of this review. Various mechanisms have been proposed, including the stimulation by E_2 of the expression of genes critical for regulating the cell cycle (43, 44). E_2 may bind to nuclear estrogen receptors and thus initiate this gene expression. Estrogen binding to plasma membrane receptors may also participate in the stimulation of cell proliferation (45). Alternatively, E_2 has been proposed to bind to a regulatory plasma protein and thus cancel the inhibition of cell proliferation exerted by this protein (46). Whatever the mechanistic details, the inhibition of E_2-induced proliferation of human tumor cells by hormone antagonists clearly demonstrates the role of the estrogen receptor in cell proliferation and hormone-dependent tumor growth (47). *In vivo*, the hormone antagonists also inhibit E_2-induced tumor development as illustrated by the inhibition of renal carcinogenesis in Syrian hamsters (48). In that model, tamoxifen clearly inhibits tumor appearance by receptor-mediated processes, since early events such as estrogen-induced DNA alterations are not affected by this treatment. These data demonstrate that estrogen-regulated proliferation of hormone-responsive transformed or tumor cells may fix any spontaneous or induced DNA damage and thus establish a potentially malignant tumor.

III. E_2 as Epigenetic Carcinogen

Estrogens including E_2 have been classified as epigenetic nongenotoxic carcinogens based on their failure to induce mutations in a series of bacterial and mammalian gene mutation assays (49, 50). For instance, E_2, E_1, and other estrogens do not display any mutagenic activity in the Ames (*Salmonella typhimurium*) assay with or without an extrinsic metabolizing system (51–53). E_2 and E_1 also failed to induce mutations in V79 Chinese hamster cells when tested in the 10^{-9} to 10^{-4} M concentration range (54, 55). Moreover, E_2 did not induce sister chromatid exchanges in human lymphocytes, whereas diethylstilbestrol generated such alterations (56). This lack of apparent mutagenic activity of E_2 led several researchers to propose various epigenetic pathways of tumor induction by estrogens as an explanation of the role of estrogen in breast cancer and other human tumors. Several of these pathways are presented and discussed below.

A. Uncontrolled cell proliferation by E_2

Tumorigenesis by uncontrolled stimulation of mammary epithelial cell proliferation has been proposed by Furth (57). A more recent modification of this mechanistic proposal is the hormone-dependent receptor-mediated proliferation of mammary epithelial cells carrying spontaneous replication errors (23). The absence of estrogen receptors in proliferating human mammary epithelial cells (58, 59) provides evidence against this mechanistic pathway, at least in the form proposed. It is possible that estrogens stimulate growth factors by receptor-mediated pathways in neighboring cells, which in turn stimulate mammary epithelial cell proliferation (44). However, the development of synthetic estrogens such as 17α-ethinylestradiol or 2-fluoroestradiol with well maintained hormonal potency but significantly reduced carcinogenic activity in animal models (10, 13, 60) indicates that the background of spontaneous replication errors of normal cells may not be sufficient for tumors to develop solely in response to a proliferative stimulus. More likely, tumors may arise by hormone receptor-mediated proliferation of cells transformed by specific genetic damage in addition to background lesions. This view is consistent with the ability of estrogens to induce various genetic lesions as described below.

B. Carcinogenesis by covalent modification of E_2 receptors

Fishman, Bradlow, and co-workers (61, 62) proposed the induction of breast cancer by a covalent modification of E_2 receptors resulting in a permanent uncontrolled stimulation of mammary epithelial cell proliferation by receptor-mediated processes. According to this hypothesis, 16α-hydroxyestrone, an E_1 metabolite, covalently binds to amino groups of proteins, including the estrogen receptor protein, and thus permanently stimulates the receptor and induces hormone-responsive processes, including gene expression and cell proliferation, in an uncontrolled manner (63). In support of this mechanism, many studies have been conducted with the aim of correlating 16α-hydroxylation of estrogens with tumor induction in laboratory rodents (61), with incidence of breast cancer and other diseases in humans (62–65), and with other parameters of tumorigenesis, such as induction of oncogene expression (66–70). In most of the early studies of Bradlow, Fishman and associates (71, 72), 2- and 16α-hydroxylation of E_1 were assayed by tritium release from [2-^{3}H]- and [16α-^{3}H]estrone as substrates, respectively (71, 72). These assays have never been fully validated against established product isolation assays of estrogen metabolism but have been questioned because of spurious release of tritium from ^{3}H-labeled E_1 (73–77). In addition, the positive correlation between elevated 16α-hydroxylation rates and breast cancer risk observed by Fishman and Bradlow and associates (62) and Osborne *et al.* (78) could not be validated in other laboratories by other researchers (26, 79, 80). Because of this lack of validation of the assay of E_1 2- and 16-hydroxylation and because of inadequate corroboration of the molecular epidemiology results by other laboratories using validated product isolation assays, further work is needed to determine the validity of the mechanistic hypothesis of breast cancer induction as proposed by Fishman and Bradlow.

