Tamoxifen versus Aromatase Inhibitors for Breast Cancer Prevention

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ABSTRACT

Long-term exposure to estradiol is associated with an increased risk of breast cancer, but the mechanisms responsible are not firmly established. The prevailing theory postulates that estrogens increase the rate of cell proliferation by stimulating estrogen receptor (ER)–mediated transcription, thereby increasing the number of errors occurring during DNA replication. An alternative theory suggests that estradiol is metabolized to quinone derivatives, which directly remove base pairs from DNA through a process called depurination. Error-prone DNA repair then results in point mutations. We postulate that both processes act in an additive or synergistic fashion. If correct, aromatase inhibitors would block both processes, whereas antiestrogens would only inhibit receptor-mediated effects. Accordingly, aromatase inhibitors would be more effective in preventing breast cancer than antiestrogens. Our initial studies showed that catechol-estradiol metabolites are formed in MCF-7 human breast cancer cells in culture. We then used an animal model that allows dissociation of ER-mediated function from the effects of estradiol metabolites and showed formation of genotoxic estradiol metabolites. We also examined the incidence of tumors formed in these ERα knockout mice bearing the Wnt-1 transgene. The absence of estradiol markedly reduced the incidence of tumors and delayed their onset. In aggregate, our results support the concept that metabolites of estradiol may act in concert with ER-mediated mechanisms to induce breast cancer. These findings support the possibility that aromatase inhibitors might be more effective than antiestrogens in preventing breast cancer.

INTRODUCTION

Experimental, clinical, and epidemiologic data suggest that estrogens contribute to the development of mammary cancer, but the mechanisms responsible remain incompletely understood (1–9). The generally accepted hypothesis is that estrogens bind to estrogen receptor (ER) α or β and stimulate the transcription of genes involved in cell proliferation (refs. 8, 9; Fig. 1, left). With each cycle of new DNA synthesis during mitosis, the chances for error in DNA replication without adequate repair increase. As the proliferative process continues, several mutations accumulate (8–11). When these mutations disrupt critical regions required for cellular proliferation, DNA repair, angiogenesis, or apoptosis, neoplastic transformation results (12).

An alternative hypothesis, which has remained controversial, is that estradiol can be converted to genotoxic metabolites and directly damage DNA (refs. 10, 11, 13, 14; Fig. 1, right). The putative genotoxic pathway involves cytochrome P450 1B1, which catalyzes the hydroxylation of estradiol to 4-OH-estradiol. This compound is then further converted to the estradiol-3,4-quinone, which can bind covalently to guanine or adenine, resulting in destabilization of the glycosyl bond that links these purine bases to the DNA backbone (Fig. 1, right). Consequently, adenine and guanine, which are bound to the estradiol quinone, are released from the DNA backbone as 4-OH-estrone (estradiol)-1-N7-guanine (Fig. 1, right) or 4-OH-estrone (estradiol)-1-N-3-adenine. With detachment of these two adducts from the DNA backbone, a apurinic site is left behind in the DNA. Through the process of error-prone DNA repair, these sites now form point mutations that serve as potential initiators of neoplastic transformation (14, 15). Our working hypothesis is that estradiol acts on both pathways shown in Fig. 1 in an additive or synergistic fashion to induce breast cancer.

In vitro studies provide experimental evidence supporting the estradiol genotoxicity hypothesis. Liehr and colleagues, using the V-79 cell carcinogenicity assay, found that low doses of estradiol in the 0.01 to 0.1 nmol/L range cause a 3.8- to 4.2-fold increase in the rate of genetic mutations (16). As additional evidence of mutagenicity, Russo et al. administered estradiol to benign MCF-10F breast cells in vitro in doses ranging from 0.007 nmol/L to 1 μmol/L (17, 18) and found that even very low estradiol concentrations induced loss of heterozygosity at chromosomal sites that often contain loss of heterozygosity in human breast tumors (i.e., 11q23.3, 11q23.1-25, 3p21, 3p21-21.2, 3p21.1-14.2, and 3p14.2-14.1). They also documented the neoplastic transformation of these cells by demonstrating an increase in anchorage-independent colony formation and loss of duct differentiation.

Our studies sought further evidence of the validity of the estrogen genotoxic hypothesis. We used MCF-7 cells containing a stably transfected aromatase gene and measured genotoxic products after incubation with estrogen substrates (13, 19, 20). We also used an ERα knockout animal model that lacks ERβ in mammary tissue (21–24). We reasoned that any neoplastic changes induced by estrogens must therefore work through ER-independent pathways. Taken together, these studies showed that mammary cancer cells can convert estradiol to genotoxic metabolites and that non-receptor-mediated mechanisms involving estradiol can modulate the process of mammary cancer development.


