Normal mammary gland morphology in pubertal female mice following in utero and lactational exposure to genistein at levels comparable to human dietary exposure

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Abstract

The objective of the study was to determine the effect of in utero and lactational exposure to genistein (0, 0.1, 0.5, 2.5 and 10 mg/kg/day) on mammary gland morphology in female B6D2F1 mice at levels comparable to or greater than human exposures. The effect of diethylstilbestrol (DES; 0, 0.1, 1, 10 μg/kg/day) on the mammary gland was also examined as a positive estrogenic control. Pregnant females were treated by daily gavage from gestational day 12 to postnatal day (PND) 20. Female offspring were weaned on PND21 and mammary gland whole mounts were examined for growth (length and area of the epithelial tree), proliferation (number of terminal end buds (TEBs)), and differentiation (density of alveolar buds (ABs)) on PND49. The highest dose of DES induced a significant increase in mammary gland growth (P < 0.05) and also decreased the number of TEBs (P < 0.06). The density of ABs was not significantly affected by DES. By contrast to DES, genistein had no effect on mammary gland morphology at any dose. These results suggest that in utero and lactational exposure to genistein at levels comparable to or greater than human exposures do not adversely affect mammary gland development in pubertal female B6D2F1 mice. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

It has been suggested that exposure to a high level of estrogen in utero increases the risk of developing mammary tumors in female offspring (Trichopoulos, 1990). This hypothesis is supported by epidemiological studies linking breast cancer in women and indicators of high estrogen
Exposure in utero (Ekborn et al., 1992; Weiss et al., 1997). Rodent studies have also demonstrated that maternal subcutaneous exposure to estradiol, or factors which increase maternal estrogen levels, significantly increases the incidence and decreases the latency of carcinogen-induced mammary tumorigenesis (Hilakivi-Clarke et al., 1997b, 1998). Offspring of estradiol-treated mothers have an increased density of terminal end buds (TEBs), which are the predominant site of carcinogen-induced malignant transformation (Russo and Russo, 1978, 1987). Estradiol also induced a decrease in the differentiation of epithelial structures to alveolar buds (ABs). Since epithelial differentiation protects the mammary gland from malignant transformation (Russo and Russo, 1987), the increased risk of mammary tumorigenesis in offspring exposed to a high level of estrogen in utero is attributed to an increase in TEBs and/or a decrease in their differentiation to ABs. Maternal subcutaneous exposure to the estrogenic isoflavone genistein during gestation also mimics the effect of estradiol on mammary gland development in female mouse offspring and carcinogen-induced mammary tumorigenesis in female rat offspring (Hilakivi-Clarke et al., 1998, 1999a). This has raised concern that dietary exposure of pregnant women to soy products, which are known to contain a high level of genistein, may also increase the risk of developing mammary tumors in the female offspring.

In contrast to the effects of in utero genistein exposure on mammary tumorigenesis, it is well established that Asian women who consume a high level of isoflavones from soy, compared to women consuming a Western diet, have a lower risk of breast cancer (Lee et al., 1991; Ziegler et al., 1993). In addition, genistein administered to rats through the diet during the gestational period does not alter the incidence and multiplicity of carcinogen-induced mammary tumors in female offspring (Lamartiniere et al., 2000), in contrast to the effect of subcutaneous exposure to genistein (Hilakivi-Clarke et al., 1998, 1999a). Furthermore, early postnatal exposure to genistein at pharmacological doses (Lamartiniere et al., 1995b; Murrill et al., 1996), or at total body dose comparable to the level typical of Asian countries (≈ 1 mg/kg) (Hilakivi-Clarke et al., 1999b), or via the diet at levels that produce similar serum genistein concentrations as human males on a high soy diet (Fritz et al., 1998), actually reduces carcinogen-induced mammary tumorigenesis. These effects are associated with a decrease in the number of TEBs at the time of carcinogen treatment (~ day 50), and in some instances, an increase in ABs. The proposed mechanism for the protective effects of early genistein exposure is from increased cell proliferation in the terminal ductal structures resulting in enhancement of mammary gland maturation and differentiation, with a concomitant decrease in the number of sites susceptible to carcinogen-induced tumorigenesis.

