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The etiology and prevention of breast cancer

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Abstract

Metabolism of estrogens via the catechol estrogen pathway is characterized by a balanced set of activating and protective enzymes (homeostasis). Disruption of homeostasis, with excessive production of catechol estrogen quinones, can lead to reaction of these quinones with DNA to form depurinating estrogen-DNA adducts. Some of the mutations generated by these events can lead to initiation of breast cancer. A wealth of evidence, from studies of metabolism, mutagenicity, cell transformation and carcinogenicity, demonstrates that estrogens are genotoxic. Women at high risk for breast cancer, or diagnosed with the disease, have relatively high levels of depurinating estrogen-DNA adducts compared to normal-risk women. The dietary supplements *N*-acetylcysteine and resveratrol can inhibit formation of catechol estrogen quinones and their reaction with DNA to form estrogen-DNA adducts, thereby preventing initiation of breast cancer.

Introduction

Chemical carcinogenesis became a distinct discipline of cancer research when James and Elizabeth Miller established that chemical carcinogens react covalently with the nucleophilic groups of DNA, RNA and proteins [1–3].

On the basis of cellular functions, it is logical to hypothesize that DNA is the key macromolecule reacting with carcinogens. In fact, the development of malignant cells after transfection of mice with DNA from cells malignantly transformed by chemicals points indeed to the mutagenic basis of cancer initiation [4]. The fact that genetic changes in cells are irreversible and that malignant changes are transmitted from one generation of cells to another support DNA as the crucial cellular target for cancer initiation. Furthermore, detailed knowledge of the mechanism of cancer initiation provides the strongest basis from which the prevention of human cancers can be approached.

Covalent reaction of electrophilic metabolites of carcinogens with DNA is the major cause of DNA damage leading to cancer-initiating mutations. Generally this process involves formation of carcinogen-DNA adducts. There are two types of adducts when chemical carcinogens react with DNA: stable adducts that remain in DNA, unless removed by repair

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(e.g. adducts at the exocyclic NH₂ group of Ade or Gua), and depurinating adducts that are released from DNA with formation of apurinic sites (e.g. adducts at the N3 and N7 of Ade, N7 of Gua, and sometimes C8 of Gua [5–8]). The N7 position of Gua, and the N3 and N7 positions of Ade are among the most nucleophilic sites in DNA [9]. When DNA is exposed to carcinogens, these are the positions where covalent bonds are most abundantly formed.

Among the chemical carcinogens, polycyclic aromatic hydrocarbons (PAH) have two major mechanisms of metabolic activation to produce ultimate carcinogens: one is formation of radical cations and the other is formation of bay-region diol epoxides [5,6]. Evidence that depurinating PAH-DNA adducts play a major role in cancer initiation derives from a correlation between the sites of depurinating adducts in DNA and the sites of mutations of the Harvey(H)-*ras* oncogene in mouse skin papillomas and in preneoplastic mouse skin [10–12].

For compounds containing one or two benzene rings, there is a third mechanism of metabolic activation, which produces extremely weak ultimate carcinogens. In these compounds, activation occurs via metabolic formation of electrophilic catechol quinones, which react with DNA by Michael addition to form DNA adducts.

This mechanism of activation has been demonstrated to occur with benzene [13,14], naphthalene [15,16], estrone (E₁) and estradiol (E₂) [17–21], diethylstilbestrol [22], hexestrol [20,23,24] and possibly bisphenol A [25] (Fig. 1). In this mechanism, the benzene ring is enzymatically hydroxylated to form a phenol. Then, a second hydroxylation leads to formation of a catechol, followed by oxidation to produce the electrophilic ultimate carcinogenic *ortho*-quinone metabolite (Fig. 1). The *ortho*-quinone reacts with the purine bases of DNA to form N3Ade and N7Gua adducts that subsequently depurinate (Fig. 1).

Estrogen genotoxicity mechanism in the etiology of breast cancer

Exposure to estrogens has been epidemiologically associated with increased risk of breast cancer in premenopausal and postmenopausal women [26,27].

For years, the scientific community did not accept estrogens as chemical carcinogens, because these compounds were not found to induce mutations in bacterial and mammalian test systems [28–32]. These findings led scientists to classify E₁ and E₂ as epigenetic carcinogens that operate mainly by stimulating abnormal cell proliferation via estrogen receptor (ER)-mediated processes [29,33–37]. The stimulated cell proliferation would generate more opportunities for mutations leading to carcinogenesis [33,37,38]. The ER-mediated events can be involved in accelerating the process of carcinogenesis, but they do not play any role in cancer initiation because the hypothesized mutations obtained during cell proliferation are random.

