Prepubertal estradiol and genistein exposures up-regulate BRCA1 mRNA and reduce mammary tumorigenesis

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Introduction

Animal studies and epidemiological research indicate that childhood and adolescence might be particularly sensitive periods for breast cancer initiation (1–4). These periods might also be sensitive to exposures that provide a persistent protection towards breast cancer. For example, high soy intake in Asian countries was initially thought to explain the substantially lower breast cancer risk among Asian than Caucasian women, but more recent findings indicate that it does not reduce breast cancer risk if consumed only in adulthood (5). Prepubertal exposure to soy or genistein, a weak estrogenic compound present in soy products, reduces breast cancer risk in animal models (6–8) and in human populations (5,9).

It is not clear whether exposure to estrogens before puberty affects breast cancer risk. On the one hand, early puberty onset increases breast cancer risk (10,11), suggesting that earlier ovarian estrogen production increases breast cancer risk. On the other hand, indirect evidence in human studies provides evidence that peripubertal exposure to estrogens reduces later breast cancer risk (12–15). Specifically, women who were overweight around puberty are at a reduced risk of developing breast cancer later in life.

Several plausible mechanisms exist to explain why estrogen exposure prior to the onset of puberty might affect breast cancer risk. First, mammary gland morphology influences vulnerability to malignant transformation and estrogen exposure before puberty may alter the normal pattern of mammary epithelial proliferation and differentiation. Particularly, in rats there is an association between the number of highly proliferative terminal end buds (TEBs) and the likelihood of developing mammary tumors when the gland is exposed to a carcinogen (16,17). In contrast, a gland containing a high number of differentiated lobules is resilient to carcinogen-induced mammary tumorigenesis (16). The highest number of TEBs is seen around post-natal weeks 5–7, and eventually TEBs differentiate to alveolar buds and lobules (16). Earlier studies show that prepubertal exposure to genistein reduces the number of TEBs and increases the density of lobulo-alveolar units (6,7).

Second, changes in mammary gland morphology are likely to occur in parallel with changes in specific signaling pathways and may involve genes that, for example, regulate mammary cell proliferation and differentiation. 17β-Estradiol (E2) and other estrogens bind primarily to estrogen receptor (ER)-α and ER-β, which, in turn, mediate the effects of estrogens on the mammary gland. Specifically, ER-α is believed to mediate the proliferative actions of estrogens, although in the normal gland the effect may be indirect, as proliferating cells do not contain ER-α (18). The specific functions of ER-β in the breast are not known, but there is some evidence that this receptor may inhibit cellular proliferation by antagonizing the actions of ER-α (19–21). It is to be noted that ER-β is the predominant form in the normal rat mammary gland (22).

Estrogens also regulate the expression of the tumor suppressor BRCA1, at least in normal mouse mammary gland and in human breast cancer cell lines (23–28). Loss of the wild-type BRCA1 allele is linked to inherited breast cancers (29,30), probably because this gene participates in DNA damage repair and recombination processes related to maintenance of
genomic integrity, control of cell proliferation and regulation of gene transcription (31,32). Not surprisingly, BRCA1 has been found to regulate the expression of several genes identified as important players in affecting breast cancer risk, including cyclin D1, c-myc, and the STAT-1AK pathway (32). In addition, BRCA1 inhibits the signaling of the ligand-activated ER-α (26) and can also inhibit this receptor in a ligand-independent manner (27). Thus, BRCA1 protein may function to suppress mammary epithelial proliferation by inhibiting ER-α mediated pathways.

This study investigated whether prepubertal exposures to E2 or genistein modify mammary gland BRCA1, ER-α and ER-β expression.

Materials and methods

Animals
Female Sprague-Dawley rats were obtained from Charles River Laboratories on day 10 of gestation. The animals were housed singly and were fed Purina 5001 laboratory chow. Two days after the offspring were born, the females were cross-fostered, weaned on post-natal day 22 and thereafter housed in groups of 3-5 animals.