C. Estrogen-induced chromosomal abnormalities

Barrett and co-workers (81, 82) have reported the neoplastic transformation of Syrian hamster embryo cells by E_2 and by the synthetic estrogen diethylstilbestrol without detectable concomitant gene mutations at the ouabain resistance and 6-thioguanine resistance loci. In contrast, there was a consistent correlation of cell transformation with aneuploidy. Both chromosome losses and gains were observed,

suggesting a nondisjunctional mechanism (81, 82). The lack of detectable gene mutations at defined loci by synthetic and natural estrogens and the occurrence of aneuploidy concomitant with cell transformation led Barrett and co-workers (83–85) to propose an epigenetic pathway of estrogen-induced carcinogenesis with the following features: synthetic or natural estrogens including E_2 may disrupt microtubule organization of cells, resulting in anaphase abnormalities and nondisjunction. The resulting chromosomal aneuploidy subsequently may induce cell transformation. However, in a study of the genetic changes occurring during the rare spontaneous progression of Syrian hamster embryo (SHE) cells from normal to immortalized and further to neoplastic transformed cells, Endo *et al.* (86) observed chromosomal abnormalities in cells that were not capable of inducing tumors in nude mice. Thus, these authors (86) concluded that other genetic changes (mutations) were necessary in addition to chromosomal abnormalities for cells to acquire tumorigenicity. This view is also consistent with the concept of Lengauer *et al.* (87) that aneuploidy is a part of multiple types of genetic alterations, including base substitutions, deletions, insertions, gene amplifications, numerical chromosomal changes, and chromosomal translocations that together make up the genetic instability leading to human cancer.

D. Epigenotoxic mechanism of estrogen carcinogenesis

Li and co-workers proposed an "epigenotoxic," multistage scheme for estrogen carcinogenesis in the hamster kidney (88–91). They defined an epigenotoxic carcinogen as "an agent that is not involved in direct (covalent) or indirect interactions with genetic material but, nevertheless, is able to elicit heritable changes by alternative mechanisms" (91, 92). According to this hypothesis, which has been developed mainly by studying the hamster kidney model, estrogen-induced carcinogenesis involves estrogen-mediated cathepsin D and peroxidase induction, reparative cell proliferation, aneuploidy and inappropriate protooncogene and suppressor gene expression such as amplification of c-*myc* (91, 92–95). The sustained overexpression of early estrogen response genes such as c-*fos* and c-*myc* is thought to be related to estrogen-induced genomic instability as manifested by amplification of c-*myc* (95), which is a mechanism of activation of this gene to a transforming oncogene. Tumors are thought to arise from the distinct growth advantage of cells overexpressing c-*fos*, c-*myc*, and c-*jun* and other early estrogen response genes.

Li *et al.* (94, 96–98) postulated the induction of genetic instability by mechanisms other than direct covalent or indirect interactions of estrogen metabolites with genetic material because they detected only very low rates of metabolic conversion of E_2 to the catechol metabolites 2- and 4-hydroxyestradiol, the precursors of reactive semiquinone and quinone intermediates (as discussed below). Their hypothesis is also based on their inability to confirm the formation of estrogen-induced DNA adducts (99) by ^{32}P-postlabeling assay as described earlier by Liehr and co-workers (100, 101). Unfortunately, rates of metabolic conversion of estrogens to catechol metabolites determined by Li and co-workers (94, 96–98, 102) were measured using an unvalidated, indirect radioenzymatic assay that converts the unstable catecholestrogens to more stable methoxyestrogens catalyzed by catechol-*O*-methyltransferase (103, 104). This assay has been shown to underestimate rates of catecholestrogen formation by 2 to 3 orders of magnitude (105). Specifically, 4-hydroxylation of estrogens cannot be detected by this radioenzymatic assay in microsomal preparations expressing both estrogen 2- and 4-hydroxylase activity (105), because 2-hydroxyestradiol inhibits the catechol-*O*-methyltransferase-mediated methylation of 4-hydroxyestradiol and thus inhibits formation of assayable product (106).

In contrast, much higher rates of catechol formation than those described by Li *et al.* (94, 96–98, 102) were obtained in target organs where estrogens induce tumors using product isolation assays fully validated and cross-checked in several laboratories (105, 107–112). In these studies, assays were validated in the same (Liehr) laboratory using two different product isolation procedures, a gas chromatography-based, and a TLC-based method or in two different laboratories (Liehr and Weisz) using the same hamster microsomal preparations (105, 109, 111). Finally, the rates of 2- and 4-hydroxylation of E_2 determined in these studies are consistent with rates published by other authors [as reviewed by Zhu and Conney (112)]. Moreover, the covalent binding of estrogens including catecholestrogen metabolites to DNA, initially published by Liehr and associates (100, 101), has now been confirmed by Cavalieri *et al.* (see discussion below) and by Hayashi *et al.* (113).

Li and associates (89, 90, 94) questioned the ability of estrogens and their metabolites to induce DNA damage in the carcinogenesis process on the grounds that insufficient concentrations of E_2 are present in target tissues of hormonal cancer and that rates of its conversion to catecholestrogens are too low to result in significant amounts of genotoxic metabolites. This critique is based upon the measurement of plasma E_2 levels (114) and the assumption of concordance between plasma and tissue E_2 levels (115). However, this assumption is clearly not correct, since, for instance, in premenopausal women, the ratio of mammary tissue to plasma E_2 levels approximates 1:1, whereas in postmenopausal women the ratio is 10–50:1 (116). Thus, local concentrations of E_2 in human mammary tissue and in breast tumors depend more likely on the aromatase activity of individual mammary cells (autocrine or paracrine action) than on the ovarian hormone supply. Further evidence in support of a predominant local production of hormone is provided by the high aromatase activity of individual mammary cells (117–120). The importance of mammary aromatase activity for local E_2 concentrations has also been documented by studies in nude mice inoculated on one side with MCF-7 breast cancer cells stably transfected with aromatase and on the other side with sham-transfected cells (121). Administration of the aromatase substrate androstenedione stimulated the proliferation only of the aromatase-positive MCF-7 tumors. The relative importance of *in situ* production of E_2 *vs.* uptake from plasma was examined by administering SILASTIC implants of this hormone (121). The E_2 levels were more than 4-fold higher in aromatase-positive than -negative tumors. These experiments identify the local production of E_2 in hormone-responsive tissue including mammary gland as a more im-

