Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats

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Genistein, a component of soy, was administered to prepubertal female Sprague–Dawley CD rats and investigated for chemoprevention against mammary cancer. Genistein, at 500 μg/g body wt or an equivalent volume of the vehicle, dimethylsulfoxide (DMSO), was injected (s.c.) on days 16, 18 and 20 post-partum. At day 50 post-partum all animals were exposed to 80 μg dimethylbenz[a]anthracene (DMBA) per g body wt. Animals treated prepubertally with genistein as compared to DMSO had reduced incidence and significantly fewer adenocarcinomas per animal. Mammary whole mount analysis showed that prepubertal genistein treatment resulted in mammary glands of 50-day-old rats developing fewer terminal end buds and more lobules II. Cell proliferation studies with bromodeoxyuridine (BrdU) showed that terminal end buds from mammary glands of 50-day-old females treated prepubertally with genistein had significantly fewer cells in S-phase of the cell cycle. Serum genistein concentrations in 21- and 50-day-old females following prepubertal genistein treatment were 4.2 ± 0.6 μM and 102 ± 30 nM, respectively. Animals treated prepubertally with genistein had significantly fewer cells in S-phase of the cell cycle. Genistein, in soybeans and soy products as compared to DMSO had reduced incidence and significantly fewer adenocarcinomas per animal.

Introduction

During 1994 it was expected that almost 182 000 new cases of breast cancer would be diagnosed in the US and that the disease would kill 46 000 American women. This accounted for a death rate of 22.4/100 000, which was almost five times higher than the rate found in China (1). It has been suggested that in the developed countries of the Western hemisphere this discrepancy could possibly be due to a high level of exposure to chemicals in the environment or workplace (2,3). Another possible correlation is the difference in diet between the West and Orient. Epidemiologic studies have demonstrated a relationship between a diet high in soy-foods and a low incidence of breast cancer (4,5). A traditional diet consumed by an oriental female is high in soy products. One of the components of soy is genistein, a phytoestrogen. It had been hypothesized that genistein is the active component of soy that is providing this protective/chemopreventive effect (6–8) and we were the first to directly verify this hypothesis (9,10).

Genistein (4',5,7-trihydroxyisoflavone), an isoflavonoid, is found naturally in soy as the β-glucoside, genistin, and other more complex glycosidic conjugates (11). Microflora in the intestine are able to hydrolyze the glucoside to genistein. Isoflavonoids occur mainly in soybeans and soy products (tofu, soy-flour, milk, tempeh, etc). Genistein is a diphenolic planar molecule with an aromatic A-ring, a second oxygen atom 11.5 Å from the one in the A-ring, and has a molecular weight similar to that of the steroidal estrogens. It has been shown to compete with 17β-estradiol in receptor binding assays (12,13) and to have estrogenic properties in cell culture and uterine weight assays (14–16). Genistein can also inhibit the estrogenic effects of estrone, estradiol, and diethylstilbestrol, i.e. it is a partial estrogen antagonist (13,16,17). It has been shown to inhibit topoisomerase II (18), platelet-activating factor/EGF-induced expression of c-fos (19), diacylglycerol synthesis (20), and tyrosine kinases (21). Additionally, genistein has been shown to have antioxidant and antipromotional activities (22). Genistein has been shown to induce gland differentiation (23–27) and it is an inhibitor of angiogenesis (28). It was recently demonstrated that an immunonjugate composed of genistein, linked to an antibody specific for the B-cell leukemia ED19 receptor, was >99% effective at eliminating leukemia cells in an in vivo system (29).

In 1990, Barnes et al. (6) demonstrated that rats, on soy-based diets from puberty onwards, developed a lower number of chemically-induced mammary tumors. More recently our laboratory has demonstrated that genistein given during the neonatal period of development protects against DMBA-induced mammary adenocarcinomas (9,10). Female Sprague–Dawley CD rats treated with genistein on days 2, 4, and 6, post-partum, developed fewer adenocarcinomas and had an increased mean-time to tumor appearance as compared to vehicle-treated controls. However, evidence of toxicity was seen in follicular development and significantly reduced circulating progesterone levels were present in these animals (10).