Estrogenic exposures
On post-natal day 7, the rat pups were divided into three groups and received either 10 μg E2 (Sigma Chemical Co., St. Louis, MO), 50 μg genistein (Sigma Chemical Co.) or vehicle, administered as s.c. injections in a volume of 0.05 ml E2 and genistein were dissolved in peanut oil, which also served as the vehicle. The injections were repeated daily between post-natal days 8 and 20. Since the animals weighed ~45 g on post-natal day 7 and 40 g on day 21, we estimated that the doses administered to the rats started at a level of 0.67 μg/g body wt E2 and 3.3 μg/g body wt genistein and were 0.25 μg/g E2 and 1.25 μg/g genistein on the last day of exposure.

Thirty animals from the control group and 30 animals from the E2-treated group were given 7,12-dimethylbenz[a]anthracene (DMBA) to induce mammary tumors. Because prepubertal genistein exposure has been shown to reduce carcinogen-induced mammary tumorigenesis in rats (6-8), this endpoint was not studied here.

The remaining animals in each treatment group were killed at 3 weeks (at the end of the E2 or genistein treatments) (n = 6-7/group) and at 16 weeks of age (no genistein-exposed animals were studied at this time point) (n = 7/group). We collected the second and third inguinal and fourth abdominal mammary glands to study changes in morphology to obtain mRNA and protein for gene/protein expression assays.

Reproductive factors
We determined vaginal opening (as an indicator of the onset of puberty) and measured uterine wet weights and serum E2 levels. In the final analysis, E2 levels were measured uterine wet weights and serum E2 levels. In the final analysis, E2 levels were measured.

Mammary tumorigenesis
At 47 days of age some rats from the E2-treated and vehicle groups were given a single dose of 10 mg DMBA by oral gavage to induce mammary tumors (~50 mg/kg body wt). The dose used in this experiment is a sub-optimal dose that induces tumors in approximately two-thirds of the control group and thus enables assessment of both reductions and increases in the end-points of tumorigenesis. As described by Russo and Russo, >75% of the tumors induced by 10 mg DMBA are adenocarcinomas (34); in our previous experiments, the proportion of adenocarcinomas in the control group has been 80-100% (6,35).

The animals were examined for mammary tumors by palpation once per week. The end-points for data analysis were (i) week of tumor appearance (latency), (ii) number of animals with tumors (tumor incidence), (iii) number of tumors per animal (tumor multiplicity) and (iv) tumor growth. Tumor sizes were measured once a week by recording the tumor diameters with a caliper and determining the length of the longest axis and the width perpendicular to the longest axis. The animals were killed when detectable tumor burden approximated 10% of total body weight, as required by our institution. All surviving animals, including those that did not appear to develop mammary tumors, were killed 17 weeks after carcinogen administration.

The time to tumor presentation (tumor-free survival, TFS) was measured as the number of weeks from DMBA exposure to the time the first tumor per animal could be palpated. Estimations of TFS were calculated by the methods developed by Kaplan and Meier (36). Differences between the two arms (vehicle and E2) were tested using an extension of the log rank test and both Gehan’s and Peto and Peto’s generalized Wilcoxon tests as implemented in STATISTICA (37). The differences were considered significant if P < 0.05. All probabilities were two-tailed. Differences in latency of tumor appearance and multiplicity were analyzed using one-way ANOVA.

Mammary gland morphology
Mammary morphology of the right fourth abdominal gland was assessed in whole mounts from rats that had not been exposed to DMBA. The mammary glands were stained with carmine aluminum and processed as previously described (17). The total number of terminal end buds (TEBs) was counted in each whole mount. Further, the relative density of lobulo-alveolar units (LAUs) was determined using a 5 point visual scale (0 = absent, 5 = numerous) that we validated earlier (17,38). Identification of each of these epithelial structures was based on the guidelines of mammary gland morphology by Russo and Russo (16). All the analyses were done double-blind under an Olympus dissecting microscope. Differences in mammary morphology (TEBs, LAUs and density of mammary epithelium) were analyzed using two-way ANOVA, with treatment and age as independent variables.

