Effects of Daidzein, Genistein, and 17β-Estradiol on 7,12-Dimethylbenz[a]anthracene-Induced Mutagenicity and Uterine Dysplasia in Ovariectomized Rats

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Abstract: Phytoestrogens, primarily isoflavones daidzein (DZ) and genistein (GE), are increasingly used by postmenopausal women as an alternative to hormone replacement therapy due to reports that estrogen therapy increases the risk of breast and endometrial cancers. These compounds, as estrogen receptor agonists, may influence chemical carcinogenesis in estrogen-responsive tissues such as the uterus. We utilized ovariectomized (OVX) rats to model menopause and assessed the effects of dietary DZ, GE, or 17β-estradiol (E2) on carcinogen-induced mutagenesis and carcinogenesis in the rat uterus. Big Blue® transgenic rats (derived from Fischer 344 strain) were exposed to 7,12-dimethylbenz[a]anthracene (DMBA) in the presence or absence of the supplements. At 16- or 20-wk sacrifice, the uteri were removed and processed to determine mutant frequencies (MFs) and immunohistochemical or histopathological parameters, respectively. In rats treated with DMBA alone, a significant increase in lacI MFs (P < 0.01) in both OVX and intact (INT) rats was observed. The DMBA-induced MFs were not significantly altered by dietary DZ, GE, or E2 in both OVX and INT rats. Although dysplasia was not induced in the uterus of OVX and INT rats treated with DMBA alone, it was detected in 55% of OVX rats fed E2 alone and in 100% of OVX rats fed E2 along with DMBA exposure. Cell proliferation also was significantly higher in OVX rats fed E2 and treated with DMBA. In rats fed the isoflavones and treated with DMBA, the incidence of dysplasia was either reduced or virtually absent in both OVX and INT groups. These results indicate that a high incidence of dysplasia was associated with E2 feeding with or without DMBA treatment in the OVX rats, whereas the incidence was low in rats fed DZ or GE and treated with DMBA, suggesting a weak estrogen receptor agonist of DZ or GE in the rat uterus. The absence of dysplasia in OVX rats exposed to DMBA alone also suggests, in part, a promotional mechanism via estrogen- or isoflavone-driven cell proliferation.

Introduction

The undesirable symptoms of menopause and other disorders such as cardiovascular disease and osteoporosis linked to decreased levels of estrogen have brought about the increased use of hormone replacement therapy (HRT) among women. However, recent reports have indicated that HRT exacerbates the development of breast cancer, endometrial cancer, heart disease, and other diseases (1–6). To manage menopausal symptoms, many women are embracing alternative forms of therapy such as soy isoflavones (7,8). Soy isoflavones are heterocyclic phenols with structural similarity to 17β-estradiol (E2) and are selective estrogen receptor modulators (9). The major constituents of soy isoflavones are daidzein (DZ) and genistein (GE). Both DZ and GE can bind to estrogen receptors (ERα and β) and induce various estrogenic and anti-estrogenic responses in target tissues (10,11).

Isoflavones are found in high concentrations in legumes, and they have been shown to be effective as chemopreventive agents for certain types of cancers, particularly breast and prostate cancers (10,12,13). Evidence also points to the beneficial effects of isoflavones in prevention of cardiovascular disease and osteoporosis (14,15). In addition, soy products are the source of other constituents, such as lignans, protease inhibitors, saponins, phytosterols, coumestans, and phytates, all of which are known for their health-promoting benefits (16). However, recent studies have shown that dietary GE, a component of soy, enhances chemically induced carcinogenesis in the colon (17) and in the mammary gland (18,19). In addition, phytoestrogens can induce gene mutations, chromosome aberrations, aneuploidy, or DNA adducts, suggesting the possible involvement of phytoestrogens in chemical carcinogenesis (20,21). Although the uterus is an estrogen-sensitive organ and postmenopausal estrogen therapy is associated with the increase of endometrial cancer risk (6), research on the effects of phytoestrogens in chemical

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carcinogenesis in the uterus is rare and the roles of phytoestrogens in the estrogen-dependent malignancies in the uterus are not extensively investigated.

