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The incidence of breast cancer is increasing dramatically in the United States. Man-made environmental agents such as pesticides, polychlorinated biphenyls (PCBs), phthalate esters, and dioxin have been implicated in this increase. Many xenobiotics such as DDT and PCBs have weak estrogenic activity and may enhance breast cancer formation by an estrogenic effect on breast epithelial cell growth. These agents can also inhibit gap junctional intercellular communication (GJIC); this reduction may also be involved in enhanced growth and breast cancer formation by these agents. The studies outlined in this proposal will determine whether there is a link between xenobiotic inhibition of human mammary epithelial cell GJIC, growth, and estrogenicity. These studies are highly relevant to the prevention of breast cancer. An understanding of the relationship between xenobiotic inhibition of GJIC, estrogenic activity, and the enhancement of growth in human breast epithelial cells will lead to more widely acceptable, mechanism-based arguments that xenobiotics are involved in the etiology of human breast cancer. This should lead to more focused regulatory efforts to reduce exposure to these agents.

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BACKGROUND:

The incidence of breast cancer is increasing dramatically in the United States. Man-made environmental agents such as pesticides, polychlorinated biphenyls (PCBs), phthalate esters, and dioxin have been implicated in this increase (1-5). Several studies have identified increased levels of xenobiotics such as *p,p'*-DDE, the major human metabolite of DDT, in women with breast cancer (2-5). Many such xenobiotics have weak estrogenic activity and have been suggested to enhance breast cancer formation by an estrogenic effect on breast epithelial cell growth (6,7). However, the role of such xenoestrogens in human breast carcinogenesis is highly controversial. Several studies indicate no association between tissue levels of xenobiotics and breast cancer (8,9) and not all of the xenobiotics associated with breast cancer are estrogenic (9). Therefore, if synthetic chemicals are to be accepted as etiologic factors in breast cancer, relevant mechanisms must be demonstrated. Few mechanistic hypotheses have been tested, however.

Many of the xenobiotics that have been linked to human breast cancer inhibit gap junctional intercellular communication (GJIC) in nonbreast cells *in vivo* and *in vitro* (10,11). Substantial recent evidence indicates that a reduction of GJIC contributes to cell proliferation in xenobiotic-treated tissues and to the loss of growth control in neoplastic cells (12,13). Therefore, one potential mechanism for how xenobiotics might enhance breast cancer formation is that they inhibit breast epithelial cell GJIC resulting in enhanced growth. The studies outlined below will test this hypothesis.

Gap junctions are plasma membrane channels that connect the interiors of neighboring cells (12,13). The channels are approximately 1.5-2 nM in diameter and comprised of proteins known as connexins. At least thirteen connexins have been identified in mammals (13). Gap junction channels permit small (<1,000 Da) ions, nutrients, second messengers, metabolites, and other molecules to diffuse between neighboring cells, but prevent larger molecules and macromolecules from passing. The functions of this gap junctional intercellular communication (GJIC) include homeostasis, electrical synapsing (in certain neurons and muscle cells), and the regulation of cell growth (13). This latter function may be mediated by the intercellular exchange of signals such as cAMP that inhibit cellular growth, although these have not been identified (13).

Evidence that GJIC plays a role in growth regulation and neoplasia has been accumulating over the past three decades. Many neoplastically transformed cells have fewer gap junctions, reduced connexin expression, and decreased capacity for GJIC compared to nontransformed cells (14). A variety of growth enhancers, tumor promoters, and oncogenic agents inhibit GJIC whereas many growth inhibitors and differentiating agents enhance it (13). Stable transfection of gap junction-deficient neoplastic cells with connexin cDNAs has resulted in reduced growth *in vitro* and tumorigenicity *in vivo* of the transfected cells (15-19). Reduction of nontransformed cell GJIC by treatment with antisense connexin DNA enhanced cell growth *in vitro* (18) or blocked the cell's ability to suppress the growth of cocultured, transformed cells (21,22). These studies suggest that connexins are tumor suppressor genes.

