Dietary Estrogens Stimulate Human Breast Cells to Enter the Cell Cycle

Craig Dees,1 James S. Foster,2 Shamila Ahamed,2 and Jay Wimalasena2

1Risk Analysis Section, Health Sciences Research Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee; 2Department of Obstetrics and Gynecology, University of Tennessee Hospital, Knoxville, Tennessee

It has been suggested that dietary estrogens neutralize the effect of synthetic chemicals that mimic the effects of estrogen (i.e., xenoestrogens, environmental estrogens). Genistein, a dietary estrogen, inhibits the growth of breast cancer cells at high doses but additional studies have suggested that at low doses, genistein stimulates proliferation of breast cancer cells. Therefore, if dietary estrogens are estrogenic at low doses, one would predict that they stimulate estrogen-receptor positive breast cancer cells to enter the cell cycle. Genistein and the fungal toxin zearalenone were found to increase the activity of cyclin dependent kinase 2 (Cdk2) and cyclin D1 synthesis and stimulate the hyperphosphorylation of the retinoblastoma susceptibility gene product pRb105 in MCF-7 cells. The steroidal antiestrogen ICI 182,780 supressed dietary estrogen-mediated activation of Cdk2. Dietary estrogens not only failed to suppress DDT-induced Cdk2 activity, but were found to slightly increase enzyme activity. Both zearalenone and genistein were found to stimulate the expression of a lucifase reporter gene under the control of an estrogen response element in MVLN cells. Our findings are consistent with a conclusion that dietary estrogens at low concentrations do not act as antiestrogens, but act like DDT and estradiol to stimulate human breast cancer cells to enter the cell cycle. — Environ Health Perspect 105(Suppl 3):633-636 (1997)

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Estrogenic chemicals present in the human diet or environment have been called xenoestrogens or environmental estrogens. Recently it has been suggested that xenoestrogens may have a role in the etiology of breast cancer (1,2,3). Compounds that have been suggested to be xenoestrogens include pesticides, dyes, pollutants, plasticizers and food preservatives (2,4-7). For example, the pesticide DDT and the food colorant Red Dye No. 3 have been shown to bind to the estrogen receptor (ER) and to stimulate proliferation of ER-positive breast cancer cells (2). At the molecular level, DDT and Red Dye No. 3 mimic the effects of estradiol by stimulating breast cancer cells to enter the cell cycle (2,8). Entry into the cell cycle requires cyclin D1 synthesis, activation of cyclin-dependent kinase 2 (Cdk2), and retinoblastoma protein (pRb105) hyperphosphorylation (2,8). Phosphorylation of pRb105 by activated cyclin-dependent kinase can be detected as a migration shift using Western blot analysis after breast cancer cells are treated with estradiol, Red Dye No. 3, or DDT (8-11).

Whether xenoestrogens have a role in the etiology of human breast cancer remains controversial (1,12-16). It has been suggested that exposure to xenoestrogens cannot produce adverse effects on reproductive tissue because they are neutralized by estrogenic compounds derived from dietary sources (17). Therefore, if dietary estrogens have chemopreventive activity by antagonizing the effects of estrogen, one would predict that cellular processes associated with entry into the cell cycle (e.g., cyclin-dependent kinase activation, cyclin D1 synthesis) would be blocked by dietary estrogens. Genistein has recently been shown to be a potent inhibitor of ER-positive breast cancer cell growth when added at high concentration (>10 μM) (18,19). However, genistein at lower concentrations has also been shown to increase the growth of ER-positive cells (19,20).

In this study, we examined the effects of dietary estrogens on cyclin D1 synthesis, Cdk2 activity, and pRb105 phosphorylation. By performing Cdk2 assays on MCF-7 breast cancer cells that were treated with low concentrations of genistein or zearalenone in addition to DDT, we also determined if genistein or zearalenone exhibited antiestrogenic activity. The ability of zearalenone and genistein to stimulate the expression of a reporter gene under the control of an estrogen response element in human breast cells stably transfected with this construct was also determined.

