Soy Isoflavones Increase Latency of Spontaneous Mammary Tumors in Mice

Zeming Jin and Ruth S. MacDonald

Genetics Area Program and Food Science and Human Nutrition, University of Missouri, Columbia, MO 65211

ABSTRACT Soy protein, with and without isoflavones, is being added to foods by manufacturers in response to the Food and Drug Administration (FDA)-approved health claim for cardiovascular protection. Furthermore, soy isoflavones are increasingly consumed by women in the United States as an alternative to hormone replacement therapy. The role of these phytoestrogens in breast cancer is controversial. Although exposure of rodents to soy isoflavones during the perinatal period appears to reduce mammary cancer formation, exposure in utero or during adulthood may increase tumor growth. The mouse mammary tumor virus (MMTV)-neu mouse spontaneously develops mammary tumors due to overexpression of the neu oncogene occur in 20–40% of human breast cancers. We fed MMTV-neu mice AIN-93G diets containing no isoflavones, 250 mg/kg genistein, 250 mg/kg daidzein or an isoflavone mixture (NovaSoy, equivalent to 250 mg genistein/kg) from 7 wk of age. Mammary tumor latency was significantly delayed in mice fed isoflavones compared with the control. Once tumors formed, however, the isoflavones did not reduce the number or size of tumors such that at 34 wk of age there were no differences in tumor burden among the treatment groups. Hence, in the MMTV-neu mouse, soy isoflavones delayed mammary tumorigenesis. Further studies are warranted to define the cellular mechanisms through which these compounds affect mammary tumorigenesis in this model.

KEY WORDS: soy • isoflavones • genistein • daidzein • mammary cancer • mice • diet

Decreased risk of mammary cancer has been associated with factors that reduce life-long exposure to estrogen, including childbirth at young age, breastfeeding, late age of onset of menses and early age of menopause (1,2). Recent evidence suggests that lifelong consumption of plant foods containing estrogicn compounds, particularly soy isoflavones, may reduce breast cancer risk (3). From data collected in the Shanghai Breast Cancer Study, the excretion of total isoflavones was significantly lower in women with newly diagnosed breast cancer than in matched controls without cancer (4), suggesting a protective effect of diets containing isoflavones. In experimental animal models of breast cancer, the role of dietary phytoestrogens, such as genistein and daidzein from soy, is equivocal. When genistein was injected subcutaneously into newborn rat pups, the risk of developing mammary cancer after carcinogen exposure during adulthood was reduced (5,6). Rats fed genistein during the prepubertal period also had reduced carcinogen-induced mammary tumors as adults (7,8). In contrast, we reported that mice fed genistein from weaning and treated with the chemical carcinogen dimethylbenz[a]anthracene (DMBA) had more advanced cancer than mice fed no phytoestrogens (9). Hilakivi-Clarke et al. (10), found that carcinoma-induced mammary tumorigenesis in female rats was increased by genistein exposure in utero, whereas Lamariniere et al. (11) observed no effect of in utero genistein exposure on mammary tumorigenesis. Using implanted MCF-7 cells in an athymic mouse model, dietary genistein increased tumor cell growth in a dose-dependent manner (12,13). In contrast, the tumorigenic potential of mammary tumor cells implanted into nude mice was impaired if the cells were treated with genistein before implantation (14). Hence, genistein has been found to both reduce and promote mammary cancer development in different animal models. Messina and Loprinzi (15) recently reviewed the literature describing the controversies in this area.

Genistein and daidzein have been shown to suppress sister chromatid exchanges in bone marrow cells and DNA adduct formation in liver and mammary glands of mice induced by DMBA (16). However, it is not known at present whether the response of mammary cancer to phytoestrogens is dependent upon the genetic mutations associated with tumorigenesis. The mouse mammary tumor virus (MMTV)-neu/ErbB-2 transgenic mouse develops spontaneous mammary cancer with a long latency due to overexpression of the neu protooncogene (17). Overexpression of neu-ErbB-2 has been reported to

2 Funding provided by the Missouri-Illinois Biotechnology Alliance and the University of Missouri Food for the 21st Century.
3 To whom correspondence should be addressed. E-mail: macdonaldr@missouri.edu.
4 Abbreviations used: DMBA, dimethylbenz[a]anthracene; EGF, epidermal growth factor; MMTV, mouse mammary tumor virus.
occur in 20–40% of human mammary cancers (18). Therefore, this mouse model offers a unique opportunity to examine mammary cancer development in response to dietary intervention. The neu gene was first identified in rat neuroblastoma, and was found subsequently to be homologous to genes associated with erythroblastosis in chickens (ErbB) and human growth factor receptors HER (19). Hence, neu/ErbB-2 which is equivalent to HER2, is a member of the HER family of receptors, which includes the epidermal growth factor (EGF) receptors HER1, HER2, HER3 and HER4. HER2 is an important regulator of normal mammary growth and development. In this study, we fed MMTV-neu transgenic mice diets containing no phytoestrogens, genistein, daidzein or an isoflavone mixture from 7 wk of age to examine the response of mammary tumor development to soy phytoestrogens.