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RESULTS

Aromatase-Transfected MCF-7 Cells. We initially determined whether enzymes responsible for formation of depurinating DNA adducts were present in aromatase-transfected human MCF-7 human breast cancer cells (Fig. 2A). The methodology used to detect metabolites has been used extensively and published elsewhere (13–15) and involves a Coularray, high-performance liquid chromatography, electron capture, multarray detector with confirmation by mass spectrometry. Substrates (i.e., 4-OH estradiol and testosterone) were added to culture medium and incubated with cells for 24 hours before collecting medium for later measurement of the various metabolites. As shown in Fig. 2A, we detected large amounts of 4-methoxy-estradiol as well as substantial amounts of the quinone conjugates (glutathione, N-acetylcysteine, and cysteine) and the depurinated species, 4-OH-estrone (estradiol)-1-N7-guanine, in aromatase-transfected MCF-7 cells incubated with 4-OH-estradiol. We then determined whether these cells could aromatize a sufficient amount of testosterone to estradiol to result in formation of the depurinating species. As shown in Fig. 2B, we detected 131 pg/mL of estrogen, indicating the production of estrogens by aromatization. The 4-OH-estrone (estradiol)-1-N7-guanine species was also present at a total concentration (estrone plus estradiol) of 0.17 pg/mL, as were the glutathione, cysteine, and N-acetylcysteine conjugates of estradiol-3,4-quinone. Finally, the aromatase inhibitor letrozole at a dosage of 1 μmol/L inhibited estrogen formation from a total of 131 pg/mL of estrone and estradiol (Fig. 2C) to 2.8 pg/mL and inhibited their downstream metabolites to undetectable levels in most cases.

Measurements in Human Breast Tissue. The subjects for this study were recruited from women undergoing breast biopsies. They included a control group of 49 women subsequently diagnosed histologically as having benign breast disease (40 Caucasian, 3 African American, 2 Hispanic, 1 Asian, and 3 unknown; age range, 31-82 years, median age, 52 years). The cancer case group included 28 women, 12 subsequently diagnosed as having carcinoma in situ and 16 with invasive carcinoma (15 Caucasian, 2 African American, 2 Asian, and 9 unknown; age range, 36-88 years, median age, 54 years). Figure 3A and B shows that the level of estradiol plus estrone was 5 pmol/g in breast tissue from women with breast cancer and 2 pmol/g in control normal breast tissue. Expressed as picogram per milliliter, these values would represent 1,650 and 660 pg/mL (25). As evidence that the human breast can synthesize the quinone derivatives of estradiol, the total estrone and estradiol conjugates of the estrogen quinones are in the range of 2 pmol/g in the control normal tissue and 8 pmol/g in nontumor tissue from the breast with carcinoma. Statistical differences between breast cancer cases and controls were found for 4-OH estrogens (P = 0.01) and quinone conjugates (P = 0.003) using the Wilcoxon rank sum test.

Measurements in ER Knockout/Wnt-1 Mammary Tissue. Mammary tissue in ER knockout (ERKO)/Wnt-1 transgenic mice exhibits an altered metabolic balance (26). Formation of 4-OH-estrogen metabolites is favored over those of the 2-OH species, and the catechol-O-methyl-transferase pathway seems to be relatively inactive (Fig. 4). We detected 10.9 pmol/g of 4-OH-estradiol and 4-OH-estrone in mammary tissue as well as a total of 2.3 pmol/g of total conjugated quinone. No 4-methoxy-estrogen metabolites were present.

Tumor Incidence in ERKO/Wnt-1 Animals. Bocchinfuso et al. had previously shown that ERKO/Wnt-1 animals exhibit a delayed onset of tumor development compared with animals expressing the ERα. Nonetheless, they observed a nearly 100% incidence of mammary tumors even in the absence of ER α and β (21, 22). To directly determine the effect of estradiol in the absence of ER, we castrated animals at 15 days of age and treated half with silastic implants containing estradiol and the other half with implants of cholesterol. The 7.5-mm implants resulted in calculated plasma levels of estradiol of 300 pg/mL, which approximate the levels in intact ERKO animals. Because the negative feedback effects of estradiol on the hypothalamus and pituitary are lacking in ERKO animals, luteinizing hormone and estradiol levels are quite high in these animals. The 2.5-mm implants produced levels of ~75 pg/mL, a concentration one-third of circulating levels in intact ERKO animals and 7-fold higher than in wild-type female mice. After 100 weeks of observation, the high-dose, estradiol-treated animals (i.e., 7.5-mm implants) developed more tumors (12 of 15 versus 4 of 10), which appeared earlier than in the animals receiving cholesterol implants (50% of tumors at 50 weeks versus 25% of tumors at 100 weeks; P < 0.004, Fig. 5). Tumor formation in animals receiving lower doses of estradiol (i.e., 2.5-mm implants) developed tumors at a rate intermediate between that
of high-dose estradiol-treated and castrate animals. Lower concentrations of estradiol did not enhance the rate of tumor formation. These data provide evidence that estradiol exerts effects through both an ERα-dependent pathway and an ERα-independent pathway to produce breast tumors. The levels of estradiol used in these studies are similar to those found in premenopausal women (i.e., 50-600 pg/mL depending on the phase of the menstrual cycle). On the other hand, normal female mice have circulating levels of estradiol, which peak at only 30 pg/mL at the time of ovulation.