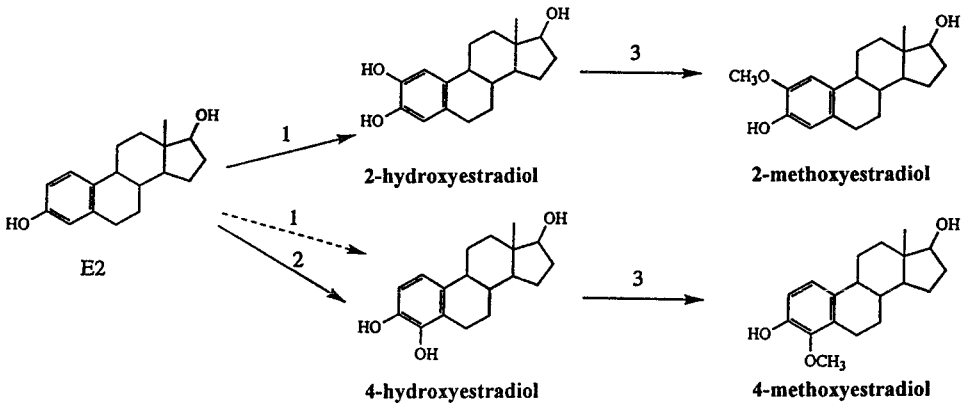

FIG. 1. Metabolic conversion of E_2 to catecholestrogens. Hepatic cytochrome P450 3A and extrahepatic cytochrome P450 1A enzymes [1] convert E_2 mainly to 2-hydroxyestradiol and approximately 15%–20% 4-hydroxyestradiol (127–130). Cytochrome P450 1B [2] of uterus, mammary gland, testis and other tissue converts E_2 mainly to 4-hydroxyestradiol (131). These catechol metabolites are methylated by catechol-*O*-methyltransferase [3] to corresponding methoxyestrogens.

portant determinant of tissue E_2 levels than the hormone supplied by circulation.

The metabolic conversion of E_2 to catecholestrogen metabolites has been underestimated by Li *et al.* as discussed above. A specific conversion of E_2 to the carcinogenic catechol metabolite 4-hydroxyestradiol by a specific cytochrome P450 has been detected in organs of rodents where estrogens induce tumors and in human breast and uterine tissue, as discussed below. This specific metabolic process may also result in elevated local concentrations of catecholestrogen metabolites. Additional research is needed to correlate local tissue and cellular estrogen and estrogen metabolite concentrations with tumorigenesis.

E. Conclusion: E_2 as epigenetic carcinogen

In summary, the proposals of estrogen as an epigenetic (epigenotoxic) carcinogen as discussed above all emphasize features that most likely participate in, but may not be sufficient for, the development of hormone-responsive cancers. There is widespread agreement that the action of estrogens as hormones by receptor-mediated processes is necessary for oncogenesis. Also, the induction by estrogens including E_2 of genetic lesions such as c-*myc* gene amplification or aneuploidy is a part of genetic changes necessary for the induction of carcinogenesis, as postulated by Lengauer *et al.* (87) and discussed below. The early reports of a lack of DNA reactivity and of mutational effects of estrogens or their metabolites, which served as the basis for the epigenetic mechanistic hypotheses outlined above, may have been based on inadequate experimental design and/or insufficiently sensitive detection technology. In more recent studies from various laboratories, sufficient evidence has been obtained, which demonstrates the ability of estrogens to undergo metabolic activation and to directly or indirectly modify DNA as discussed below.

Several studies in support of epigenetic mechanistic hypotheses have been carried out with poorly validated and inadequate assays. For instance, values for 16α-hydroxylation and catecholestrogen formation by radiometric or radioenzymatic assays have been obtained with unvalidated assays and have not been corroborated in other laboratories. Moreover, the roles of local formation and local concentrations of estrogens and their metabolites have not been fully examined in relation to the carcinogenesis process. Finally, breast cancer is a complex disease. It is more likely that estrogens act in a dual function as hormones, as outlined above, and as carcinogens, as outlined below, with both these characteristics necessary for completion of tumor development.

IV. E_2 as Genotoxic Carcinogen

The genotoxicity studies are focused on catecholestrogen metabolites, because catecholestrogens are hydroquinones that may readily be oxidized to DNA-reactive quinones and semiquinones. These investigations of DNA damage by steroidal estrogens via catecholestrogen metabolites received additional impetus with the discovery of the carcinogenic activity of 4-hydroxyestradiol, comparable to that of E_2 in the hamster kidney tumor model (52, 122, 123). More recently, 4-hydroxyestradiol administered to CD-1 female mice in the first 5 days after birth induced a 9-fold higher incidence of uterine adenocarcinoma than was observed with E_2, whereas 2-hydroxyestradiol was approximately as carcinogenic as the parent hormone (124). Therefore, the formation of catecholestrogens and their metabolic activation to reactive intermediates is discussed below in addition to the various types of DNA damage they may induce *in vitro* and *in vivo*.

A. Metabolic conversion of E_2 to catecholestrogens

2-Hydroxylation of steroidal estrogens is the major metabolic oxidation of estrogenic hormones in most mammalian species as illustrated in Fig. 1 (112, 125, 126). In human or hamster liver, this oxidation is catalyzed by cytochrome P450 3A enzymes, whereas cytochrome P450 1A enzymes are the predominant estrogen 2-hydroxylases in extrahepatic tissues (127–130). These estrogen 2-hydroxylases convert E_2 to approximately 80–85% 2-hydroxyestradiol and, due to a lack of specificity of the enzyme(s), to 15–20% 4-hydroxyestradiol (76, 109). In contrast, specific estrogen 4-hydroxylase(s), which convert E_2 mainly to 4-hydroxyestradiol (131), have been identified (107–109) in those organs of rodents in which chronic estrogen exposure induces malignant or benign tumors: hamster kidney (10), mouse uterus (124, 132), or rat pituitary (133). The specific and local formation of 4-hydroxylated estrogens is important, because 4-hydroxyestradiol is as carcinogenic as E_2 in the hamster kidney tumor model (52, 122, 123), whereas in the mouse uterus the 4-hy-

droxylated estrogen was 9 times more carcinogenic than the parent hormone (124).