Mutation induction, as an early biomarker of carcinogenesis, has played a significant role in predicting the carcinogenicity of most environmental toxicants. However, the assessment of mutation in certain tissues such as the uterus was hampered by the lack of a relevant animal model. With the availability of transgenic animal models such as the Big Blue® (BB) rat, which harbors mutational targets in all tissues of the body, it is possible to assess tissue-specific mutations or tumors. The strength of this model is that it permits direct comparison between carcinogenesis and mutagenesis in the same tissue. In this study, BB transgenic rats were treated with a single dose of the model mutagen and carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) or vehicle, and they were ovariectomized (OVX) to model the menopausal condition or the ovaries remained intact (INT). BB transgenic rats were derived from the Fischer 344 strain, and, although they are not as sensitive to DMBA exposure as Sprague-Dawley rats, they respond positively to the carcinogen (22,23), and their use in the present study facilitates the assessment of in vivo mutations in the uterus.

Mutagenesis and carcinogenesis studies were conducted in rats fed the control diet alone and control diets containing the isoflavones or E2 in the presence or absence of DMBA treatment. It should be noted that the process of tumorigenesis, the route from DNA damage to cancer (tumor), encompasses multi–end points including mutations, dysplasia or abnormal cell growth, and neoplasia (abnormal mass or colony of cells with uncontrollable growth or tumor) that can be determined by various assays. In this study, we assessed mutation as an early biomarker of DNA damage and dysplasia because it is prevalent among women and is considered to be a precancerous condition. We sought to address the following issues: 1) whether or not the phytoestrogens or E2 can modulate chemically induced mutations in the uterus of OVX and INT rats and 2) whether or not E2 or phytoestrogen intake can increase the incidence of uterine dysplasia.

Materials and Methods

Chemicals

DZ (lot 1-FSS-31–1) and GE (lot 6-ECGW-83–2) were purchased from Toronto Research Chemicals (Toronto, Canada). Purity as determined by nuclear magnetic resonance, desorption electron ionization, gas chromatography/mass spectrometry, and high-performance liquid chromatography–ultraviolet (detector) analyses was >99%. DMBA was from Sigma Chemicals (St. Louis, MO). Transpack™ in vitro λ-phase packaging extract and 5-bromo–4-chloro–3-indolyl–b-D-galactopyranoside (X-gal) were obtained from Stratagene (La Jolla, CA). Mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody was from Clone PC10, Dako Corporation (Carpinteria, CA). Biotinylated goat anti-mouse immunoglobulin G (IgG) was purchased from Boehringer-Mannheim (Indianapolis, IN), and streptavidin-conjugated horseradish peroxidase was supplied by Jackson Immunoresearch Laboratories (West Grove, PA).

Animals and Treatments

Female (3-wk-old) BB transgenic rats were obtained from Taconic Farms (Germantown, NY). On arrival at the National Center for Toxicological Research (NCTR), they were quarantined for 2 wk before use in the experiments. The Institutional Animal Care and Use Committee at NCTR approved the animal handling, maintenance, and experimental protocol for this study. The rats were placed on an isoflavone–alfalfa-free diet (NIH-31C) and had free access to water. The NIH-31C diet has the same basic formulation as the standard NIH-31 diet (24) except that the protein contributed by soy meal and alfalfa was replaced by casein and the soy oil by corn oil. The diet was analyzed by liquid chromatography/mass spectrometry and was free of isoflavones at a detection limit of 0.5 µg.

