Estrogenicity of Metabolites of Benzophenone Derivatives Examined by a Yeast Two-Hybrid Assay

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The estrogenic activities of S-9 metabolites of benzophenone derivatives (benzophenone, 2-hydroxy-4-methoxybenzophenone, 2,2′-dihydroxy-4-methoxybenzophenone, 2-hydroxy-4-octyloxybenzophenone, 2,4-dihydroxybenzophenone and 2,3,4-trihydroxybenzophenone) and benzhydrol were examined with a yeast two-hybrid screening system. After chemicals were incubated in an S-9 mix at 37°C for 4 hr prior to their incubation with the yeast strain, the S-9 mix containing metabolites was assayed for the estrogenic activity by the yeast two-hybrid assay. Benzophenone, 2-hydroxy-4-methoxybenzophenone and 2,2′-dihydroxy-4-methoxybenzophenone exhibited estrogenic activities after incubation with the S-9 mix. The estrogenic metabolites of 2-hydroxy-4-methoxybenzophenone were fractionated by high-performance liquid chromatography, one of which was identified as 2,4-dihydroxybenzophenone. This assay will be a useful tool for detecting proestrogens.

Key words —— yeast two-hybrid assay, benzophenone, estrogenic metabolite

INTRODUCTION

Benzophenone is listed among the "chemicals suspected of having endocrine disrupting effects" by the World Wildlife Fund, the National Institute of Environmental Health Sciences in the U.S.A., and the Ministry of Environment in Japan. However, benzophenone is an important compound in everyday life because of its ability to absorb and dissipate ultra violet (UV) light.[1] Its twelve derivatives, designated as benzophenone-1 through benzophenone-12, are used in cosmetics and sunscreens to protect human skin and hair from UV irradiation. 2-Hydroxy-4-methoxybenzophenone (benzophenone-3, BZ-3) is one of the most widely used UV absorbers for sunscreens on the market. Orally or topically administered BZ-3 is converted to at least three metabolites, 2,4-dihydroxybenzophenone (benzophenone-1, BZ-1), 2,3,4-trihydroxybenzophenone and 2,2′-dihydroxy-4-methoxybenzophenone (benzophenone-8, BZ-8).[2–5] BZ-1 and 2,3,4-trihydroxybenzophenone exhibited estrogenic activities in an *in vitro* assay system using MCF-7 cells.[6] Benzophenone is converted to an estrogenic metabolite, *p*-hydroxybenzophenone.[7] Thus, benzophenone derivatives can be categorized as proestrogens. However, the estrogenic activities of the metabolites of benzophenone derivatives have not been fully elucidated.

We have developed a novel assay procedure for detecting the hormonal activities of chemicals using a yeast two-hybrid system.[8] We tested the estrogenic activities of various chemicals, and found that a phenol with a hydrophobic moiety at the para-position is the key structural moeity of estrogenic chemicals.[9] The phenyl or phenylether residues of lipophilic chemicals can be converted to a phenol residue by drug metabolizing enzymes. These facts imply that some chemicals exert their estrogenic activities by metabolic activation *in vivo*. The endocrine activities of pesticides and natural products can be affected by metabolism.[10,11] For example, methoxychlor (MXC) is metabolized to 2,2-*bis*(hydroxyphenyl)-1,1,1-trichloroethane to exert estrogenic activity.[12] The EDSTAC final report recommends that the evaluation of chemicals using *in*

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vitro high throughput prescreens should be performed in the presence and absence of metabolically active extracts to detect proestrogens.[13] There are few reports of assay procedures that are able to evaluate the estrogenic activity of metabolites.[14–16] In mutagenicity testing, incubation with an S-9 extract mixture has been the standard method for *in vitro* metabolic activation.[17] Here we apply a yeast two-hybrid assay for detection of estrogenic activity after metabolic activation by incubation with an S-9 extract mix (S-9 mix), and examine the estrogenic activities of metabolites of benzophenone derivatives.

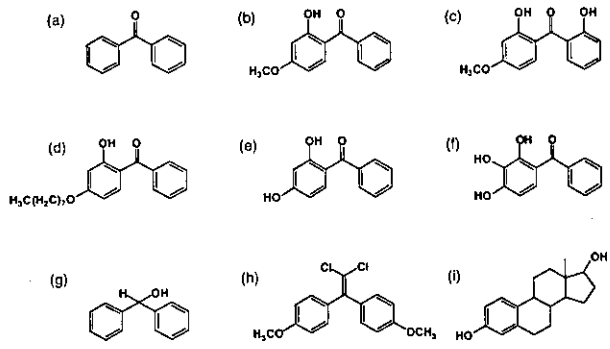

Fig. 1. The Structure of Chemicals Examined in This Assay

a), benzophenone; b), BZ-3; c), BZ-8; d), 2-hydroxy-4-octyloxybenzophenone (BZ-12); e), 2,4-dihydroxybenzophenone (BZ-1); f), 2,3,4-trihydroxybenzophenone; g), benzhydrol; h), MXC, i), E_2.

MATERIALS AND METHODS

Chemicals —— 17-β-Estradiol (E_2, > 97.0%), MXC (> 97.0%), benzophenone (> 98.0%), 2,4-dihydroxybenzophenone (> 98.0%), 2,3,4-trihydroxybenzophenone (> 98.0%), and benzhydrol (> 98.0%) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2-Hydroxy-4-methoxybenzophenone (> 98.0%), 2,2′-dihydroxy-4-methoxybenzophenone (> 98.0%) and 2-hydroxy-4-octyloxybenzophenone (> 98.0%) were purchased from Aldrich Chem. Co. (Milwaulkee, WI, U.S.A.). All other chemicals were reagent grade, obtained from commercial sources, and used without further purification.

Activation by an S-9 Fraction —— S-9 extracts (rat liver 9000 × *g* supernatant fraction induced with phenobarbital and 5,6-benzoflavone) and glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). To a tube containing 990 μl of the S-9 mix (S-9 mix: 20 μl S-9, 0.8 μmol NADPH, 0.8 μmol NADH, 1.0 μmol glucose-6-phosphate, 0.4 U G6PDH, 20 μmol Na_2HPO_4, 20 μmol NaH_2PO_4, 6.6 μmol KCl and 1.6 μmol $MgCl_2$) 10 μl of each test chemical dissolved in dimethyl sulfoxide (DMSO) was added and then incubated at 37°C for 4 hr. The chemicals, after incubation with the S-9 mix, were stored at –80°C until their application to the yeast two-hybrid strain. The heat-inactivated S-9 extract was prepared by incubation at 95°C for 5 min, and used for the negative control experiments. The structures of chemicals examined in this paper are shown in Fig. 1.