In humans, the predominant conversion of E_2 to 4-hydroxyestradiol has been detected in microsomes of uterine myometrium and fibroids, *i.e.*, in benign uterine myomas (134), and in benign and malignant mammary tumors and normal mammary tissue (135). In addition, a specific estrogen-4-hydroxylase activity occurs in MCF-7 breast cancer cells and is induced further in these cells by 2,3,7,8-tetrachlorodibenzo-p-dioxin, a common environmental pollutant (136). This human estrogen-4-hydroxylase activity has been identified as cytochrome P450 1B1, a novel extrahepatic isozyme detected specifically in mammary tissue, ovary, adrenal gland, uterus, and several other tissues (131, 137, 138). In one reported measurement of estrogen metabolite concentrations in a human breast cancer extract, the ratio of 4-hydroxyestradiol to 2-hydroxyestradiol metabolite concentrations was 4:1 (139). The same 4:1 ratio was detected for the rates of formation of these catechols by breast cancer microsomes (135). It was concluded from all these studies that in rodent or human organs prone to estrogen-associated cancer, the predominant metabolic conversion of E_2 to 4-hydroxyestradiol might result in raised concentrations of this carcinogenic estrogen metabolite in these tissues. Local tissue catechol estrogen concentrations need to be measured in future studies to examine this possibility.

B. Metabolic activation of catecholestrogens

Catecholestrogens are capable of metabolic redox cycling as illustrated for 4-hydroxyestradiol in Fig. 2. This process consists of the organic hydroperoxide-dependent oxidation of the catecholestrogen (the hydroquinone) to the quinone, and the NADPH-dependent cytochrome P450 reductase-catalyzed reduction of the quinone intermediate back to the hydroquinone (140). The semiquinone free radical is an intermediate in each of these metabolic conversions. The estrogen semiquinone is a reactive species and may react with molecular oxygen and form quinone and superoxide radicals (141). Alternatively, nonenzymatic redox couples between copper ions and catecholestrogens also generate reactive oxygen radicals (142, 143). Thus, metal ion-catalyzed or enzyme-mediated redox cycling is a mechanism of metabolic activation resulting in the continuous formation of free radicals from possibly small amounts of catecholestrogen substrates that are reused in this process. This cycling reaction may go on indefinitely, depending on the availability of catechol substrate and organic hydroperoxide cofactor or metal ion for the oxidation step of the cycle.

In this context, it is noteworthy that the hormone antagonist tamoxifen stimulates quinone reductase (144, 145), which reduces estrogen quinone metabolites to hydroquinones (catechols) by two-electron reduction (140, 141). This direct reduction of quinones to hydroquinones bypasses the semiquinone radical intermediates and thus decreases free radical generation. Tamoxifen may thus protect from breast cancer by inhibiting hormone receptor-mediated proliferation of breast cancer cells and, in addition, by decreasing the toxicity and potential mutagenicity caused by quinones including estrogen quinone metabolites.

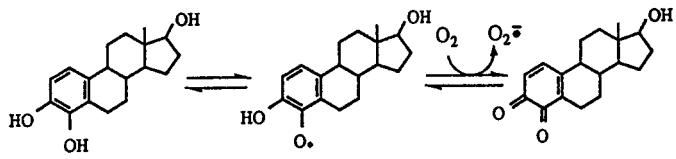

FIG. 2. Metabolic redox cycling of catecholestrogens. 4-Hydroxyestradiol (as shown) is capable of metabolic redox cycling between quinone and hydroquinone (catechol) forms. Catechols are oxidized by organic hydroperoxide-dependent cytochrome P450 1A enzymes or other peroxidases, whereas quinones are reduced by NADPH-dependent cytochrome P450 reductase or NADH-dependent cytochrome b_5 reductase (140). Both oxidation and reduction proceed via the semiquinone intermediate, which may react with molecular oxygen and form superoxide anion (141). 2-Hydroxyestradiol or other catecholestrogens (not shown) may undergo metabolic redox cycling in an analogous manner.

C. Free radical-mediated DNA damage induced by estrogens

Several types of free radical-mediated DNA damage are induced by estrogens and/or their metabolites and are listed in Table 1. For instance, DNA single-strand breaks are induced in MCF-7 human breast cancer cells in culture by 3,4-estrone quinone (146, 147), formed by oxidative metabolism of 4-hydroxyestrone. This type of DNA damage is also induced in ΦX-174 RFI plasmid DNA by 2-hydroxyestradiol and 10 μM Cu(II)sulfate and *in vivo* in the kidney of Syrian hamsters treated with either E_2 or 4-hydroxyestradiol many months before the development of neoplasms in this organ (142, 148). A tissue-specific induction of DNA single-strand breaks was observed in the dorsolateral prostates of Nobel rats treated with E_2 plus testosterone for 16 weeks before the development of E2 + testosterone-related prostate cancer in this tissue (149). In contrast, this lesion was not detected in ventral prostate, where cancers do not develop under these conditions, and was not induced in either tissue by androgen treatment alone.

