Carcinogenesis vol.17 no.7 pp.1451–1457, 1996

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

W.Britton Murrill, Nadine M.Brown, Jun-Xuan Zhang, Patricia A.Manzolillo, Stephen Barnes, and Coral A.Lamartiniere[1]

Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL 35294–0019, USA

[1]To whom correspondence should be addressed

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 as compared to vehicle spent more time in the estrus phase of the estrus cycle, although all animals did cycle. In 50-day-old females, circulating estradiol-17β and progesterone concentrations were not significantly altered by the prepubertal genistein treatment. Oocyte/follicle counts and numbers of atretic follicles and corpora lutea were not significantly different between the genistein- and vehicle-treated animals. We conclude that genistein treatment during the prepubertal period can suppress the development of chemically-induced mammary cancer without significant toxicity to the endocrine/reproductive system.

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 immunoconjugate 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.* (8) 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-

*Abbreviations: BrdU-IHC, bromodeoxyuridine-immunohistochemistry; HPLC, high pressure liquid chromatography; DMBA, dimethylbenz[*a*]-anthracene; DMSO, dimethylsulfoxide.

ing estrogen and progesterone levels in female rats treated prepubertally with genistein.

Materials and methods

Chemicals

Genistein was purified (30) from a concentrate derived from soy molasses supplied to us by Protein Technologies International (St Louis, MO). Purity was determined to be >98% as analyzed by High Pressure Liquid Chromatography (HPLC). Dimethylbenz[*a*]anthracene (DMBA), dimethylsulfoxide (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 phytoestrogens (limit of detection ~10 nM). Diet and water were supplied *ad libitum*. Animals were kept in a climate-controlled room with a 12 h light/12 h dark cycle. Animals were sexed at birth and litters were reduced so that each dam had 10 pups (4–6 females/dam). On days 16, 18, and 20 post-partum one-half of the females from each litter received 500 µg genistein/g body wt via s.c. injections. This was a genistein dose approximately equivalent to the one used in our neonatal chemoprevention protocol i.e. 5 mg genistein/rat on days 2, 4 and 6 post-partum (9,10). A 4 day old rat weighed ~10 g. The other females received an equivalent volume of the vehicle, DMSO, only. Animals were weaned at day 22 post-partum.

Tumorigenesis

For the tumorigenesis experiments 50-day-old female rats (27 genistein-treated and 25 vehicle-treated females) received 80 µg DMBA (in sesame oil)/g body wt, via oral gavage. All animals were palpated twice weekly for mammary tumors until they were 200-days old, until the tumors reached 3.0 cm in diameter, or until they were moribund. The location, relative size, and date of appearance of each tumor were charted for each animal. Representative sections of each tumor were placed in 10% neutral buffered formalin. Tissues for microscopic examination were trimmed, embedded in paraffin, cut into sections 5 µm thick, and stained with H&E. Coded slides were evaluated histopathologically by Dr Roger Thompson, a board certified veterinary pathologist, as to tumor type, tissue of origin, differentiation, and degree of invasiveness.

Gland differentiation

Using the abdominal mammary gland (gland pair number 4) (31), mammary whole mounts were prepared. Mammary glands were removed at the time of sacrifice, spread on a microscope slide, and then placed in neutral buffered formalin for 8 h (22-day old animals) or overnight (33- and 50-day-old animals). Glands were de-fatted in acetone for 4 h (22-day old animals) or overnight (33- and 50-day-old animals), placed in 70% alcohol (30 min), hydrated in water (15 min), and stained with alum carmine (8 h for 22-day old animals or overnight for 33- and 50-day-old animals). After staining, glands were run through a series of graded alcohols (35–100% ethanol) and placed in xylene, which 'clears' the tissue. Glands were then compressed between two glass slides for 24 h, released and allowed to expand for at least 8 h, then mounted using a glass cover slip and Permount (Fisher Scientific, Atlanta, GA).

