Mammary Gland Morphology in Sprague-Dawley Rats following Treatment with an Organochlorine Mixture in Utero and Neonatal Genistein

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Received on June 27, 2003; accepted on September 11, 2003

In a related reproductive toxicology study designed to investigate the effects of in utero exposure to environmental toxicants and potential interaction with postnatal genistein, gross enlargement of thoracic mammary glands was observed in female offspring at 200 days of age. Therefore, the objective of this study was to analyze the effect of in utero exposure to a mixture of toxicants on mammary gland morphology. Time-mated Sprague-Dawley rats were treated on days 9–16 of gestation with vehicle or a mixture of environmental toxicants at 1× the acceptable daily intake. Furthermore, it is unclear whether postnatal exposure to phytoestrogens in soy formulas poses breast cancer benefit or risk, and potential interactions with environmental toxicants are unknown. Therefore, half the female pups from each treatment group received either subcutaneous vehicle or genistein (10 μg/g body weight [bw]/day) on postnatal days 2–8. Following necropsy at 200 days of age, a pathologist, blinded to treatment groups, examined mammary gland histopathology. Only mild histological changes were found in mammary glands of rats exposed to the mixture in utero while pronounced ductal hyperplasia, lactational changes, and fibrosis were observed in mammary glands from the genistein group and were more prominent in the mixture + genistein group. Mammary glands of the control group were histologically normal. Collectively, our results reveal that postnatal exposure to pharmacological levels of genistein induces profound morphological changes in the mammary glands of adult female rats, and that high levels of phytoestrogens possess the potential to modulate the toxicological effects of toxicant mixtures.

Key Words: mixtures; genistein; mammary; tumors; development.

Breast cancer etiology is not fully understood, but recent data suggest that gene mutations account for only a small percentage (5%) of all of the cases of documented breast cancer (American Cancer Society, 2002). Furthermore, it has been reported that only 50% of breast cancer risk can be attributed to established risk factors including age, nationality, family, and reproductive history (Snedeker, 2001; Wolff and Weston, 1997). It has also been noted that substantial differences exist in risk of breast cancer for Asian-American women born in the United States compared to Asian-American women born in Asia (Ziegler et al., 1993). Taken together, these results suggest that exposure to a Western lifestyle profoundly alters breast cancer risk. Two factors thought to play an important role in the pathogenesis of breast cancer are exposure to environmental toxicants and consumption of dietary phytoestrogens. Although exposure to environmental toxicants has been linked with increased risk of breast cancer (DeWailly et al., 1994; Romieu et al., 2000; Wolff and Weston, 1997), others have failed to find an association (Gammon et al., 2002; Krieger et al., 1994). Thus, the role of environmental toxicants in the pathogenesis of breast cancer remains uncertain, and the effect of exposure to mixtures of environmental toxicants is unknown. Similarly, the role of dietary phytoestrogens in breast cancer remains controversial, and the potential interaction between exposure to environmental toxicants and phytoestrogens is unknown.

Synthetic halogenated organic compounds, such as dichlorodiphenyldichloroethene (DDE) and its persistent metabolite p,p’-dichlorodiphenoxy-dichloroethylene (p,p’-DDE), hexachlorobenzene (HCB), dioxins and furans, polychlorinated biphenyls (PCBs), other organochlorine pesticides, and metals such as lead and cadmium have been found as contaminants in tissues collected from the human population globally (Davies and Mes, 1987; Frank et al., 1988; Mes, 1992; Mes et al., 1990; Newsome et al., 1995; Szymczynski et al., 1981a,b). Detectable levels of suspected endocrine toxic chemicals such as p,p’-DDE, a-hexachlorocyclohexane, and selected PCB congeners in second trimester human amniotic fluid have been reported (Foster et al., 2000), indicating fetal exposure to these...
compounds. However, the effect of these toxicants, when present together in a mixture on development or reproductive function, are poorly understood. Cell culture experiments with mixtures of common organochlorines have provided evidence that these chemicals in combination can have additive and synergistic effects as measured by MCF-7 cell proliferation (Payne et al., 2000, 2001). Taken together, these data suggest that environmental toxicants can interact to augment the growth of mammary gland cells, function, and, ultimately, pathophysiology.