Moreover, concentrations of 8-hydroxyguanine DNA bases, formed by hydroxy radical reaction with guanine bases, are increased over control values in DNA incubated either with catecholestrogens and copper(II) sulfate (143), with 4-hydroxylated estrogen metabolites and a microsomal activating system (150), with diethylstilbestrol and horseradish peroxidase (151), or *in vivo* in the DNA of Syrian hamsters treated with diethylstilbestrol (152), E_2, or 4-hydroxyestradiol (153). An analogous increase in hydroxy radical damage to DNA has been identified in human mammary tissue of breast cancer patients compared with controls (154, 155). Other forms of estrogen-induced free radical action are consistent with the DNA damage described above and include increased protein oxidation (156), lipid peroxidation in kidneys of estrogen-treated hamsters (157, 158) and in dorsolateral prostates of Noble rats treated with E_2 plus testosterone (149), and in low-density lipoprotein (LDL) (159). The role of estrogen-induced free radical generation and action in carcinogenesis is further supported by the decrease in E_2-induced hamster kidney tumor incidence by ascorbic acid (vitamin C) (160), which is a free radical scavenger and is

TABLE 1. Estrogen-induced direct or indirect DNA damage *in vitro* or in rodents

Type of DNA damage	Estrogen used	In cell free systems or cells in culture (reference)	*In vivo* (ref.)
Single-strand breaks			
	Estrone-3,4-quinone	MCF-7 cells (146, 147)	
	E_2		Hamster (148)
	2- or 4-Hydroxyestradiol	ΦX 174 RFI DNA (142)	Hamster (148)
	E_2 plus testosterone		Rat prostate (149)
8-Hydroxylation of guanine bases			
	2- or 4-Hydroxyestradiol	DNA, Cu(II)SO_4 (143)	
	4-Hydroxyestradiol	DNA, microsomes (150)	Hamsters (153)
	4-Hydroxyestrone	DNA, microsomes (150)	
	Equilenine-3,4-quinone	DNA, microsomes (150)	
	E_2		Hamsters (153)
Bulky DNA adducts (unknown structure)			
	E_2		Hamsters (162)
E_2-induced malondialdehyde-DNA adducts			
	E_2		Hamster (158)
Estrogen-DNA adducts			
	Estrone-3,4-quinone	DNA (165–168)	
	Estrone-3,4-quinone	COIII gene (171)	
	Estradiol-3,4-quinone	DNA (165–168)	Rat (167)
	4-Hydroxyestradiol	DNA, peroxidase (167)	Rat (167), hamster (175)
	4-Hydroxyestrone	DNA, peroxidase (167)	
	4-Hydroxyequilenine		
	Semiquinone	DNA (173, 174)	

known to reduce estrogen quinones to hydroquinones (161) but does not have any known estrogenic hormone antagonist activity.

D. Indirect DNA adduct formation induced by E_2

In addition to the direct free radical-initiated DNA damage described above, estrogen exposure also results in indirect DNA adduct formation (158, 162, 163). Some of these adducts have been formed by reactive aldehydes such as malondialdehyde, which are generated by decomposition of lipid peroxides produced by estrogen treatment of the animals. For instance, malondialdehyde-DNA adduct levels were increased over control values in hamsters treated with E_2 (158). Adducts of this type have also been identified in mammary DNA of breast cancer patients (164).

E. Direct estrogen DNA adducts

In addition to indirect DNA adduct formation, estrogen metabolites also are capable of direct covalent binding to DNA. As shown in Fig. 2, catecholestrogens may be oxidized to quinone intermediates, which may covalently bind to DNA *in vitro* (165, 166). The adducts of estrone-3,4-quinone, formed by oxidation of 4-hydroxyestrone, are unstable and decompose to form apurinic sites (166–168) consistent with adduction characteristics of carcinogenic hydrocarbons (169, 170). In contrast, the DNA adducts of estrone-2,3-quinone, formed by oxidation of 2-hydroxyestrone, are chemically stable and do not generate appreciable amounts of apurinic sites. The formation of the mutagenic apurinic sites by the carcinogenic 4-hydroxyestrogen metabolites and the generation of stable DNA adducts by the weakly or noncarcinogenic 2-hydroxyestrogen metabolites is consistent with adduct patterns of carcinogenic *vs.* weakly carcinogenic or noncarcinogenic hydrocarbons, respectively (169, 170). This adduct pattern has been taken as evidence for a mechanism of carcinogenesis by unstable adduct formation of 4-hydroxylated estrogens, induction of gene mutation, and subsequent tumor initiation (167, 169, 170). In incubations of estrone-3,4-quinone with the COIII gene, the estrogen metabolite was covalently bound mainly to guanine (171). Furthermore, the *in vitro* replication of the COIII template containing these adducts was obstructed, indicating an arrest of DNA polymerase by these estrogen metabolite-guanine lesions. 4-Hydroxyequilenin, a metabolite of the equine steroidal estrogen equilenin (172), which is a component of the common estrogen replacement medication Premarin (Ayerst Laboratories, New York, NY), forms unusual cyclic adducts with DNA *in vitro* (173, 174). Taken together, these data demonstrate that steroidal estrogens may be metabolically activated and form estrogen-DNA adducts *in vitro* (167, 168) and *in vivo* (175).

V. E_2-Induced Chromosomal or Genetic Mutations

Numerous genetic lesions affecting growth-controlling genes are part of a general genetic instability resulting in tumor development (87). These multiple types of genetic alterations include: 1) subtle sequence changes such as base substitutions, deletions, or insertions; 2) alterations in chromosome number such as losses or gains of whole chromosomes; 3) chromosome translocations; and 4) gene amplifications (87). The latter three of these types of genetic lesions have clearly been shown to be inducible by the natural hormone E_2 as discussed below: 1) numerical chromosomal alterations such as aneuploidy with or without apparent DNA damage; 2) structural chromosomal aberrations; and 3) c-*myc* gene amplications. In addition, there is preliminary evidence of estrogen-induced gene mutations and gene deletions. These events will be discussed in this order and are also listed in Table 2.