Coded whole mounts were evaluated via light microscopy using the criteria established by Russo *et al.* (32–34). The outer fringe of the mammary gland was evaluated (1.45 mm inward for 22-day old animals and 2.78 mm inward for 33- and 50-day-old animals). This represents the location of most of the actively growing and carcinogen-susceptible terminal ductal structures in the gland (32–34) at these ages. Whole mounts were evaluated for the number of terminal end buds, terminal ducts, and lobules type I and II. A terminal end bud was designated as a club-shaped terminal ductal structure >100 µm in diameter which had 3–6 epithelial cell layers in the periphery of the bud. A terminal duct had a diameter <100 µm and had 1–3 epithelial cell layers between the ductal lumen and outside edge of the structure. Lobules type I were comprised of 5–10 alveolar buds and lobules type II had 11–20 alveolar buds.

Mammary gland size was determined from whole mounts using an image analysis system linked to a video camera and a 486 computer. The carmine-stained mammary glands were projected to a video screen and prints were made using a Seikosha video printer. The parameter of the gland was outlined on the video print, then the perimeter was traced by a sonic digitizer system (Graf Bar, Science Accessories Corporation, Southport, CT). The instrument records multiple x and y coordinates each second during a tracing, and these coordinates are used in an in-house developed computer program to determine the area in mm^2. The system was calibrated with a micrometer photographed with the glands.

Cell proliferation

Cell proliferation was studied in the contralateral abdominal mammary gland using BrdU. Animals were injected with a single i.p. pulse of BrdU (100 µg BrdU/g body wt dissolved in DMSO), 2 h prior to sacrifice. The contralateral abdominal gland was removed and placed in 10% neutral buffered formalin for 24 h. The tissue was embedded in paraffin, sliced into 5 µm sections, and placed on SuperfrostPlus (Fisher) microscope slides. Tissue sections were deparaffinized in xylene and re-hydrated using a series of graded alcohols (100–70%). The tissue sections were immersed in 3.5 N HCl for 15 min and then digested for 4 min using 0.01% trypsin. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Slides were incubated 20 min with 10% horse serum to block non-specific binding of the antibody, followed by a 30 min incubation (37°C) with BrdU primary antibody (Dako, Carpenteria, CA), a 20 min incubation (room temperature) with biotinylated horse anti-mouse secondary antibody (Signet, Dedham, MA), and a 20 min incubation (room temperature) with an Ultra Streptavidin detection system (Signet). Color was then developed with the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma) and the tissue was counter-stained with Gills no 2 hematoxylin (Sigma). Cell nuclei staining brown with the DAB were scored as being in S-phase, while those staining blue with hematoxylin were scored as not in the S-phase. Tissue sections were evaluated using a Nikon light microscope and Sony video camera and monitor.

Serum hormone and genistein concentrations

Progesterone and estradiol-17β concentrations were analyzed from sera samples collected from 50-day-old genistein- and vehicle-treated animals. Samples were assayed by Dr Larry Boots, OB/GYN Research and Diagnostics Laboratory at The University of Alabama at Birmingham. Radioimmunoassay-kits were purchased from Pantex (Santa Monica, CA). Genistein and its metabolites were analyzed from blood sera of 50-day-old animals using HPLC-MS (11).

Vaginal smears

Vaginal smears were prepared from genistein-treated and vehicle-treated animals on days 43–50 post-partum. The aspirate was collected and spread on a microscope slide, allowed to air dry, and then Papanicolaou-stained with Orange G-6 (Surgipath, Richmond, IL), EA-50 (Surgipath), and hematoxylin no 2 (Sigma) (35). Coded slides were evaluated using a light microscope. The criteria used to determine the different stages of the estrus cycle were those described by Hafez (36).

Follicular development

The ovaries from 50-day-old genistein-treated and vehicle-treated animals were removed, fixed in neutral buffered formalin, paraffin embedded, and sectioned to 5 µm (retaining every 20th section for evaluation). Slides were stained with hematoxylin and eosin and evaluated under a light microscope. The ovaries were evaluated and scored for the number of follicles present at each of the following stages of development: primordial-normal, growing-normal, growing-atretic, antral-normal, antral-atretic, and corpora lutea (37,38). The final follicle numbers determined do not represent the absolute follicle numbers but are relative to the counting procedure.