The animal treatment protocol is displayed in Fig. 1. Starting at 50 days of age, animals were randomly divided into 4 sets, each set containing 7 different treatment groups (5 rats for mutagenesis study and 10 rats for carcinogenesis study per group), and fed NIH-31C diet or diets containing 250 ppm or 1,000 ppm DZ, 250 ppm or 1,000 ppm GE, a combination of 1,000 ppm DZ and 1,000 ppm GE, or 5 ppm E2 until termination of the experiments. Based on food consumption calculations, a dose of 1,000 ppm DZ or GE, the highest dose fed to the rats, represents approximately 20 mg of daily ingested isoflavones per rat; this intake falls within the estimated daily range, that is, 20–50 mg/day consumed per person in Asian populations where consumption of soy in the diet is associated with health-promoting benefits (11,25,26). At the same time, two sets of rats were gavaged with a single dose (1 ml) of 80 mg DMBA/kg, and the other two sets were gavaged with an equal volume of sesame oil. This dose of DMBA is within the range that produces significant mutant frequency (MF) in multiple tissues of BB rats (23,27). In addition, 80 mg/kg of DMBA is equally effective in inducing 50% tumors in the mammary gland of F344 rats (28).

At 9 wk of age, one set of rats from DMBA treatment or vehicle control was bilaterally OVX under 100 mg/kg ketamine and 15 mg/kg xylazine, and the other two sets remained with INT ovaries. Food consumption and body weight were recorded weekly. The animals were sacrificed, and the uteri were harvested and weighed for mutagenesis study at 23 wk of age (16 wk following DMBA treatment) and at 27 wk (20 wk following DMBA treatment) for carcinogenesis study. The 23-wk termination time for the mutagenesis was based on previous mammary study that indicated that DMBA-induced mutants peak between 18 and 23 wk after treatment, whereas a DMBA dose of 80 mg/kg has been shown to induce mammary tumors in 50% of the treated rats (28). With the exception of samples for histopathological
analyses, the tissues were immediately frozen in liquid nitrogen and stored at –80°C until processed for lacI assay.

lacI Mutagenesis Assay

The animals were sacrificed at 16 wk post-DMBA treatment when lacI MFs were high and the assay was conducted as previously described for the rat (23). Briefly, DNA extraction, λ packaging, and plating for lacI mutant plaques were carried out in a “blocked” manner so as to minimize bias from day-to-day variations in experimental procedures. The lacI containing λ shuttle vector was recovered by mixing the genomic DNA extracted from uterine tissues with Transpack™ in vitro λ phage packaging extract. The resulting phages were pre-adsorbed to Escherichia coli SCS-8 cells for 20 min at 37°C, mixed with prewarmed NZY top agar containing 1.5 mg/ml of X-gal, and poured into 250-mm assay trays containing BB media. The plates were incubated overnight at 37°C and scored for mutant blue plaques. Color control mutants were included in all plating, and the results were accepted only if mutant CM1 could be detected.

Packaging and plating were repeated for the DNA samples until at least 2 × 10⁵ plaques were scored for each data point. The mutant blue plaques were picked into individual tubes containing 500 µl of SM buffer and 50 µl of chloroform. To confirm the mutant phenotypes, all recovered putative mutant phages from the 250-mm assay plates were diluted 1:100 and replated on 100-mm plates with 3.5 ml of top agarose containing 1.5 mg/ml of X-gal. The sectored plaques were also verified for their phenotype as specified in previous experiments (27), and the confirmed sectored plaques were separately scored but not included in the MF calculation. The lacI MF was calculated by dividing the number of verified mutant plaques by the total number of plaques analyzed.