PURPOSE:

Many environmental chemicals such as pesticides, phthalate esters, polychlorinated biphenyls, and dioxins have been demonstrated to be nongenotoxic carcinogens (tumor promoters) in two-stage rodent carcinogenesis bioassays. The model tissues have usually been the skin or liver. In some cases, xenobiotics may enhance tumor formation by increasing

initiated cell growth relative to surrounding noninitiated cells (25). Many of these agents also inhibit gap junction function and/or connexin expression *in vivo* and *in vitro* (10,11), demonstrating a link between the upregulation of growth and the downregulation of GJIC.

Several of the xenobiotics that have been implicated as etiologic factors in human breast cancer are also weakly estrogenic (6,9). No studies, however, have examined whether xenobiotics affect GJIC in human mammary epithelial cells and the relationship to growth and estrogenic activity. The inhibition of GJIC might lead to increased cell growth and the enhancement of neoplastic transformation in the breast. **The purpose of this project is to determine whether xenobiotics block GJIC and/or enhance growth in human mammary epithelial cells and how these effects correlate with estrogen receptor (ER) status, neoplastic transformation, and xenobiotic estrogenicity.**

SCOPE OF THE RESEARCH:

We are testing several DDT-related compounds (*p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD, and *o,p'*-DDT) for their effects on GJIC and growth of ER^+ and ER^- normal and neoplastic human mammary epithelial cells. These agents inhibited GJIC in rodent fibroblasts (26,27) and a recent study (28) showed that *p,p'*-DDT blocked GJIC in NHMEC, but did not examine growth effects. These latter cells were obtained from reduction mammoplasties. We are also using fenarimol, which is a pesticide structurally similar to DDT but a noninhibitor of GJIC (29), and ß-estradiol, an estrogenic steroid, as negative and positive controls respectively. We are correlating changes in GJIC with cell growth, estrogenicity, estrogen-receptor status, and neoplastic transformation. We will also identify the biochemical and molecular mechanisms of action of these compounds on GJIC in the mammary cells. Specifically, we will determine whether connexin expression and/or localization are affected by these compounds. This will enable comparisons with other tissues and cell types and will facilitate the development of antagonists. Finally, by correlating xenobiotic effects on GJIC with changes in mammary cell proliferation and estrogenic activity, we will provide information on the role of altered GJIC in breast epithelial cell growth and neoplasia and how this is related to xenoestrogenicity.

PREVIOUS STUDIES RELATED TO THIS RESEARCH:

Connexin43 (Cx43) and connexin26 (Cx26) are expressed in normal human mammary epithelial cells (NHMEC) (23,24). When gene expression in neoplastic breast epithelial cells was compared to nontransformed breast epithelial cells using the technique of subtractive hybridization, Cx26 was identified as a gene whose expression was reduced in the neoplastic cells (23). Subsequently, GJIC and connexin expression in several human breast carcinoma cell lines and primary breast carcinomas were analyzed and the results demonstrated that GJIC and the expression of both Cx26 and Cx43 were nearly undetectable in the neoplastic cell lines and tumors (24). Thus, the reduction of connexin expression and GJIC appears to contribute to the neoplastic phenotype of human breast carcinoma cells as in other neoplastic cells.

As mentioned above, DDT-related compounds have been shown to block GJIC in many types of cells including NHMEC (28). No studies, however, have correlated this with ER status of the cells or with effects on cellular growth. As reported below, data generated during the first year of this project indicate that DDT-related compounds block GJIC and enhance cell growth in two ER^+ cells lines, MCF-7 and MCF-10A/neoT. However, in ER^- NHMEC, *p,p'*-DDT also blocked GJIC but did not enhance growth.

Recent studies from our group (29-33) and others (34,35) are defining how xenobiotics alter GJIC *in vivo* and *in vitro*. Our data indicate that the inhibition of GJIC by *p,p'*-DDT in WB-F344 rat liver epithelial cells which express Cx43 occurs as both rapid and prolonged effects (29,32). Rapid inhibition occurs within 15 min of exposure of WB cells to *p,p'*-DDT without changes in gap junction number, gap junction size, Cx43 content, or Cx43 phosphorylation and is most likely due to closure or blockage of gap junction channels. The rapid reduction in channel permeability may be due to increased intracellular Ca^{+2}. Inhibition after prolonged exposure (0.5-6 h) to *p,p'*-DDT involves decreases in Cx43 content and in the number of gap junctions. This is due to gap junction endocytosis and degradation in lysosomes. These changes also appear to be posttranscriptional since no appreciable changes in Cx43 mRNA levels were detected (29,32).In this project, we are using similar approaches to determine whether DDT-related xenobiotics affect human mammary epithelial cell gap junctions in similar ways.