Statistical analysis

Data from the tumorigenesis study was analyzed using the mathematical model proposed by Kokoska *et al.* (39). Standard analysis was conducted using the Wilcoxon Rank Sum test and the Fisher Exact test. The Armitage test, as suggested by NCI guidelines (40), was used as an alternative perspective to the results obtained via the Kokoska approach. The analysis strategy followed for the mathematical modeling consisted of three parts. First, the distributional characteristics of the data were examined. Using the goodness-of-fit test suggested by Freedman *et al.* (41), the Poisson distribution was selected as the most appropriate model for the number of tumors per animal and the Weibull distribution was selected to describe the distribution of tumor appearance times. Second, the parameters associated with the model were estimated. The third step was to test the overall experimental effect of a change in the number of induced tumors and/or the time of tumor appearance (global test).

Cell proliferation, gland differentiation, and ovary histology data were analyzed using the Student's *t*-test (independent).

Results

Tumorigenesis study

Female rats treated prepubertally with genistein developed almost 50% fewer DMBA-induced tumors as compared to

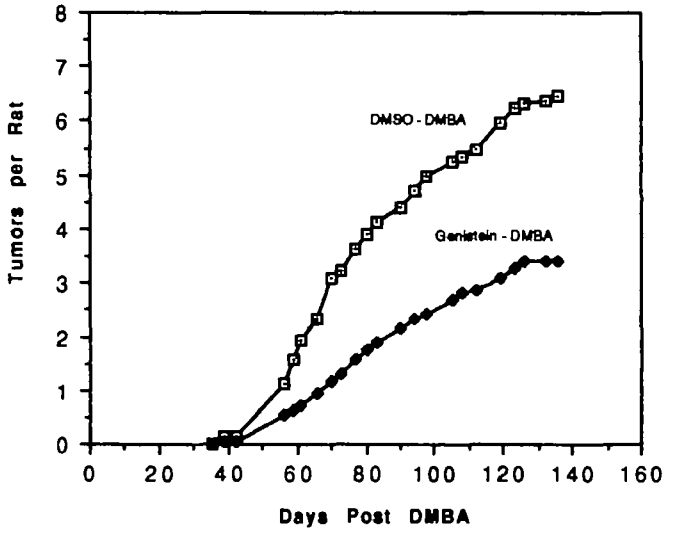

Fig. 1. Ontogeny of palpable mammary tumors in female rats treated prepubertally with genistein and DMSO, and at day 50 post-partum with DMBA. Female Sprague–Dawley CD rats were injected s.c. with 500 μg genistein/g body wt or an equivalent volume of the solvent, DMSO, on days 16, 18, and 20 post-partum. DMBA was administered by gavage at 80 μg/g body wt. The DMSO-DMBA group and the genistein-DMBA group contained 25 and 27 female rats/group, respectively.