Animal experiments suggest that developmental-stage exposure to environmental toxicants is an important determinant of toxicant-induced changes in reproductive-tract and mammary-gland development and the pathogenesis of mammary tumors. Specifically, prenatal and perinatal exposures to environmental toxicants are associated with an increase in mammary tumor formation (Brown et al., 1998a; Desaulniers et al., 2001; Fenton et al., 2002; Markey et al., 2001) while toxicant exposure after differentiation of the mammary gland has been linked with either no effect or decreased risk of mammary gland tumor development (Holcomb and Safe, 1994; Nesaretnam et al., 1998; Ramamoorthy et al., 1999). Mammary gland morphogenesis begins in utero and proceeds through several discrete stages as the animal ages (Russ and Russo, 1978). At birth, the mammary gland consists of an epithelial ductal rudiment embedded in stroma tissue from which terminal end buds (TEBs) are formed during the first week of life. Estrogen stimulation, acting through estrogen receptor-α (ERα) induces rudimentary ducts to elongate and bifurcate to generate increasing numbers of TEBs that reach a maximum at 21 days of age. Gene-knockout studies have demonstrated that mammary glands fail to develop beyond the prepubertal stage in mice lacking ERα or the enzyme aromatase (Fisher et al., 1998; Korach et al., 1996). ERβ also has been demonstrated to be required for normal lobuloalveolar development as suggested by the finding of larger alveoli and less secretory epithelium in ERβ-/- mice compared to wild-type controls (Förster et al., 2002). With the onset of sexual maturity and the addition of progesterone and prolactin, increased side branching and formation of alveolar buds occurs with each estrous cycle until 63 days of age in the rat. During pregnancy, progesterone has been shown to contribute to ductal branching and lobuloalveolar development capable of milk production (Conneely and Lydon, 2000; Humphreys et al., 1997; reviewed by Hovey et al., 1999). In virgin mice, prolactin has been shown to exert indirect effects on ductal side branching and terminal end bud regression, whereas in pregnant animals, it acts directly on the mammary epithelium to induce lobuloalveolar development (Brisken et al., 1999). A prolactin receptor-knockout mouse model revealed that a functional PRLR is required for mammary gland development and milk production during pregnancy (Kelly et al., 2002). Hence, estrogens promote ductal elongation and progesterone-receptor expression whereas progesterone and prolactin promote lobuloalveolar development.

We therefore propose that in utero exposure and early postnatal exposure to environmental toxicants will act on the undifferentiated cells of the mammary gland to alter mammary development and increase risk of tumor formation. Indeed, in utero exposure to environmental toxicants such as TCDD and bisphenol A has been associated with delayed differentiation of the mammary gland, which has been associated with increased sensitivity to carcinogens (Fenton et al., 2002; Markey et al., 2001). Mammary gland tumor numbers were augmented by prenatal treatment with TCDD (Brown et al., 1998a). Similarly, a recent study (Band et al., 2002) demonstrated a significant increase in risk for breast cancer for women who commence cigarette smoking during the pubertal transition or before a first full-term pregnancy compared to those that start smoking later in life. These data suggest that the critical window for carcinogen-induced mammary tumors is prior to complete differentiation of the mammary gland. Hence, we propose that in utero exposure to environmental toxicants with a long half-life will result in exposure of the developing mammary gland prior to differentiation of the TEB into alveolar buds and subsequently into lobules.