In order to determine whether the chemoprevention occurred as a consequence of exposure during this selected window of development only (neonatal) and because of our concern about toxic effects from exposure during this critical period, we investigated the effects of a prepubertal exposure to genistein for suppressing chemically-induced mammary tumors. In addition to investigating mammary tumorigenesis, we also examined gland development in whole mounts, cell proliferation via bromodeoxyuridine-immunohistochemistry (BrdU-IHC*), the estrus cycle, follicular development, and circulat-
ing estrogen and progesterone levels in female rats treated prepuber tally with genistein.

Materials and methods

Chemicals

Genistein was purified (95%) from a concentrate derived from soy molasses supplied to us by Protein Technologies International (St Louis, MO). Purity was determined to be >95% as analyzed by High Pressure Liquid Chromatography (HPLC). Dimethyl benzanthracene (DMBA) dimethyl sulfoxide (DMSO), sesame oil, and BrdU were purchased from Sigma Chemical Company (St Louis, MO).

Animals

Female Sprague-Dawley (CD) rats (Charles River Breeding Laboratories, Raleigh, NC) were bred in the UAB Animal Resources Facility. Dams were fed ProLab 3000 animal diet (Agway Inc., Syracuse, NY) until parturition and then transferred to AIN-76A diet (Harlan Teklad, Madison, WI). AIN-76A is a semi-purified diet containing no detectable phytoestrogen. DMBA was administered (100 mg/kg body weight) as a single i.p. pulse on day 5. Animals were placed in a climate-controlled room with a 12 h light/12 h dark cycle. Animals were weaned at birth and litter sizes were reduced so that each dam had 10 pups (4-6 females/sex). Daily weights were recorded until most of the pups were 100 g and then transferred to AIN-76A diet (Harlan Teklad, Madison, WI). AIN-76A is a climate-controlled diet with a 12 h light/12 h dark cycle. Animals were kept in a climate-controlled room with a 12 h light/12 h dark cycle. Animals were placed on a video camera (Surgipath, Richmond, IL), and their positions were recorded using the Student's t-test (independent) and the Weibull distribution was selected as the most appropriate model for the number of tumors per animal. The ovaries were evaluated and scored for the number of follicles present in the ovaries of each animal. Data from the tumorigenesis model was analyzed using the mathematical model proposed by Kokeska et al. (39). Standard analysis was conducted using the Wilcoxon Rank Sum test and the Fisher Exact test. The null hypothesis was evaluated and scored for the number of follicles present in each of the following stages of development: primordial, normal growing-normal, growing-atretic, atretic-normal, atretic-atretic, and corpora lutea (37, 38).

Results

Tumorigenesis study

Female rats treated prepuber tally with genistein developed almost 50% fewer DMBA-induced tumors as compared to
animals treated prepubertally with vehicle, 3.93 ± 0.69 tumors/animal versus 7.36 ± 0.95 tumors/animal \( (P < 0.01) \), respectively (Figure 1). No significant difference in mean time to tumor development was observed between the two groups. Animals treated with genistein and DMBA showed an 85% incidence of tumors while animals treated with vehicle and DMBA developed a 92% incidence of tumors.

Sixty-one percent of the mammary tumors were located in the thoracic region while 30% and 9% were located in the abdominal and inguinal regions, respectively. All tumors of 1 cm diameter or greater were prepared for histopathological evaluation. Ninety-three percent of the mammary tumors from the DMSO- and genistein-treated rats were found to be adenocarcinomas. The remaining tumors were fibroadenomas. Of the adenocarcinomas, 29% consisted of cells forming tubular or ductal structures, 7% consisted of cells arranged as alveolar structures, and the others were variable combinations of cells forming tubules, ductules and alveolar structures. In reference to tumor invasiveness, 82% were infiltrative, 3% in situ, and 7% not definitive. The majority (84%) of the tumors were found to be moderately differentiated, while 3% were well differentiated and 13% were poorly differentiated. There were no statistical differences between groups on tumor location, tumor classification, structure origin, and degree of invasiveness or differentiation.