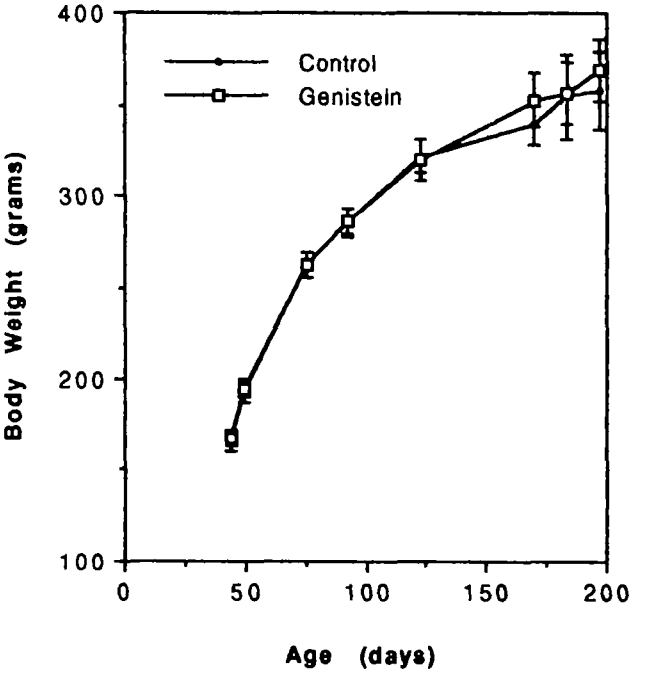

Fig. 2. Body weights of female rats treated prepubertally with genistein or DMSO and at day 50 with DMBA. Values represent the mean ± SEM for 27 genistein-treated and 25 vehicle-treated female rats.

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,

Table I. Mammary gland size and and uterine weights of female rats treated prepubertally with genistein

Age-treatment (no./group)	Gland size (mm^2)	Uterine weight (mg)
22 days-DMSO (8)	53±3	49±3
22 days-Genistein (8)	64±2[a]	91±5[b]
33 days-DMSO (8)	214±22	180±29
33 days-Genistein (8)	250±13	192±16
50 days-DMSO (7)	548±9	295±34
50 days-Genistein (8)	598±29	284±34

Gland size was determined from whole mounts using an image analysis system connected to a video camera. Values represent mean ± SEM. [a]$P < 0.05$, [b]$P < 0.001$, as compared to DMSO-treated 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

Age-treatment (no./group)	Percentage of terminal ductal structures			
	Terminal end buds	Terminal ducts	Lobules I	Lobules II
22 days DMSO (8)	55 ± 7	44 ± 4	1 ± 0	0
22 days Genistein (8)	70 ± 4	29 ± 3[a]	1 ± 1	0
33 days DMSO (8)	77 ± 8	8 ± 2	14 ± 3	1 ± 1
33 days Genistein (8)	72 ± 4	8 ± 2	19 ± 3	1 ± 1
50 days DMSO (7)	33 ± 3	25 ± 2	26 ± 1	17 ± 1
50 days Genistein (8)	18 ± 2[b]	21 ± 2	26 ± 3	34 ± 3[b]

Terminal ductal structures were evaluated using the criteria proposed by Russo *et al.* (32–34). The outer fringe of the mammary gland (1.45 mm inward for 22 day-old animals, 2.78 mm for 33 and 50 day animals) was evaluated using a light microscope and video camera. Values represent mean ± SEM. [a]$P < 0.05$ [b]$P < 0.001$ as compared to age-matched DMSO-treated animals.

Table III. Number of cells in S-phase in terminal ductal structures of mammary glands of rats treated prepubertally with genistein

Age-treatment (no./group)	No. of cells in S-phase			
	Terminal end buds	Terminal ducts	Lobules I	Lobules II
22 days DMSO (8)	1729 ± 283	1241 ± 129	*	*
22 days Genistein (8)	1972 ± 109	720 ± 84[a]	*	*
33 days DMSO (8)	1728 ± 126	66 ± 20	120 ± 25	*
33 days Genistein (8)	1458 ± 85	63 ± 8	100 ± 17	*
50 days DMSO (7)	1428 ± 110	756 ± 93	264 ± 37	44 ± 1
50 days Genistein (8)	765 ± 57[b]	901 ± 87	195 ± 11	168 ± 51[a]

Sprague–Dawley CD rats were injected s.c. with 500 μg genistein/g body wt or an equivalent volume of vehicle on days 16, 18 and 20 post-partum. Two hours prior to sacrifice, all animals were injected i.p. with 100 μg BrdU/g body wt. Proliferation was determined in three of each of the terminal ductal structures/gland. Values represent mean ± SEM of cells in S-phase/terminal ductal structures multiplied by the number of terminal ductal structures/gland. [a]$P < 0.05$, [b]$P < 0.01$ compared to age-related DMSO-treated animals. *Insufficient numbers to analyze.

(146 ± 5 μm versus 112 ± 4 μm cross-section, respectively). In 50-day-old genistein-treated females, there was a significant decrease 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 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).