It is unclear if the effects of environmental toxicant mixtures on mammary tumor development occur at concentrations below the no-effect level for the individual components of the mixture. In addition, concerns over the possible effects of environmental toxicants with estrogenic activity have led to questions about the safety of phytoestrogens in breast milk and soy-based infant formula (Irvine et al., 1995), as well as the use of isoflavone supplements. Maternal transfer of dietary genistein to offspring has been demonstrated in animal and human studies. In lactating rats fed genistein (250 mg/kg body weight [bw] in the diet), total genistein concentration in the serum and milk of dams 7 days postpartum were 418 ± 198 and 137 pmol/ml, respectively (Fritz et al., 1998). The total genistein concentrations in stomach milk, serum, and mammary gland of 7-day-old offspring in this study were 4439 ± 1109 pmol/ml; 726 pmol/ml, and 440 ± 129 pmol/ml, respectively. Phytoestrogen concentrations in human milk can increase 10-fold when the mother consumes soy products (Slavin, 1996). One woman who ingested a moderate challenge with 20 g of roasted soybeans (equivalent to 37 mg isoflavones) had total isoflavones (daidzein, genistein, and glycitein) in plasma, breast milk, and urine of approximately 0.2 mM/l, 2.0 mM/l, and 3.0 mM/l, respectively (Franke et al., 1998). Because infants can digest and absorb dietary phytoestrogens in active forms (Irvine et al., 1998a), and since neonates are generally more susceptible than adults to perturbations of the sex steroid milieu (Irvine et al., 1998b), we propose that exposure to these naturally occurring estrogenic compounds may pose a developmental hazard to estrogen-sensitive target tissue functions later in life. Therefore, half the animals in this study were also administered genistein postnatally and potentially interaction between the environmental toxicant mixture and
genistein was investigated. Our overall objective was to determine the effect of in utero exposure to a mixture of common chemical contaminants to which humans are exposed on re-productive function at four distinct developmental life stages. In this study, gross enlargement of the mammary glands was detected in animals at 200 days of age only. Therefore, the objective of the present study was to document the effects of in utero exposure to a mixture of environmental toxicants and postnatal genistein on mammary gland histology in the 200-day-old Sprague-Dawley rat.

MATERIALS AND METHODS

Animals. Time-mated Sprague-Dawley rats were purchased from Charles River (St. Constant, Quebec) and acclimated to holding facilities for 1 week. Animals were caged in pairs in clear plastic cages containing wood chips for bedding and maintained under controlled temperature (24°C), humidity (30–70%), and light (12/12 light/dark). All animals were provided standard laboratory rat chow (8804 Harlan Teklad, Madison, WI) and water ad libitum. Animal care and handling were in accordance with Canadian Council for Animal Care guidelines. Dams received corn oil (vehicle control, n = 9) or the 1× treatment mixture (n = 10) in a volume of 1 μl/g bw daily by gavage from days 9 through 16 of gestation. On postnatal days 2 through 8, half of the pups in each dose group received 10 μg genistein/g bw/day, subcutaneously. Oral gavage was thought to be too stressful for the neonatal rats and too labor intensive; thus, genistein was administered subcutaneously to prevent stressing of the pups. Prior studies have used widely divergent doses of genistein ranging from 4 μg/g (Lewis et al., 2002) to 500 μg/g bw on days 16, 18, and 20 (Brown et al., 1998b; Cotroneo et al., 2002; Fritz et al., 1998; Murrill et al., 1996). Hence, a dose of 10–μg/g bw/day on days 2–8 was thought to be adequate to provide developmental exposure to a dietary estrogen without inducing toxic effects.

Mixture formulation. The formulation of the mixture and selection of dose levels is described elsewhere (Wade et al., 2002). Briefly, the contaminant mixture was formulated to reflect the types of persistent organic and inorganic contaminants for which there is evidence of exposure in Canadian populations. The dose levels of each component in the mixture reflect the currently promulgated safe levels of exposure as published by the ATSDR (minimum risk level, MRL), Canadian Environmental Protection Act Chemical Assessments (tolerable daily intake; TDI) or U.S. EPA (reference dose, RfD), or at the lowest NOEL available in the scientific literature (Table 1). The dosing solutions provided a daily dose of each component equivalent to 1× their daily safe or no-effect levels of exposure.