Yeast Two-Hybrid Assay for Detecting Estrogenic Activity after Metabolic Activation —— In this study, we used the yeast two-hybrid system with the estrogen receptor, estrogen recepter α (ERα), and the coactivator, transcriptional intermediary factor 2 (TIF2), as previously described.[8,9] Yeast cells carrying the pGBT9-estrogen receptor ligand binding domain (pGBT9-ERLBD) and pGAD424-TIF2 plasmids were grown overnight at 30°C with vigorous shaking in selective medium (S.D. medium lacking tryptophan and leucine). The yeast cells, resuspended in 2 × S.D. medium made up at twice the usual concentration, were mixed at a 1 : 1 (v/v) ratio with the test chemicals, which had been treated with the S-9 mix, and then incubated at 30°C for 4 hr. Aliquots of cells were withdrawn and washed by centrifugation. The cell density was determined by measurement of the absorbance at 595 nm. A lysate was prepared by enzymatic digestion of the cells with 1 mg/ml Zymolyase 20T at 37°C for 15 min. The lysate (200 μl) was mixed with 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (40 μl) and incubated at 30°C for 30 min. The enzymatic reaction was stopped by the addition of 1 M Na_2CO_3 (100 μl). β-Galactosidase activity was calculated as described previously.[8] Estrogenic activity was also tested by MCF-7 proliferation assay[18] and a reporter gene assay using HeLa cells.[19]

HPLC Analysis of BZ-3 Metabolites —— BZ-3 (1.0×10^{-4} M) was incubated with the S-9 mix as mentioned above. The metabolites after S-9 activation (1.5 ml) were extracted twice with 3.0 ml ethylacetate. The extracts were dried under an N_2 stream and dissolved in 30 μl methanol. The extracts (15 μl) were applied to a reverse phase HPLC column (Cadenza CD-C18, 4.6 × 250 mm, 3 μm; Imtakt, Kyoto, Japan). LC-10AD pumps were used with a DGU-14A degassing unit and C-R7A integrator (Shimadzu, Kyoto, Japan). The HPLC column was eluted with a 75% methanol/water at a flow rate of

1.0 ml/min. The eluate was monitored at 230 nm with an SPD-10AV detector (Shimadzu). Each 0.5 ml fraction was collected and dried under an N_2 stream, and dissolved in 10 μl DMSO to be applied to the yeast two-hybrid strain for testing estrogenic activity.

Liquid Chromatography/Mass Spectrometry Analysis of BZ-3 Metabolites —— Liquid chromatography/mass spectrometry (LC/MS) analysis was performed on an API3000 (Applied Biosystems, Foster City, CA, U.S.A.) equipped with an electrospray ionization (ESI) interface and an Agilent 1100 series HPLC from Agilent Technologies (Waldbronn, Germany). The HPLC system consisted of a G1312A HPLC binary pump, a G1367A autosampler and a G1379A degasser. The column used was a reverse phase HPLC column (Cadenza CD-C18, 2.0 × 100 mm, 3 μm; Imtakt). The mobile phases consisted of 100% acetonitrile (A) and 1% aqueous acetic acid (B). Elution was performed using a linear gradient from 30% A to 80% A during 30 min at 0.2 ml/min. The ESI interface was control by Analyst software (v.1.2). ESI-MS was operated in negative or positive ion mode. The heated capillary and voltage were maintained at 500°C with and –/+4.2 kV (negative/positive mode), respectively. Mass spectra were measured from *m/z* 50 up to *m/z* 300.

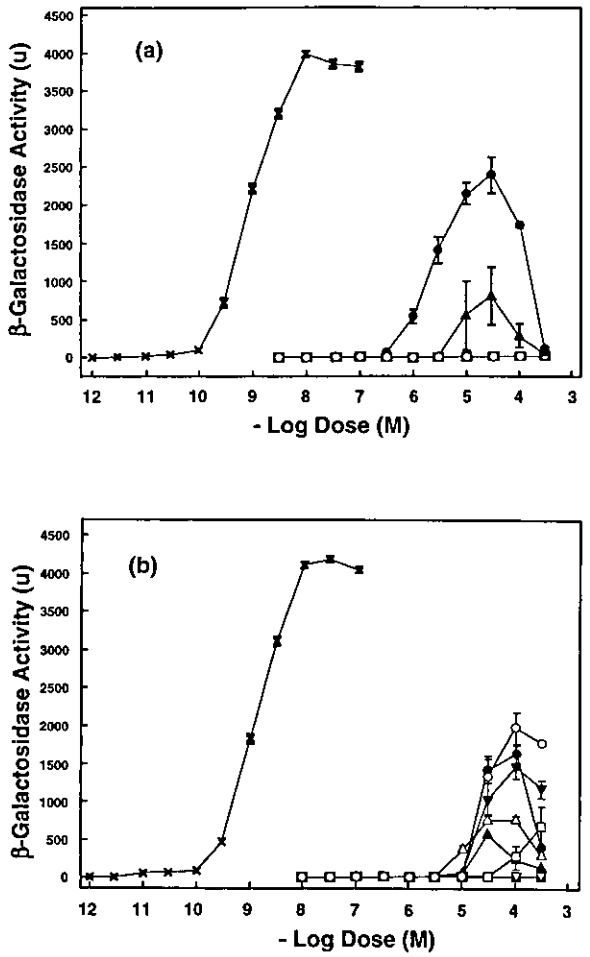

Fig. 2. Dose–Response Curve for Chemicals Incubated in Inactive S-9 Mix (a) and in Active S-9 Mix (b)

Values are means of three separate experiments (bars: S.D.). Benzophenone, (□); BZ-3, (○); BZ-8, (△); BZ-12, (▽); BZ-1,(●); 2,3,4-trihydroxybenzophenone, (▲); benzhydrol, (■); MXC, (▼); E2, (×).

RESULTS

Estrogenic Activity of Metabolites of Benzophenone Derivatives

The negative control experiments were performed using an S-9 mix containing inactive S-9 extracts (an inactive S-9 mix). Serial dilutions of E_2 were incubated with the inactive S-9 mix at 37°C for 4 hr, and then the reaction mixtures were applied to the yeast two-hybrid assay. Maximum β-galactosidase activity induced by incubation with E_2 was obtained at concentrations of 1.0×10^{-8} M and higher (Fig. 2a). The concentration of E_2 showing 10% of the 1.0×10^{-7} M activity (relative effective concentration, REC_{10}) was 1.7×10^{-10} M. Under these conditions BZ-1 and 2,3,4-trihydroxybenzophenone exhibited estrogenic activities. Their REC_{10} values were 6.5×10^{-7} and 6.8×10^{-6} M, respectively. Benzophenone, BZ-3, BZ-8, BZ-12, benzhydrol and MXC did not exhibit estrogenic activities.

The metabolic activation experiments were performed using an S-9 mix containing active S-9 extracts (an active S-9 mix). Maximum β-galactosidase activity induced by incubation with E_2 was obtained at concentrations of 1.0×10^{-8} M and higher (Fig. 2b). The REC_{10} of E_2 was 2.5×10^{-10} M. MXC, benzophenone, BZ-3 and BZ-8 exhibited estrogenic activities after incubation with the active S-9 mix. Their REC_{10} values were 2.5×10^{-5}, 1.5×10^{-4}, 1.4×10^{-5} and 1.0×10^{-5} M, respectively. The estrogenicities of BZ-1 and 2,3,4-trihydroxybenzophenone were reduced by incubation with the active S-9 mix. Their REC_{10} values were 1.5×10^{-4} and 2.1×10^{-5} M, respectively.