BRCA1 mRNA
Expression of BRCA1 mRNA in the rat mammary glands was analyzed by RNase protection assay (RPA), as described elsewhere (39). The rat BRCA1 cDNA fragments for the riboprobe construct were RT-PCR amplified from testis total cellular RNA. The total RNA was reverse transcribed and PCR amplified with rat BRCA1 primers: (i) BRCA1-187, 5′-TCCACAAAGTGCGACGCCC-3′ and (ii) BRCA1-366, 5′-GGCCGGTTTGCTGGTACCC-3′. The RT-PCR amplified cDNA fragments were subcloned into pGEM-T easy vector (Promega). The values were standardized against values of 36B4 and data were expressed as fold difference from the control values (set to 1) for each age.

Radioactivity was visualized by autoradiography or exposed to a Phosphor-Imager cassette and quantitated with Imagequant Software (Molecular Dynamics). Differences in expression of mRNA BRCA1 were analyzed using two-way ANOVA, with treatment and age as independent variables.

ER-α and ER-β expression

Immunohistochemistry. ER-α and ER-β expression in the mammary gland were analyzed by immunocytochemistry (40). Briefly, formalin-fixed tissue sections (5 μm) were cut from paraffin blocks and mounted onto pre-coated slides. Sections were deparaffinized in two 10 min changes of xylene and rehydrated through graded alcohols to distilled water. Sections were microwave heated for antigen retrieval in 10 mM citrate buffer (pH 6.0) for a total of 10 min, cooled to room temperature and washed three times for 5 min in phosphate-buffered saline (PBS). Sections were then treated with 3% H2O2 in water for 10 min to block endogenous peroxidase activity, washed with PBS and incubated with 20% goat serum in 1% BSA for 30 min. After blocking, excess solution was removed and sections were incubated overnight (4°C) with the ER-α antibody (MC-20 rabbit polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA) or the ER-β antibody (PAl-313 rabbit polyclonal IgG; Affinity Bioreagents Inc., Golden, CO) at 1:100 and 1:500 dilutions, respectively. After several washes, sections were treated with biotinylated goat anti-rabbit IgG (1:200 in 1% BSA; Vector Laboratories, Burlingame, CA) for 1 h, followed by a 30 min incubation with streptavidin-peroxidase conjugate (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA). Antigen-antibody complex was visualized by incubation with 3,3′-diaminobenzidine. Finally, sections were slightly counterstained with Gill’s hematoxylin stain, dehydrated through graded alcohol and mounted.

Specificity of immunodetection. ER-α and ER-β proteins in the mammary epithelial cells was tested by pre-absorbing the primary antibodies with ER-α and ER-β blocking peptides.

The level of expression on each slide was assessed in three separate lobular and three ductal fields by determining the intensity of staining and percentage of cells stained. This was done under light microscopy at 25× magnification. A scale of 0 (none) to 3 (most intense) was used to estimate intensity. The percentage of positively stained cell nuclei in mammary glands and ducts was assessed on a scale from 0 to 5. The two scores were combined and a mean score for lobules and ducts in each slide was calculated. ER-α and ER-β expression levels in the three groups were then compared using one-way ANOVA.
Western blot analysis. Mammary glands were homogenized in 500 μl of ice-cold homogenization buffer containing 25 mM Tris–HCl (pH 7.4), 2 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and the protease inhibitors leupeptin (1 μM), pepstatin (1 μM) and aprotinin (1 μg/ml). The amount of protein present in the cells was determined using the BCA Protein Assay (Pierce, IL).

Protein from tissue extracts was electrophoresed under reducing conditions in 8% Tris–glycine gels (Novex, San Diego, CA) and transferred to a nitrocel lulose membrane (Protran; Schleicher & Schuell). ER-α and ER-β recombinant proteins (obtained from Panvera) were included as negative and positive controls, respectively. The membrane was blocked for 1 h at room temperature in 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 to block non-specific binding. Blots were then incubated overnight at 4 °C with a 1:4000 dilution of the ER-β antibody raised against the ligand-binding domain of the hER-β receptor (obtained from J.-A. Gustafsson’s Laboratory, Karolinska Institute, Huddinge, Sweden). The membranes were washed and incubated with a secondary antibody linked to horseradish peroxidase-labeled anti-rabbit IgG (Amersham Pharmacia Biotech) for 1 h. After extensive washing, blots were developed using ECL (Amersham Pharmacia Biotech) for 1 min and exposed to X-Omat AR film (Eastman Kodak Co.).