Necropsy. Of the rats remaining on the study at postnatal day 200, one female from each litter was anesthetized with isofluorane and, after recording terminal body weight, was sacrificed by exsanguination via cardiac puncture followed by decapitation. Animals were killed between 9:00 A.M. and 1:00 P.M. on either of 2 consecutive days, with the number of animals per treatment group balanced between days. At necropsy in animals that had received postnatal genistein, thoracic mammary glands were observed to be grossly distended with evidence of milk production; thus, mammary glands were collected from a subset of animals for histopathological analyses. Mammary glands were collected from rats assigned to the control (n = 4), and treatment groups: 1× mixture on gestation days 9–16 (n = 4; MIX), 10 μg genistein/g/day (postnatal days 2–8; GEN; n = 5), and the combination of mixture and genistein (1× mixture + 10 μg genistein/g/day; MIXGEN; n = 7).

Mammary gland histomorphology. The first right thoracic mammary gland from each animal was carefully dissected and fixed in neutral buffered formalin for routine histopathological analysis. Briefly, mammary glands were dehydrated through a graded ethanol series, embedded in paraffin, and 5-μm-thick sections cut and stained with hematoxylin and eosin. Each slide was scored on a 4-point scale ranging from 0 (normal) to 4 (severe changes) by a pathologist blinded to treatment groups. Histopathological changes were further scored according to distribution of changes (0.25 focal, 0.5 (locally diffuse), and 0.75 (diffuse)). Therefore, the most severely affected sections with severe diffuse pathological changes would be scored a maximum of 4.75.

Statistical analyses. All statistical procedures were performed using SigmaStat (SPSS, Inc., Chicago, IL). Histopathological scores were summarized by descriptive statistics and checked for normality. Where the normality test failed, data were compared by ANOVA on ranks and treatment effects determined by multiple comparisons using Dunn’s method. The accepted level of significance was set at p ≤ 0.05.

RESULTS

Histopathology of Mammary Glands

Control group (cont.). Normal breast parenchyma, with no evidence of fibrocystic change, and any atypical features or carcinoma were noted in mammary glands of any animals in

### TABLE 1

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
<th>Purity (%)</th>
<th>MRL/RfD/TDI1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldrin</td>
<td>Accustandard</td>
<td>98.6</td>
<td>30 ng 2</td>
</tr>
<tr>
<td>p,p-DDT</td>
<td>Sigma</td>
<td>98</td>
<td>30 ng 2</td>
</tr>
<tr>
<td>p,p-DDE</td>
<td>Sigma</td>
<td>99</td>
<td>570 ng 2</td>
</tr>
<tr>
<td>Dieldrin</td>
<td>Sigma</td>
<td>97</td>
<td>50 ng 2</td>
</tr>
<tr>
<td>Endosulfan</td>
<td>Accustandard</td>
<td>99.6</td>
<td>50 ng 2</td>
</tr>
<tr>
<td>Heptachlor</td>
<td>Polysciences Corp.</td>
<td>Technical</td>
<td>0.5 μg g 2</td>
</tr>
<tr>
<td>Hexachlorobenzene</td>
<td>Aldrich</td>
<td>99+</td>
<td>0.3 μg g 2</td>
</tr>
<tr>
<td>Hexachlorocyclohexane</td>
<td>Polysciences Corp.</td>
<td>Technical</td>
<td>0.3 μg g 2</td>
</tr>
<tr>
<td>Mirex</td>
<td>Gift</td>
<td>98</td>
<td>0.8 μg g 2</td>
</tr>
<tr>
<td>Methoxychlorohexydrine</td>
<td>Aldrich</td>
<td>95</td>
<td>2 μg g 2</td>
</tr>
<tr>
<td>1,2,3-Trichlorobenzene</td>
<td>Aldrich</td>
<td>97</td>
<td>0.77 μg g 2</td>
</tr>
<tr>
<td>1,2,4-Trichlorobenzene</td>
<td>Aldrich</td>
<td>99</td>
<td>2.3 μg g 2</td>
</tr>
<tr>
<td>1,2,3,4-Tetrachlorobenzene</td>
<td>Aldrich</td>
<td>98</td>
<td>0.2 μg g 2</td>
</tr>
<tr>
<td>Pentachlorobenzene</td>
<td>Aldrich</td>
<td>98</td>
<td>0.5 μg g 2</td>
</tr>
<tr>
<td>TCDD</td>
<td>Accustandard</td>
<td>99</td>
<td>1 ng g 2</td>
</tr>
<tr>
<td>PCB (as Arochlor 1254)</td>
<td>Accustandard</td>
<td>Technical</td>
<td>1 μg g 2</td>
</tr>
<tr>
<td>Cadmium chloride</td>
<td>Fisher</td>
<td>99+</td>
<td>0.7 μg g 2</td>
</tr>
<tr>
<td>Lead chloride</td>
<td>Fisher</td>
<td>99+</td>
<td>0.1 ng g 2</td>
</tr>
</tbody>
</table>