HPLC Analysis of the Metabolites of BZ-3

After incubation of 1.0×10^{-4} M BZ-3 with the active S-9 mix, the metabolites were extracted with ethylacetate and then fractionated by HPLC. Three

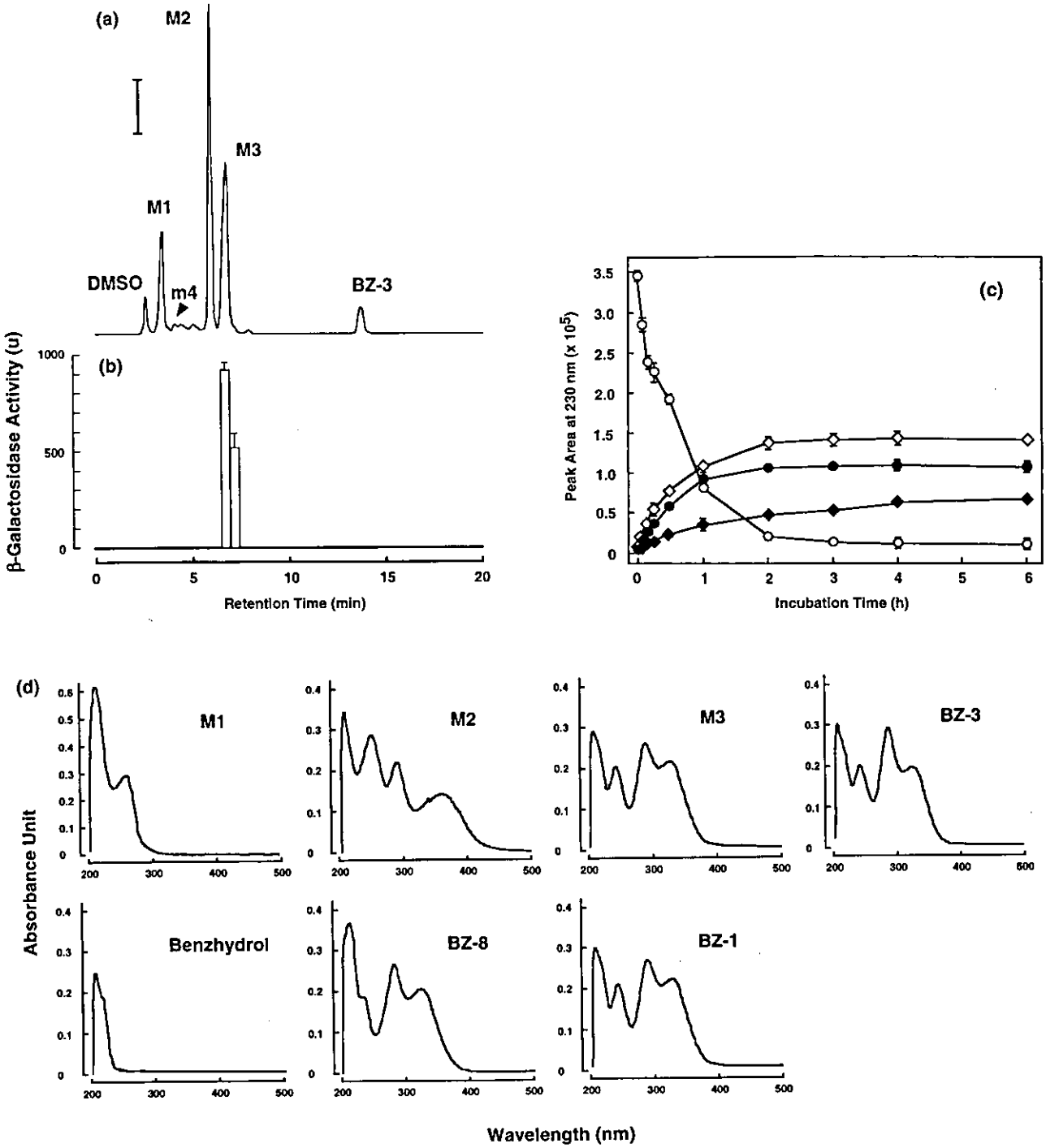

Fig. 3. The HPLC Analysis of the Metabolites of BZ-3 (a)

Bar expresses 0.2 auf. at 230 nm. The retention times were as follows (min): DMSO, 2.6; M1, 3.5; m4 (2,3,4-trihydroxybenzophenone), 4.1; M2, 5.9; M3 (BZ-1), 6.8; BZ-8, 8.1; BZ-3, 13.8. The estrogenic activities of the fractions (b). Values are means of three separate experiments (bars: S.D.). Changes in the levels of BZ-3 and its major metabolites, M1, M2 and M3 (c). Points express the peak area of recorded at 230 nm. Values are means of three separate experiments (bars: S.D.). BZ-3, (○); M1, (◆); M2, (◇); M3,(●). The concentration of BZ-3 at zero time was $9.5 \pm 0.1 \times 10^{-5}$ M. The UV spectra of M1, M2, M3, BZ-3, benzhydrol, BZ-8 and BZ-1 in methanol (d).

major metabolites (M1, M2 and M3) were detected (Fig. 3a). The retention time of a minor metabolite (m4) corresponded to that of 2,3,4-trihydroxybenzophenone. Each fraction was also examined for its estrogenic activity using the yeast assay system. The fractions containing M3 exhibited estrogenic activities (Fig. 3b). M1, M2 and M3 produced from BZ-3 concurrently (Fig. 3c). UV spectra of metabo-