Equivalences of loading and transfer were checked by staining with Ponceau S and by re-probing with β- actin antiserum. The density of bands was quantified with ChemiImages 5500 (Alpha Innotech Corp.). The ratio between the densities of ER-β bands and β-actin was used to analyze the results. The results were expressed as fold difference compared to vehicle control rats. ER-β expression levels in the groups were then compared using two-way ANOVA.

Results

Physical and reproductive development of the E2-treated rats

We have earlier studied the effect of prepubertal genistein exposure on these end-points (6), and therefore did not investigate them here. Daily exposure to E2 between post-natal days 7 and 20 did not affect body weight gain during the treatment (mean ± SEM on post-natal day 21: vehicle, 59.2 ± 0.6 g; E2, 58.6 ± 0.9 g) or later (during week 8: vehicle, 177 ± 1 g; E2, 173 ± 1 g).

Vaginal opening occurred on day 33.1 ± 0.4 in the vehicle controls. All the E2-exposed rats, however, had vaginal opening on post-natal day 20. Thus, an indicator of puberty onset occurred significantly earlier in the E2-exposed rats than in the control vehicle rats ($t = 34.8, \text{df} = 58, P < 0.0001$).

Uterine wet weights, both unadjusted and adjusted for body weight, were similar in the two groups (data not shown). Variability in uterine wall thickness indicated that rats in the E2 and vehicle groups exhibited all stages of estrus cycling and did not stay in constant estrus, which is seen to happen in rats exposed to a higher level of E2 during the first 30 days of life (41). Further, serum E2 levels during week 8 were not significantly different between the rats exposed to vehicle (67.4 ± 7.4 pg/ml; $n = 4$) or E2 (74.4 ± 7.4 pg/ml; $n = 4$) during the prepubertal period.

Mammary tumorogenesis after E2 treatment

The incidence of mammary tumors was determined weekly. The first tumors in the vehicle group appeared on week 6 and in the E2-treated group on week 8. However, no significant differences in the mean latency to tumor appearance between the two groups were seen (vehicle, 9.5 ± 0.7 weeks; E2-exposed, 9.8 ± 1.2 weeks). At the end of the study, i.e. on week 17 following DMBA administration, the percentage of rats with mammary tumors was 68% (15/22) in the vehicle-treated group and 15% (3/20) in the group exposed to E2 ($\chi^2 = 10.0, \text{df} = 1, P < 0.0015$). Results of survival analysis indicated that the two groups differed significantly with regard to incidence of tumor presentation ($\chi^2 = 12.22, \text{df} = 1, P < 0.0005$) (Figure 1). Tumor multiplicity was not different between the vehicle and E2 groups (1.6 ± 0.2 tumors/tumor-bearing rat for the vehicle and 1.3 ± 0.3 for the E2 group).

Mammary whole mounts

Differences in mammary gland morphology were assessed at the ages of 3, 8 and 16 weeks. The numbers of TEBs and LAUs and mammary gland epithelial density were not affected by an estrogenic treatment at the age of 3 weeks (Figure 2A–C). At week 8, however, rats exposed to E2 and genistein at prepuberty had significantly less TEBs than the vehicle controls ($F[2,20] = 5.6, P = 0.012$). Density of LAUs was increased ($F[2,23] = 6.73, P = 0.005$) and density of the mammary epithelium decreased in the glands exposed prepubertally to genistein ($F[2,23] = 4.10, P < 0.030$).

Mammary gland morphology at 16 weeks was assessed only in the E2 and vehicle-exposed rats (data not shown). The results indicated that the prepubertally E2 exposed rats had more LAUs at this age than the controls ($F[1,10] = 10.94, P = 0.008$), but no differences in mammary epithelial density were seen.

BRCA1 expression in the mammary glands

Figure 3A shows the expression levels of BRCA1 mRNA in different rat tissues as determined by RPA. As previously reported, the highest levels were found in the testis and uterus (25).