The findings that specific oxidative metabolites of estrogens, catechol estrogen quinones, can be formed in estrogen metabolism and can react with DNA led to and support the hypothesis that these estrogen metabolites can become endogenous chemical carcinogens by generating the mutations leading to the initiation of cancer [8,18,19,39,40]. This paradigm suggests that specific mutations generate abnormal cell proliferation leading to cancer,

rather than ER-mediated abnormal cell proliferation giving rise to random mutations [28,33–37]. The specificity of the crucial mutations derives from the preliminary intercalating physical complex between the estrogens and DNA before formation of a covalent bond between them. This has been demonstrated by studying the mechanism of cancer initiation by the human carcinogen diethylstilbestrol [22].

Compelling evidence from studies of estrogen metabolism, formation of DNA adducts, mutagenicity, cell transformation and carcinogenicity led to and supports the hypothesis that specific estrogen metabolites, the catechol estrogen quinones, can react with DNA to form estrogen-DNA adducts in crucial genes, initiating the process that leads to breast cancer development [8,40]. The major initiating pathway is shown in Fig. 2. E_1 and E_2 can be metabolically converted into 4-hydroxy $E_1(E_2)$ by cytochrome P450 (CYP) 1B1. Oxidation of the catechol estrogens leads to the corresponding $E_1(E_2)$ -3,4-quinone [$E_1(E_2)$ -3,4-Q], which can react with DNA to form small amounts of stable adducts remaining in the DNA, unless removed by repair, and predominant amounts of the depurinating adducts 4-OHE $_1(E_2)$ -1-N3Ade and 4-OHE $_1(E_2)$ -1-N7Gua (Fig. 2), which detach from DNA, leaving behind apurinic sites. Possible errors in the repair of these sites can lead to the crucial mutations initiating breast and other cancers [8,40].

Metabolism of estrogens

Metabolic formation of estrogens occurs by aromatization of androstenedione and testosterone, catalyzed by CYP19 (aromatase), to produce E_1 and E_2 , respectively (Fig. 3). E_1 and E_2 are interconverted by 17 β -hydroxysteroid dehydrogenase. When an excess of estrogen is produced, it is stored as estrone sulfate. Estrogens are metabolized via two main pathways: formation of the 16 α -OHE $_1(E_2)$ (not shown in Fig. 3) and formation of the catechol estrogens 2-OHE $_1(E_2)$ and 4-OHE $_1(E_2)$ (Fig. 3) [41]. CYP1A1 hydroxylates E_1 and E_2 preferentially at the 2 position, whereas CYP1B1 catalyzes the hydroxylation almost exclusively at the 4 position [42–44]. The two catechol estrogens are inactivated by conjugation to glucuronides and sulfates, especially in the liver (not shown in Fig. 3). In extrahepatic tissues, the most common path of conjugation of catechol estrogens is *O*-methylation, catalyzed by catechol-*O*-methyltransferase (COMT) [45,46]. A low activity of COMT renders more competitive oxidation of the catechol estrogens to $E_1(E_2)$ -2,3-Q and $E_1(E_2)$ -3,4-Q catalyzed by CYP or peroxidase (Fig. 3).

Oxidation of semiquinones to quinones can also be obtained by molecular oxygen. Reduction of estrogen quinones to semiquinones by CYP reductase completes the redox cycle (Fig. 3). In this process, the molecular oxygen is reduced to superoxide anion radical, and then converted by superoxide dismutase to hydrogen peroxide. In the presence of Fe^{2+} the hydrogen peroxide is converted to hydroxyl radical. Reaction of the hydroxyl radical with lipids yields lipid hydroperoxides [47] (not shown in Fig. 3), which can serve as unregulated cofactors for the oxidation of catechol estrogens by CYP. Thus, redox cycling can become a major contributor to the formation of catechol estrogen quinones, which are the ultimate carcinogenic metabolites of estrogens.

4-OHE₁(E₂) are more potent carcinogens than 2-OHE₁(E₂) [48–50]. This property cannot be attributed to formation of hydroxyl radicals from redox cycling, because the 2-OHE₁(E₂) and 4-OHE₁(E₂) have the same redox potential [51,52]. The greater carcinogenic potency of 4-OHE₁(E₂) is related to the much higher levels of depurinating DNA adducts formed by the E₁(E₂)-3,4-Q compared to the E₁(E₂)-2,3-Q (Fig. 4) [21]. This is due to different mechanisms of adduction. The E₁(E₂)-3,4-Q react via a proton-assisted 1,4-Michael addition [53], whereas the E₁(E₂)-2,3-Q rearrange to *para*-quinone methides, which then react via a 1,6-Michael addition [54].

Following the formation of catechol estrogen quinones (Fig. 3), they can be inactivated by conjugation with glutathione (GSH). Another inactivation pathway for the quinones is reduction to their respective catechols by quinone reductase [55,56], a protective enzyme that can be induced by a variety of compounds [57]. If all the protective processes are insufficient, the catechol estrogen quinones can react with DNA to form predominantly depurinating adducts.