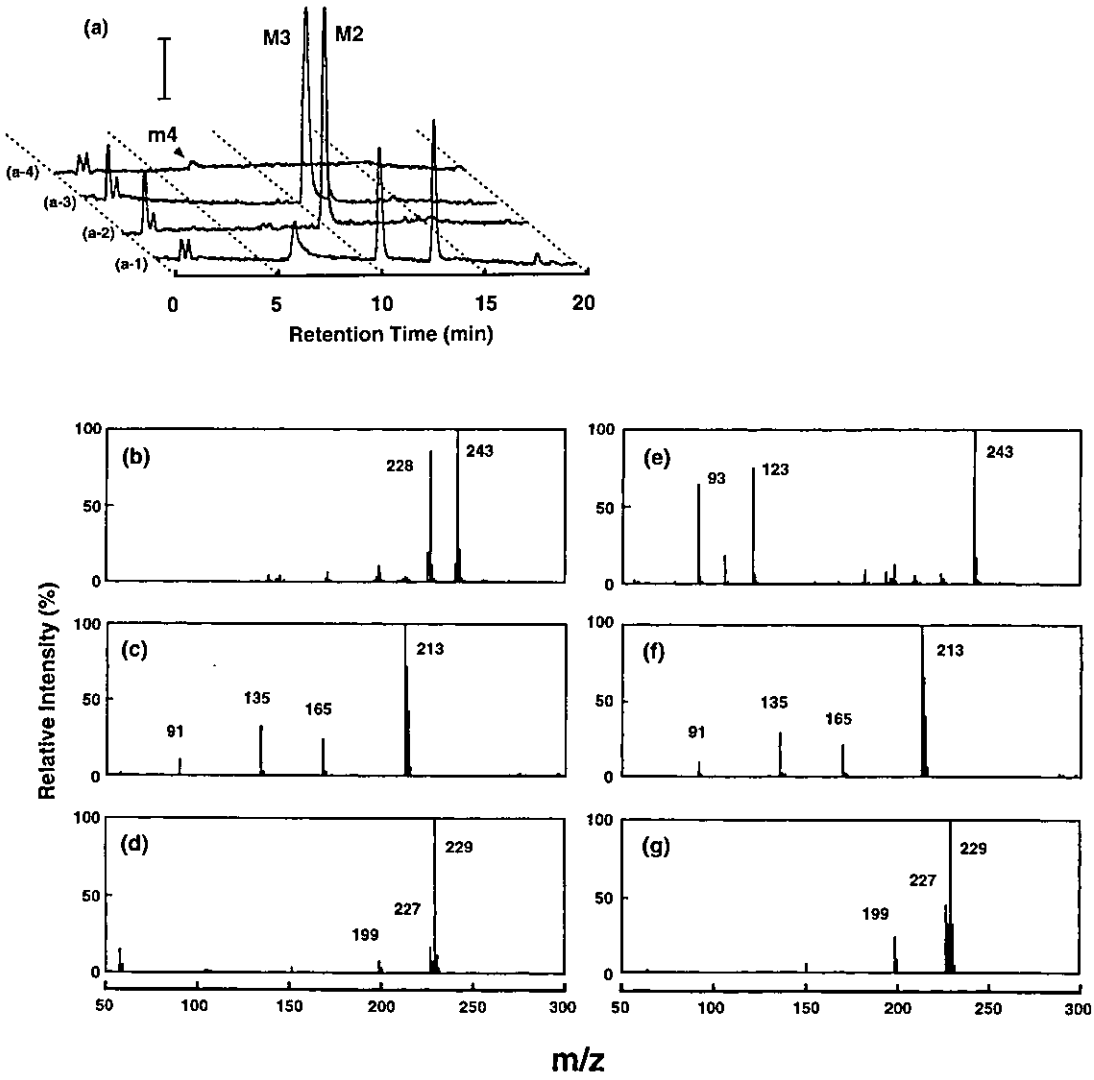

Fig. 4. Total Ion Chromatograms of BZ-3, BZ-8, BZ-1, 2,3,4-Trihydroxybenzophenone, M2, M3 and m4 (a)

Mixture of benzophenone derivatives containing 2.0×10^{-5} M BZ-3, BZ-8, BZ-1, and 2,3,4-trihydroxybenzophenone (a-1), M2 (a-2), M3 (a-3), m4 (a-4). Bar expresses the intensity of 1.0×10^{8} cps. The retention times were as follows (min): m4 (2,3,4-trihydroxybenzophenone), 6.7; M2, 10.0; M3 (BZ-1), 10.9; BZ-8, 13.5; BZ-3, 18.5. The mass spectra of M2 (b), M3 (c), m4 (d), BZ-8 (e), BZ-1 (f) and 2,3,4-trihydroxybenzophenone (g).

lites, BZ-3, benzhydrol, BZ-8 and BZ-1 in methanol were exhibited in Fig. 3d. Both benzhydrol and M1 did not have absorption above 280 nm. M2 absorbed from 200 to 440 nm with maxima at 290 and 360 nm and minima at 277 and 326 nm. M3 absorbed from 200 to 400 nm with maxima at 289 and 325 nm and minima at 262 and 312 nm. The profiles of UV spectrum and retention time in HPLC analysis of M3 were identical to those of BZ-1. After incubation of 1.0×10^{-4} M BZ-3 with the active S-9 mix for 4 hr, the concentrations of M3 (BZ-1) and BZ-3 were $3.3 \pm 0.1 \times 10^{-5}$ and $2.0 \pm 0.2 \times 10^{-6}$ M, respectively. To obtain further information about the metabolites, LC/MS analysis was also performed. The total ion chromatograms (negative ion mode) of M2, M3 and m4 were exhibited Fig. 4a. The mass spectra of M2, M3, m4, BZ-1 and 2,3,4-trihydroxybenzophenone were shown in Figs. 4b–4g, respectively. The base peak was detected at *m/z* 243 in the mass spectrum of M2. The mass spectra of M3 and m4 were identical to those of BZ-1 and 2,3,4-trihydroxybenzophenone, respectively. M1 did not detectable in both negative and positive ion mode.

DISCUSSION

P450 enzymes in the active S-9 mix are able to convert E_2 to its oxidative metabolites.[20] The dose–

response curves of E_2 after the incubation with the active S-9 mix were almost identical to that after the incubation with the inactive S-9 mix containing medium (Figs. 2a and b). Under these conditions, degradation of E_2 in the active S-9 mix containing medium was not observed (data not shown). The active S-9 mix fraction is able to mix with yeast cells suspended in 2 × S.D. medium without the need for additional measures.

The hydroxyl group of E_2 at the 3-position plays an important role in activating the ER.[21] Without the S-9 mix activation, benzophenone derivatives with a hydroxyl group at the 4-position, BZ-1 and 2,3,4-trihydroxybenzophenone, exhibited estrogenic activities. Some compounds containing phenol residues, such as *p*-alkylphenols, parabens and bisphenol A, exhibit estrogenic activities.[9,22–24] The phenol residues of these compounds are believed to participate in mimicking E_2 at the ER ligand-binding domain.[9] The phenol residue in benzophenone derivatives would also play such a role in activating ER. MXC, benzophenone, BZ-3 and BZ-8 exhibited estrogenic activities after incubation with the active S-9 mix. MXC is designated as a proestrogen for its conversion to an estrogenic metabolite, 2,2-*bis*(hydroxyphenyl)-1,1,1-trichloroethane.[12] It was also confirmed by us that benzophenone after metabolic activation showed estrogenic activity as well in the proliferation assay and the reporter gene assay using cultured cells (data not shown). This assay system is applicable for the detection of the proestrogens. We demonstrated that BZ-3 was converted to an estrogenic metabolite BZ-1 by incubation with the active S-9 mix. BZ-8 has an additional hydroxyl group at 2′-position of BZ-3. The estrogenic activities of its metabolites would depend on 2,2′,4-trihydroxybenzophenone and/or metabolites with hydroxyl group at a 4′-position. With both incubation with the inactive and the active S-9 mix, BZ-12 exhibited no estrogenic activity. The bulky octyloxy group moiety may prevent hydroxylation and/or ER activation.

BZ-3 is metabolized to BZ-1, BZ-8 and 2,3,4-trihydroxybenzophenone *in vivo*.[2–5] In the assay system, BZ-3 was metabolized to M1, M2, M3 (BZ-1) and m4 (2,3,4-trihydroxybenzophenone). The structures of M1 and M2, non-estrogenic metabolites, were inferred by examination of their UV spectra and LC/MS analysis. M1 was assumed to be a benzhydrol derivative for its lack of absorption above 280 nm. Benzhydrol could be one of the major metabolites of benzophenone in hepatocytes,[2–5] and had no absorption above 250 nm in methanol. Instead of formation of BZ-8, an unknown metabolite, M2, was detected. Compared to BZ-3, the UV spectrum of M2 was shifted to a longer wavelength. The mass spectrum suggested a molecular weight (M.W.) for M2 of 244. The M.W. of BZ-3 is 228.2. The UV and mass spectra of M2 were quite different from those of BZ-8 (Figs. 3d, 4b and 4e). M2 would be formed by the hydroxylation in the aromatic ring with methoxy and hydroxyl groups of BZ-3.

BZ-3 is used in many cosmetics and sunscreens as a UV-absorber.[1] The compound can be absorbed topically and converted to an estrogenic metabolite, BZ-1.[25,26] We demonstrated that BZ-3 was converted to the estrogenic metabolite, BZ-1, in a 33% yield by incubation with S-9 mix for 4 hr. At the same condition, 2,3,4-trihydroxybenzophenone was produced in a less than 1% yield (data not shown). Thus, the yield of non-estrogenic metabolites including M1 and M2 based on BZ-3 was approximately 60%. From 1 to 10% of BZ-3 in cosmetic products penetrates human skin.[25,26] These facts suggest that BZ-1 is produced *in vivo* by those applying a sunscreen or a cosmetic containing BZ-3. UV absorbers are increasingly used as a result of growing concern about UV irradiation and skin cancer. Schlumpf, *et al.* reported that other UV absorbers, such as 4-methyl-benzylidene camphor and octyl-methoxycinnamate, also exhibit estrogenic activity.[27] Studies of the effects on endocrine systems by UV absorbers should be performed more extensively, because of their use in children.