A. E_2-induced chromosomal aberrations

Changes in the number of chromosomes (numerical chromosomal aberrations or genome mutations) may be induced by E_2 and other estrogens in cells in culture (81, 85, 176) or in laboratory animals (93, 177, 178). In addition, E_2 is a potent inhibitor of mitosis *in vitro* and is capable of inducing genomic mutations in cultured cells (176, 179, 180). Potential targets for inducing numerical changes in the chromosome are the spindle apparatus (microtubules and centrioles), the DNA, regulating proteins, and centromeres. Alterations of these cellular components may be induced by estrogen metabolites directly via covalent binding or indirectly by free radical generation as discussed above.

Synthetic and natural estrogens including E_2 also induce structural chromosomal aberrations in addition to the numerical changes discussed above. For instance, perinatal exposure of rodents to estrogen results in chromosomal aberrations in the same target tissues in which tumors subsequently develop (181, 182). Treatment of Syrian hamsters with E_2 also leads to structural chromosomal aberrations such as deletions, inversions, and translocations in kidney cells long before tumors develop in this organ (93, 177, 178). The lower frequency of chromosomal aberrations in the hamster kidney cortex induced by 17α-ethinylestradiol compared with frequencies induced by E_2 or diethylstilbestrol (178) points to a role of catechol metabolites in the genesis of this lesion, because the rate of conversion of this synthetic estrogen to 2- and 4-hydroxylated metabolites by hamster kidney microsomes is one third the rate observed with the natural hormone (110) and correlates with the low carcinogenic activity compared with that of E_2 (10). In summary, E_2 induces aneuploidy and structural chromosomal changes (81, 85, 93, 176–183), which may be viewed as part of a larger pattern of various types of covalent damage to genetic material at the DNA or chromosome level occurring *in vitro* or *in vivo*. These types of chromosomal aberrations by themselves may not be sufficient for tumors to develop (86) but may contribute to tumorigenesis by compromising the integrity of the genetic material (87).

B. E_2-induced gene mutations

The mutagenic potential of estrogens including the natural hormone E_2 has been highly controversial. Early studies of the mutagenic activity of estrogens were all negative, *i.e.*, neither E_2 nor its catechol metabolites induced point mutations in the Ames bacterial reversion test (51–53), in Syrian hamster embryo cells (81–85), or in V79 Chinese hamster cells (53–55) in the concentration ranges tested. Estrogens including E_2 were classified as nonmutagenic and nongenotoxic based on this failure to induce gene mutations (49, 50, 83, 84). However, these results are not consistent with the various types of DNA damage discussed above, which are known to be potentially mutagenic.

More recent observations point to estrogen-induced gene mutations in several test systems. For instance, diethylstilbestrol induces mutations at the Na^+/K^+-ATPase locus (184). Moreover, either E_2 or the synthetic estrogen diethylstilbestrol are mutagenic and inactivate the gpt transgene of the Chinese hamster G12 cell line (185, 186). Specifically, the inactivation of the gpt transgene is caused by a pattern of mutations unique for a given mutagen. Diethylstilbestrol induces approximately 37% deletion and 25% methylation silencing among independent 6-thioguanine-resistant clones, whereas E_2 produced 53% deletions and only a few methylation-silenced mutants (186, 187). 4-Hydroxyestrone and 16α-hydroxyestrone both induce methotrexate resistance in MCF-7 breast cancer cells with an enhancement factor of 88 and 2-hydroxyestrone with an enhancement factor of 33 (188). In contrast, the parent hormone E_2 showed only a slight effect with an enhancement factor of 3.2. These data clearly implicate the metabolic activation of parent estrogens to catecholestrogens in the induction of this type of mutation. The induction of methotrexate resistance did not correlate with receptor-mediated responses (188). Both E_2 and 16α-hydroxyestrone stimulated expression of the pS2 gene, whereas 2- and 4-hydroxyestrone did not do so. The authors concluded that the development of methotrexate resistance was possible in the absence of estrogen receptors (188).

The testing of E_2 at various concentrations demonstrated a low frequency of mutations of the hprt gene by this hormone at the lowest dose assayed (10^{-10} M E_2) in V79 Chinese hamster lung cells, whereas at higher doses this effect was not observed (55). This mutagenic activity of E_2 at that low dose but not at elevated doses was independently confirmed (T. Albrecht and J.G. Liehr, unpublished). Moreover, Markides *et al.* (159) provided an explanation for this concentration dependence of the mutagenic activity of E_2 by demonstrating that only the catecholestrogen metabolites 2-

TABLE 2. E_2-induced genetic mutations

Type of genetic mutation	Test system (ref.)
Numerical chromosomal aberrations (aneuploidy)	
	Syrian hamster embryo cells (81, 85, 176)
	Human fibroblasts (183)
	Syrian hamster kidney (177, 178)
Structural chromosomal aberrations	
	Mouse genital tract (181, 182)
	Syrian hamster kidney (177, 178)
Gene mutations	
	gpt Transgene, Chinese hamster G12 cells (185, 186)
	hprt Gene, Chinese hamster V79 cells (55)
	Methotrexate resistance gene, MCF-7 human breast cancer cells (188)
Gene amplification	c-*myc* Gene, hamster kidney tumors (95)
Microsatellite instability	Syrian hamster kidney (189)

and 4-hydroxyestradiol exhibit prooxidant characteristics and only at low physiological concentrations and in the presence of metal ions. In contrast, at higher micromolar concentrations, all estrogens, including catecholestrogen metabolites, act as antioxidants. These data may provide an explanation for the failure of estrogens to induce mutations in previous studies, because only micromolar concentrations of E_2 have been examined in these previous assays (54, 55, 81–85).