Depurinating estrogen-DNA adducts in the etiology of breast cancer

Carcinogens react with DNA to form two types of adducts: stable adducts and depurinating adducts. Investigators in chemical carcinogenesis have always considered only stable adducts, which remain in DNA unless removed by repair. These adducts are usually detected, but not identified, by the ³²P-postlabeling technique.

In general, metabolically activated PAH and estrogens predominantly produce adducts with DNA at the most nucleophilic sites of Ade and Gua, with destabilization of the glycosyl bond and subsequent depurination. In the Watson-Crick DNA model (Fig. 5), the backbone is constituted by deoxyribose and phosphate, the Gua is hydrogen-bonded to cytosine, and Ade is hydrogen bonded to thymine. The Gua has an exocyclic NH₂ group that can react with electrophiles to form a stable adduct (Fig. 5, hollow arrow). If reaction occurs at the N-7 and sometimes C-8 of Gua, depurinating adducts are formed (Fig. 5, solid arrows). In the case of Ade, reaction of an electrophile at the exocyclic NH₂ group forms a stable adduct (Fig. 5, hollow arrow), whereas depurinating adducts are obtained at the N-3 and N-7 sites (Fig. 5, solid arrows). Following the reaction at the N-3 of Ade, destabilization of the glycosyl bond occurs via formation of an intermediate oxocarbenium ion with subsequent depurination and generation of an apurinic site in the DNA [58].

The first evidence that depurinating DNA adducts play a major crucial role in cancer initiation was obtained from a correlation between the level of depurinating PAH-DNA adducts and oncogenic H-*ras* mutations in mouse skin papillomas [10]. The potent carcinogens 7,12-dimethylbenz[*a*]anthracene (DMBA) [59] and dibenzo[*a,l*]pyrene (DB[*a,l*]P) [60,61] yield predominantly depurinating Ade adducts that induce A to T transversions in codon 61 [10]. By comparison, the weaker carcinogen benzo[*a*]pyrene (BP) forms twice as many Gua depurinating adducts as Ade depurinating adducts [62], corresponding to twice as many codon 13 G to T transversions as codon 61 A to T transversions [10], respectively.

A similar correlation between the sites of formation of depurinating DNA adducts and *H-ras* mutations was found in mouse skin and rat mammary gland treated with the ultimate carcinogenic metabolite E₂-3,4-Q [63,64] (see below). When E₁(E₂)-3,4-Q react with DNA, they form 99% depurinating adducts 4-OHE₁(E₂)-1-N3Ade and 4-OHE₁(E₂)-1-N7Gua by the 1,4-Michael addition mechanism (Figs 2 and 3) [17–19,21], whereas E₁(E₂)-2,3-Q produce much lower levels of 2-OHE₁(E₂)-6-N3Ade by the 1,6-Michael addition mechanism that occurs after tautomerization of the E₁(E₂)-2,3-Q to the E₁(E₂)-2,3-Q methide [21,54]. The levels of DNA adducts formed by the two catechol estrogen quinones are in agreement with the greater carcinogenic potency of 4-OHE₁(E₂) compared with the borderline carcinogenic activity of 2-OHE₁(E₂) [48–50].

Mutagenicity of estrogens

To understand how estrogen-DNA adducts induce mutations leading to cancer, one needs to start by looking at the relationship between PAH-DNA adducts and oncogene mutations in mouse skin. A correlation was found between the proportion of depurinating adducts and *H-ras* mutations formed in mouse skin treated with one of three carcinogenic PAH: BP, DMBA and DB[*a,l*]P. Both DMBA and DB[*a,l*]P form predominantly depurinating Ade adducts, 79% and 81%, respectively (Table 1) and induce CAA to CTA transversions in codon 61 of the *H-ras* gene in the tumors [10]. BP, however, is different. BP forms depurinating Gua (46%) and Ade (25%) adducts in the mouse skin tumors, which contain 48% GGC to GTC mutations at codon 13 and 24% CAA to CTA mutations at codon 61 in the *H-ras* gene [10]. Similar results were obtained with BP by Colapietro *et al.* [65]. Thus, the oncogene mutations correlate with the level of depurinating adducts at Ade or Gua.

Correlation of the levels of depurinating Ade and Gua adducts with the mutated base in the *H-ras* oncogene was subsequently found in preneoplastic mouse skin within 12 hr of treatment with BP, DMBA or DB[*a,l*]P [11]. This finding led to two discoveries. First, these mutations arise by misrepair, not misreplication, of the adducted DNA, because misreplication requires two rounds of DNA replication, which would take three days [12]. Second, the mutations occur at apurinic sites generated by loss of the depurinating DNA adducts [12].