STATEMENT OF WORK:

The technical objectives of this project are:

1. Characterize the dose-responsive effects of DDT-related xenobiotics on GJIC in human mammary epithelial cells.
2. Determine whether DDT-related xenobiotics affect the growth of human mammary epithelial cells.
3. Identify the biochemical and molecular mechanism(s) by which DDT-related xenobiotics alter GJIC in human mammary epithelial cells.

EXPERIMENTAL METHODS:

1. Culture of human mammary epithelial cells: Proliferating cultures of NHMEC are obtained from Clonetics, Corp. (San Diego, CA) and cultured in serum-free/phenol red-free growth medium also obtained from Clonetics. These cells are derived by Clonetics from reduction mammoplasties and consist of cells from the mammary gland terminal ducts, the most common site of breast carcinoma development (36). NHMEC proliferate for at least 15 population doublings *in vitro* and are cultured by routine methods.

Several immortalized human mammary epithelial cell lines have been obtained from Dr. Bonnie Sloane (Wayne State University) and are also being used. These include nontransformed MCF-10A, H-ras-transfected MCF-10A/neoT, neoplastic MCF-7, and neoplastic BT20 cells. All of these lines are cultured in phenol red-free Dulbecco's MEM supplemented with 10% fetal bovine serum and gentamicin (40 μg/ml).

2. Determination of xenobiotic toxicity in mammary epithelial cells: The toxic effects of the DDT-related compounds was determined in these cells by trypan blue dye staining. Test agents were first dissolved in dimethylsulfoxide (DMSO) then applied to the cells (1 μl/ml culture medium). Control cultures were treated with DMSO (1 μl/ml). The cultures were sampled 1, 3, and 7 d after treatment and stained with 0.4% trypan blue. Viable and nonviable (blue) cells were identified and counted microscopically as we have reported (30).

3. Dye microinjection assay for GJIC: GJIC in these cells is assayed by microinjection of fluorescent Lucifer Yellow (LY) dye as we have described (29). The cells are cultured in 35 mm dishes, treated with the test agents for 1-7 d then microinjected. Cells are impaled with LY-filled glass micropipets and dye is loaded into the cells by iontophoresis. Cells are observed under the fluorescent microscope for evidence of dye transfer to neighboring cells. GJIC is

quantified as the percentage of neighboring cells adjacent to microinjected cells that take up dye. Ten cells per dish are injected for each treatment dose and duration and triplicate dishes are run per treatment group.

4. Xenobiotic effects on mammary epithelial cell growth: Xenobiotic effects on mammary cell growth *in vitro* is determined as we have reported (37). Cells are plated into 24 well multi-well dishes (25,000 cells/well), treated with xenobiotics, and the number of cells per well is determined on day 7 of treatment by trypsinizing the cells and counting them with a hemacytometer. Triplicate wells are sampled per xenobiotic dose.

5. Immunostaining of Cx43 and Cx26 gap junctions: We have obtained highly specific mouse monoclonal and rabbit polyclonal antibodies to both Cx43 and Cx26 from Zymed Corp. (South San Francisco, CA). These will be used to stain Cx43 and Cx26 gap junctions in xenobiotic-treated mammary epithelial cells by indirect immunofluorescence as we have reported (29). After staining, gap junction spots will be counted morphometrically (29) and changes in the number of gap junction spots per cell will be correlated with reductions in dye-coupling. As noted with rat liver epithelial cells (29-33), we expect to see dose-dependent reductions of Cx43 and Cx26 gap junction number in mammary epithelial cells and that these changes will only occur after prolonged (several hours) treatment.