MATERIALS AND METHODS

Female MMTV-neu/ErbB-2 transgenic mice [n = 72; FVB/N-TgN(MMTVneu)202MUL; Jackson Laboratories, Bar Harbor, ME] were purchased at 4–5 wk of age. The mice were housed individually in plastic boxes with cob bedding in an air-conditioned room with a 12-h light-dark cycle. Room temperature was 21°C with 50% humidity and tap water was provided. The mice were acclimated for 2 wk during which they were fed a casein-based control diet [AIN93G; (20)]. The mice were then assigned to one of the following four diet groups ([n = 18]: casein control, genistein (250 mg/kg diet), daidzein (250 mg/kg diet) or isoflavone mixture (NovaSoy, Archer Daniels Midland, Decatur, IL; 250 mg genistein equivalent/kg diet). The mice were subjected to a 23 wk of age (Fig. 1). In contrast, mice fed diets containing genistein, daidzein or the isoflavone mixture did not develop mammary tumors until wk 27. Analysis of the curves by the LIFETEST procedure of SAS found that the control differed from the genistein, daidzein and isoflavone mixture groups, with no differences among the three isoflavone groups. Tumor latency (age of tumor appearance) was significantly increased in mice fed the soy phytoestrogen compared with the control group (Fig. 2). Once tumors developed, the growth rate, as determined by weekly caliper measurements, was not affected by dietary treatment (data not shown). At termination of the study, there were no differences in the percentage of mice with mammary tumors among the dietary groups (89, 94, 88 and 100% in control, genistein, daidzein and isoflavone mixture groups, respectively). At this time, no differences in total tumor burden, mean tumor size or mean tumor number per mouse were observed because of the dietary treatments (data not shown). Although there were no differences in tumor burden associated with the dietary treatments, the location of tumors was influenced by the soy isoflavones. Mice fed genistein had more tumors in the left thoracic quadrant compared with controls, whereas mice fed daidzein had fewer tumors in the right thoracic and gonadal quadrants compared with controls (Fig. 3). The percentage of mice with lung metastases did not differ due to the dietary treatments (data not shown).

RESULTS

The mice fed the experimental diets gained weight at similar rates and there were no differences in final body weight among the treatment groups (Table 1). Food intake was monitored periodically throughout the study and did not differ among the treatment groups (data not shown). The development of palpable tumors was first observed in the control mice at 23 wk of age (Fig. 1). In contrast, mice fed diets containing genistein, daidzein or the isoflavone mixture did not develop mammary tumors until wk 27. Analysis of the curves by the LIFETEST procedure of SAS found that the control differed from the genistein, daidzein and isoflavone mixture groups, with no differences among the three isoflavone groups. Tumor latency (age of tumor appearance) was significantly increased in mice fed the soy phytoestrogens compared with the control group (Fig. 2). Once tumors developed, the growth rate, as determined by weekly caliper measurements, was not affected by dietary treatment (data not shown). At termination of the study, there were no differences in the percentage of mice with mammary tumors among the dietary groups (89, 94, 88 and 100% in control, genistein, daidzein and isoflavone mixture groups, respectively). At this time, no differences in total tumor burden, mean tumor size or mean tumor number per mouse were observed because of the dietary treatments (data not shown). Although there were no differences in tumor burden associated with the dietary treatments, the location of tumors was influenced by the soy isoflavones. Mice fed genistein had more tumors in the left thoracic quadrant compared with controls, whereas mice fed daidzein had fewer tumors in the right thoracic and gonadal quadrants compared with controls (Fig. 3). The percentage of mice with lung metastases did not differ due to the dietary treatments (data not shown).