The understanding of how depurinating PAH-DNA adducts generate cancer-initiating mutations led rapidly to the discovery that the overwhelmingly predominant depurinating estrogen-DNA adducts generate oncogene mutations in mice and rats (Table 2) [63,64].

Although early studies indicated that estrogens were not mutagenic [66], more appropriate assays have demonstrated the ability of estrogens to directly induce mutations. A survey of the IARC *p53* database suggests that estrogens can induce mutations in breast cancer in humans [67]. When sporadic breast cancers are compared to germline cases and cancer in hormone-independent tissues such as lung, bladder and brain, mutational hotspots are observed at codons 163 and 179 in the *p53* gene. These hotspots have an increased frequency of A.T to G.C mutations. In addition, when cultured human breast epithelial cells were transformed by E₂ or 4-OHE₂, a 5-bp deletion in exon 4 of TP53 on chromosome 17 was observed [68]. Furthermore, women with familial breast cancer exhibiting BRCA

mutations have similarly increased frequencies of A.T to G.C mutations and hotspots at codon 163, among others, in the *p53* gene [69].

Clear evidence of the mutagenicity of estrogens has been obtained using the Big Blue[®] (BB[®]) rat2 embryonic cell line. This cell line is transfected with approximately 60 copies of the Lambda-LIZ vector, which enables detection of mutations in the *lacI* and or *cII* genes. Multiple treatments of BB[®] rat2 cells with 4-OHE₂ induced a dose-dependent, statistically significant increase in the mutant fraction of cells [70]. E₂-3,4-Q was similarly mutagenic, but 2-OHE₂ was not [70].

Further evidence for the mutagenicity of estrogens was obtained by implanting female BB[®] rats, a Fisher 344 strain containing approximately 80 copies of the Lambda-LIZ vector in each cell, with E₂ and/or 4-OHE₂. The vector has no effect on the physiology or biochemistry of the rats. Treatment with the estrogens induced mutations in the *cII* gene. 4-OHE₂ specifically induced A.T to G.C mutations, consistent with the formation of 4-OHE₂-1-N3Ade adducts [40].

Treatment of female ACI rats by intramammary injection of E₂-3,4-Q induced A.T and G.C mutations [64], and produced predominantly depurinating 4-OHE₂-1-N3Ade and 4-OHE₂-1-N7Gua adducts (Table 2) [19]. These findings are consistent with error-prone repair of depurinating adduct-generated apurinic sites being the mechanism of induction of mutations leading to breast cancer.

In summary, a variety of types of data from both *in vitro* and *in vivo* experiments have demonstrated that the estrogen metabolites 4-OHE₂ and E₂-3,4-Q induce mutations in rats, mice and human-derived breast epithelial cells. Some of these mutations can play a role in the initiation of breast cancer.

Imbalances of estrogen metabolism in cancer initiation

The metabolism of estrogens via the catechol estrogen pathway is characterized by a balanced set of activating and protective enzymes (homeostasis), which minimize the oxidation of catechol estrogens to quinones and their reaction with DNA (Fig. 3). Initiation of cancer by estrogens is based on estrogen metabolism in which homeostasis has been disrupted. A variety of endogenous and exogenous factors can disrupt estrogen homeostasis. These include diet, environment, lifestyle, aging and genetic factors.

Before describing various imbalancing factors in estrogen metabolism, it is appropriate to report a known factor that can help maintain estrogen homeostasis. This is the feedback inhibition exerted by methoxy estrogens on the expression of CYP1A1 and CYP1B1 [71], which helps regulate the levels of catechol estrogens.

One factor that can imbalance estrogen metabolism is excessive synthesis of estrogens by overexpression of CYP19 (aromatase) in target tissues [72–74] and/or the presence of unregulated sulfatase that converts excess stored E₁-sulfate into E₁ (Fig. 3) [75,76]. A second factor that can imbalance estrogen homeostasis may be the production of high levels of 4-OHE₁(E₂), due to overexpression of CYP1B1, which converts E₁(E₂) predominantly to

4-OHE₁(E₂) (Fig. 3) [43,44,77,78]. Higher levels of 4-OHE₁(E₂) could result in more oxidation to the major ultimate carcinogenic metabolites E₁(E₂)-3,4-Q. An analogous effect could be produced by a lack of or low level of COMT activity due to polymorphic variation [46,79]. Insufficient activity of this enzyme would be translated into low levels of methylation of 4-OHE₁(E₂) and subsequent increase of the competitive oxidation of 4-OHE₁(E₂) to E₁(E₂)-3,4-Q (Fig. 3). Higher levels of E₁(E₂)-3,4-Q can also be obtained by polymorphism of quinone reductase (NQO1) that leads to decreased conversion of quinones into catechols [80] (Fig. 3). Similarly, low cellular levels of GSH, which reacts efficiently with the quinones, can produce higher levels of E₁(E₂)-3,4-Q.