6. Western blot assay of Cx43 and Cx26 protein: Cx43 and Cx26 protein levels in xenobiotic-treated mammary cells will be analyzed by Western blotting as we have described (29) using the above antibodies to Cx43 and Cx26. Based upon data with several other types of cells (29-35), we expect to detect three prominent Cx43 bands in control mammary cell blots. The two most slowly migrating bands (44 and 46 kDa) represent phosphorylated Cx43 with the 46 kDa band being associated with gap junction plaques (38,39). A more rapidly migrating 42 kDa band is also present and represents nonphosphorylated Cx43 (38,39). Phosphorylation of Cx43 occurs at the plasma membrane but the kinases have not been identified and the role of phosphorylation in gap junction assembly and function is also unclear (38,39). Cx26, on the other hand, is not phosphorylated and migrates as a single band at approximately 21 kDa (40). Treatment of mammary epithelial cells with xenobiotics at GJIC-inhibitory concentrations should decrease the content of phosphorylated Cx43 and of Cx26 as we have noted with other types of cells (29-33).

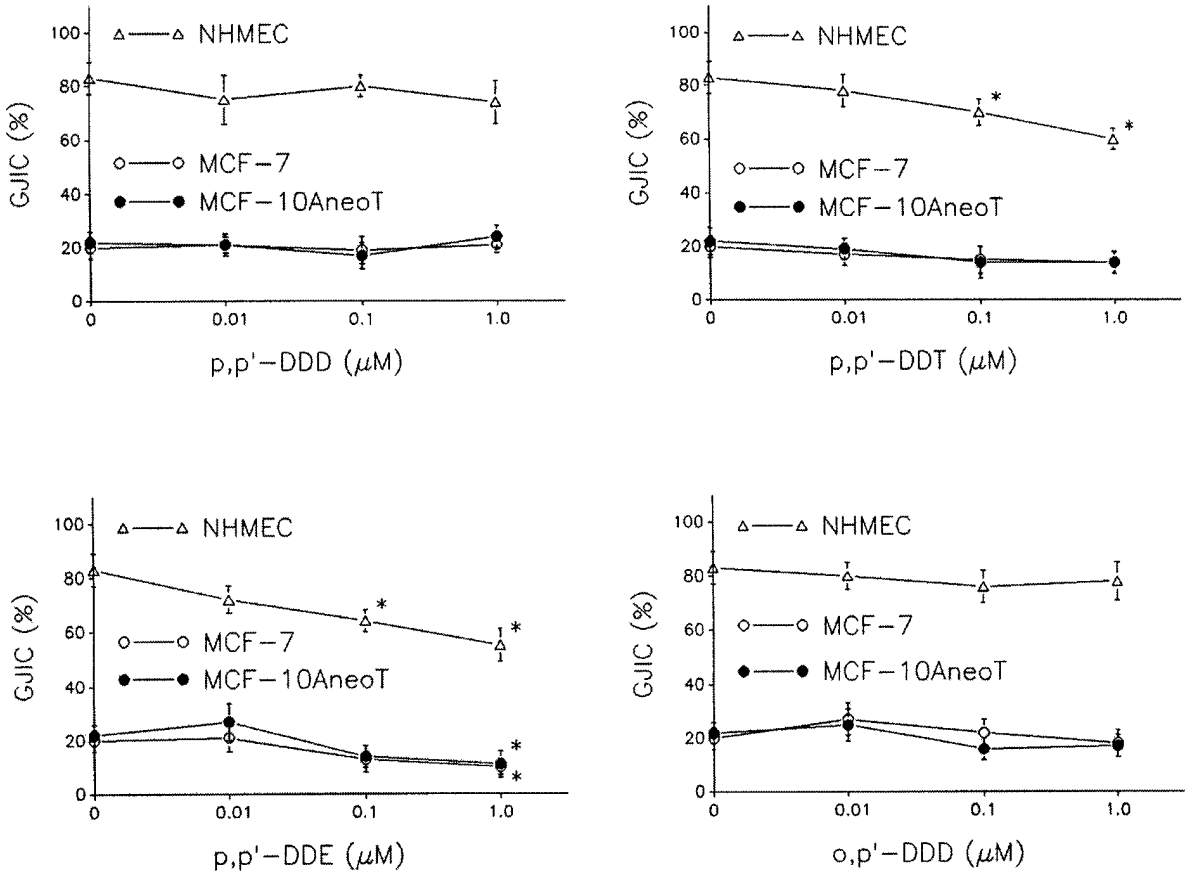
7. Northern blot assay of Cx43 and Cx26 mRNA: Steady-state levels of Cx43 and Cx26 mRNA in xenobiotic-treated mammary epithelial cells will be analyzed by Northern blotting as we have described (29). The blots will be hybridized with probes generated by random primer labeling of Cx43 and Cx26 full-length cDNAs available in my laboratory. The blots will be stripped and rehybridized with a glyceraldehydephosphate dehydrogenase (GAPDH) probe to check RNA loading and transfer. Cx43 and Cx26 mRNA bands will be measured by densitometry and normalized to GAPDH. We predict that xenobiotics will not affect Cx mRNA levels (29).