1× dose, equivalent to the estimated dose in mass/kg body weight/day of daily exposure that will not cause adverse health effects in humans or experimental NOAEL. Where MRL for chronic exposure differed from MRL for intermediate exposure (as for HCB), the intermediate value was used.

Minimum risk level (Agency for Toxic Substances and Disease Registry, Atlanta, ATSDR).


TDI, tolerable daily intake (CEPA).

PTDI, provisional tolerable daily intake (Health Canada; Grant, 1983).

NOAEL, no observable adverse effect level (Feeley and Grant, 1993).

MRL for DDT and DDE includes all isomers and metabolites of DDT: Relative levels of DDT and DDE in mixture reflect relative concentrations in serum of Canadian women (Jarrell et al., 1993; Foster, W. G., unpublished).

Gift from Dr. L. S. Kaminsky, Dept. of Health, Albany, N.Y. to Dr. I. Chu, Health Canada.
the control group (Fig. 1a). Rare focal mild ductal epithelial hyperplasia was seen in the section from one animal of the control group only. No calcifications were found in slides from any of the tissues in the control group (Table 2).

**Mixture group (MIX).** No significant pathological changes were found in any of the tissues from animals in this group, and in particular, no cystic dilatations or calcifications were seen in any of the tissues from animals of the mixture group (Fig. 1b). In addition, no atypical cells or carcinoma cells were identified in any of the sections examined. Slight focal stromal fibrosis and minimal focal secretory changes were found in the mammary glands in two of four animals from dams fed the test mixture (Table 2).

**Genistein group (GEN).** The mammary glands of animals in the neonatal genistein-treatment group were characterized by lactational changes, prominent cystic ductal dilatations, florid and atypical epithelial hyperplasia (Fig. 1c), and prominent microcalcifications (Table 2). Atypical cytological features with enlarged pleomorphic nuclei and occasional prominent nucleoli were found in the cells lining the ducts and the lobules. In two animals from the genistein group, *in situ* ductal carcinoma of the comedo type was detected, with focal microinvasion and stromal reaction. Expanded ducts with cancer cells showing high nuclear cytoplasmic ratio, nuclear pleomorphism, prominent nucleoli, and a high mitotic activity (with a mitotic rate of more than 20 per 10 high-power fields; × 40 objective) were also observed (Fig. 1d). Atypical mitotic figures were also identified throughout sections of mammary glands from these animals. Comedo necrosis was very prominent and was accompanied by calcifications. Intraluminal secretions and lactational changes such as secretory changes in epithelial cells, lobular expansion, and secretory product in the

**FIG. 1.** Representative hematoxylin and eosin-stained, 5-µm-thick paraffin-embedded sections of mammary glands from animals of (a) the vehicle control group showing normal ducts, epithelia, and stroma; (b) the **MIX**-treatment group, which reveal small ducts with focal stromal fibrosis and minimal secretory changes; (c) the **GEN**-treatment group revealing lactational changes and epithelial hyperplasia; (d) higher magnification of boxed region in c illustrating atypical mitotic figures (arrow heads); (e) dilated ducts with calcification and fibrosis typical of sections from the **MIXGEN**-treatment group; and (f) grossly dilated ducts and calcification with papillary invaginations shown in the figure inset.
Summary of Semiquantitative Changes in Mammary Glands of PND 200 Female Rats Treated in Utero with a Mixture of Environmental Toxicants