Table IV. Estrus cycle in female rats treated prepubertally with genistein

Treatment (no./group)	Percentage of time spent in each phase of estrus			
	Proestrus	Estrus	Metestrus	Diestrus
DMSO (8)	22 ± 4	23 ± 3	0	55 ± 2
Genistein (8)	17 ± 4	36 ± 4	1 ± 1	46 ± 3

Female Sprague–Dawley CD rats were treated prepubertally with genistein or DMSO. Daily vaginal smears (days 43–50 post-partum) were analyzed. Values represent mean ± SEM.

Table V. Serum estradiol 17-β and progesterone concentrations in 50-day-old female rats treated prepubertally with genistein

Age-treatment (no./group)	Estradiol-17β (pg/ml)	Progesterone (ng/ml)
50 days-DMSO (8)	436 ± 112	264 ± 36
50 days-Genistein (8)	350 ± 126	197 ± 31

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.

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

TableVI. Follicular analysis in female rats treated prepubertally with genistein

Treatment (no./group)	Numbers of follicular structures					
	Primordial normal	Growing normal	Growing atretic	Antral normal	Antral atretic	Corpora lutea
DMSO (8)	155±19	108±8	14±2	48±6	31±5	42±4
Genistein (8)	137±21	93±11	13±1	46±6	31±4	38±4

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 1/1000 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 intravenously.

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).

Cell proliferation as measured by the BrdU-IHC labelling index revealed that the proliferative compartment of terminal end buds of genistein-treated animals was smaller than those from vehicle-treated animals. A decrease in total number of undifferentiated terminal end bud cells in S-phase, which are considered to be of maximal sensitivity for transformation (32,44–47), may be an explanation for a lower rate of mammary tumorigenesis.

Genistein as an endocrine modulator

Prepubertal genistein treatment resulted in precocious vaginal openings. Evaluation of the estrous cycle from days 43–50 post-partum revealed that genistein-treated females had longer cycles (5 day cycles as opposed to 4 day cycles for controls) and spent more time in the estrus phase (36%) and less time in proestrus (17%) and diestrus (46%) phases. Nevertheless the genistein-treated animals did cycle. These data on the effects of genistein on the estrus cycle in rats are comparable with those of women on a diet supplemented with soy protein and the menstrual cycle. Soy protein given daily for 1 month to premenopausal women significantly increased follicular phase length by an average of 2.5 days (48). Mitotic rate for breast tissue is almost 4-fold greater during the luteal phase than during the follicular phase (49,50). Menstrual cycle length is also longer in Asian women than in Western women (49,51) and Asian women have a lower incidence of breast cancer (7). This may be due in part to ingestion of soy containing genistein. A retrospective assessment of cycle length also revealed shorter cycle lengths for breast cancer patients compared with control subjects (51).

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 prepubertal 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.

Acknowledgements

The authors wish to thank Dr Sanford Bishop (UAB Department of Pathology) for the use of the image analysis system and Charles Kim for his excellent technical assistance Additionally, Mr Murrill would like to thank Dr John Waterbor and Dr Doug Heimburger at UAB, and the National Cancer Institute's Cancer Prevention and Control Training Program for fellowship support This research was supported by the following grants NIH-RO1-CAG1742, NIH-RO1-ES07273 and AICR 92SG12 The genistein concentrate used in the isolation and purification of genistein was donated by Protein Technologies International, St Louis, MO The mass spectrometer used in the analysis of genistein was purchased by funds from a NIH instrumentation grant (S10RR06487) and from this institution Operation of the shared facility has been supported in part by a NCI Core Research Support (grant to the UAB Comprehensive Center P03-CA13148-24)

References

1 The American Cancer Society (1994) *Cancer Facts and Figures* Atlanta, GA

2 Kuratsune,M., Ikeda,M., Nakamura,Y. and Hirohata,T. (1987) A cohort study on mortality of 'Yusho' patients: a preliminary report *Int. Symp. Princess Takamatsu Cancer Res. Fund*, **18**, 61–66