RESULTS:

1. Culture of human mammary epithelial cells: We have been successfully culturing NHMEC and the immortalized mammary cell lines as described above. The NHMEC can be passaged by trypsinization 3-4 times before they senesce; in the studies described below, we use the cells at passages 1 or 2.

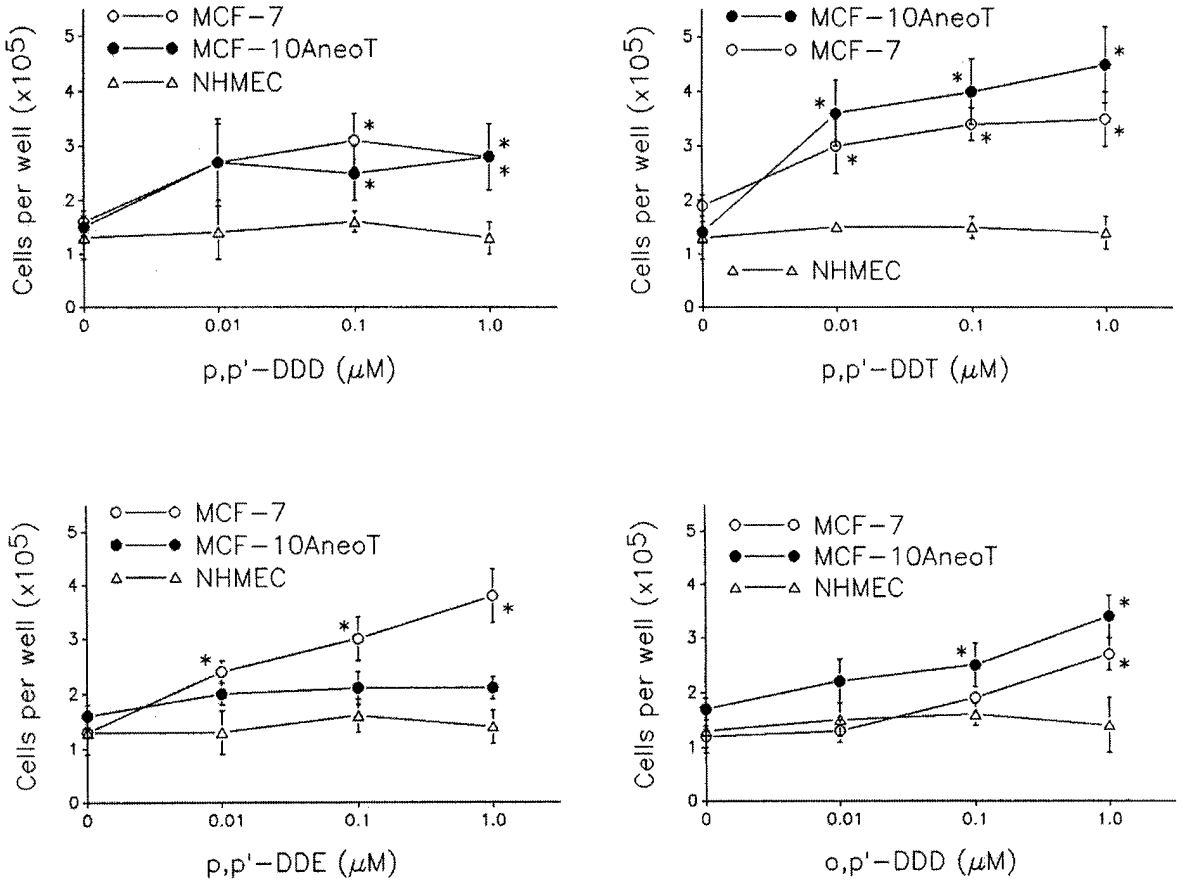
2. Determination of xenobiotic toxicity in mammary epithelial cells: The toxic effects of *p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD, *o,p'*-DDT, and fenarimol at concentrations of 0.001, 0.01, 0.1, and 1 μM were determined in the above cell lines by trypan blue dye staining. Control cultures were treated with DMSO (final concentration of 0.1%). None of the DDT-related compounds were toxic (i.e., they did not increase trypan blue staining) after 1 and 7 d of treatment (data not shown).