Materials and Methods

Chemicals

ICI 182,780 was a gift from Alan Wakeling, Zeneca Pharmaceuticals (Macclesfield, England). Dietary estrogens and estradiol were purchased from Sigma Chemicals (St. Louis, MO). Dietary estrogens and DDT were concentrated solutions in absolute ethanol. An equal amount of ethanol was added to all control cells.

Human Breast Cancer Cells

MCF-7 cells were maintained in Dulbecco’s Modified Minimal essential medium/ Ham’s F12 1:1 without phenol red (Sigma Chemicals). Both cell lines were incubated at 37°C in a 5% CO2 atmosphere with 10% fetal bovine serum (FBS). Prior to studies on Cdk2 activation, MCF-7 cells were grown without removal of serum and transfer into methionine-free medium for 72 hr before exposure. During dietary

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Address correspondence to Dr. C. Dees, PhotoGen LLC, 7327 Oak Ridge Highway, Suite A, Knoxville, TN 37931. Telephone: (423) 539-9975. Fax: (423) 539-9654. E-mail: genase@Bcom

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Abbreviations used: AVG, albumin-globulin; ATP, adenosine triphosphate; Cdk2, cyclin-dependent kinase 2; ER, estrogen receptor; FBS, fetal bovine serum; PBS, phosphate-buffered saline; pRb105, retinoblastoma susceptibility gene product; tp, time zero.
Estrogenic Assay

The ability of zearalenone and genistein to stimulate the expression of luciferase production under the control of an estrogen response element was performed by depriv-ing MVLN cells of estrogen. Estrogen deprivation in MVLN cells was accom-
mplished by growing them in culture with phenol red-free DMEM/F12 medium con-
taining 5% charcoal-stripped, delipidated calf serum. After gradual withdrawal of the serum, the cells were left in the medium alone for 24 hr more and then treated with estradiol (1 nM), estradiol (1 nM) and ICI 182,780 (100 nM), zearalenone (10 nM), zearalenone (10 nM) and ICI 182,780 (100 nM), genistein (0.1 μM), or genistein (0.1 μM) and ICI 182,780 (50 nM). The medium alone was used as the control. After 24 hr of treatment the cells were lysed and the luciferase assay performed using Promega’s Luciferase Assay kit (Madison, WI) per the kit protocol. Light emission from treated MVLN cell extracts was measured using a scintillation counter in out-of-coincidence mode.

Cyclin-dependent Kinase 2 Assays

MCF-7 cells for Cdk2 analysis were exposed to estrogens for 20 hr. After incu-
bation, cells were washed twice with ice-
cold phosphate-buffered saline (PBS) and lysed by the addition of cold lysis buffer (Tris 20 mM, pH 7.5, NaCl 250 mM, 0.1% NP-40, NaF 10 mM, NaVO3 1 mM, PMSF 1 mM). After 15 min on ice, the lysates were centrifuged at 20,000 x g for 15 min (4°C). Cdk2 was precipitated from equal amounts of cell extracts using purified rabbit anti-Cdk2 (Santa Cruz Biotechnology, Santa Cruz, CA) and pro-
tein albumin-globulin (A/G) agarose. Immunoprecipitates were washed three times with the lysis buffer and twice with kinase buffer (Tris 40 mM, pH 7.5, MgCl2 10 mM). The immunoprecipitates were suspended in 30 μl of kinase buffer supple-
mented with 400 μg/ml histones (Sigma Chemicals type II-SS), 5 μM adenosine
triphosphate (ATP), 0.5 mM dithiothreitol, 0.5 mM ethylene glycol tetraacetic acid, and 5 μCi γ-[32P]-ATP for 20 min at room temperature. The reaction was stopped using gel electrophoresis sample buffer, and the reaction products were separated on a 14% polyacrylamide gel (Novex, San Diego, CA).