In other more recent studies, a 2.4- to 3.6-fold amplification of the c-*myc* gene was detected by Southern blot analysis in 67% of primary renal tumors induced by E_2 or diethylstilbestrol treatment of Syrian hamsters (95). The c-*myc* gene was localized to hamster chromosome 6qb by fluorescence *in situ* hybridization. This chromosome 6 has a high frequency of trisomies and tetrasomies in the kidney of hamsters treated for at least 5 months and in renal tumors (95). Li *et al.* (95) concluded that estrogen-induced genomic instability, as demonstrated by c-*myc* gene amplification and concurrent chromosomal changes, was a key element in carcinogenic processes induced by estrogens (95). In the same animal model, E_2 has been shown to alter tandem repeat sequences of DNA (microsatellite instability) in premalignant kidney of hamsters treated with this hormone for 3 and 4 months and subsequently in kidney tumors that had developed after 7 months (189). This type of mutation has been shown to be inducible by free radicals (190) and may have been generated by metabolic redox cycling of estrogen metabolites (140, 141). This type of mutation is important because microsatellite instability has been detected in 100% of genital tract tumors induced in the daughters of women treated with the transplacental carcinogen and synthetic estrogen, diethylstilbestrol (191).

Taken together, these data demonstrate that estrogens, including the natural hormone E_2, induce multiple forms of genetic lesions including DNA microsatellite instability, DNA sequence deletions, gene amplification, chromosomal aberrations, and changes in the number of chromosomes. Such genetic alterations have recently been proposed by Lengauer, Kinzler, and Vogelstein (87) to be the basis of most human cancers. It is possible that estrogens may only be weak mutagens. However, only a low frequency of mutations is expected from natural circulating hormones. Thus, this area of research requires additional studies with more refined assay conditions designed to detect weak mutagens. Moreover, several types of mutations, such as DNA microsatellite instability or gene amplification, may have been missed by classical gene mutation assays because these tests are designed to detect only single-point mutations in only one specific gene.

VI. Indirect Evidence for the Genotoxic and Mutagenic Activity of E_2

In addition to the genotoxic and mutagenic activity of E_2 discussed above, other indirect biochemical and genetic evidence supports the role of genotoxicity and gene mutations in the induction of tumors by the natural hormone E_2 and contradicts a mechanism of oncogenesis based solely on hormonal receptor-mediated pathways. The following examples illustrate this dual role of estrogens as hormones and carcinogens:

1. There are several synthetic estrogens such as 2-fluoroestradiol and 17α-ethinylestradiol, which exhibit comparable hormonal potency, yet poor carcinogenicity compared with E_2, which induces a 100% tumor incidence in the Syrian hamster kidney model (10, 13, 60). These poorly carcinogenic estrogens, 2-fluoroestradiol and 17α-ethinylestradiol, have a decreased catecholestrogen formation compared to that of the parent estrogens (101, 110, 192). The existence of such poorly carcinogenic, yet hormonally potent, synthetic estrogens directly contradicts tumor incidence mediated solely by hormone receptor pathways. Their altered metabolism implicates catecholestrogen metabolites to play a crucial role in tumor initiation.

2. The induction of kidney tumors in hamsters by E_2 may be completely prevented by coadministration of α-naphthoflavone, an inhibitor of cytochrome P450 1A-mediated catecholestrogen formation, or inhibited by ascorbic acid (vitamin C), a free radical scavenger and reductant of the DNA-reactive catecholestrogen quinone metabolites (160, 161, 193). This modulation of E_2-induced carcinogenesis by decreasing concentrations of catecholestrogen or catecholestrogen quinone metabolites further supports the concept of tumor initiation by reactive metabolic intermediates of this hormone.

In this context, it is noteworthy that Ah receptor agonists such as 2,3,7,8-tetrachlorodibenzo-p-dioxin, which induce the metabolic conversion of E_2 to 4-hydroxyestradiol (136), do not appear to induce mammary carcinogenesis. To the contrary, in rats exposed to this chemical, spontaneous mammary and uterine tumorigenesis is decreased over controls, and the sizes of chemically induced tumors are reduced (194, 195). In humans, short-term exposure to this organochlorine compound (*e.g.*, after an explosion of a chemical manufacturing facility in Seveso, Italy) may provide protection from mammary cancer (196), whereas long-term occupational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin slightly elevates the risk for breast cancer (197–199). These apparently conflicting results may be due to various biological effects of this organochlorine chemical on the carcinogenesis process. In addition to stimulation of estrogen hydroxylation via the Ah receptor, it may also act as an antiestrogen and inhibit a variety of hormone receptor-mediated responses [reviewed by Safe (200)]. Thus, it is possible that this compound may stimulate tumor initiation by inducing metabolic activation, but then may inhibit the completion of tumor development by its hormone antagonism. These data illustrate that chemical modulators of estrogen-induced carcinogenesis may be useful for the study of mechanistic aspects only if they alter narrowly defined biological parameters. Mechanistic conclusions cannot be drawn from studies of agents with multiple biochemical and endocrine effects.

3. Estrogen receptors in the human mammary epithelium are localized in cells distinct and different from cells expressing markers of cell proliferation (58, 59). Moreover, SHE cells, which have been used to study the mechanism of estrogen-induced cell transformation (81–85), do not express measurable levels of estrogen receptor and estrogen treatment is not mitogenic to these cells (201). In this cell line, either estrogens or the hormone antagonists tamoxifen or ICI 164,384 induce morphological transformation and aneuploidy (176). These

data indicate that estrogen-induced cell transformation and aneuploidy arise in cells early in the carcinogenesis process and do not require estrogen receptors. Moreover, receptor-mediated processes may be linked indirectly rather than directly to mammary cell proliferation during mammary oncogenesis as discussed in *Section III.A*.