The contrasting effects of genistein appear to be related to the route and timing of exposure (discussed in Lamartiniere et al., 2002). The relevance of the rodent studies for estimating the risk of genistein exposure to humans is also confounded by the form of genistein exposure. For example, injection of the aglycone form of genistein is not equivalent to the form of genistein found in breast milk (glucuronides/sulfates) or soy foods (glucoside). These discrepancies may lead to differences in systemic absorption and bioactivity. These factors make estimating the risks and/or benefits of soy consumption during pregnancy and lactation difficult to ascertain. Therefore, the objective of this study was to determine if combined in utero and lactational exposure to genistein causes alterations in mammary gland morphology, which is known to predict the sensitivity of the pubertal mammary gland to carcinogen-induced tumorigenesis (Russo and Russo, 1978, 1987). The level of genistein exposure in Asian populations consuming a soy rich diet has been reported to range from ~ 1 to 30 mg/day, or ~ 0.02 to 0.55 mg/kg/day for a 55 kg woman, and even less in Western populations (Fukutake et al., 1996; Yamamoto et al., 2001). Therefore, pregnant dams were treated orally with genistein at levels comparable to human exposures (0.1 and 0.5 mg/kg/day) so that developing offspring would be exposed to a more relevant form and dose of genistein both in utero and through breast milk. Higher doses of genistein (2.5 and 10 mg/kg/day) were also selected to mimic potentially
higher exposures due to dietary supplementation. Diethylstilbestrol (DES) was also included as a positive estrogenic control at doses approximately 1000 times lower than genistein. This is consistent with the 1000-fold higher affinity of DES for estrogen receptor α relative to genistein (Matthews et al., 2000).

2. Materials and methods

All mice were obtained from Charles River Laboratories (Portage, MI) and housed in polycarbonate cages with cellulose fiber chips (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensburg, NY) as bedding and maintained in a humidity (30–40%) and temperature (23 °C) controlled room on a 12 h light–dark cycle. All animals were given free access to deionized water in glass bottles with rubber stoppers and AIN-76A rodent feed ad libitum (Research Diets, New Brunswick, NJ). This diet is a casein-based open-formula purified diet with non-detectable levels of the estrogenic isoflavones genistein, diadzein or glycitein (unpublished data, and Odum et al., 2001; Thigpen et al., 1999). DES was purchased from Sigma (St. Louis, MO) and genistein (> 98% pure) from Indofine Chemical Company (Somerville, NJ).

2.1. Time course of mammary gland development

An initial study of the development of the mammary gland in female B6D2F1 mice was carried out prior to the treatments detailed below. This initial study was done primarily to determine the optimal time point for assessing mammary gland development across treatment groups and to establish a baseline of mammary gland development in this strain of mouse. Twelve mature virgin C57BL/6 female mice (F0) were obtained from the vendor and housed two per cage upon arrival. Each pair of females was housed with a DBA/2 proven breeder male within a week of arrival. This hybrid strain was chosen since this study was designed to examine the effects on the F1 male offspring (Fielden et al., 2002), and it has been previously established that a high level of fertilization can be obtained with this strain in in vitro fertilization assays (Fielden et al., 2001; Huang et al., 1998). Following evidence of pregnancy, dams were separated from the males, housed individually and sequentially assigned to the treatment groups. For the DES experiment, F0 mice were treated by daily gavage from gestational day (GD) 12 to postnatal day (PND) 20 with 0.1 ml of corn oil vehicle (Sigma) or a nominal dose of 0.1, 1, and 10 μg DES per kg of maternal body weight. For the genistein experiment, F0 mice were treated by daily gavage from GD12 to PND20 with 0.1 ml of corn oil vehicle or a nominal dose of 0.1, 0.5, 2.5 or 10 mg genistein per kg of maternal body weight. On PND21, the offspring were weaned and housed with same sex littermates. There were a total of 46 female pups from the 12 litters. Within each litter, the pups were split and examined for mammary gland development at one of five time points; 3, 4, 5, 7 or 10 weeks of age. Each age group was represented by 5–9 animals, which were all derived from different litters.