Imbalances in estrogen metabolism have been observed in animal models for estrogen carcinogenicity: the kidney of male Syrian golden hamsters [81], the prostate of Noble rats [82] and the mammary gland of ER- α knockout mice [83]. Imbalance of estrogen homeostasis can also be observed by comparing analysis of breast tissue from women with and without breast cancer [84]. In normal breast tissue from women with breast carcinoma, the levels of 4-OHE₁(E₂) were nearly four-times higher than the levels in breast tissue from women without breast cancer. The 4-catechol pathway contributes more than 95% to the initiation of cancer (see below). In addition, the levels of catechol estrogen-GSH conjugates in normal breast tissue from women with breast carcinomas were three-times the level in breast tissue from control women [84]. The excessive amounts of catechol estrogen-GSH conjugates detected were used as surrogates for the formation of depurinating estrogen-DNA adducts. Further evidence of imbalance in estrogen homeostasis derives from the greater expression of estrogen-protective enzymes (COMT and NQO1) (Fig. 3) in breast tissue of women without breast cancer and higher expression of estrogen-activating enzymes (CYP19 and CYP1B1) (Fig. 3) in breast tissue of women with breast cancer [85].

The imbalance of estrogen metabolism is not only governed by endogenous factors, but also by environmental factors. These factors include substances we ingest by mouth, skin and nose. It is logical to hypothesize that there are environmental compounds capable of affecting estrogen metabolism with increased formation of catechol estrogen quinones. For example, dioxin induces expression of the activating enzyme CYP1B1 [77,78] (Fig. 3). These compounds do not act as direct carcinogens themselves, but can make the estrogens become carcinogenic by disrupting homeostasis.

Estrogen-DNA adduct biomarkers in women with and without breast cancer

If estrogens initiate breast cancer by a genotoxic mechanism, formation of depurinating estrogen-DNA adducts should be significantly higher in women at high risk for breast cancer, as well as women diagnosed with breast cancer. This would be a fundamental characteristic of women at high risk for breast cancer. Indeed, observation of higher levels of estrogen-DNA adducts in women at high risk for breast cancer would indicate that formation of these adducts is a causative factor in the etiology of breast cancer and not a consequence of the cancer itself.

Three studies have been conducted of women at normal and high risk for breast cancer, as well as women diagnosed with the disease. In the first two studies, a spot urine sample was

obtained from each woman and analyzed by ultraperformance liquid chromatography/tandem mass spectrometry for 38 estrogen metabolites, estrogen conjugates and depurinating estrogen-DNA adducts [86,87]. For each subject, the ratio of depurinating estrogen-DNA adducts, 4-OHE₁(E₂)-1-N3Ade plus 4-OHE₁(E₂)-1-N7Gua or 2-OHE₁(E₂)-6-N3Ade, to their respective estrogen metabolites and conjugates (see formula in Fig. 6) was calculated and compared. High levels of estrogen-DNA adducts have been seen in the first two studies analyzing urine samples [86,87] and in the third study analyzing serum [88] from women that are at high risk of breast cancer or have the disease (Fig. 6) [86–88]. Highly significant differences in relative levels of estrogen-DNA adducts were observed when urine or serum samples from normal-risk women were compared to those from high-risk women or those with breast cancer (Fig. 6). In the ratio of estrogen-DNA adducts to estrogen metabolites and conjugates, the adducts arising from reaction of E₁(E₂)-3,4-Q with DNA play the predominant role (97%), while the adducts arising from reaction of E₁(E₂)-2,3-Q play a minimal role, that is, less than 3% [86–88]. This finding is consistent with the minimal ability of E₂-2,3-Q to form DNA adducts compared to E₂-3,4-Q [21] and the borderline carcinogenic activity of the 2-OHE₁(E₂) compared to that of the 4-OHE₁(E₂) [48–50]. These studies showed that elevation of estrogen-DNA adduct levels through imbalanced estrogen metabolism is associated with high risk of developing breast cancer.

Breast tissue from women with breast cancer has higher levels of the activating enzymes, aromatase (CYP19) and CYP1B1, while breast tissue from women without breast cancer has higher levels of the protective enzymes, COMT and NQO1 [85]. These findings support the idea that imbalances in estrogen metabolism can lead to the initiation of breast cancer.