### TABLE 1

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body weight</th>
<th>Abdominal fat pad</th>
<th>Uterus + ovaries</th>
<th>Kidneys</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.1 ± 0.6</td>
<td>0.33 ± 0.05</td>
<td>0.11 ± 0.02a</td>
<td>0.34 ± 0.04</td>
<td>1.29 ± 0.20c</td>
</tr>
<tr>
<td>Genistein</td>
<td>24.1 ± 0.6</td>
<td>0.33 ± 0.05</td>
<td>0.14 ± 0.04b</td>
<td>0.33 ± 0.02</td>
<td>1.12 ± 0.16ab</td>
</tr>
<tr>
<td>Daidzein</td>
<td>24.4 ± 0.6</td>
<td>0.36 ± 0.05</td>
<td>0.13 ± 0.02abc</td>
<td>0.35 ± 0.02</td>
<td>1.23 ± 0.21bc</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>23.5 ± 0.6</td>
<td>0.26 ± 0.05</td>
<td>0.14 ± 0.04b</td>
<td>0.35 ± 0.05</td>
<td>1.05 ± 0.13a</td>
</tr>
</tbody>
</table>

1 Values are mean ± SEM, n = 18. Values within a column with different letters differ, P < 0.05.
2 Final body weight.
However, mice fed the isoflavone mixture tended to have \((0.1 < P < 0.25)\) a lower metastatic rate (50%) compared with the other groups (75% control; 71% genistein; 80% daidzein).

Despite no differences in growth rate or final body weight, liver weights of mice fed the control diet were significantly greater than those fed genistein or the isoflavone mixture (Table 1). The mean liver weight of mice fed the isoflavone mixture was also less than in mice fed daidzein, but did not differ from those fed genistein. Mice fed genistein or the isoflavone mixture had greater mean weight of uterus plus ovaries compared with the control fed mice (Table 1). The mean weight of the uterus plus ovaries in mice fed daidzein was intermediate and not different from either the control or the other two isoflavone-fed groups. No differences in abdominal fat pad or kidney weights were observed due to the dietary treatments (Table 1).

Overall, there were no differences in the number of lobule types due to dietary treatment (data not shown). The number of Type 3 lobules per gland was 2.34, 2.41, 1.46 and 1.67 in control, genistein-, daidzein- and isoflavone-fed mice, respectively; the number of Type 1 lobules per gland was 2.19, 3.76, 2.92 and 3.07 in control, genistein-, daidzein- and isoflavone-fed mice, respectively.

**DISCUSSION**

The MMTV-neu/ErbB-2 spontaneous mammary tumor mouse provides a suitable model with which to examine the influence of dietary compounds on breast cancer risk. Over-expression of the neu/ErbB-2 oncogene is commonly found in human breast cancer (18). We found a significant and physiologically relevant delay in tumor development in these mice when soy isoflavones were fed from early adulthood. Hence, this study supports a protective effect of soy isoflavones on breast cancer. Although tumor development was delayed in the mice fed soy isoflavones, these compounds did not prevent tumor formation, nor did they affect the rate of tumor growth. A similar delay in tumorigenesis without prevention of tumor development was observed in MMTV-neu mice treated with a retinoid analog (22). Recently, Mizunuma et al. (23) observed a decrease in mammary tumor development in MMTV-neu mice fed biochanin A, which is converted to genistein by gut microflora. When MMTV-neu mice were rendered germ free to eliminate gut microorganisms, no protection from mammary carcinogenesis occurred when the mice were fed biochanin A, which suggests that genistein was the active agent in reducing tumor development. We tested two known estrogenic compounds, genistein and daidzein, because they are the dominant isoflavones found in soy. Although both of these compounds have affinity for the estrogen receptor (24), genistein but not daidzein is also a potent inhibitor of tyrosine kinases (25). Tyrosine kinases are essential for cell signaling networks and are strictly controlled in normal cells. Elevated tyrosine kinase activity disturbs cellular signaling and increases malignant transformation (26); therefore, reduced tyrosine kinase activity may prevent transformation. Dalu et al. (27) reported that feeding genistein to rats inhibited EGFR receptor phosphorylation and decreased expression of the ErbB-2/neu receptor in prostate tissue. It is possible that a similar suppression occurred in the mammary tissue of the mice in our study. However, daidzein and genistein were equivalent in delaying mammary tumor development, thus suggesting that the re-
response was not due to inhibition of tyrosine kinase activity, although this remains a possible mechanism.

NovaSoy is increasingly being used by food manufacturers as an ingredient to increase the health benefits of foods, and it is also available as a dietary supplement. NovaSoy is an extract of soybeans that contains the isoflavones, primarily in the glycosylated form, saponins, some carbohydrates and other undefined compounds. In this study NovaSoy was tested at a dose that provided an amount of genistein (aglycone equivalents) equivalent to that in the genistein diet. We found a similar response to all three diets in delaying tumor development, which suggests that a common mechanism was involved. However, there were subtle differences in tumor development associated with the dietary treatments. Mice fed the genistein diet tended to develop smaller but more numerous tumors per mouse compared with those fed daidzein. The location of tumors differed among the dietary treatments (Fig. 3). Hence, further studies are warranted to define the cellular mechanism affected by these soy isoflavones.