2.2. F0 treatment

The results reported here for female offspring are derived from two independent experiments examining the effects of gestational and lactational exposure to DES (Fielden et al., 2002) and genistein (Fielden et al., submitted) on male reproductive development and sperm quality. The initiation of both experiments was separated in time by approximately 6 months. Each experiment consisted of its own vehicle control group and was analyzed independently.

Eleven week old virgin C57BL/6 female mice (F0) were housed two per cage upon arrival and acclimatized for at least 3 days. Each pair of females was housed with a DBA/2 proven breeder male within a week of arrival. This hybrid strain was chosen since this study was designed to examine the effects on the F1 male offspring (Fielden et al., 2002), and it has been previously established that a high level of fertilization can be obtained with this strain in in vitro fertilization assays (Fielden et al., 2001; Huang et al., 1998). Following evidence of pregnancy, dams were separated from the males, housed individually and sequentially assigned to the treatment groups. For the DES experiment, F0 mice were treated by daily gavage from gestational day (GD) 12 to postnatal day (PND) 20 with 0.1 ml of corn oil vehicle (Sigma) or a nominal dose of 0.1, 1, and 10 μg DES per kg of maternal body weight. For the genistein experiment, F0 mice were treated by daily gavage from GD12 to PND20 with 0.1 ml of corn oil vehicle or a nominal dose of 0.1, 0.5, 2.5 or 10 mg genistein per kg of maternal body weight. There was a 1-day interruption of treatment on the day of parturition (PND0). The dose of test chemical
was adjusted daily to body weight for each dam before dosing. Offspring were weaned on PND21 when F0 mice were euthanized.

2.3. Offspring development

Offspring body weight was measured on PND7 and PND21. Anogenital distance (AGD; the length of the perineum from the base of the genital tubercle to the proximal edge of the anus when the skin was naturally extended without stretching) was measured on PND7 and 21. Measurements were to the nearest 0.1 mm and obtained with a dissecting scope equipped with an ocular micrometer (Nikon, Melville, NY). AGD was measured by a single person to increase precision and reduce technical error. Offspring were weaned on PND21, housed with same sex littermates until PND49 when terminal body weight and mammary gland development were analyzed.

2.4. Whole mount analysis of mammary gland development

Mammary whole mounts were prepared from the fourth abdominal gland on the right side. Glands were spread on Nitex fabric (Sefar America, Kansas City, MO), fixed in ethanol:acetic acid (3:1 v/v) for 1 h, rehydrated in 70% ethanol (15 min), rinsed in water (5 min), and stained in carmine alum (2 g/l) overnight (14–18 h) at 4 °C. The stained gland was progressively dehydrated in ethanol (70–100%) in 3 steps (15 min/step) and cleared in toluene (15 min). The stained gland was then removed from the Nitex fabric and stored in methyl salicylate (Sigma) in glass scintillation vials, coded and stored at room temperature until analysis.

Mammary whole mounts were examined under a light microscope (10–20 ×, equipped with ocular micrometer) for epithelial growth, length of mammary tree, number of TEBs and density of ABs. All whole mounts were evaluated blind to the identity of the treatment group by two people and averaged. Mammary growth was evaluated as a percentage of the mammary epithelium that occupied the mammary fat pad. Mammary gland size was determined by mammary tree length, which was measured as the length in millimeters between the nipple and the most distal terminal branches. The identification of terminal structures was based on the classification by Russo and Russo (1978). Club-shaped terminal ductal structures greater than 100 μm in diameter were classified as TEBs, while terminal structures less than 100 μm in diameter were considered terminal ducts. ABs were identified as terminal or lateral buds that had differentiated by septation, cleavage and further sprouting into 3–5 smaller buds. No attempt was made to distinguish ABs from lobules. ABs were assessed qualitatively for density on a scale of 0–3, where 0 = absent, 1 = low, 2 = moderate, and 3 = abundant (Hilakivi-Clarke et al., 1997a).