<table>
<thead>
<tr>
<th></th>
<th>Cystic dilatation</th>
<th>Calcification</th>
<th>Lactational changes</th>
<th>Atypical epithelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>0 ! 0</td>
<td>0 ! 0</td>
<td>0 ! 0</td>
</tr>
<tr>
<td>Mixture</td>
<td>4</td>
<td>0.65 ! 0.70</td>
<td>0 ! 0</td>
<td>1.10 ! 1.18</td>
</tr>
<tr>
<td>Genistein</td>
<td>5</td>
<td>2.69 ! 1.38</td>
<td>2.44 ! 2.19</td>
<td>3.44 ! 1.03</td>
</tr>
<tr>
<td>Mixture + genistein</td>
<td>7</td>
<td>4.43 ! 0.85 ( \times )</td>
<td>4.14 ! 0.88 ( \times )</td>
<td>3.86 ! 1.15 ( \text{c, e} )</td>
</tr>
</tbody>
</table>

Note. See Table 1 for the concentrations of individual chemical constituents, neonatal genistein, or the combination of the mixture plus genistein compared to vehicle-treated controls. Histological sections from the first thoracic mammary gland were scored on a 4-point scale ranging from 0 (normal) to 4 (severe changes) by a pathologist blinded to treatment groups. Histopathological changes were further ranked according to distribution of changes (0.25 focal), 0.5 (locally diffuse), and 0.75 (diffuse).

\( \times \) Significant difference between mixture + genistein treatment and control groups, \( p = 0.001. \)

\( \times \) Significant difference between mixture + genistein treatment and mixture groups, \( p = 0.001. \)

\( \times \) Significant difference between mixture + genistein treatment and control groups, \( p = 0.002. \)

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DISCUSSION

As part of a study designed to determine the consequences to reproductive development and function resulting from in utero exposure to a mixture of environmental toxicants, at levels currently considered to be without effect by regulatory bodies in North America (United States Environmental Protection Agency and Health Canada), and interaction with postnatal genistein, gross morphological changes were detected in the thoracic mammary glands of female mice at 200 days of age. Therefore, the purpose of this study was to analyze the histological changes in the mammary glands induced by in utero exposure to the environmental toxicant mixture and postnatal genistein exposure. Our results demonstrate that developmental exposure to the environmental toxicant mixture alone does not alter mammary gland histopathology in the adult rat. In contrast to our expectations and evidence in the literature of a protective effect of dietary phytoestrogens (Fritz et al., 1998; Hilakivi-Clarke et al., 2002; Katadre et al., 2002; Murrill et al., 1996), genistein treatment during postnatal days (PND) 2–8 induced profound changes of two rats in mammary gland structure, including cystic dilatation, lactational changes, calcification, and atypical hyperplasia with comedo-type cancer in situ. Moreover, our results revealed interaction between the toxicant mixture and postnatal genistein treatment, as shown by enhanced lactational changes and calcification together with prominent cystic dilatation and focal fibrosis. Mammary glands from animals of the control group did not contain any histopathological changes, indicating that the observed changes in the GEN and MIXGEN groups are not the consequence of aging. Moreover, a prior study (Eldridge et al., 1999) has shown that spontaneous mammary tumors only begin to rise in Sprague-Dawley rats beyond 18 months of age, and this increase is related to persistent estrous in this strain of rats. Since our animals continued to cycle normally, we conclude that the changes in mammary gland cytoarchitecture documented in the current study are not the result of ageing or ovarian failure. Collectively, our data suggest that maternal exposure to environmental toxicants, at daily exposure levels in the upper range of the levels currently considered to be without adverse effect by North American regulatory bodies, does not induce structural changes at the light microscope level in the mammary glands of the offspring. Since the present study was limited to light microscopy, we cannot confirm that the mammary glands of these animals were unresponsive to the individual components of the toxicant mixture. However, the profound structural changes induced by the combination of in utero mixture-exposure and postnatal administration of genistein suggest that low concentrations of environmental toxicants can interact with hormonally active agents postnatally to alter mammary gland cytoarchitecture. To our knowledge, this is the first study to describe the effects of in utero exposure to a mixture of environmental toxicants at concentrations considered to be without adverse effect by North American regulatory bodies, on mammary gland morphology, as well as the effect of combining in utero toxicant-mixture exposure with postnatal exposure to dietary phytoestrogens. Our results therefore expand the current knowledge base and demonstrate that interaction of subthreshold concentrations of environmental toxicants and pharmacological doses of dietary estrogens can interact to induce profound structural changes in the mammary gland in the adult rat.