Results

Stimulation of Estrogen Receptor-controlled Luciferase Production

We hypothesized that if dietary estrogen exposures were mitogenic, they acted through the ER. Therefore, the expression of a reporter gene (luciferase) under the control of an estrogen response element must be increased in MVLN cells exposed to dietary estrogens. (Figure 1) shows that exposure of MVLN cells to genistein (0.1 μM) or zearalenone (10 nM) stimulates the production of a luciferase reporter gene that is under the control of a Xenopus estrogen response element. The steroid antiste-
rogen ICI 182,780 (100 nM) inhibited luciferase production in MVLN cells stimu-
lated by all of the estrogens, demonstrating the requirement for ER in xenoestrogen action (Figure 1).

Stimulation of Cyclin D1 Synthesis

Synthesis of cyclin D1 occurs early in G1 phase prior to activation of Cdk2 (9–11). Estradiol and xenoestrogens (e.g., DDT, Red Dye No. 3) (2) induce increased syn-
thesis of cyclin D1 in G1-arrested MCF-7 cells (8). Increased synthesis of cyclin D1 protein induced by genistein and zeara-
lenone confirm that both dietary estrogens regulate expression of this protein critical for progression through the cell cycle at low concentration (0.5 μM) (Figure 2).
Stimulation of Cdk2 Activation

Subsequent to cyclin D1 synthesis and activation of Cdk4, transit of cells through G1 and entry into S phase requires Cdk2 activation (9–11). Both genistein and zearalenone inhibited Cdk2 activity when added to growth-arrested MCF-7 cells (Figure 3A). Increased Cdk2 activity induced by genistein or zearalenone could be detected as early as 12 to 16 hr after they were added to MCF-7 cells (data not shown). However, maximum levels of dietary estrogen-stimulated Cdk2 activity occurred 18 to 22 hr after they were added to human breast cancer cells (Figure 3A).

The steroidal antiestrogen ICI 182,780 (50 nM) partially inhibits Cdk2 activity stimulated by estradiol (10 nM) and completely inhibits Cdk2 activity by DDT (0.3 μM), genistein (1 μM), and zearalenone (1 nM) (Figure 3B). Both genistein and zearalenone slightly increased Cdk2 activity induced in MCF-7 cells by DDT (Figure 4).

Phosphorylation of pRb105

Cdk activation results in the hyperphosphorylation and inactivation of pRb105, allowing for release of transcription factors of the E2F family, which are required for S phase entrance (10). Therefore, if dietary estrogens mimic the effects of estradiol on the cell cycle, then increased phosphorylation of pRb105 should occur in breast cancer cells treated with dietary estrogens. Figure 5 shows that low levels of dietary estrogens (0.5 μM) induce hyperphosphorylation of pRb105 that can be detected as a mobility shifted form of pRb105 by Western blot analyses.

Discussion

The role, if any, that xenoestrogens have in the etiology of human breast cancer is controversial (1,2,15,17,22). Some investigators have proposed that exposure to xenoestrogens may enhance the risk of developing breast cancer (1,2,22). Some epidemiologic studies have supported this hypothesis (3), but others do not find any correlation with xenoestrogen exposure and breast cancer (14). Additionally, it has been proposed that exposure to estrogenic chemicals in the diet may neutralize or somehow prevent xenoestrogens from having any significant biologic effect (17).

Human diets contain plant-derived nonsteroidal estrogenic compounds (e.g., genistein). Other estrogenic molecules in the diet may include compounds produced by fungi (e.g., zearalenone) (20). Additional estrogenic compounds in the diet may include zeranol, a synthetic derivative of zearalenone, which has been used as a hormonal growth-promoter in cattle (20). Dietary derived estrogenic compounds like genistein have been proposed to act in preventing proliferation of ER-positive breast cells (17). However, one previous report failed to find any chemopreventive action of genistein or zearalenone because these compounds stimulated the growth of MCF-7 human breast cancer cells (20). More recent reports suggest that genistein is a potent inhibitor of MCF-7 cells (18,19). The apparent disparity in the reported effects of genistein on MCF-7 cells may depend on the concentration of genistein used. Genistein is a potent inhibitor of protein tyrosine kinases and can inhibit cell cycle progression in tumor cells independent of action at the ER (23,24). Other inhibitors of MCF-7 cells have been shown to have concentration-dependent estrogenic effects and can also display antiestrogenic activity (25). We surmise that dietary estrogens may have estrogenic activity at low concentrations and antiestrogenic activity or toxicity at higher concentrations.