**Body and uterine weights and mammary gland size**

There was no significant effect on body weights between the genistein-treated and vehicle-treated groups at all ages (Figure 2). At 22 days of age genistein-treated females had significantly larger uterine wet-weights and mammary gland sizes (Table I). At 33- and 50-days post-partum, uterine wet-weights and mammary gland sizes of genistein-treated females were not significantly different from those of controls.

**Mammary gland differentiation**

The predominant terminal ductal structure of the vehicle-treated virgin Sprague–Dawley CD rat was the terminal end bud (55%, 77% and 33% in 22-, 33- and 50-day-old animals, respectively; Table II). In 22-day-old females there was also a high percentage of terminal ducts (44%) and very few lobules. The terminal end buds and terminal ducts accounted for 99% of the terminal ductal structures in the periphery of the 22-day-old female abdominal gland. In 33-day-old animals we observed more terminal end buds (77%) and fewer terminal ducts (8%) and an increase in lobules, especially lobules I (14%). Nevertheless, the undifferentiated structures (terminal end buds and terminal ducts) still comprised 85% of the total terminal ductal structures. At day 50 post-partum, the terminal end buds, terminal ducts, lobules I and lobules II comprised 33%, 25%, 26%, and 17%, respectively, of the terminal ductal structures in the periphery of the abdominal mammary gland. By day 50 post-partum undifferentiated structures represented only 58% of the peripheral terminal ductal structures.

Prepubertal genistein treatment resulted in a slightly increased percentage of terminal end buds and a significantly decreased percentage of terminal ducts in 22-day-old females (Table II). Furthermore, it was observed that mammary terminal end buds of 22-day-old genistein-treated animals were significantly larger \( (P < 0.05) \) than those of vehicle-treated animals.
Table II. Mammary terminal ductal structures in female rats treated prepubertally with genistein or vehicle

<table>
<thead>
<tr>
<th>Age-treatment (no/group)</th>
<th>Percentage of terminal ductal structures</th>
<th>No. of cells in S-phase in terminal ductal structures of mammary glands of rats treated prepubertally with genistein</th>
</tr>
</thead>
</table>
|                         | Terminal ducts | Lobules I | Lobules II | S
| 22 days DMSO (8)        | 55 ± 7        | 44 ± 10   | 1 ± 0      | 0
| 22 days Genistein (8)   | 70 ± 4        | 29 ± 7    | 1 ± 1      | 0
| 33 days DMSO (8)        | 77 ± 7        | 82 ± 8    | 14 ± 4     | 1 ± 1
| 33 days Genistein (8)   | 72 ± 6        | 80 ± 3    | 10 ± 5     | 1 ± 1
| 50 days DMSO (7)        | 15 ± 3        | 25 ± 2    | 26 ± 1     | 17 ± 1
| 50 days Genistein (8)   | 18 ± 2        | 21 ± 2    | 26 ± 3     | 34 ± 3

Terminal ductal structures were evaluated using the criteria proposed by Russo et al. (132-134). The outer ridge of the mammary gland of 25 mm long and 22 day-old animals. 278 mm for 33 and 50 day-old animals was evaluated using a light microscope and video camera. Values represent mean ± SEM as compared to age-matched DMSO-treated animals.

Discussion

Chemoprevention

We have demonstrated that genistein administered subcutaneously on days 16, 18 and 20 post-partum suppressed the development of DMBA-induced mammary tumors. Female rats treated with genistein developed almost 50% fewer tumors as compared to control animals. There was no significant difference in the latency period between genistein-treated and non-treated animals, but there was a significant effect on the multiplicity (almost 2:1). The location of the DMBA-induced mammary tumors (61%, 30%, and 9% in the thoracic, abdominal, and inguinal regions, respectively) is consistent with our recent report (10) and those of others (32,33,42). As in our previous work (10), we found that the identical DMBA treatment resulted in >90% of the mammary tumors being adenocarcinomas. Prepubertal genistein treatment inhibited to the same extent the degree of invasiveness or differentiation of these DMBA-induced adenocarcinomas. Historical data from our laboratory revealed that female Sprague–Dawley rats not treated with DMBA did not typically develop adenocarcinomas.