Some environmental toxicants are thought to promote the development of mammary tumors directly via estrogenic effects in vivo on breast epithelial cell proliferation (Bradlow et al., 1995; Davis et al., 1993; Wolff and Weston, 1997), whereas others act indirectly by altering the expression of...
genes responsible for estrogen synthesis and metabolism (Cournoul et al., 2001; Mucci et al., 2001). Alternative mechanisms involve induction of DNA adducts (Kuljukka-Rabb et al., 2001; Rundle et al., 2000) or direct effects of the toxicants on epidermal growth factor or insulin-like growth factor-I-regulated proliferation-signaling pathways (Tannheimer et al., 1997; 1998). Moreover, results from in vitro studies suggest that environmental toxicant mixtures can interact in an additive, synergistic, or inhibitory manner to modify the risk of breast cancer by altering cancer-cell proliferation and estrogen signaling (Du et al., 2000; Payne et al., 2000, 2001; Suzuki et al., 2001). Environmental toxicants may also inhibit tumor development or pathophysiology through antiestrogenic effects (Arcaro et al., 1999). Thus, given similar or even competing mechanisms of action, it is difficult to predict the effect of environmental-toxicant exposure on mammary gland morphogenesis and cancer risk from studies in which toxicants have been tested in isolation. Therefore, we tested the effect of a mixture of environmental toxicants for which human exposure has been documented (Davies and Mes, 1987; Frank et al., 1988; Mes, 1992; Mes et al., 1990; Newsome et al., 1995; Szymczynski et al., 1981a,b) and in utero exposure demonstrated (Foster et al., 2000). Components of our mixture are known to possess divergent mechanisms of action with some known to be estrogenic, such as methoxychlor, while others such as TCDD have mixed estrogenic and antiestrogenic effects. Chemical components of our mixture may therefore either act in an additive or even synergistic manner to augment effects on mammary gland morphology. Alternatively, it is possible that these chemicals can inhibit the actions of each other with the result that there is no detectable effect. In the current study, histopathological changes documented in the mixture group were unremarkable and did not differ significantly from the control animals, indicating that the toxicant mixture does not produce morphological alterations in the mammary gland. We cannot exclude the possibility that, if we had followed the animals past 200 days of age, we may have seen morphological alterations and mammary tumor development. On the basis of several in vitro cell-based assays, it has been suggested that dietary intake of hormonally active toxicants such as organochlorine pesticide residues in food have little estrogenic activity (Gaido et al., 1998; Safe, 1995), and thus, it is unlikely that environmental toxicants would contribute markedly to the pathogenesis of breast cancer. Although our results appear to support the above view, evidence of interaction between our toxicant mixture and postnatal genistein are taken to suggest that treatments have disrupted mammary-gland morphogenesis directly, which are unmasked by postnatal phytoestrogen exposure prior to mammary gland differentiation and may involve changes in gonadal-steroid receptor or growth-factor receptor expression in the mammary gland.

In the current study, postnatal genistein treatment on PND 2–8 induced profound alterations in mammary gland morphol-
potentially tumorigenesis. Taken together, we propose that neonatal exposure to genistein induces morphological alterations in mammary glands and increased risk of mammary tumorigenesis, whereas prepubertal exposure to pharmacological doses of genistein are associated with increased differentiation of the mammary gland and decreased risk of mammary tumors (Cotroneo et al., 2002). Since mammary gland morphogenesis is a hormone-dependent process (Conneely and Lydon, 2000; Fisher et al., 1998; Förester et al., 2002; Hovey et al., 1999; Humphreys et al., 1997; Korach et al., 1996) that progresses from birth into adulthood (Russo and Russo, 1978), with cyclical changes occurring with each estrous cycle as well as pregnancy and lactation (Hovey et al., 1999), defining the potential risks vs. benefits of genistein, will require attention to the developmental stage of the mammary gland during exposure as well as dosage and route of administration.