2.5. Statistical analysis

All data analysis was performed using SAS version 8.0 (SAS Inc, Cary, NC). To estimate experimental error, the litter was considered the experimental unit. Body weight and AGD were analyzed with a repeated measures analysis of variance using the MIXED procedure of SAS. Using an analysis of covariance, litter size was found to account for a significant source of the variation (P < 0.05). Therefore, for the analysis of body weight, the model included dose, time, and dose × time interaction as fixed effects and litter size as a covariate. Likewise, for the analysis of AGD, body weight was included in the model as a covariate since body weight was found to account for a significant source of the variation (P < 0.05). For comparison, AGD was also analyzed as a ratio of AGD to the cube root of body weight (Gallavan et al., 1999). Mammary gland length was also analyzed by ANOVA using the MIXED procedure of SAS. Body weight was also included as a covariate since it was found to account for a significant source of the variation (P < 0.05). Due to the unbalanced nature of the ANOVA, and to adjust group means for the covariate, comparisons between control and treated groups were performed on LS-means and adjusted for multiple comparisons by Dunnett’s
method, while comparisons between different age groups in the time-course experiment were determined by Tukey’s method. Arithmetic means, standard errors and n values for litters are reported in the results. Percent of fat pad growth, number of TEBs and AB density were analyzed by a non-parametric ANOVA using the NPAR1WAY procedure of SAS. Comparisons between groups were made with the Kruskal-Wallis test. The level of significance was set at $p < 0.05$.

### 3. Results

#### 3.1. Time course of mammary gland development in B6D2F1 female mice

Mammary gland growth and length of the epithelial tree increased in an age-dependent manner (Fig. 1A and B). At 3 weeks of age the mammary tree was barely established and constituted less than 5% of the size of the fat pad. A stalk...
originating from the nipple averaged less than 2 mm in length. The mammary gland then increased to an average of 17, 35, and 79% of the fat pad at 4, 5 and 7 weeks of age, respectively. This corresponded to an average mammary tree length of 3, 5, and 13 mm from the nipple to the most distal branches. By 10 weeks of age, mammary tree growth was at a maximum of 100% with a tree length averaging 18 mm.

To assess the proliferative status of the mammary gland, the number of TEBs was determined (Fig. 1C). At 3 weeks of age, there were no TEBs apparent. The terminal branches consisted primarily of terminal ductal structures less than 100 μm in diameter. TEBs increased in number at 4 weeks of age and peaked by 5 weeks of age. By week 7, the number of TEBs was still elevated significantly \( P < 0.05 \) above that of 4 week old mice but did not differ significantly from the 5 week time point. By week 10, however, TEB number decreased significantly \( P < 0.01 \) relative to 7 week old mice. There was no evidence of differentiated mammary glands in 3–7 week old females. At 10 weeks of age, however, 2–6 females had AB density values of 3 (Fig. 1D), while the other 4 females had no evidence of differentiation. Based on the size of the mammary tree and the number of TEBs, the 7 week time point was chosen as a suitable age to assess treatment-related increases and decreases in growth and proliferation. Although decreases in differentiation could not be observed at this time point, we expected in utero and lactational exposure to estrogens to increase the number of ABs. More importantly, this age is consistent with many other studies investigating the morphological effects of estrogenic chemicals on mammary gland development (Hilakivi-Clarke et al., 1998, 1997a,b; Lamartiniere et al., 2002; Vassilacopoulou and Boylan, 1993).