3 Manz,A., Berger,J., Dwyer,J.H., Flesch-Janys,D., Nagel,S. and Waltsgott,H. (1991) Cancer mortality among workers in a chemical plant contaminated with dioxin *Lancet*, **338**, 959–964

4 Lee,H.P., Gourley,L., Duffy,S.W., Esteve,J., Lee,J. and Day,N.E. (1991) Dietary effects on breast cancer risk in Singapore *Lancet* **337**, 1197–1200

5 Adlercreutz,H., Honjo,H., Higashi,A., Fotsis,T., Hamalainen,E., Hasegawa,T. and Okada,H. (1991) Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet *Am. J. Clin. Nutr.* **54**, 1093–1100

6 Setchell,K.D.R., Borriello,S.P., Hulme,P., Kirk,D.N. and Axelson,M. (1984) Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease *Am. J. Clin. Nutr.*, **40**, 569–578

7 Adlercreutz,H. (1990) Western diet and western diseases: some hormonal and biochemical mechanisms and associations *Scand. J. Clin. Lab. Invest.*, **50** (201), 3–23

8 Barnes,S., Grubbs,C., Setchell,K.D.R. and Carlson,J. (1990) Soybeans inhibit mammary tumors in models of breast cancer *Prog. Clin. Biol. Res.*, **347**, 239–253

9 Lamartiniere,C.A., Moore,J., Holland,M. and Barnes,S. (1995) Neonatal genistein chemoprevents mammary cancer *Proc. Soc. Exp. Biol. Med.*, **208**, 120–123

10 Lamartiniere,C.A., Moore,J.B., Brown,N.M., Thompson,R., Hardin,M.J. and Barnes,S. (1995) Genistein suppresses mammary cancer in rats *Carcinogenesis*, **16**, 2833–2840

11 Barnes,S., Kirk,M. and Coward,L. (1994) Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC-mass spectrometry *J. Agric. Food Chem.*, **42**, 2466–2474

12 Shutt,D.A. and Cox,R.I. (1972) Steroid and phytoestrogen binding to sheep uterine receptors *in vitro* *J. Endocrinol.* **52**, 299–310

13 Mathieson,R.A. and Kitts,W.D. (1980) Binding of phytoestrogen and estradiol-17β by cytoplasmic receptors in the pituitary gland and hypothalamus of the ewe *J. Endocrinol.*, **85**, 317–325

14 Cheng,E., Story,C.D., Yoder,L., Hale,W.H. and Burroughs,W. (1980) Estrogenic activity of isoflavone derivatives extracted and prepared from soybean oil meal *Science*, **118**, 164–165

15 Bickoff,E.M., Livingston,A.L., Hendrickson,A.P. and Booth,A.N. (1962) Relative potencies of several estrogen-like compounds found in forages *J. Agric. Food Chem.*, **10** (5), 410–412

16 Folman,Y. and Pope,G.S. (1966) The interaction in the immature mouse of potent estrogens with coumestrol, genistein and other utero-vaginotrophic compounds of low potency *J. Endocrinol.*, **34**, 215–225

17 Martin,P.M., Horowitz,K.B., Ryan,D.S. and McGuire,W.L. (1978) Phytoestrogen interaction with estrogen receptors in human breast cancer cells *Endocrinology*, **103**, 1860–1867

18 Okura,A., Arakawa,H., Oka,H., Yoshinari,T. and Monden,Y. (1988) Effect of genistein on topoisomerase activity and on the growth of [Val12] Ha-*ras*-transformed NIH 3T3 cells *Biochem. Biophys. Res. Commun.*, **157**, 183–189

19 Tripathi,Y.B., Lim,R.W., Fernandez-Gallardo,S., Kandala,J.C., Guntaka,R.V. and Shukla,S.D. (1992) Involvement of tyrosine kinase and protein kinase C in platelet-activating-factor-induced c-*fos* gene expression in A-431 cells *Biochem. J.*, **286**, 527–533