It has been previously demonstrated that estrogen induces Cdk2 activity, cyclin D1 synthesis, and hyperphosphorylation of pRb105 in growth-arrested MCF-7 cells (8). Two xenoestrogens also have been shown to mimic estrogen’s ability to stimulate MCF-7 cells to enter the cell cycle (2). Therefore, using Cdk2 activation, cyclin D1 synthesis, and hyperphosphorylation of pRb105, we examined the effects of low concentrations of genistein and zearalenone on growth-arrested MCF-7 cells.

In all assays the dietary estrogens stimulated molecular changes, indicating that they stimulated cell cycle entry. Additionally, rather than antagonizing the effects of DDT, both dietary estrogens stimulated Cdk2 activation. Hyperphosphorylation of pRb105 was induced by both genistein and zearalenone. Flow cytometric data has confirmed that both genistein and zearalenone induce S phase entry in MCF-7 cells as has been demonstrated with estradiol (8) (data not shown). Therefore, at low concentrations, dietary estrogens appear to stimulate MCF-7 cells to enter the cell cycle. In addition, the steroidal antiestrogen ICI 182,780 was found to inhibit stimulation of Cdk2 activity by genistein and zearalenone (Figure 3B). Genistein and zearalenone also stimulated the production of a reporter molecule under the control of an estrogen response element that was also inhibited by ICI.
182,780 (Figure 1). Therefore, genistein and zearalenone stimulation of human breast cancer cells to enter the cell cycle is mediated through the dietary estrogens’ effects on the ER and transcriptional control via estrogen-responsive elements. Further studies are required to determine if genistein and zearalenone inhibit Cdk2 activity, cyclin D1, synthesis, and hyperphosphorylation of pRb105 at higher concentrations.

Our studies using molecular assays to evaluate the effects of dietary estrogens agree with previous reports (19,20) that at low concentrations genistein and zearalenone produce proliferative effects on human breast cancer cells. The effects appear to be concentration dependent, which would agree with a recent study that shows stimulation of MCF-7 cell growth at genistein concentrations of less than 10 μM. Our studies do not support the suggestion that dietary estrogens neutralize the effects of DDT. In contrast, the effects of dietary estrogens at low concentrations on DDT-induced Cdk2 activation appear to be additive or perhaps synergistic (Figure 4).

Under the proper conditions and concentrations, genistein has been reported to be a potent inhibitor of MCF-7 cell growth (18,19). However, our studies suggest that women should not consume particular foods (e.g., soy-derived products) to prevent breast cancer. Further, our results suggest that low concentrations of genistein may stimulate MCF-7 cells to enter the cell cycle. If the amount of estrogens that can be derived from the dietary sources does not contribute a level high enough to suppress ER-positive cell growth, dietary estrogens may increase the risk of breast cancer, especially in combination with dietary derived xenoestrogens. The risk of exposure to xenoestrogens and low levels of dietary estrogens may be further increased if developing fetal tissues are exposed (23).

The molecular effects of dietary derived and xenoestrogens on ER-positive breast cancer cells appear to be complex. The effects of dietary estrogens may be concentration dependent and may interact with synthetic and natural estrogens. It may be premature at this time to suggest dietary changes that significantly alter the amount of dietary derived estrogens until additional research can fully elucidate the effects they have on reproductive tissues in terms of dose, tissue-specific effects (like tamoxifen), and potential interactions with other estrogenic compounds. It remains to be determined if dietary estrogens are beneficial or, as suggested by our in vitro studies, an additional carcinogenic risk factor for tissues where proliferation is controlled by estrogens.

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