Prepubertal genistein treatment did not significantly alter body weights, but it did result in significant increases in the genistein-treated animals had vaginal openings by 27 days post-partum as compared to 37 days post-partum for the vehicle-treated animals. Animals treated prepubertally with genistein as compared to vehicle spent more time in the estrus phase of the estrus cycle (36% versus 23%, respectively) (Table IV). Nevertheless, all animals cycled. In 50-day-old female rats treated prepubertally with genistein, circulating progesterone and estradiol-17β concentrations were determined to be slightly, but not significantly lower (Table V).

Evaluation of the ovaries for genistein toxicity did not reveal any alteration in numbers of oocytes/follicles, atretic follicles and corpora lutea (Table VI).

Total genistein (aglucones and conjugates) concentrations in sera from 21- and 50-day-old female rats treated on days 16, 18 and 20 post-partum were 4.2 ± 0.6 µM and 102 ± 30 nM, respectively. No genistein was detected in the sera from animals not injected with genistein.

50-day-old genestein-treated females, there was a significant increase in number and percentage of terminal end buds. Also, at day 50, prepubertal genistein treatment resulted in a significant increase in lobules II.

Cell proliferation

Cell proliferation was evaluated using BrdU-IHC. Taking into consideration the total proliferative compartment (number of cells in S-phase per terminal ductal structure multiplied by the number of terminal ductal structures per gland), it was calculated that the terminal ducts of 22-day-old genistein-treated animals had 42% fewer total cells in S-phase (Table III). The proliferative compartment of terminal end buds in 22-day-old female rats were not significantly different. At 33 days of age there were no significant differences in all terminal ductal structures. However, the terminal end buds of 50-day-old genistein-treated female rats had significantly fewer total cells (46% less) in S-phase and more cells in S-phase in lobules II of genistein-treated animals than in vehicle-treated animals.

Endocrine studies

Animals treated prepubertally with genistein reached sexual maturity earlier than control animals. One hundred percent of
Treatment (no./group) Numbers of follicular structures

<table>
<thead>
<tr>
<th>Treatment (no./group)</th>
<th>Primordial normal</th>
<th>Growing normal</th>
<th>Growing atretic</th>
<th>Antral normal</th>
<th>Antral atretic</th>
<th>Corpora lutea</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO (8)</td>
<td>155±19</td>
<td>108±8</td>
<td>14±2</td>
<td>48±6</td>
<td>31±5</td>
<td>42±4</td>
</tr>
<tr>
<td>Genistein (8)</td>
<td>137±21</td>
<td>93±11</td>
<td>13±1</td>
<td>46±6</td>
<td>31±4</td>
<td>38±4</td>
</tr>
</tbody>
</table>

Female Sprague-Dawley CD rats were treated prepubertally with genistein or DMSO. Ovaries from 50-day-old females were prepared for histopathological evaluation. Values represent the mean ± SEM.

mammary gland size and uterine weights in 22-day-old animals. Hence, acute exposure to genistein appears to have caused estrogen-like proliferative actions (23,43). The high circulating genistein concentration (4.2 μM) 24 h after the last of three injections points to the potential of the genistein dose to stimulate cellular differentiation, mammary gland growth and uterine weight. At 33- and 50-days of age, however, the effects on gland size and uterine weight were no longer significantly manifested, presumably due to the diminution of genistein concentrations over time (10,23). The circulating genistein concentration in 50-day-old female rats injected subcutaneously with 500 μg genistein/g body wt on days 16, 18 and 20 post-partum was 102 nanomolar (27.5 ng/ml). Considering that genistein is ~1/10 000 to 1/10 000 as potent an estrogen as estradiol-17β (12-17), 102 nanomolar of genistein would translate to ~3–30 pg/ml ‘equivalent estrogen’ concentration. Radioimmunoassay of estradiol-17β concentrations from serum of 50-day-old control females (Table V) was 43.6 pg/ml, i.e. the genistein ‘estrogenicity’ would be ~6–60% of the estradiol concentration. It is therefore plausible that prior to, and perhaps at day 50 post-partum, the circulating genistein would be able to modulate physiological functions. This is to be contrasted to the neonatal genistein treatment where no genistein was detected in the serum of 50-day-old female rats (10). Nevertheless, with both protocols, circulating genistein concentrations are diminished with time and would probably not be a direct effector as the animals age and the chemoprevention persists. The prolonged presence of genistein in sera is probably a result of the subcutaneous route of administration since genistein is rapidly cleared from the blood when administered intra-venously.