3. Dye microinjection assay for GJIC: GJIC was assayed by fluorescent dye microinjection in NHMEC, MCF-10A/neoT, and MCF-7 cells and was found to be greatest in NHMEC. The effects of DDT-related compounds on GJIC in these cells were also determined. As shown below, *p,p'*-DDT and *p,p'*-DDE decreased GJIC in MCF-7 cells, MCF-10A/neoT cells, and NHMEC in a dose-related manner after 7 d treatment. *p,p'*-DDD and *o,p'*-DDD did not affect GJIC in these cells. We are currently examining the effects of the other agents on GJIC in these cells and are initiating similar studies in MCF-10A and BT20 cells.



4. Xenobiotic effects on mammary epithelial cell growth: The effects of DDT-related compounds on the growth of MCF-7 cells, MCF-10A/neoT cells, and NHMEC were determined by counting trypsinized cells. As shown below, all of the agents increased the growth of MCF-7 cells in a dose-responsive manner. Three of the four compounds (with the exception of *p,p'*-DDE) also increased the growth of MCF-10A/neoT cells. In contrast, none of the DDT-related

agents affected the growth of NHMEC. We are also examining the effects of the other agents on growth in these cells and are initiating similar studies in MCF-10A and BT20 cells.



5, 6, and 7. Immunostaining of Cx43 and Cx26 gap junctions; Western blot assay of Cx43 and Cx26 protein; and Northern blot assay of Cx43 and Cx26 mRNA: These studies are in progress.

DISCUSSION:

The initial data we have generated indicate that GJIC was greatest in normal human mammary epithelial cells (NHMEC) compared to preneoplastic (MCF-10A/neoT) and neoplastic (MCF-7) human mammary epithelial cells and that *p,p'*-DDT and *p,p'*-DDE blocked GJIC in these cells, but *p,p'*-DDD and *o,p'*-DDD did not. The data also indicate that these agents enhanced cell growth in MCF-7 and MCF-10A/neoT cells, but not in NHMEC.

MCF-7 and MCF-10A/neo T cells are ER^+ whereas NHMEC are ER^-. Thus, these initial data suggest an association between the ER status of a cell and the enhancement of cell growth by DDT-related agents. Since DDT-related agents may have estrogenic activity, their ability to enhance growth in the ER^+ MCF-7 and MCF-10A/neo T cells may be due to an estrogenic mechanism. This awaits direct proof, however, especially since the relative estrogenicities of these agents are controversial (6,7).

The data also indicate the lack of an association between the inhibition of GJIC, growth enhancement, and ER status. The lack of growth enhancement in the NHMEC may be due not only to their lack of ER, but also to the possibility that they have other functional growth control mechanisms that prevent growth in the presence of blocked GJIC. These additional controls on cell proliferation may be absent in MCF-7 and MCF-10A/neoT cells.

The data also suggest that the inhibition of GJIC by DDT-related agents does not involve the ER since inhibition occurred in ER^- NHMEC (28). Other factors are probably involved in the inhibition of GJIC such as the elevation of intracellular Ca^{+2} and effects on cAMP metabolism (reviewed in 10,11).

Finally, these agents stimulated growth and inhibited GJIC in human mammary epithelial cells at micromolar concentrations which are comparable to DDT levels in blood and breast tissue (4,5). This suggests similar effects could occur *in vivo* and contribute to neoplastic disease in the breast.

CONCLUSIONS:

These initial data indicate that the reduction of GJIC by xenobiotics does not necessarily result in enhanced cell growth and that the estrogen receptor status of the cells is important in the growth stimulation by xenobiotics.

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