4. The strongest evidence for an additional (carcinogenic) role of estrogens in hormone-induced oncogenesis is provided by experiments in transgenic mice. Mice overexpressing the Wnt-1 gene produce elevated amounts of a protein important in cell signaling during embryonal development. These mice develop mammary tumors with high incidence within a few months after birth (202). These transgenic mice have been cross-bred with estrogen receptor-α knockout (ERKO) mice to examine the role of estrogen receptors in breast tumor incidence (203). The incidence of mammary tumors was delayed, but not eliminated, in the cross-bred animals (48 weeks) compared with mice only overexpressing the Wnt-1 gene (24 weeks). When the Wnt-1 overexpressing/estrogen receptor-α knockout cross-bred animals were ovariectomized to reduce their E_2 production, the mammary tumor incidence was significantly reduced (203). The authors concluded that ectopic expression of the Wnt-1 protooncogene induces mammary tumors in transgenic mice in the absence of estrogen receptors. Moreover, decreases in circulating E_2 concentrations achieved by ovariectomy of these animals decrease this tumor incidence. The data support a role of genotoxicity of E_2 in mammary carcinogenesis and contradict oncogenesis in this organ mediated solely by hormone receptor pathways.

All these data are consistent with and support the conclusion that genotoxic processes and gene mutations participate and play a tumor-initiating role in the induction of mammary tumors by the natural hormone E_2. These data are inconsistent with tumor induction solely based on hormonal receptor-mediated processes (as postulated previously (23, 49, 50).

Estrogen-induced carcinogenesis in the mammary gland and in other organ sites likely is complex and requires *both* receptor-mediated and genotoxic events for neoplastic development. Indirect evidence in support of this dual role of estrogens as hormones and as tumor-initiating chemicals is the inhibition of tumor incidence either by: 1) hormone antagonists interfering with receptor-stimulated cell proliferation (44, 47, 48); or 2) inhibitors of metabolic activation of estrogens (160, 161, 193). An important aspect of this proposed action of estrogens is that inhibition of either of these events will inhibit oncogenesis, albeit at a different stage of neoplastic development. The modulation of receptor-mediated tumor cell proliferation by hormone antagonists thus may leave intact the accumulated genetic lesions induced by estrogens and/or other carcinogens. This concept is supported by the inhibition of estrogen-induced renal carcinogenesis in Syrian hamsters by tamoxifen without concomitant decrease in estrogen-induced DNA adduct levels (48). In contrast, inhibitors of metabolic activation of estrogens are proposed to act by inhibiting the accumulation of potentially mutagenic DNA alterations induced by estrogens. This concept is supported by the inhibition of estrogen-induced renal tumorigenesis in hamsters by α-napthoflavone or ascorbic acid (vitamin C) (160, 161, 193). It is also supported by the action of poorly carcinogenic, yet hormonally potent, synthetic estrogens 2-fluoroestradiol or 17α-ethinylestradiol (10, 13, 60). Inhibitors of estrogen metabolism have not yet been explored for the prevention of breast and other hormone-associated cancer in humans and may offer an attractive alternative to hormone antagonists, because they may inhibit mammary tumorigenesis at an early stage.

VII. Summary and Conclusion

The data outlined above clearly demonstrate that the natural hormone E_2 is a carcinogen in humans and in animals (1–40). Multiple forms of DNA damage are induced by E_2 *in vitro*, in cells in culture, and in laboratory animals (142–153, 158, 162–178). Several of these estrogen-induced DNA lesions have also been detected in human tissue (154, 155, 164, 183). In addition, E_2 induces at least a low frequency of gene mutations (55, 95, 185–189). The failure to detect mutagenic activities of steroidal hormones reported previously may have been due to either inappropriate assay conditions, which could not have identified a weak mutagen, or due to an inappropriate choice of assays not designed to detect the type(s) of mutations induced by E_2.

The multiple forms of DNA damage induced by catecholestrogen metabolites after metabolic activation to quinone-reactive intermediates provide strong support for the conclusion that the natural estrogenic hormone E_2 exerts genotoxicity most likely via metabolic activation to catecholestrogens. The induction of gene mutation by estrogens outlined above also supports this conclusion but requires further work and experimental detail. We do not yet know which critical genes are mutated by estrogen or their metabolites in the oncogenesis process and the mechanism of induction of mutations. Much additional research is needed to sketch the mechanistic events resulting in hormone-associated cancer.

Despite these deficiencies in our knowledge of the mutagenic activity of E_2, the human epidemiological studies point to estrogen as a weak carcinogen adding approximately 3% breast cancer risk/year of estrogen exposure (39, 40). These human data are in line with animal carcinogenicity and cell culture data. They are also in agreement with the more moderate levels of DNA modification by estrogen compared with the substantial genotoxicity of potent carcinogens such as benzo[a]pyrene or 7,12-dimethylbenzanthracene (169, 170). The weak mutagenic activity of E_2 at the hprt locus of V79 cells also points to E_2 as a weak mutagen/carcinogen (55). In a comparison of the induction of aneuploidy by E_2 in human and hamster fibroblasts, Tsutsui *et al.* noted the much weaker induction of this genetic instability in the human compared with the rodent cells (183). This weak mutagenic activity of E_2 explains the difficulties of previous workers to detect any mutational events and the underlying genotoxicity induced by E_2 and makes understandable the resulting eagerness to classify estrogens as epigenetic, nonmutagenic carcinogens. However, this classification will have to be reconsidered in light of the more recent evidence cited above. The weak mutagenic activity of E_2 is also understandable in view of the

role of this endogenous hormone in many physiological processes. A high mutagenic and carcinogenic activity of E_2 would not have permitted the existence of many higher life forms including that of the human species.

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