The delay in mammary tumor development in this transgenic animal model in response to dietary phytoestrogens is dramatic and illustrates a protective effect of these compounds. The phytoestrogens were fed throughout adult life, at a dosage comparable with the dietary exposure found in Asian cultures (28) and used by other researchers in animal models (7,8,10–13). It has been estimated (29) that Chinese adults consume 77–102 mg of total isoflavone aglycones/d (equivalent to 0.039–0.051 mg/kcal assuming 2000 kcal/d). In our study, the mice consumed ∼0.75 mg isoflavones/d (0.052 mg/kcal). Lamartinie et al. (5,6) first reported that genistein injected into neonatal rats suppressed the development of DMBA-induced mammary tumors. This observation was corroborated recently (30). A similar protective effect occurred when genistein was administered during the prepubertal period (7,8). This effect may be a result of genistein causing undifferentiated terminal end buds to differentiate into mature lobules, which are less sensitive to mutagenesis by DMBA (31,32). These studies led to the conclusion that the critical window of time for chemoprevention by genistein is during the perinatal period (11). Contrasting effects have been reported on the response to genistein when administered in utero. When genistein was injected into pregnant dams, the incidence of DMBA-induced mammary tumors in female offspring was greater than in vehicle-treated controls (10). This response was not observed, however, when genistein was fed to dams, rather than injected (33). Lamartinie et al. (11) suggested that the route of administration of genistein influences its biological action.

In contrast to the protective effects of perinatal genistein, lifelong exposure to genistein has been found both to increase and to have no effect on DMBA-induced tumor development in animal models. In previous work, we found an increase in DMBA tumor progression in C57/JB6 mice fed diets containing 1 g genistein/kg diet from weaning (9). Although there was no difference in the number of tumors per mouse or tumor size, the histological stage of tumors from mice fed genistein was more advanced than from mice fed no genistein. In a similar study design, but using a lower dose of genistein (250 mg/kg), no protection from DMBA-induced tumors was observed when genistein was fed throughout life (11).

In the present study, despite providing a delay in development of palpable mammary tumors, dietary phytoestrogens did not prevent tumor development. This observation suggests that tumor initiation was delayed by the dietary compounds, or that growth of the tumors was inhibited for a short period. The growth of the tumors was apparently not affected after this period because the size and number of mammary tumors at termination was not different among the dietary treatment groups. A possible explanation for the delay in mammary tumor development would be an altered rate of mammary gland maturation. A characteristic of MMTV-neu mammary gland histology is incomplete involution of lobules after lactation (17). Although we found no significant difference in mean number of Type 1, 2 or 3 lobules among the dietary treatments, mice fed the isoflavones tended to have higher Type 1:Type 3 ratios (control 0.94 vs. genistein 1.56, daidzein 2.0 and isoflavone mix 1.84). This observation agrees with a previous report that genistein increased the number of Type 1 lobules in rats fed genistein from 21 d of age (33). The similarities in our study did not begin consuming the soy isoflavones until 7 wk of age, supposedly beyond the stage of sexual maturation. This suggests that soy isoflavones may influence mammary gland development during the adult period.

Mammary tumors in MMTV-neu mice have been reported to metastasize to the lungs in ∼60% of mice (34). We observed a similar high rate of lung metastases in our mice. It was interesting to note that only 50% of mice fed the isoflavone mixture developed lung metastases. Although the data were not significant, there is a suggestion that the combination of soy isoflavones may have reduced the metastatic potential of the mammary tumors, but genistein and daidzein did not. Genistein was reported previously to reduce invasion of mammary carcinoma cells in vitro (35), although our data suggest that a compound other than genistein in the isoflavone mixture provided this protection.

The effect of soy phytoestrogens on organ weights was a surprising observation in this study. The mean body weights of the mice were not affected by dietary treatment. However, liver weights were significantly reduced by genistein and the isoflavone mixture. It has been observed that genistein-glucoside inhibited liver oxidative damage (36), which may influence total liver weight. As observed previously (37), dietary genistein increased the weight of the uterus plus ovaries, demonstrating an estrogenic effect on these organs. A similar increase was observed with the isoflavone mixture, which provided an equivalent amount of genistein; however, daidzein did not affect the weight of the reproductive tract.

In conclusion, dietary soy isoflavones, genistein, daidzein and a commercial soy extract, delayed tumorigenesis in the MMTV-neu mouse but did not prevent tumor development. Because overexpressed ErbB-2/neu is a common mutation in human breast cancer, further studies are warranted to examine the role of soy phytoestrogens in breast cancer prevention.

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LITERATURE CITED


JIN AND MACDONALD

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