20 Dean,N.M., Kanemitsu,M. and Boynton,A.L. (1989) Effects of the tyrosine-kinase inhibitor genistein on DNA synthesis and phospholipid derived second messenger generation in mouse 10T1/2 fibroblasts and rat liver T51B cells *Biochem. Biophys. Res. Commun.*, **165**, 795–801

21 Akiyama,T., Ishida,J., Nakagawa,S., Ogawara,H., Watanabe,S., Itoh,N., Shibuya,M. and Fukami,Y. (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases *J. Biol. Chem.*, **262**, 5592–5595

22 Wei,H., Bowen,R., Cai,Q., Barnes,S. and Wang,Y. (1995) Antioxidant and antipromotional effects of the soybean isoflavone genistein *Proc. Soc. Exp. Biol. Med.*, **208**, 124–130.

23 Brown,N.M. and Lamartiniere,C.A. (1995) Xenoestrogens alter mammary gland differentiation and cell proliferation in the rat *Environ. Health Perspect.*, **103**, 708–713

24 Kiguchi,K., Constantinou,A.I. and Huberman,E. (1990) Genistein-induced cell differentiation and protein-linked DNA strand breakage in human melanoma cells *Cancer Commun.*, **2**, 271–277

25 Watanabe,T., Kondo,K. and Oishi,M. (1991) Induction of *in vitro* differentiation of mouse erythroleukemia cells by genistein, an inhibitor of tyrosine protein kinases *Cancer Res.*, **51**, 764–768

26 Kondo,K., Tsuneizumi,K., Watanabe,T. and Oishi,M. (1991) Induction of *in vitro* differentiation of mouse embryonal carcinoma (F9) cells by inhibitors of toposomerases *Cancer Res.*, **51**, 5398–404

27 Miller,D.R., Lee,G.M. and Maness,P.F. (1993) Increased neurite outgrowth induced by inhibition of protein tyrosine kinase activity in PC12 pheochromocytoma cells *J. Neurochem.*, **60**, 2134–2144

28 Fotsis,T., Pepper,M., Adlercreutz,H., Hase,T., Montesano,R. and Schweigerer,L. (1995) Genistein, a dietary ingested isoflavonoid, inhibits cell proliferation and *in vitro* angiogenesis *J. Nutrition*, **125** (Suppl. 3), 790S–797S

29 Uckun,F.M., Evans,W.E., Forsyth,C.J., Waddick,K.G., Ahlgren,L.T., Chelstrom,L.M., Burkhardt,A., Bolen,J. and Myers,D.E. (1995) Biotherapy of B-cell precursor leukemia by targeting genistein to CD19-associated tyrosine kinases *Science*, **267** 886–891

30 Coward,L., Barnes,N.C., Setchell,K.D.R. and Barnes,S. (1993) Genistein and daidzein, and their β-glycoside conjugates: anti-tumor isoflavones in soybean foods of the American and Asian diets *J. Agric. Food Sci.*, **41**, 1961–1967

31 Boorman,G., Eustin,S., Elwell,M., Montgomery,C. and MacKenzie,W. (1990) *Pathology of the Fischer Rat: Reference and Atlas* Academic Press, New York