Carcinogenic activity of estrogens in cultured human breast epithelial cells and animal models

Treatment of cultured human breast epithelial cells with estrogens has provided a good model for studying the mechanism of carcinogenesis by estrogens. The immortalized, nontransformed cell line MCF-10F does not contain ER- α ; when these cells are treated with E₂ or 4-OHE₂, depurinating estrogen-DNA adducts are formed [77,89,90] and transformation of the cells is detected by growth in soft agar [91–93]. The catechol estrogen 2-OHE₂ can also transform these cells, but to a much lesser extent. The presence of an anti-estrogen such as tamoxifen or ICI-182,780 does not inhibit the estrogen-induced transformation of MCF-10F cells [94]. These results indicate that the cells are transformed through the genotoxic effects of E₂ and 4-OHE₂, not through ER-mediated processes. If the transformed MCF-10F cells are put through a selection process based on their invasiveness and the aggressive cell lines are implanted into severely compromised immunodeficient mice, tumors develop [93,95], demonstrating that the cells have been transformed into cancer cells.

The ability of estrogens to induce cancer was first demonstrated in laboratory animals. When male Syrian golden hamsters were implanted with E₁, E₂, diethylstilbestrol or hexestrol, kidney tumors were induced [96]. It was then discovered that 4-OHE₁(E₂), but not 2-OHE₁(E₂), also induced kidney tumors in the hamsters [48,49]. The 2-OHE₁(E₂) were

eventually shown to have borderline activity in inducing uterine tumors in CD-1 mice, but the 4-OHE₁(E₂) had much higher activity in this model [50]. The lack or very low level of carcinogenicity of the 2-OHE₁(E₂) is consistent with the much smaller ability of the 2,3-quinones to react with DNA to form adducts, compared with that of the 3,4-quinones [21].

Nonetheless, studies in the above animal models did not answer questions about the role of ER- α -mediated events in the induction of tumors by estrogens. To address these questions, a strain of transgenic mice with ER- α knocked out was developed by Bocchinfuso *et al.* [97,98], the ERKO/*wnt-1* mouse. Despite the presence of the *wnt-1* transgene, these mice were not expected to develop mammary tumors because of the lack of ER- α . By contrast, 100% of the female ERKO/*wnt-1* mice developed mammary tumors, although at a slower rate than the parent female *wnt-1* mice [98]. The catechol estrogens 4-OHE₁(E₂) and the GSH conjugates formed by E₁(E₂)-3,4-Q were detected in mammary tissue from the female ERKO/*wnt-1* mice, but no methoxy estrogens were detected [83], suggesting that these mice have little protection from oxidation of the 4-catechol estrogens to their reactive quinones.

To further our understanding of the mechanism of tumor initiation by estrogens, female ERKO/*wnt-1* mice were ovariectomized at 15 days of age, removing the major source of estrogens, and implanted with one of several doses of E₂. Mammary tumors developed in a dose-dependent manner [99,100], even when the mice were implanted with E₂ plus the anti-estrogen ICI-182,780 [101]. Taken together, these results provide strong evidence that estrogens initiate cancer through a genotoxic mechanism, rather than by ER-mediated events.

Key factors in the catechol estrogen metabolic pathway

The metabolism of estrogens via the catechol estrogen pathway is regulated by a balanced set of activating and protective enzymes that keeps the system in homeostasis. Under these conditions, formation of catechol estrogen quinones and their reaction with DNA is minimal (Fig. 3). When the homeostatic conditions are disrupted, higher levels of catechol quinones and estrogen-DNA adducts are formed, and cancer can be initiated. This hypothesis has received strong support from three studies in which the levels of estrogen-DNA adducts were determined in urine and serum samples from women. Women at normal risk of breast cancer had relatively low levels of adducts compared to women at high risk of breast cancer or diagnosed with the disease (Fig. 6) [86–88]. Conversely, the levels of estrogen metabolites and conjugates were higher in women at normal risk of breast cancer compared to the women at high risk or with breast cancer (Fig. 6) [86–88].

The levels of estrogen metabolites, estrogen conjugates and depurinating estrogen-DNA adducts rely on the expression of five estrogen-metabolizing enzymes. The first activating enzyme is CYP19 (aromatase), which catalyzes the conversion of androgens into estrogens (Fig. 3). An excessive amount of estrogen produced by over-expression of CYP19 [72–74] can imbalance their metabolism. The second activating enzyme is CYP1B1, which almost exclusively converts E₁(E₂) to 4-OHE₁(E₂) (Fig. 3). Oxidation of 4-OHE₁(E₂) leads to E₁(E₂)-3,4-Q, the predominant ultimate metabolites in the initiation of cancer by estrogens. A protective enzyme is COMT, which catalyzes the methylation of catechol estrogens,

thereby preventing their oxidation to semiquinones and quinones (Fig. 3) [46,77,90]. A second protective enzyme is the quinone reductase, NQO1 or NQO2, which reduces catechol estrogen quinones back to catechol estrogens (Fig. 3) [55,56,102].