Proliferating Cell Nuclear Antigen/Apoptosis Analysis in Uterine Tissues

Uterine tissues derived from animals sacrificed at 27 wk of age (20 wk following DMBA treatment) were excised, weighed, and fixed in 10% buffered formalin for 48–72 h and processed for 8 h on a Shandon Pathcenter Tissue Processor (Shandon, Inc., Pittsburgh, PA). They were embedded in paraffin, sectioned at 4 µm, and mounted on positive (+) charged slides. Cell proliferation indices were determined for all the tissues by immunohistochemical localization of PCNA, slightly modified from Foley et al. (29). Uterine tissues were placed in xylene to remove paraffin, rehydrated in a series of alcohol with decreasing water content, and immersed in phosphate-buffered saline. Endogenous peroxidase was quenched with 3% H₂O₂ containing 0.1% sodium azide. The sections were placed in antigen-retrieval solution consisting of 1% zinc sulfate in deionized water and heated for 7.5 min in a 700-watt microwave oven on full power. Routine streptavidin procedures were then performed, beginning with 0.5% casein to block nonspecific binding of subsequent antibody and sequential incubation of sections in a mouse monoclonal anti-PCNA antibody, biotinylated goat anti-mouse IgG (Boehringer-Mannheim, Indianapolis, IN), and streptavidin-conjugated horseradish peroxidase. The PCNA-positive cells were visualized by incubating the sections in 3,3′-diaminobenzidine hydrochloride chromogen followed by counterstaining with Mayer’s hematoxylin. Although unstained nuclei were identified as cells in the G₀ phase of the cell cycle, those stained dark red represented S-phase cells, with the nuclei that have a light reddish tint stained counted as cells in the G₁ phase. The number of cells in each stage of the cell cycle was counted per a total of 1,000 cells. For the enumeration of apoptotic cells, another set of

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**Figure 1.** Animal treatment protocol. Fifty-day-old rats were treated with either 80 mg/kg DMBA or vehicle and maintained on isoflavones, estradiol, or isoflavone/estradiol-free diets until termination of the experiments. At 16 or 20 wk after DMBA treatment, animals were sacrificed to conduct mutagenesis and carcinogenesis assays, respectively. *Note that animals were exposed to DMBA 2 wk prior to ovariectomy.
slides was stained with the terminal deoxynucleotide transferase-mediated dUTP nick-end labeling as described by Gavrieli et al. (30).

**Histopathological Analysis**

At necropsy, the uterine and other tissues were examined grossly, removed, wet-weighed, and preserved in 10% neutral-buffered formalin. Lesion descriptions were recorded on the Individual Animal Necropsy Record Form. Tissues were trimmed, processed, and embedded in Tissue Prep II, sectioned at 4–6 µm, and stained with hematoxylin and eosin. In addition, select tissues were harvested for PCNA, apoptosis assay, and in situ hybridization as described previously. They were microscopically examined, and, when applicable, non-neoplastic lesions were graded for severity.

**Statistical Analysis**

MFs as a function of dose were analyzed by a one-way analysis of variance (ANOVA) that included the effect of the different doses. Contrasts were constructed to make the comparison of interest as well as the effect of each treatment with control, and the P values were adjusted by Holm’s modifications (31) of the Bonferroni procedure to correct for multiple comparisons. In addition, a t-test was conducted to compare the means between the OVX and INT groups for any significant difference between the groups. PCNA data and dysplasia were analyzed by ANOVA to determine the significant effects of the treatments.

**Results**

**Mortality and Mean Body Weights**

Two DMBA-treated OVX rats fed 250 ppm and 1,000 ppm DZ died early in the study, and the cause of death was attributed to surgery or gavage error rather than treatment effect. Another rat from the INT group fed E2 and exposed to DMBA also died early in the study, but the cause of death was undetermined. Six other rats in various groups treated with DMBA were found to be moribund and euthanized. All of these animals had gross mammary gland mass that was diagnosed as adenocarcinoma. Food intakes and body weights were measured during the course of the study. Interestingly, food consumption was essentially similar, and no statistical differences were found among the treatment groups (data not shown). As shown in Fig. 2 (A–D), DZ and GE feeding did not significantly affect body weight gain. However, in E2-fed rats from both OVX and INT groups with or without DMBA treatment, body weight gain was markedly reduced relative to the other treatment groups (Fig. 2A–D). Also, E2 treatment increased average uterine weight in OVX rats, whereas, in the INT group, all the treatment groups including DZ and GE demonstrated uterine weight comparable with that of the animals fed the control diet (data not shown).