This assay system was able to detect the conversion of BZ-3 to an estrogenic metabolite in a minimum number of steps. This yeast two-hybrid system is able to evaluate the effects of chemicals on thyroid hormone receptors and androgen receptors by changing pairs of the receptors and coactivators to the relevant pairs.[8] Studies of the thyromimetic and anti-thyromimetic activities of metabolites of chemicals are ongoing in our laboratory. This assay system will be a useful tool for the detection of prohormonal activities of chemicals.

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Prepubertal Zearalenone Exposure Suppresses *N*-Methyl-*N*-nitrosourea–Induced Mammary Tumorigenesis but Causes Severe Endocrine Disruption in Female Sprague-Dawley Rats

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Abstract: The effect of prepubertal exposure to zearalenone, an estrogenic mycotoxin, on N-*methyl*-N-*nitrosourea (MNU)-induced mammary tumorigenesis and its influence on reproductive organs were examined in female Sprague-Dawley rats. Prepubertal rats were treated daily with either 0.1 or 10 mg/kg body weight of zearalenone between 15 and 19 days of age and compared with zearalenone-untreated animals (30 rats in each group). Six rats in each group were autopsied at 28 days of age, and their growth was evaluated. All remaining rats were given 50 mg/kg body weight MNU at 28 days of age and followed by monitoring for occurrence of mammary tumors ≥1 cm in diameter. Zearalenone did not affect body weight increase, and mammary glands showed similar development at 28 days of age (time at carcinogen administration). Both low- and high-dose zearalenone treatment significantly reduced incidence of mammary tumors ≥1 cm in diameter but did not influence latency (time between MNU administration and harvest of mammary tumor ≥1 cm in diameter) compared with untreated controls. Zearalenone dose dependently suppressed the number of histologically detected tumors (carcinomas) and multiplicity; the suppression was significant with high-dose treatment. However, high-dose treatment caused significantly earlier vaginal opening, both low- and high-dose treatment significantly caused irregularity of estrous cycle (persistent estrus or prolonged diestrus) at 8 to 10 wk of age, and zearalenone dose dependently increased the number of anovulatory rats (ovaries without newly formed corpora lutea) at 37 wk of age. Thus, short-duration zearalenone treatment in the prepubertal period suppressed subsequent mammary cancer occurrence but also severely damaged ovarian functions. This suggests that ingestion of foods containing zearalenone in the infantile period can have dramatic effects in later life.*

Introduction

Zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcyclic acid-lactone] is a mycotoxin synthesized by *Fusarium* molds. Zearalenone is found in a variety of host plants and debris from soil around the mold and is present as a natural contaminant in food as a result of infection of grain by *Fusarium* species. Contamination of grains by zearalenone has been reported worldwide (1). Thus, humans may be directly exposed to considerable quantities of zearalenone by ingestion of contaminated grain products (2). Zearalenone is often a contaminant of grains commonly fed to livestock and, once ingested by animals, is reduced to zeranol by 3α-hydroxysteroid dehydrogenase (3). Zearalenone resembles 17β-estradiol, allowing it to bind to estrogen receptors in target cells, and exerts estrogenic action (4,5); zeranol has greater estrogenic potency (6). Zearalenone and zeranol have been widely used to promote growth of livestock in the United States due to their potent anabolic effects (7–10). Thus, zearalenone or zeranol could enter the food chain via contaminated meat products, resulting in indirect exposure of humans.

Estrogens have long been recognized as important mitogens in the breast and are associated with increased risk of breast cancer. Estrogens and estrogenic compounds can have considerable effects on humans and animals during critical periods of development. The perinatal period is most sensitive to estrogenic chemicals, and human data indicate that high maternal exposure to estrogen during pregnancy increases the risk of breast cancer among daughters (11,12). Zearalenone is estrogenic, stimulates growth of MCF-7 human breast cancer cells (13), and functions as an antiapoptotic agent (14). When administered to suckling rats, zearalenone increases frequency of spontaneous mammary tumors (15) but reduces frequency of 7,12-dimethylbenz(α)anthracene (DMBA)–induced mammary tumors (16). The

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available experimental evidence regarding effects of zearalenone consumption in the early period of development on breast cancer risk is inconsistent.

There has been increasing concern about the impact of exposure to environmental compounds with hormone-like action during critical periods on human development and reproductive health. Zearalenone disrupts reproductive function when given to neonatal mice (17). Risk assessment of endocrine disruption by xenoestrogens and their metabolites requires accurate evaluations of their estrogenic potency. However, effects of zearalenone on breast cancer risk and reproductive system functions have not been analyzed simultaneously. The object of the present study was to examine effects of prepubertal administration of zearalenone on occurrence of *N*-methyl-*N*-nitrosourea (MNU)–induced mammary tumors and reproductive function in female Sprague-Dawley rats.

Materials and Methods

Animals

Ninety 14-day-old female Sprague-Dawley rats (10 pups per nursing mother) were obtained from Charles River Japan (Atsugi). To avoid endocrine-disrupting chemicals in the breeding condition, rats were housed in standard rat polyisopentene cages (TPX, Charles River Japan) with sterilized white pine chips (White Flake, Charles River Japan, Yokohama) as bedding. To avoid phytoestrogens in the diet, rats were fed the low-phytoestrogen diet NIH-07 PLD (Oriental Yeast, Chiba, Japan), which effectively reduces adverse endocrine-disrupting actions (18), and water was supplied in polycarbonate bottles with rubber stoppers throughout the experiment. Thus, known endocrine-disrupting agents were eliminated from the environment of the rats.

Chemicals

Zearalenone was purchased from Sigma (St. Louis, MO). The purity was 99%, as analyzed by thin-layer chromatography. It arrived in powder form and was kept at 0°C in the dark. Immediately before use, zearalenone was dissolved in 100% dimethylsulfoxide (DMSO; purity ≥ 99%, Nacalai Tesque, Kyoto) and stored at 4°C.

MNU was obtained from Nacalai Tesque. Upon arrival, it was kept at –20°C in the dark. MNU was dissolved in physiological saline containing 0.05% acetic acid.

Experimental Procedures

Animals were randomly divided into 3 groups of 30 animals each. From 15 to 19 days of age, two groups received a daily subcutaneous injection of zearalenone at 0.1 (low-dose group) or 10 (high-dose group) mg/kg body weight, and the third group (untreated controls) received an equal volume of the vehicle (100% DMSO). Human exposure to zearalenone in the United State is 1–5 mg/day (0.02–0.1 mg/kg body weight per day) (19). Thus, a physiological dose (0.1 mg/kg/day) and a pharmacological dose 100 times greater than the physiological dose (10 mg/kg/day) were selected for the present study. Rats were weaned at 21 days of age, and animals were then observed daily for vaginal opening (puberty onset). At 28 days of age, six randomly selected rats per group were autopsied to assess effects of zearalenone. On the same day, the remaining rats (24 rats per group) received an intraperitoneal injection of freshly prepared MNU at 50 mg/kg body weight. They were then housed in groups of three or four animals and examined weekly for mammary tumor development. Body weight was recorded weekly from the 2nd wk of age to the end of the experiment, and growth rate was compared among groups. The Animal Experimentation Committee at Kansai Medical University approved all procedures involving animals.