Breast tissue from women without breast cancer has been found to have high levels of the protective enzymes COMT and NQO1, and low levels of expression of the activating enzymes CYP19 and CYP1B1, whereas breast tissue from women with breast cancer shows high levels of the activating enzymes CYP19 and CYP1B1 and low levels of the protective enzymes COMT and NQO1 [85]. Another protective enzyme is glutathione-S-transferase (GST), which renders more efficient the reaction between catechol estrogen quinones and GSH, thereby avoiding reaction of the quinones with DNA (Fig. 3).

Prevention of breast cancer initiation by *N*-acetylcysteine and resveratrol

Estrogen homeostasis in the catechol estrogen pathway can be maintained or re-established by the use of specific compounds, *N*-acetylcysteine (NAC) and resveratrol (Res), which are particularly effective in blocking formation of estrogen-DNA adducts [103]. NAC is the acetyl derivative of the amino acid cysteine (Fig. 7), which is one component of the tripeptide GSH. Res, 3,5,4'-hydroxystilbene (Fig. 7), is a natural antioxidant present in grapes, wine, peanuts and other plants. It has anticarcinogenic effects in several *in vitro* and *in vivo* systems [104,105]. These compounds can prevent oxidative and/or electrophilic damage to DNA by inhibiting formation of the electrophilic catechol estrogen quinones and/or reacting with them.

The antimutagenic and anticarcinogenic properties of NAC are attributed to multiple protective mechanisms, such as its nucleophilicity, antioxidant activity and inhibition of DNA adduct formation [106,107]. Hydrolysis of NAC by acylase in the liver and gut yields cysteine, the precursor to the formation of intracellular GSH. This guarantees replenishment of this crucial tripeptide. Changes in GSH homeostasis have been implicated in the etiology and progression of cancer and other human diseases [108]. GSH cannot be used as a preventive agent because it does not cross cell membranes, and the use of cysteine as a preventive agent is limited by its toxicity in humans. NAC has very low toxicity, and it can cross the blood-brain barrier [106,107]. NAC reacts with the electrophilic E₁(E₂)-3,4-Q [109,110] to prevent their reaction with DNA to form adducts (Fig. 3). Like cysteine, NAC reduces catechol estrogen semiquinones to catechol estrogens (Fig. 3) [111]. NAC prevents formation of estrogen-DNA adducts and malignant transformation of the human MCF-10F cells, as well as the mouse E6 mammary cells treated with 4-OHE₂ [112,113].

Res exerts chemopreventive effects in various *in vitro* and *in vivo* systems [105,114]. These properties are attributed to the easy hydrogen abstraction from the 4'-OH bond, with formation of an oxy radical [115]. The easy abstraction is due to the great resonance stabilization energy of the intermediate. Res is a modulator of CYP1B1 [77,78,116] and an inducer of quinone reductase [78,117]. Res also reduces estrogen semiquinones to catechol estrogens [78]. When MCF-10F cells are cultured in the presence of both 4-OHE₂ and Res, formation of depurinating estrogen-DNA adducts is inhibited in a dose-dependent manner [78,118].

To investigate whether the effects of NAC and Res on the formation of estrogen-DNA adducts are additive or synergistic, MCF-10F cells were cultured in the presence of 4-OHE₂ plus NAC or Res or NAC and Res together (Fig. 7) [119]. It was observed that the effects of NAC and Res combined together were additive in inhibiting formation of the depurinating estrogen-DNA adducts, and the two combined compounds could completely inhibit formation of the adducts [119]. At low concentrations, NAC and Res had similar inhibitory effects, but at high concentrations, the effects of Res were about 50% greater than those of NAC [119].

In summary, NAC and Res are both able to reduce estrogen semiquinones to catechol estrogens (Fig. 3). They also have complementary abilities, with NAC reacting with catechol estrogen quinones and replenishing cellular GSH, and Res inducing quinone reductase and modulating CYP1B1 activity (Fig. 3). Thus, together they inhibit formation of estrogen-DNA adducts and maintain estrogen homeostasis.

Conclusions

Metabolism of estrogens in the catechol estrogen pathway is characterized by a balanced set of activating and deactivating enzymes. Under these conditions, little formation of the catechol estrogen quinones, the ultimate carcinogenic metabolites of estrogens, occurs, and breast cancer cannot be initiated. When homeostasis is disrupted, however, excessive oxidation of catechol estrogens to quinones can take place. The quinones can react with DNA to form predominantly the depurinating adducts 4-OHE₁(E₂)-1-N3Ade and 4-OHE₁(E₂)-1-N7Gua. The apurinic sites derived from the loss of these adducts from DNA lead to mutations that can initiate breast cancer. This mechanism involves metabolism of the phenols to catechols, their oxidation to quinones, and reaction of the quinones with DNA via a 1,4-Michael addition. This genotoxic mechanism also operates for the weak carcinogens benzene, naphthalene, diethylstilbestrol and hexestrol (Fig. 1).