**lacI Mutagenesis in Uterus**

The uterine tissues from both OVX and INT rats were processed for lacI mutagenesis assay as described in Materials and Methods. As shown in Table 1, DZ, GE, and E2 intakes alone did not cause any appreciable mutagenic response in the uterus (MFs ranged from 11 to 25 ± 1.6–4.0 × 10^-6). The DMBA-induced lacI MFs were significantly higher in both the OVX (83 ± 6.3 × 10^-6) and the INT (100 ± 6.2 × 10^-6) rats compared with the control and the isoflavone diet groups (P < 0.01, Table 1). In comparison with the OVX animals treated with DMBA, lacI MFs were significantly higher in the DMBA-treated INT group (P < 0.05: OVX vs. INT). With the exception of a reduced DMBA lacI MF seen in the rats fed the isoflavone mixture, dietary DZ or GE resulted in variable changes in lacI MFs in both OVX and INT rats; however, the responses were not statistically significant. In addition, in rats fed either DZ or GE diet alone the lacI MF in the uterus was not statistically significant compared with the MF in the rats fed the control diet. Taken together, neither DZ nor GE diet significantly modified the mutagenicity of DMBA or spontaneous MF in the uterus (Table 1).

**PCNA/Apoptosis in the Uterus**

The effect of dietary DZ, GE, or E2 either separately or combined with DMBA treatment on cell proliferation and apoptosis was assessed by PCNA immunohistochemistry (Fig. 3A–D). Compared with the animals fed the control diet, the percentage of proliferating cells observed in rats fed both doses of DZ, the low dose of GE, the mixture, and E2 either alone or in the presence of DMBA was higher (P < 0.05) in the OVX rats (Fig. 3A and B). Accumulation of cells at the G1 phase also was significantly increased in rats fed the isoflavone and E2 diets, indicating cell cycle arrest. Apoptosis was, however, inhibited in OVX rats fed the isoflavone and E2 diets alone. In the INT animals, apoptosis indices were comparatively increased in all the treatment groups; however, neither the dietary isoflavones nor DMBA exposure independently influenced the responses in this group (Fig. 3C and D).

**Uterine Histopathology in OVX and INT Rats**

Histopathological analysis of uterine samples from the OVX group revealed no neoplastic transformation, malignant or nonmalignant, in the DMBA-treated rats alone or in the isoflavone-fed rats treated with DMBA. However, a developmental malformation was seen in the uterus of one rat treated with DMBA and fed the E2 diet in this group. As shown in Fig. 4, uterine atrophy was prominent in this group with or without the supplements or DMBA treatment as expected in ovariotectomy. Although the percentage of OVX rats with atrophy as depicted in Fig. 4 appears to be essentially similar in the treatment groups, except E2, the degree of severity of atrophy graded as 3–4 (high) and 2 or below (low).
was high in rats receiving the control diet and those fed the isoflavones or treated with DMBA alone. Interestingly, the severity was markedly reduced in DMBA-treated rats fed the isoflavones, and it was completely eliminated in E2-fed rats exposed to DMBA, suggesting that E2 was more potent at reducing the severity of atrophy than the isoflavones. In addition, whereas uterine dysplasia was virtually absent in rats fed phytoestrogens or DMBA alone in both OVX (Fig. 4 and INT not shown) groups, dietary E2 caused a high incidence of dysplasia in OVX rats (55% in E2 fed alone and 100% in E2 fed plus DMBA treatment). Furthermore, in the OVX group the combination of DMBA exposure and some concentrations of the phytoestrogens (that is, 250 and 1,000 ppm DZ and 250 ppm GE) only produced marginal incidences (10–18%) of dysplasia (Fig. 4), suggesting a remarkably low estrogenic potency of the phytoestrogens that may be indicative of their health-promoting benefits.