**DISCUSSION**

A variety of evidence suggests that estrogens can contribute to the production of breast cancer. The commonly held theory is that estrogens stimulate cell proliferation, increase the number of genetic mutations in proportion to the number of mitotic divisions, and promote the propagation of these mutations by stimulating growth (9, 27). An alternate hypothesis suggests that estrogens may be metabolized directly to genotoxic compounds (10, 11, 13–15, 28). Our working construct is that these two pathways act in concert in an additive or synergistic fashion to cause breast cancer (Fig. 1). In support of this hypothesis, we showed that MCF-7 cells can convert 4-OH-estradiol and testosterone both to the 4-OH-estrone (estradiol)-1-N7-guanine depurinated product and to estrogen quinones. We also showed that the incidence of breast tumor development in ERα knockout transgenic animals could be enhanced by administration of estradiol to oophorectomized animals. These new data provide direct new evidence of the biological importance of the genotoxic pathway.

Our first aim was to show that human breast cancer cells convert testosterone or 4-OH-estradiol to genotoxic products. We clearly showed this in a MCF-7 cell model system by using a highly sensitive and specific assay for steroid measurements. A commonly expressed criticism of the genotoxic hypothesis is that supraphysiologic amounts of estrogen are needed to form genotoxic metabolites of estradiol (11). Our *in vitro* experiments can be criticized on the same basis. However, we believe that biological end points of this process provide a higher level of sensitivity than do biochemical measurements. This reasoning is supported by studies that examined the biological effects of estrogen under similar *in vitro* conditions. Russo et al. have

![Fig. 2](image_url)
shown that 0.007 nmol/L estradiol can induce neoplastic transformation as evidenced by increased colony formation in benign MCF-10F cells that lack a functional ER (18). Similar concentrations induce loss of heterozygosity in benign, non-ER-containing breast cells at hotspots for loss of heterozygosity in breast cancer tissue. Such low concentrations can also induce mutations in V-79 cells (16). As further evidence of the ability of physiologic amounts of estrogen to serve as precursors for these genotoxic metabolites, human breast tissue from women with and without breast cancer contain large amounts of these metabolites. Taken together, the tissue measurement data and the findings from incubated cells in vitro clearly show that human breast tissue can form substantial amounts of the genotoxic metabolites of estradiol.

The ERKO/Wnt-1 animals provide a powerful model for studying the effect of estrogen in the absence of a functioning ER. These animals have circulating estradiol levels in the range of 325 pg/mL. This is ~30- to 50-fold higher than normal as a consequence of the absence of estradiol negative feedback on the pituitary and the resultant increase in luteinizing hormone levels. In addition, the breast tissue from these animals seems to convert little 4-OH-estradiol to 4-methoxy-estadiol, a metabolite that is thought to be inactive, and to obviate further conversion to genotoxic metabolites (26).

We consider it highly relevant with respect to prevention of breast cancer to determine whether the genotoxic pathway is biologically important. Antiestrogens act only to block ER-mediated function, whereas the aromatase inhibitors reduce estradiol levels and consequently block both ER-mediated and genotoxic pathways (Fig. 1). Theoretically then, aromatase inhibitors would be much more efficacious for prevention of breast cancer than the antiestrogens (8). Data from the recently reported ATAC trial can be interpreted in light of the genotoxic hypothesis (29, 30). In this trial, the aromatase inhibitor anastrozole resulted in a 50% greater reduction of invasive contralateral breast cancer (**P < 0.05) at 4 years than did the
antiestrogen tamoxifen. In addition, the Ma.17 and International Exemestane Study trials also showed fewer contralateral tumors in the aromatase inhibitor-treated patients (31, 32). Although there are other explanations for these differences, the magnitude of greater effect of the aromatase inhibitor is substantial. This observation, when taken together with the biological data presented in this article, highlight the compelling need to determine conclusively whether the genotoxic hypothesis of estradiol-induced carcinogenesis is operative.

The ERKO animals used in this study continue to express a truncated form of the ERα. This may be capable of mediating estradiol effects via nongenomic mechanisms. There may also be some residual ERβ in breast tissue that is not detected with current techniques. To address these theoretical issues, we are currently administering fulvestrant to the ERKO/Wnt-1 animals to completely block and down-regulate residual ER. In addition, we plan to administer an ERα-specific estrogen analogue that cannot be converted to a quinone to examine directly the requirement for formation of quinones in the carcinogenic process.