The results showed that the levels of BRCA1 mRNA were significantly elevated at all ages in the glands of rats exposed prepubertally to E2 or genistein when compared with vehicle controls ($F[2,36] = 5.91, P = 0.006$) (Figure 3B–D).

ER-α and ER-β protein expression

Immunohistochemistry. ER-α and ER-β proteins were expressed predominantly in epithelial cells (Figure 4). Some cytoplasmic staining was present, as previously reported (42), but its significance remains unclear and therefore only positive nuclear immunostaining was scored.

The number of cells that stained positively for ER-α protein was lower in the glands of rats that were exposed to E2 during prepuberty than in the vehicle controls ($F[1,40] = 5.19, P < 0.028$) (Figure 5A), both in lobules and ducts (Figure 5B).

Prepubertal genistein exposure, however, induced a long-lasting up-regulation of ER-α expression (lobules, $F[2,19] = 9.65, P = 0.001$; ducts, $F[2,18] = 8.59, P = 0.002$) (Figure 5B).
ERβ staining was seen in almost all epithelial cells and the intensity of stain was very homogeneous. To confirm these results we performed western blot assays. No protein from prepubertally genistein-exposed groups was available for this assay.

Western blot. Expression of ERβ protein was determined with a polyclonal antibody obtained from Dr Gustafsson’s laboratory, which was raised in rabbits using a peptide within the ligand-binding domain of hERβ. This antibody recognizes rat, human and mouse ERβ and does not show any cross-reactivity with ERα. We verified the specificity of the ERβ antibody by using recombinant ERα and ERβ antibodies and blocking peptides.

We found that ERβ protein from tissues obtained from different organs migrated at ~62 kDa under our western blotting conditions (data not shown), which is consistent with the size predicted from the full sequence of the receptor (long form).

The results showed that mammary ERβ levels in the rats that were treated with E2 during prepuberty were increased 2-fold during both weeks 8 and 16 (P < 0.05) (Figure 6). No differences between the E2 and vehicle controls were noted during week 3.

Discussion
Prepubertal exposure to E2 reduced the susceptibility to develop carcinogen-induced mammary tumors. These findings extend the previous observations that show a reduction in carcinogen-induced mammary tumorigenesis in rats following exposure to E2, either during the first month of life (41), after puberty onset (43) or during adult life, at doses that mimic the levels seen during pregnancy (43–45). If these findings apply to humans, however, it is unlikely that women would find exposure of young girls to E2 or other hormones an acceptable way to prevent breast cancer. A more attractive way may be to use dietary estrogenic factors, such as soy, which contains high...
levels of genistein. In both human and animal studies high soy or genistein intake prior to puberty onset reduces breast cancer risk (5–7,9).

Estrogens induce cell proliferation that in turn increases the likelihood that DNA damage could occur. However, estrogens also stimulate the expression of genes that can repair the damage, including BRCA1 (23–28). Our present results provide evidence in support of a protective role of BRCA1 in the mammary gland that has been exposed to estrogenic compounds before puberty (46). BRCA1 mRNA was significantly higher in the mammary glands of prepubertally E2- and genistein-exposed rats than in their vehicle controls. The increase was seen not only immediately after the exposures, but also several weeks after the treatments, suggesting that a long-lasting up-regulation of BRCA1 may be involved in explaining the protective effect of prepubertal exposures to estrogens.

Immunohistochemical analysis revealed that the glands of the E2-exposed rats contained significantly less ER-α-positive cells than the glands of the control rats, and this reduction persisted for at least 4 months after the cessation of E2 exposure. Other studies have assessed ER-α levels at the time of estrogen exposure, mostly in vitro using human breast cancer cells (47), showing decreased expression of ER-α in response to treatment with E2. Interestingly, human data obtained through breast surgery show that ER-α levels in normal breast tissue are higher in women diagnosed with breast cancer than in women with benign lesions (48). Further, ER-α levels are significantly lower among Asian women exhibiting low breast cancer risk than among Caucasians exhibiting high breast cancer risk (49). Our data thus support the idea that reduced levels of ER-α in the mammary gland predict low breast cancer risk.