### 3.2. Body weight and AGD in female offspring

Neither DES nor genistein caused a treatment-related effect on body weight or AGD (Table 1). There was a statistically significant change in body weight in DES-exposed offspring in the 10 μg/kg dose group on PND7, however, the change
in body weight was small ( < 3%) and not considered biologically significant.

3.3. Mammary gland growth

DES caused a modest, but dose-dependent increase in mammary gland growth (Fig. 2A and B). There was a small increase in the percent of fat pad growth which was close to significance ($P < 0.06$) (Fig. 2A), while the length of the mammary tree was significantly increased ($P < 0.05$) in both the 1 and 10 μg/kg dose group (Fig. 2B). By contrast, genistein had no effect on mammary gland growth (Fig. 3A and B). The average fat pad growth and mammary gland length were also similar between all three experiments.

3.4. Mammary gland terminal end buds

The number of TEBs in DES-exposed offspring was slightly decreased from 12.0 ± 5.4 in the control group to 8.0 ± 4.2 in the 10 μg/kg group ($P < 0.06$) (Fig. 2C). The number of TEBs in the genistein-exposed offspring were not significantly different from controls (Fig. 3C). The number of TEBs was also similar between all experiments.

3.5. Mammary gland differentiation

There appeared to be an increase in the density of ABs in the DES-exposed offspring (Fig. 2D), however, the results were not significant due to a higher than normal variation between litters.

Fig. 2. Mammary gland development of female offspring on PND49 exposed to DES from GD 12 to PND 21. (A) Percent of mammary tree growth (%). (B) Length of the mammary tree (mm). (C) Number of TEBs. (D) AB density. Asterisks indicate significantly different from time-matched vehicle control ($P < 0.05$). *$P = 0.0530$; **$P = 0.0585$; ***$P = 0.0520$; ****$P = 0.0580$. Values are mean ± S.D. The number of litters in each group is shown in Table 1.
Fig. 3. Mammary gland development of female offspring on PND49 exposed to genistein from GD 12 to PND 21. (A) Percent of mammary tree growth (%). (B) Length of the mammary tree (mm). (C) Number of TEBs. (D) AB density. There were no significant treatment-related effects on mammary gland development. Values are mean ± S.D. The number of litters in each group is shown in Table 1.

There was one litter in the control group with an average AB density value of 2.5, whereas the rest of the litters had an average AB density value less than 0.5. By contrast, there were three litters in both the 1 and 10 µg/kg DES group with average AB density values equal or greater than 1.0. A large variation in litter response was also observed in the time-course experiment at 10 weeks of age (Fig. 1D). There was no significant effect due to genistein on mammary gland differentiation. In contrast to the results in the DES and genistein experiments, there were no ABs present in naïve female offspring at 7 weeks of age (Fig. 1D), thus suggesting an effect of the vehicle, or perhaps another variable that was not controlled since these experiments were not performed concurrently.

4. Discussion

The results of this study indicate that in utero and lactational exposure to genistein at levels as high, or higher, than human populations consuming a soy rich diet does not affect the morphology of the mammary gland in pubertal female mice. It is well established that the morphology of the mammary gland at the time of carcinogen exposure predicts the sensitivity to tumorigenesis (Russo and Russo, 1978, 1987). Therefore, it is likely that exposure to genistein during both in utero and early postnatal life does not contribute to an increased risk of developing mammary tumors, and previous studies in the rat suggest it may actually protect the mammary gland from tumorigenesis. For example, combined gestational
and lactational exposure to genistein through the diet reduces the number of carcinogen-induced tumors per rat and reduces the number of undifferentiated terminal ductal structures that are most susceptible to carcinogenesis (Fritz et al., 1998). This chemopreventative effect occurred at doses of genistein that produced serum genistein levels in lactating rats (40–418 pmol/ml) similar to Asian adults on a traditional soy rich diet (276 pmol/ml) (Adlercreutz et al., 1993). However, the serum genistein level in nursing pups was considerably less (86–726 pmol/ml) than the mean plasma level of genistein in infants fed a soy-based formula (2500 pmol/ml) (Setchell et al., 1997). The effects of higher serum genistein levels in infants fed a soy-based formula is difficult to predict, but may not be of concern given the protective effect of genistein administered postnatally at pharmacological doses (Lamartiniere et al., 1995a,b; Murrill et al., 1996). Taken together, these results indicate that combined in utero and early postnatal exposure of rats to genistein at human exposure levels does not adversely affect mammary gland development and susceptibility to carcinogen-induced tumorigenesis. The concordance between the results in the current study, and those in rats (Fritz et al., 1998), also suggest that genistein would not adversely affect the susceptibility of the mouse mammary gland to carcinogen-induced tumorigenesis, although this has yet to be experimentally verified.