OPEN DISCUSSION

Dr. Aman Buzdar: If this hypothesis is correct, then shouldn’t an aromatase inhibitor be effective in preventing ER-negative tumors as well?

Dr. Richard Santen: This hypothesis would suggest that estrogen is an initiator, stimulating increased proliferation, and there would have to be an estrogen receptor for estrogen to do that. But if this hypothesis is correct, you would expect that the genotoxic metabolites would hit specific genes that could result in breast cancer that would be completely independent of the estrogen receptor. So, the hypothesis would suggest that you should be able to prevent both ER positive and ER negative; that’s the implication.

Dr. Per Lønning: In regard to genotoxicity, the closest examples to that are radiation damage and also chemotherapy-induced secondary cancers. For girls who have been exposed to radiation damage early in life, the timeframe is at least 20 years before they develop clinical breast cancer. Also, we see secondary leukemias, but not secondary breast cancers, in relation to exposure to chemotherapy, which suggests that if this genotoxic damage is happening, it requires a long timeframe.

Dr. Santen: A woman is exposed to estrogen throughout her reproductive years, and we know that the incidence goes up over time, so one could think that this is accumulation of genetic mutations. In the Women’s Health Initiative Trial, it was only the individuals who had been on estrogen/progestrone prior to starting the trial who had an increased incidence of breast cancer from the therapy.

Dr. Donald McDonnell: One of the problems I have had with this hypothesis is the issue of specificity. If this is a genotoxic event, then any tissue that is rapidly proliferating should also be subjected to an increased risk of tumorigenesis, colon, particularly, and liver, but that has not been observed. How do you explain the specificity?

Dr. Santen: One of the issues is whether there are genetic differences or tissue differences in the levels of the enzymes. The key enzymes are cytochrome P450 1B1, the oxidases that convert 4-hydroxy estradiol to the 3,4-quinones, the catechol methyltransferase, and the glutathiones. We know that breast has some major differences in these enzymes compared to other tissues. In addition, if this hypothesis is correct, you would expect to find a difference in individuals who have the phenotype of low catechol methyltransferase. Catechol methyltransferase comes in high-high, low-low, and intermediate phenotypes. There have been 12 studies looking at the low-low versus the high-high; 7 out of the 12 have shown a statistically significant increased risk of breast cancer with low catechol methyltransferase. So, I think the specificity issue relates to the ability of each particular tissue to convert estradiol to these genotoxic metabolites. Some tissues convert the estrogens to the genotoxic pathway and some do not, and this could be an explanation for the toxicity in some tissues.

Dr. Vessela Kristensen: I have a comment regarding the substrate specificity of CYP1B1. The issue of the $K_{m}$ and $V_{max}$ of the CYP1B1-mediated hydroxylation of estradiol to the genotoxic and nongenotoxic pathway has been experimentally addressed in a series of publications by the group of Shimada and others. CYP1B1 is highly polymorphic and some of its polymorphic forms have much higher affinity and lower $K_{m}$ toward their substrate and are much keener to 4-hydroxylate estradiol than the others. In terms of this and other polymorphisms like the glutathione S-transferases, when you did your tumor analyses, did you observe substantial phenotypic differences, interindividual differences from tumor to tumor, that might explain a different risk?

Dr. Santen: We don’t have any data on that, I’m embarrassed to say, and it’s going to be very important to do that.

Dr. McDonnell: The fact that depurination can occur is important, but whether it occurs at the level that would produce mutagenesis is not known. I think that the studies that you’re doing are definitely going to address these issues testing a hypothesis that has been batted around for 20 years with no definitive proof.

Dr. Santen: What has now made this research possible is the availability of GC mass spectrometry to measure these levels. If this hypothesis is correct, it is going to change the way we think about preventing breast cancer. The counter argument is that the number of depurinations that occur in the human body each day are purportedly in the billions. So this is a process that goes on normally. Of course, as the counter argument to that, estrogen is a very weak carcinogen and you have to have the other components, such as tissue that has an estrogen receptor, to cause the promotional effects.

Dr. McDonnell: It’s quite possible, again proposing a hypothesis with little proof, that the estrogen receptor is actually increasing the local concentration of estrogen at a hotspot close to a specific estrogen response element.

Dr. Stephen Johnston: Coming back to the clinical issues, if you’re right, then in the ATAC study, the incidence of contralateral breast cancers in the combination arm should have been the same as the anastrozole arm.

Dr. Santen: And it wasn’t. That’s an excellent point. The tamoxifen plus anastrozole had more breast tumors than did the anastrozole alone. If tamoxifen is a partial agonist that is how we would explain why the patients clinically don’t do as well.
If our dual hypothesis pathway is correct, tamoxifen may actually be causing a bit more promotion, so that would be an explanation, potentially, for those results.

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