Evaluation of Growth at Time of Carcinogen Exposure

At the time of MNU exposure (28 days of age), six animals in each group were killed by an overdose of ether followed by cervical dislocation, and the effect of prepubertal zearalenone exposure was checked. Uterus and ovaries were weighed, and formalin-fixed samples of uterus, vagina, ovaries, and mammary gland were sectioned at 4 μm and stained with hematoxylin and eosin (HE). Mammary gland growth and differentiation were also evaluated on whole-mount sections. Mammary whole mounts were prepared using the inguinal mammary gland (gland pair number 4). Digital images of whole mounts were obtained with a flat-bed, color-image scanner (GT-9400UF, Seiko Epson, Nagano), and quantitative analysis of mammary gland dimensions was performed using Adobe Illustrator version 10.0.3 (San Jose, CA). The distance between the outermost edge of the lymph node and the furthest extension of the terminal end buds beyond the node was measured.

Mammary Tumor Detection

To determine the effect of prepubertal zearalenone exposure on mammary tumorigenesis, MNU-treated animals were examined. All animals were palpated weekly for mammary tumors, and tumor locations were recorded. A rat was autopsied when its largest tumor grew to ≥1 cm in diameter. All surviving animals were killed 33 wk after MNU, and the experiment was terminated. At autopsy, all visible mammary tumors were dissected, fixed in 10% neutral buffered formalin, and stained with HE. In addition, normal-appearing mammary glands were dissected and processed to produce routine histological preparations to detect microscopic tumors. Histopathology of all mammary tumors of all sizes was evaluated from HE-stained sections. The histological criteria for identification of mammary tumors were based on those of Russo et al. (20). Data analysis included the number of animals with gross mammary tumors (≥1 cm) and latency (from MNU administration until largest mammary tumor reached

≥1 cm in diameter). Also, numbers of histologically detected mammary tumors (total number of tumors and carcinomas) and the number of tumors and carcinomas per animal (multiplicity) were analyzed.

Evaluation of Reproductive Organs During Tumorigenesis

With MNU-treated rats, vaginal opening was checked, and estrous cyclicity was monitored by examination of vaginal smears (21) performed at the same time daily from 8 to 10 wk of age. Estrous cycles were classified as follows: 1) normal length, 4 or 5 days; 2) persistent estrus, irregular cycle in which estrus phase constitutes >50% of the observation period; and 3) prolonged diestrus, irregular cycle in which the interestrus period lasts ≤9 days. When rats were sacrificed, ovaries were weighed, and reproductive organs were fixed in neutral buffered formalin, embedded in paraffin, sectioned, and stained with HE.

Statistical Analysis

All data were expressed as mean ± SE. Cumulative incidence of gross mammary tumor (≥1 cm) was analyzed by the Mantel-Cox log rank test. Patterns of estrous cycle, weight of the ovaries with or without corpora lutea, and tumor incidence at the final time point were analyzed by χ^2 test. For all other data, after assurance of homogeneity of variance, analysis was performed using nonrepeated-measure analysis of variance parametric test or Kruskal-Wallis nonparametric test. If the P value of these pretests was <0.05, post hoc analysis was performed using Fisher's protected least significant difference test. Differences between groups were considered significant if the P value was <0.05.

Results

Body Weight Gain and Growth at Carcinogen Exposure

Prepubertal exposure to zearalenone did not affect body weight gain (Fig. 1). The doses of zearalenone used were not toxic; there were no moribund animals. At 28 days of age (time at carcinogen administration), no animals showed vaginal opening, differences in body weight among groups were not significant, and uterine–ovarian wet weight and relative uterine–ovarian weight (uterine–ovarian wet weight/body weight) were comparable among groups; histological sections of uterus, vagina, and ovaries showed no detectable difference. At that time point, whole mount preparations of mammary gland showed similar differentiation (Fig. 2); mammary ducts ended in club-shaped terminal end buds and alveolar differentiation was seen proximal to the nipple. Quantitative analysis of the growth of inguinal mammary glands revealed no statistically significant difference in mean ductal elongation (distance between outermost edge of lymph node and furthest extension of terminal end buds beyond node) among groups.

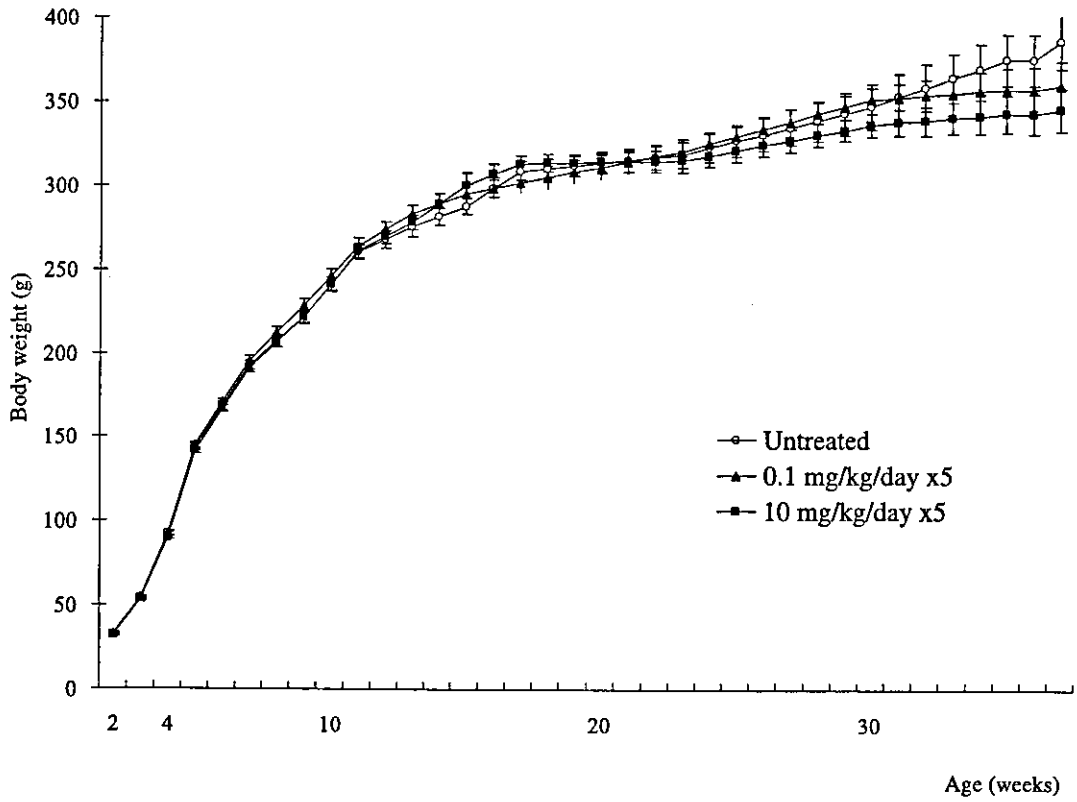

Figure 1. Body weight changes in female Sprague-Dawley rats treated daily from 15 days of age for five times with 0.1 or 10 mg/kg zearalenone or without zearalenone (untreated) and injected with 50 mg/kg MNU at 28 days of age. Values represent mean ± SE.