The route of administration, and ultimately bioavailability, appears to explain the discrepancy between both the adverse and protective effects of early genistein exposure. For example, maternal injection, but not dietary exposure, to genistein during gestation increases the incidence and multiplicity of carcinogen-induced mammary tumors (Hilakivi-Clarke et al., 1999a; Lamartiniere et al., 2000). Injections of genistein to rats have been reported to result in significantly less conjugated genistein in the circulation relative to dietary genistein exposure (Cotroneo and Lamartiniere, 2001; Fritz et al., 1998; Lamartiniere et al., 2002). Therefore, oral administration (gavage or diet) is likely to reduce the availability of free genistein to the fetus compared with subcutaneous administration. The results of in utero and lactational exposure to DES in the current study suggest that this finding cannot be generalized for other estrogens. In contrast to the lack of effects of genistein exposure reported here, in utero and lactational exposure to DES had a slight stimulatory effect on the growth of the mammary gland and the number of TEBs (Fig. 2A–C). This is consistent with subcutaneous exposure to DES or estradiol in utero (Hilakivi-Clarke et al., 1998, 1997b; Vasilacopoulou and Boylan, 1993). The discrepancy between genistein and DES may be due differences in their bioavailability, or it may indicate that the mechanism of action of DES in the mammary gland may be different from genistein. For example, genistein is known to inhibit proliferation of mammary epithelium at pharmacological doses, which is thought to be due to the ability of genistein to inhibit protein tyrosine kinase activity (Bouker and Hilakivi-Clarke, 2000). The lack of effect on mammary gland growth and TEBs suggest the level of genistein was not sufficient to inhibit tyrosine kinase activity. In addition to an increase in mammary growth and the number of TEBs induced by DES, a decrease in mammary gland differentiation would also predict an increased susceptibility to carcinogen-induced tumorigenesis. In fact, there was a slight increase in the number of ABs in the 1 µg/kg DES group, however, the effect was slightly less than significant (P < 0.06) and appeared to be restricted to only certain litters. Furthermore, the putative effect was not dose-related and may be a chance occurrence.

To summarize, gestational and lactational exposure of mice to genistein at dietary levels comparable to, and higher than, human populations on a soy-rich diet does not alter development of the mammary gland in pubertal female mice. This is consistent with data in rats showing that combined in utero and postnatal exposure to genistein, or diadzein (Lamartiniere et al., 2002), does not alter the morphology of the mammary gland in the pubertal female, or increase the susceptibility of the mammary gland to carcinogen-induced tumorigenesis. This is in contrast to the effects of gestational and lactational exposure to DES on the mammary gland, which increased the size of the mammary gland and the number of TEBs,
thus increasing the risk of carcinogen-induced tumorigenesis. Determining the risk of in utero and postnatal exposure to natural and synthetic estrogens will require both an assessment of their effects on mammary gland morphology, susceptibility to carcinogen-induced tumorigenesis, and determination of their bioavailability at doses relevant to a range of potential human exposures.

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