32. Russo,I.H. and Russo,J. (1978) Developmental stage of the rat mammary gland as determinant of its susceptibility to 7,12-dimethylbenzanthracene. *J. Natl Cancer Inst*., **61**, 1439–1449.
33. Russo,J. and Russo,I.H. (1978) DNA labelling index and structure of the rat mammary gland as determinants of its susceptibility to carcinogenesis. *J. Natl Cancer Inst*., **61**, 1451–1459.
34. Russo,I.H., Tewari,M. and Russo,J (1988) Morphology and development of the rat mammary gland. In Jones,T C , Konishi,Y. and Mohr,U. (eds), *Integument and Mammary Gland, Monograph Series on the Pathology of Laboratory Animals*. Springer-Verlag, Berlin.
35. Galbraith,W. and Marshall,P.N. (1984) Studies on papanicolaou staining. III. Quantitative investigations of orangeophilia and cyanophilia. *Stain Technol*., **59**, 133–142.
36. Hafez,E.S.E. (1970) *Reproduction and Breeding Techniques for Laboratory Animals*. Lea and Febiger, Philadelphia.
37. Heinrichs,W.L. (1985) Current laboratory approaches for assessing female reproductive toxicity. *Reprod. Toxicol*., 95–107.
38. Plowchalk,D.R., Smith,B.J. and Mattison,D.R. (1993) Assessment of toxicity to the ovary using follicle quantitation and morphometrics. *Methods Toxicol*., **3B**, 57–68.
39. Kokoska,S.M., Hardin,J.M., Grubbs,C.J. and Hsu,C. (1993) The statistical analysis of cancer inhibition/promotion experiments. *Anticancer Res*., **13**, 1357–1365.
40. Freedman,L.S., Steele,V. and Kelloff,G. (1990) *Statistical guidelines for the analysis of data from chemoprevention experiments in rodent tumor models*. Internal Report Division of Cancer Prevention and Control, National Cancer Institute, Bethesda, M.D.
41. Freedman,L.S., Midthune,D.N., Brown,C.C., Steele,V. and Kelloff,G.J. (1993) Statistical analysis of animal cancer chemoprevention experiments. *Biometrics*, **49**, 259–268.
42. Welsch,C.W. (1985) Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins. *Cancer Res*., **45**, 3415–3443.
43. Makela,S , Davis,V.L., Tally,W.C., Korkman,J., Salo,L., Vihko,R., Santti,R. and Korach,K.S. (1994) Dietary estrogens act through estrogen receptor-mediated processes and show no antiestrogenicity in cultured breast cancer cells. *Environ. Health Perspect*., **102**, 572–578.
44. Nagasawa,H., Yanai,R. and Taniguchi,H. (1976) Importance of mammary gland DNA synthesis on carcinogen induced mammary tumorigenesis in rats. *Cancer Res*., 36, 2223–2226.
45. Russo,J. and Russo,I.H (1987) Biology of disease. Biological and molecular bases of mammary carcinogenesis. *Lab. Invest*., **57**, 112–137
46. Berenblum,I. (1954) A speculative review: the probable nature of promoting action, and its significance in the understanding of the mechanism of carcinogenesis. *Cancer Res*., **14**, 471–476.
47. Russo,J. and Russo,I.H. (1980) Influence of differentiation and cell kinetics on the susceptibility of the rat mammary gland to carcinogenesis. *Cancer Res*., **40**, 2677–2687.
48. Cassidy,A., Bingham,S. and Setchell,K.D. (1994) Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. *Am. J. Clin. Nutr*., **60**, 333–340.
49. Treloar,A.E., Boynton,R.E., Behn,B.G. and Brown,B.W. (1970) Variation of the human menstrual cycle through reproductive life. *Int. J. Fertil*., **12**, 77–126.
50. Ferguson,D.J. and Anderson,T J. (1981) Morphological evaluation of cell turn-over in relation to the menstrual cycle in the 'resting' human breast. *Br. J. Cancer*, **44**, 177–181.
51. Olsson,H., Landin-Olsson,M. and Gullberg,B. (1983) Retrospective assessment of menstrual cycle length in patients with breast cancer, in patients with benign breast disease, and in women without breast disease. *J. Natl Cancer Inst*., **70**, 17–20.
52. MacMahon,B., Cole,P., Lin,T.M. *et al*. (1970) Age at first birth and breast cancer risk. *Bull. World Health Organ*., **43**, 209–221.
53. Shellabarger,C.J. and Soo,V.A. (1973) Effects of neonatally administered sex steroids on 7,12-dimethylbenz[*a*]anthracene-induced mammary neoplasia in rats. *Cancer Res*., **33**, 1567–1569.
54. Nagasawa,H., Yanai,R., Shodono,M., Nakamura,T. and Tanabe,Y. (1974) Effect of neonatally administered estrogen or prolactin on normal and neoplastic mammary growth and serum estradiol-17β level in rats. *Cancer Res*., **34**, 2643–2646.

Received on January 24, 1996, revised on March 29, 1996; accepted on April 19, 1996