A large body of evidence for the genotoxicity of estrogens has been obtained by studies conducted *in vitro*, in cell culture, in laboratory animals and in human subjects. The ER- α -negative human breast epithelial cell line MCF-10F not only forms depurinating estrogen-DNA adducts when treated with 4-OHE₂, but it is also transformed when cultured in the presence of 4-OHE₂ or E₂. These transformed cells induce tumors when injected into severely compromised immunodeficient mice, demonstrating the malignancy of the transformed cells. The 4-catechol estrogens were shown to be carcinogenic in male Syrian golden hamsters and in female CD-1 mice. Demonstration of the carcinogenicity of estrogens by a genotoxic mechanism was derived from studies with transgenic female mice that have the *wnt-1* gene inserted to induce mammary tumors, but have had the ER- α gene knocked out. The formation of mammary tumors in these mice, even in the presence of an anti-estrogen, demonstrated that the ER is not required for estrogen carcinogenicity, but that estrogen genotoxicity can account for the initiation in the development of cancer.

The depurinating N3Ade and N7Gua adducts, which are detected in serum and urine, are formed at significantly higher levels in women at high risk for breast cancer, indicating that formation of these adducts is a crucial event in breast cancer initiation. Significantly higher

levels of the depurinating estrogen-DNA adducts have also been observed in women with breast cancer. These findings suggest that the depurinating estrogen-DNA adducts can serve as biomarkers for increased risk of developing breast cancer.

In addition, knowledge of the mechanism by which estrogens initiate cancer suggests that prevention of cancer can be achieved by blocking formation of the depurinating estrogen-DNA adducts by inhibiting formation of the catechol estrogen quinones or their reaction with DNA. If the initiation of cancer is blocked, promotion, progression and development of the disease would be prevented. Evidence suggests that cancer prevention could be achieved by use of the dietary supplements NAC and Res. This preventive approach does not require knowledge of the genes involved or the series of events that follow initiation. Thus, use of these two dietary supplements could prove to be a widely applicable approach to breast cancer prevention.

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Figure 1.

Common mechanism of cancer initiation by metabolic activation to form depurinating DNA adducts for benzene, naphthalene, estrone (E₁)/estradiol (E₂), diethylstilbestrol (DES), hexestrol (HES) and bisphenol A (BPA).

Figure 2.
Major metabolic pathway in cancer initiation by estrogens.

Figure 4.

Depurinating adducts formed after 10 hours by mixtures of $\text{E}_2\text{-3,4-Q}$ and $\text{E}_2\text{-2,3-Q}$ reacted with DNA at different ratios. The levels of stable adducts formed in the mixtures ranged from 0.1% to 1% of the total adducts [21].

Figure 5. Formation of stable and depurinating DNA adducts with generation of apurinic sites.

Figure 6.

Ratios of depurinating estrogen-DNA adducts to estrogen metabolites and conjugates in (A) urine of healthy women, high-risk women and women with breast cancer – first study [86]; (B) urine of healthy women, high-risk women and women with breast cancer – second study [87] and (C) serum of healthy women, high-risk women and women with breast cancer [88].

Figure 7.

Ability of *N*-acetylcysteine (NAC), resveratrol (Res) or their combination to block formation of estrogen-DNA adducts in MCF-10F human breast epithelial cells treated with 4-OHE₂ [119]. The numbers on bars are % inhibition of depurinating adducts, compared to 4-OHE₂ alone treatment value.

Table 1Correlation of H-*ras* mutations in mouse skin papillomas with depurinating DNA adducts^a

PAH	Major DNA adducts in mouse skin	H- <i>ras</i> mutations
		No. of mutations/no. of mice
DMBA	N7Ade (79%)	4/4 CAA → CTA
DB[a,l]P	N7Ade (32%)	4/5 CAA → CTA
	N3Ade (49%)	
BP	C8Gua + N7Gua (46%)	10/21 GGC → GTC
	N7Ade (25%)	5/21 CAA → CTA

^aRef. [10].

Table 2

Mutagenesis by E2-3,4-quinone

Tissue	Depurinating adducts $\mu\text{mol/mol DNA-P}$ 4-OHE ₂ -1-N3Ade	Stable adducts $\mu\text{mol/mol DNA-P}$ 4-OHE ₂ -1-N7Gua	H-ras mutations		
			A $\rightarrow$ G Total clones	Other Total clones	Total clones
SENCAR mouse skin ^a	12.5	12.1			
6 hours			5/29		2/29
12 hours			4/30		2/30
1 day			7/50		4/50
3 days			3/40		1/40
ACI rat mammary gland ^b	81	90			
6 hours			16/29		3/29
12 hours			14/34		6/34

^aRef. [63].^bRef. [64].