In contrast, in the INT rats asynchronistic growth pattern was observed in the uterus, possibly due to exposure to the isoflavones and E2, and uterine hemangiosarcoma was detected only in one rat fed 1,000 ppm DZ and treated with DMBA. In addition, isolated incidences of mild to marked dilatations were seen in some animals exposed to DMBA with or without the isoflavones. No incidence of dilatation, mild or marked, was seen in E2-fed rats in this group. These pathologic lesions were not only sporadic but also were very low to be interpreted as treatment-related effects (data not shown).

Figure 2. Body weight gain (in grams) of ovariectomized (OVX) and intact (INT) rats fed with diets containing 0, 250, and 1,000 ppm daidzein (DZ) or genistein (GE) or 5 ppm 17β-estradiol (E2). Note that, in comparison with the other treatment groups, including the control, rats fed the E2 diet had body weight reduction irrespective of whether the ovaries were removed or INT. There were no statistical differences among the groups. A: OVX rats fed DZ, GE, or E2 alone. B: OVX rats fed isoflavones or E2 and exposed to 7,12-dimethylbenz[a]anthracene (DMBA). C: INT rats fed DZ, GE, or E2 alone. D: INT rats fed isoflavones or E2 and exposed to DMBA.
Table 1. Mutant Frequencies in the Uterus lacI Gene Measured 16 wk Post–DMBA Treatment in OVX and INT Rats Fed with Control, DZ, GE, DZG, and E2 Diets

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mutant Frequency ($\times 10^{-6}$)</th>
<th>OVX</th>
<th>INT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vehicle</td>
<td>DMBA</td>
</tr>
<tr>
<td>Control/oil</td>
<td></td>
<td>13.3 ± 2.9</td>
<td>83 ± 12.5</td>
</tr>
<tr>
<td>250 ppm DZ</td>
<td></td>
<td>16 ± 6.5</td>
<td>64 ± 47d</td>
</tr>
<tr>
<td>250 ppm GE</td>
<td></td>
<td>17 ± 4.5</td>
<td>94 ± 25d</td>
</tr>
<tr>
<td>1,000 ppm DZ</td>
<td></td>
<td>11.2 ± 4.8</td>
<td>71 ± 11.6</td>
</tr>
<tr>
<td>1,000 ppm GE</td>
<td></td>
<td>18.7 ± 7.5</td>
<td>89.5 ± 16.7d</td>
</tr>
<tr>
<td>1,000 ppm DZG</td>
<td></td>
<td>8.4 ± 2.9</td>
<td>60.5 ± 49d</td>
</tr>
<tr>
<td>5 ppm E2</td>
<td></td>
<td>23.3 ± 2.9</td>
<td>95.7 ± 37.8d</td>
</tr>
</tbody>
</table>

a: Abbreviations are as follows: DMBA, 7,12-dimethylbenz[a]anthracene; OVX, ovariectomized; INT, intact; DZ, daidzein; GE, genistein; DZG, daidzein + genistein; E2, 17β-estradiol.
b: No significant difference between OVX and INT groups as determined by t-tests ($P > 0.14$).
c: Values are mean ± SD of five rats for all treatment groups except those marked *, which had only four rats per group.
d: Significant differences were found between the rats treated with DMBA and those treated with vehicle only ($P < 0.05$). No significant changes were found among the groups treated with DMBA or among the groups treated with vehicle.