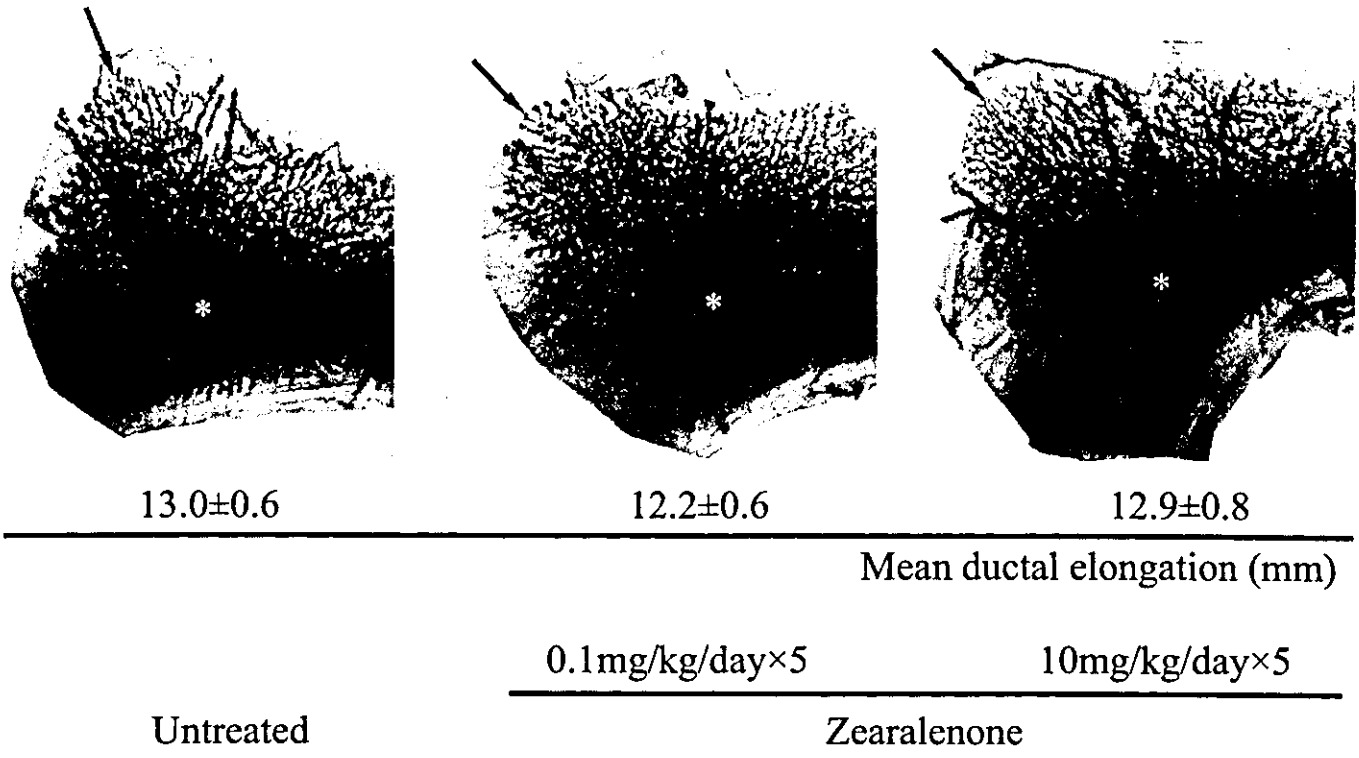

Figure 2. Representative inguinal mammary whole mounts of 28-day-old female Sprague-Dawley rats and mean ductal elongation (distance between [*], outermost edge of lymph node; [↘], furthest extension of terminal end buds beyond node). Each group consists of six rats. Values represent mean ± SE.

Mammary Tumorigenesis

As shown in Fig. 3, gross mammary tumors (≥1 cm) developed 13 wk after MNU in zearalenone-untreated controls and 15 wk after MNU in low- and high-dose zearalenone-treated groups. Log rank test analysis indicated that cumulative gross mammary tumor incidence was significantly lower in both high- and low-dose zearalenone-treated groups compared with untreated controls ($P < 0.05$). Gross mammary tumor incidence at the final time point was significantly reduced by zearalenone treatment (Table 1), whereas latency was not significantly affected. All sizes of histologically detected mammary tumors were analyzed. Histopathological examination revealed that the mammary tumors sampled were all either adenocarcinoma or fibroadenoma. Total numbers of histologically detected mammary tumors and mammary carcinomas, of all sizes, were analyzed separately. Data for histologically detected mammary tumorigenesis are summarized in Table 2. Zearalenone dose dependently inhibited mammary tumor yield as evaluated by total number of tumors and tumors per rat (tumor multiplicity); the same result was obtained when analysis was restricted to mammary carcinoma yield. High-dose zearalenone significantly suppressed mammary tumor (carcinoma) yield; low-dose zearalenone also lowered mammary tumor (carcinoma) yield but not significantly. Leukemia was observed in one low-dose zearalenone-treated mammary tumor-bearing rat sacrificed 26 wk after MNU, but no other non-mammary tumors were seen in the present experiment.

Reproductive Organ Function and Structure

Vaginal opening (puberty onset) occurred at 31 to 37 days of age. Zearalenone accelerated vaginal opening. Compared with zearalenone-untreated rats, 10 mg/kg zearalenone-treated rats (but not 0.1 mg/kg treated rats) had significantly earlier vaginal opening (Table 3). Vaginal smears taken from 8 to 10 wk of age indicated that, whereas all zearalenone-untreated animals had a normal 4- to 5-day cycle, both low- and high-dose zearalenone-treated groups had a significant decrease in the number of normal-cycling animals, with a majority of animals exhibiting persistent estrus or prolonged diestrus. In zearalenone-treated and MNU-injected rats sacrificed at the termination of the experiment (Table 4), zearalenone significantly ($P < 0.05$) and dose dependently increased the frequency of rats with no newly formed corpora lutea in the ovaries, indicating anovulation; large follicles and ceroid deposition often accompanied anovulation. Ovaries without corpora lutea tended to weigh less than those with corpora lutea, but the difference was not significant. Zearalenone treatment caused no apparent histological changes in vagina or uterus. Prepubertal zearalenone treatment disrupted endocrine function and ovarian structure in adulthood.

Discussion

Timing and dosage of exposure of immature animals to xenoestrogens influence development of mammary tumors.

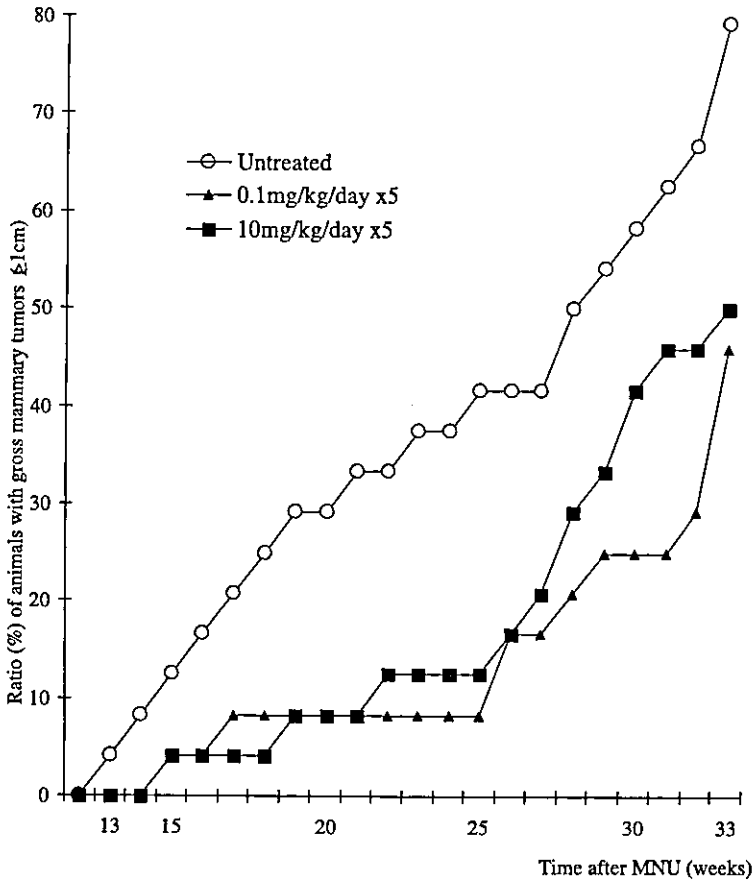

Figure 3. Cumulative incidence of gross mammary tumors (≥1 cm) in female Sprague-Dawley rats treated with 50 mg/kg MNU at 28 days of age.