Fig. 4. Immunodetection of ER-α and ER-β protein in the rat mammary gland (A and B, respectively). Specific staining was abolished when primary antibodies were preabsorbed with ER-α (C) or ER-β (D) blocking peptides. No staining was detected when slides were incubated with normal rat IgG (E).
Prepubertal genistein exposure, however, had an opposite effect on mammary ER-α expression than E2 exposure, although both reduce mammary tumorigenesis. Consistent with our data, Jefferson et al. (50) found that neonatal genistein exposure induced ER-α expression in the mouse ovary. A study by Cotroneo et al. (8), in contrast, reported a reduction in ER-α expression, as determined by staining intensity in the immunohistochemical assay in prepubertal mammary glands of rats exposed to genistein. The difference between our results and the results of Cotroneo et al. might reflect an ~200-fold higher genistein dose used in the latter study and the fact that ER-α was determined at the time of genistein exposure and not any later time.

The results of our study suggest that the expression of ER-β was increased at the time the mammary gland is most susceptible to DMBA, i.e. at 8 weeks, and that the increase lasted to the age of 16 weeks. Thus, an increase in ER-β protein levels in the mammary gland of rats exposed prepubertally to E2 may be associated with a reduction in breast cancer risk. This conclusion is also in agreement with a finding in the rat prostate showing that reduced expression of ER-β correlated with increased prostate cancer risk (51). Due to technical difficulties linked to reliability of the ER-β antibody, we could not determine whether prepubertal genistein exposure may have affected mammary ER-β levels. An earlier study found no changes in ovarian ER-β expression in mice treated with genistein neonatally (50). Nevertheless, at this point a possible role of ER-β in mediating the protective effects of prepubertal E2 or genistein exposures on mammary tumorigenesis cannot be ruled out.

Prepubertal exposures to E2 or genistein altered mammary gland development in a manner that could also be associated with a reduction in mammary tumorigenesis. These exposures increased elimination of the structures (TEBs) that are known to give rise to malignant transformation (16). We also found that the mammary glands of rats that had been treated with E2 or genistein prepubertally contained more differentiated LAUs than the glands of control rats. The differentiated mammary gland exhibits low or no susceptibility to carcinogen-induced malignancies (16), which is in accordance with the findings reported here. It remains to be seen whether the changes in the expression of BRCA1 and ER-α (and possibly ER-β) noted in the mammary glands of rats exposed to E2 or genistein during prepuberty are causally linked to the changes in the mammary gland morphology and reduced breast cancer risk.

In summary, we show that a prepubertal treatment with E2 reduces susceptibility to malignant transformation in the rat mammary gland, indicating that the estrogenicity of genistein may explain why this phytoestrogen reduces breast cancer risk when exposed during prepuberty. We also show that the reduction in mammary tumor incidence, induced by prepubertal exposure.

Fig. 5. Quantitative ER-α protein expression in mammary glands of (A) 3-, 8-, 16- and 22-week-old rats exposed to either E2 or vehicle, or (B) 8-week-old rats exposed to E2, genistein or vehicle before puberty onset, as determined by immunohistochemistry. Cell nuclei positive for ER-α was expressed as a percentage of 200 epithelial cell nuclei counted. Data were normalized against values of the control group (set to 1). Results are mean ± SEM of 6 rats per group and age. *P < 0.05, compared with controls.

Fig. 6. (A) Detection of ER-β in rat tissues by western blot. Similar amounts of homogenized tissue lysates were electrophoresed in 8% Tris–glycine gels and immunoblotted with anti-ERβ ligand-binding domain antibody (1:4000). (B) Quantitative ER-β protein expression levels in the mammary glands of 3-, 8- and 16-week-old rats after a prepubertal treatment with either E2 or vehicle, as indicated by western blot. Densitometric values of western blotting were standardized against values of β-actin, and expressed as a percentage of the control values (set to 100%). *P < 0.05, compared with controls.
exposure to either E2 or genistein, is associated with increased BRCAl expression in the mammary gland, suggesting that this tumor suppressor may play a role in mediating the protective effects of prepubertal estrogenic exposures on the breast.

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References


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