Discussion

Soy isoflavones exhibit diverse biological activities that may or may not be considered deleterious; however, most studies on these compounds focus primarily on health benefits with little or no attention given to their potential genotoxic effects such as mutation induction that is linked to cancer and other diseases. The present study evaluated the effects of DZ, GE, or E2 on the potential mutagenic and tumorigenic action of DMBA in the uterus of OVX and INT BB transgenic rats that permit concurrent assessment of mutagenesis and carcinogenesis in the same tissue. The results indicate that DMBA treatment significantly increased lacI MFs compared with the animals that were not exposed to DMBA, and DZ, GE, or E2 diets did not alter either DMBA-induced or spontaneous MFs in the uterus. Especially fascinating was the finding that uterine dysplasia was significantly induced only in OVX rats receiving E2 with or without DMBA treatment. Although DMBA treatment alone induced increased lacI MFs, it did not induce dysplasia in both OVX and INT rats, probably due, in part, to the latency of DMBA in the rat uterus or the termination time of the experiments. The experiments were terminated at 20 wk following DMBA exposure, a termination time adequate for inducing mammary tumors, and it is possible that dysplasia or even tumor might have been developed in the uterus had the termination time been extended.

The high incidence of dysplasia found in OVX rats fed E2 and treated with DMBA essentially implies that E2 does not only induce dysplasia by itself but also is capable of promoting chemically initiated cells into dysplasia. Although dysplasia itself does not cause health problems, if left untreated, it sometimes can progress to an early form of cancer. These findings have important health implications because one of the major adverse effects of estrogen replacement therapy among menopausal women is endometrial cancer (32). E2 is considered to be a weak mutagen (33); however, its metabolism by cytochrome P450 isoforms has been shown to generate catechol estrogens that can directly interact with DNA and may initiate the carcinogenesis process (34). It also has been shown that the metabolites of estrogen can directly or indirectly, through redox cycling processes, generate reactive radical species that cause oxidative DNA damage (35–37). Equally important, phytoestrogens have been identified as genotoxic agents that induce mutations, chromosome aberration, and DNA adducts in vivo (20,38,39). Our results, however, indicate that neither E2, DZ, nor GE feeding alone induced any significant mutagenic activity in the uterus, and the agents were not co-mutagenic either because they did not significantly increase the DMBA-induced MF in the uterus. Moreover, micronucleus analysis of blood samples from all of the treated and control animals by flow cytometry failed to produce significant differences in frequencies among controls and E2- or phytoestrogen-treated groups (data not shown). These results are not consistent with the positive genotoxic activities reported for phytoestrogens in vitro (18,33,34). The inconsistency is possibly due to different assay conditions or cell types used in the studies.

Dietary DZ and GE also resulted in 10–18% incidence of dysplasia in the uterus of OVX rats treated with DMBA. The low incidence of dysplasia in this group illustrates the relatively weak estrogenic potency of phytoestrogens and indicates that they may be safer compared with estradiol. However, under the conditions of the present study, the isoflavone intake did not go long enough to actually determine whether they were carcinogenic by themselves. Nonetheless, their ability to induce dysplasia even in a small scale, when a carcinogen is present, clearly indicates that they can potentially influence the growth of initiated cells in rat uterus. The underly-
Figure 3. The effect of daidzein (DZ) and genistein (GE) on cell proliferation and apoptosis in the uterus of ovariectomized (OVX) and intact (INT) rats with or without 7,12-dimethylbenz[a]anthracene (DMBA) treatment. Values are expressed as mean ± SD. *Significantly different from control diet at $P < 0.05$. **Significantly different from DMBA treatment alone at $P < 0.05$. There were no statistical differences among the groups with intact ovaries. A: OVX rats fed control diet containing DZ, GE, or 17$\beta$-estradiol (E2) alone. B: OVX rats fed isoflavones or E2 and exposed to DMBA. C: INT rats fed control diet containing DZ, GE, or E2 alone. D: INT rats fed isoflavones and exposed to DMBA.