The mechanism of genistein action in the present study is difficult to determine, due to both the multiple mechanisms through which genistein might act as well as the long latency between exposure and assessment of histological changes in the mammary gland. Genistein is recognized as a preferential ERβ agonist that has also been shown to increase the synthesis of sex hormone-binding globulin, inhibit the activity of different enzymes including aromatase, DNA-topoisomerase, and protein kinases, and possess antioxidant properties (Aldercrutz et al., 1987; Barnes, 1998; Kao et al., 1998; Petersen and Barnes, 1991). Dietary genistein (15, 150, and 300 ppm) has been shown to dose-dependently increase the growth of estrogen-dependent breast cancer cells (MCF-7) transplanted to athymic nude mice, indicating a potential estrogenic mechanism (Allred et al., 2001). Since all of the foregoing mechanisms are acute effects of genistein, it is unlikely that they contributed significantly to the histological changes in the mammary glands in our study. Therefore, while multiple mechanisms likely contribute to the mammary gland development and pathogenesis of breast cancer, we propose that in the current study, genistein exposure during the first week of life altered mammary gland programming during local changes in gonadal steroid or growth factor receptor expression in the developing mammary gland. This view is supported by several distinct reports. First, in rats fed soy proteins from PND 4 onward, there was an increase in the number of cells expressing progesterone receptors in the mammary gland TEBs; however, there were no differences in the number of cells expressing ERα or ERβ (Rowlands et al., 2002). In addition, dietary genistein exposure (300 and 800 ppm), in combination with methoxychlor (800 ppm) during gestation and lactation, has been shown to alter mammary-gland development and to increase the expression of ERα, PR, and insulin-like growth factor-1 receptor (IGF-1R) in male, but not female rats (You et al., 2002). Furthermore, we have recently demonstrated that gestational and lactational exposure to 15 mg genistein/kg bw increased progesterone-receptor expression in the glandular epithelium of the uterus (Hughes et al., 2003). These data demonstrate that early postnatal exposure to physiologically relevant concentrations of soy proteins in the diet can alter gonadal steroid-receptor expression in estrogen-sensitive target tissues, including the mammary gland.

The mechanism of the interaction between environmental toxicants and genistein is not known. Combining the chemical mixture insult in utero with exposure to pharmacological levels of genistein during early postnatal development in the current study, augmented genistein-alone-induced secretory changes and produced pronounced cystic dilatation, focal fibrosis, and calcification in mammary glands of the adult rat. Our results, therefore, demonstrate that prenatal exposure to concentrations of environmental toxicants currently considered to be without adverse effect does not induce statistically significant changes in mammary gland morphology or induce mammary gland tumors but does interact with exposure to dietary estrogens during mammary gland development to induce profound morphological alterations. The interaction of diet and prior exposure in utero to environmental toxicants has not yet been explored empirically. However, the findings of this study, although preliminary, suggest that exposure to dietary phytoestrogens at levels consistent with use of dietary supplements, during mammary gland development, induces profound changes in mammary gland morphology, and in utero exposure to estrogenic agents present in the environmental toxicant mixtures modified mammary gland response to the adverse effects of genistein.

In summary, results of the present study demonstrate that gestational exposure to mixtures of environmental toxicants on their own, at the upper range of levels currently considered to be without adverse effect, does not induce changes in mammary gland structure or increase mammary gland tumor development. However, the combination of gestational exposure to environmental toxicant mixtures and neonatal exposure to pharmacological concentrations of the dietary phytoestrogen genistein increases morphological alterations in the mammary glands of adult rats. Collectively, although preliminary, our data suggest that in utero exposure to low levels of hormonally active chemicals can produce morphological changes that may increase mammary gland susceptibility to breast cancer in adulthood.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the Canadian Chemical Producers association through the Canadian Chlorine Coordinating Committee (WF), the Great Lakes Health Effects Research Program of Health Canada (WF and MW), and by the Natural Sciences and Engineering Research Council (WF). The authors gratefully acknowledge the technical assistance of Lorraine Casavant and Dr. Alison Holloway for helpful comments during the preparation of this manuscript.

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