Mammary gland differentiation and cell proliferation

The prepubertal genistein treatment did have an immediate and significant proliferative effect on the gland size of prepubertal female rats. We also observed that there were slight, but not significant, increases in numbers of terminal end buds. Terminal end buds were larger in genistein-treated as compared to vehicle-treated animals. Coupling this with the significant decrease in numbers of terminal ducts in the 22-day-old genistein-treated animals, we interpret this to mean that the undifferentiated terminal ductal structures are preparing to progress to the more differentiated lobules. This supposition is supported by the greater percent decrease in terminal end buds and terminal ducts and the corresponding increase in lobules-I and -II from days 22 to 50 post-partum in the genistein-treated animals. By day 33 and even more by day 50 post-partum, there were significant decreases in numbers of terminal end buds. Very importantly, the 50-day-old prepubertal genistein-treated females had a significant increase in lobules II. At 50 days of age the ratio of lobules II to terminal end buds was 0.52 for DMSO-treated animals and 1.87 for genistein-treated animals, a 3.6-fold difference. We interpret these results to mean that shortly after exposure to genistein there was rapid development of the mammary gland, yielding more differentiated terminal ductal structures (lobules II) and hence, fewer undifferentiated structures (terminal end buds).

Histopathological evaluation of the ovaries, including number and morphology of corpora lutea revealed no significant difference between the treated and non-treated females. This is consistent with the circulating levels (i.e. a slight, but not significant decrease) of progesterone and estradiol 17-β concentrations. This is to be contrasted to our earlier report of neonatal genistein exposure resulting in increased antral atretic follicles and significantly decreased corpora lutea and serum progesterone levels (10).

Summary

Using genistein, an isoflavonoid found primarily in soybeans, we have demonstrated a chemopreventive effect against chemically-induced mammary cancer. Our laboratory previously demonstrated that genistein administered during the neonatal period of development (days 2, 4, and 6 post-partum) provided a chemopreventive effect against DMBA-induced
mammary adenocarcinomas (9,10). Animals treated neonatally with genistein developed fewer tumors, exhibited a longer latency period, and had a lower number of these chemically-induced mammary adenocarcinomas. However, these animals had very few functional corpora lutea and significantly reduced sera progesterone concentrations, i.e. the endocrine system of these animals was compromised. Our current study has demonstrated a chemopreventive effect resulting from genistein administered during a less vulnerable stage of development, the prepubertal period (days 16, 18 and 20 post-partum). Equally important, with this treatment we found no permanent adverse effects to the uterine weights, mammary gland size, circulating levels of estradiol-17β and progesterone, estrus cycle, or follicular development in 50-day-old genistein-treated females. There remains a need to investigate the effects of prepuberal genistein on reproduction.

The ability of genistein to enhance gland differentiation closely parallels that of gland maturation in the human female. The breast of the premenarchal female contains many undifferentiated terminal ductal structures (45). These eventually progress to more differentiated lobules during pregnancy. Women who experience a full-time pregnancy early in life have a 2-fold less likelihood of developing cancer than women who never become pregnant (52). Likewise, early exposure of rats to estrogens renders protection against mammary cancer (53,54). As evidenced by our results, genistein can accomplish this in the rat-DMBA model, a process that may be on-going in human females on a traditional oriental diet high in soy. The advantage of genistein over other estrogens may reside in its low potential for toxicity.

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References


Prepubertal genistein and mammary cancer suppression