Figure 4. Histopathology of uterine tissues derived from the ovariectomized rats sacrificed at 20 wk post–7,12-dimethylbenz[a]anthracene (DMBA) treatment. Atrophy was marked in most of the treatment groups including the control, but the incidence was less marked in rats fed 17$\beta$-estradiol (E2) diet alone. Atrophy was virtually absent when E2 feeding was combined with DMBA treatment; in this group, 100% dysplasia was detected, whereas 10–18% incidence of dysplasia was observed in rats fed the isoflavones and treated with DMBA.
ing mechanism of action of isoflavones is not clearly understood; however, they can function both as estrogen agonists and antagonists (40,41), depending on many factors, including hormonal milieu, or receptor occupancy, treatment regimen, and tissues under investigation. From a genotoxic perspective, this may imply that, when estrogen levels are low as they tend to be after ovarioectomy, isoflavones can substitute for the organism’s own estrogen and act as co-carcinogens. Several studies have suggested that isoflavones such as GE and DZ can be co-carcinogenic in tissues when administered in the presence of a carcinogen or preexisting DNA damage. An increase in DMBA-induced mammary adenocarcinoma by 1,000 ppm GE in wild-type but not in estrogen receptor-α knockout mice has been reported (42). Allred et al. (19) also found that 1-[methyl-3H]-estradiol strongly increased endometrial cancer in mice fed diets containing GE or the isoflavones. In OVX rats, isoflavones do not appear to be genotoxic. In the present study, rats fed diets containing the high dose of GE or the mixture and treated with DMBA did not produce any uterine dysplasia that is a precursor to tumor.

Because DZ, GE, and E2 did not increase DMBA-induced MFs and DMBA alone did not induce uterine dysplasia in OVX rats, the possible mechanism for dysplasia induction by E2 and/or the phytoestrogens may lie in their ability to increase cell proliferation. Cell proliferation activity occurs in a fluctuating hormonal environment, and the most widely established role of estrogen in carcinogenesis is to increase cell proliferation (43–45). In the present study, we observed that the percentage of PCNA-positive cells in S phase and G1 phase of the cell cycle in the uterus of OVX rats fed E2 diet and treated with DMBA was significantly higher than that of the other groups. There also was a corresponding increase in uterine weight in these animals in the face of ovarioectomy-related atrophy seen in the animals fed the isoflavone and the control diets. These results indicate that cell proliferation was involved in the induction of dysplasia in the uterus of OVX rats by dietary E2 and by the isoflavones and suggest that DMBA-induced cytolethality in the uterus contributed, in part, to the observed pathologic lesion.

Considering the fact that prolonged exposure to synthetic or endogenous steroidal hormones or their metabolites is causally linked to several human cancers, including endometrial cancer (43,46,47), it was surprising that dysplasia was virtually absent in the uterus of INT rats treated with DMBA or the phytoestrogens. E2 diet also did not produce any uterine dysplasia in DMBA-treated rats in this group. One explanation for this phenomenon is hormone balance. It has been shown that postmenopausal HRT using unopposed estrogens significantly increases endometrial cancer risk. This risk, however, is markedly reduced when estrogen is administered in conjunction with progestins (6). Because the ovary produces many other hormones in addition to E2, some of these hormones or their metabolites may function against estrogenic action and inhibit cell proliferation in the uterus.

In summary, using BB rats we have concurrently determined DMBA mutagenicity and possible carcinogenicity in the uterus of both OVX and INT rats fed E2, DZ, and GE diets. These agents did not appear to be genotoxic in the rat uterus because they failed to significantly alter chemically induced or spontaneous MFs. In contrast, feeding E2 with or without DMBA exposure or the phytoestrogens in the presence of DMBA caused dysplasia in the uterus of OVX rats, although to a varying degree. The absence of dysplasia in the uterus of rats treated with DMBA alone suggests that the induction of the pathologic lesion by E2 and to a lesser extent by the phytoestrogens in DMBA-treated OVX rats may operate, in part, via an estrogen- or estrogen-like–dependent cell proliferation mechanism.

Acknowledgments and Notes

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