The phytoestrogen genistein reduces frequency of DMBA-induced mammary tumors in rats when administered at prepuberty at a physiological dose (≤2 mg/kg × 4) (16) or pharmacological dose (500 mg/kg ×3) (22). Treatment with 10 mg/kg zearalenone at 7 or 14 days of age increases the incidence of spontaneous mammary tumors in Wistar rats (15), whereas treatment with ≤2 mg/kg zearalenone at 7, 14, 17, or 20 days of age decreases incidence of DMBA-induced mammary tumors in Sprague-Dawley rats (16). A high-estrogenic environment in utero may increase subsequent breast cancer risk, as indicated by the finding that frequency of mammary tumors dose dependently increases when genistein is administered in the prenatal period (23). However, in utero exposure to zearalenone did not influence incidence of DMBA-induced mammary tumors (23). Xenoestrogens may have different effects on breast cancer risk depending on the timing and dosage of exposure.

In the present study, both low (0.1 mg/kg × 5) and high (10 mg/kg × 5) doses of zearalenone administered during the prepubertal period (15–19 days of age) effectively suppressed occurrence of MNU-induced mammary tumors (carcinomas) in female Sprague-Dawley rats. This finding is consistent with those of a previous study using a DMBA model (16) in which a physiological dose (0.1 mg/kg) and a pharmacological dose (10 mg/kg) of zearalenone reduced MNU-induced mammary tumorigenesis. Increased differentiation of mammary glandular structure at the time of carcinogen exposure may play a critical role in reducing mammary cancer risk (24). Suppression of DMBA-induced mammary tumors by prepubertal zearalenone may be due to the increased differentiation of the

Table 1. Effect of Prepubertal Zearalenone Treatment on Gross Mammary Tumors (≥1 cm) Induced by MNU in Female Sprague-Dawley Rats

Zearalenone Treatment	No. of Rats	No. of Rats with Tumors ≥ 1 cm (%)	Latency (wk)[a]
Untreated	24	19 (79)	24.1 ± 1.7
0.1 mg/kg/day × 5	24	11 (46)[b]	27.7 ± 1.9
10 mg/kg/day × 5	24	12 (50)[b]	26.5 ± 1.5

a: Interval between MNU administration and the week the largest palpable tumor reached a diameter of ≥1 cm (mean ± SE).

b: $P < 0.05$ compared with untreated control.

Table 2. Effect of Prepubertal Zearalenone Treatment on Histologically Detected Mammary Tumors and Mammary Carcinomas Induced by MNU in Female Sprague-Dawley Rats[a]

Zearalenone Treatment	No. of Rats	No. of Tumors	Tumors per Rat	No. of Carcinomas	Carcinomas per Rat
Untreated	24	84	3.5 ± 0.4	74	3.1 ± 0.3
0.1 mg/kg/day × 5	24	54	2.3 ± 0.5	50	2.1 ± 0.5
10 mg/kg/day × 5	24	44	1.8 ± 0.6[b]	34	1.4 ± 0.6[b]

a: Values represent mean ± SE.
b: $P < 0.05$ compared with untreated control.

Table 3. Effect of Prepubertal Zearalenone Treatment on Reproductive Organ Function in Female Sprague-Dawley Rats Administered MNU at 28 Days of Age[a]

			Patterns of Estrous Cycle (%)		
Zearalenone Treatment	No. of Rats	Age at Vaginal Opening (days)	Normal Cycle	Persistent Estrus	Prolonged Diestrus
Untreated	24	35.3 ± 0.3	24 (100)	0	0
0.1 mg/kg/day × 5	24	34.5 ± 0.3	3 (13)[b]	11 (46)[b]	10 (41)[b]
10 mg/kg/day × 5	24	34.3 ± 0.3[c]	3 (13)[b]	13 (54)[b]	8 (33)[b]

a: Values represent mean ±SE. Estrous cycle was monitored from 8 to 10 weeks of age.
b: $P < 0.01$ compared with untreated controls.
c: $P < 0.05$ compared with untreated controls.

Table 4. Effect of Prepubertal Zearalenone Treatment on Ovarian Structure in Female Sprague-Dawley Rats Administered MNU at 28 Days of Age and Sacrificed at 37 Weeks of Age[a]

			Weight of Ovaries (mg)	
Zearalenone Treatment	No. of Rats	Rats without Corpora Lutea (%)	With Corpora Lutea	Without Corpora Lutea
Untreated	8	1 (13)	110 ± 12	57
0.1 mg/kg/day × 5	17	8 (47)	102 ± 12	79 ± 15
10 mg/kg/day × 5	13	9 (69)	89 ± 22	63 ± 6

a: Values represent mean ±SE.

mammary epithelial tree at the time of carcinogen exposure (16). Zeranol, a more potent estrogen than zearalenone, stimulates developmental growth of ovariectomized mouse mammary gland (25). Human exposure to carcinogenic stimuli can occur at any time of a woman's life. MNU can induce mammary tumors in sexually immature rats (26). In the present study, MNU was administered before the onset of puberty (at 28 days of age), and, at this time point, results of qualitative analysis of mammary gland differentiation and those of quantitative analysis of mammary gland growth did not significantly differ between females not exposed to zearalenone and those exposed daily to zearalenone at 15–19 days of age. In neonatal sex hormone–treated rats, corpora lutea were absent, showed persistent estrus, and reduced DMBA-induced mammary tumors; progesterone treatment accelerated mammary tumorigenesis (27). Although the mechanisms of suppression of mammary tumorigenesis by zearalenone require further study, altered progesterone levels may have influenced MNU-induced mammary tumorigenesis.

Zearalenone has strong estrogenicity, as indicated by in vivo bioassays (5). Thus, detailed analysis of estrogen-responsive reproductive system functions and structure after zearalenone exposure is of clinical importance. Prepubertal zearalenone treatment has been shown to have profound effects on reproductive functions of the female rat. In the present study, we found that prepubertal zearalenone exposure accelerated vaginal opening. Neonatal administration of zearalenone causes persistent estrus and sterility in mice, with structural changes in ovaries (17,28). Although we did not see structural changes in female genital organs at 28 days of age (time at carcinogen exposure), even low-dose (0.1 mg/kg) prepubertal zearalenone exposure induced profound disruption of estrous cyclicity in a considerable number of animals examined at 8 to 10 wk of age. Neonatal exposure of 3- to 5-day-old rats to zearalenone at 1.0 mg/kg produces persistent anovulatory estrus in adults, with large follicles without newly formed corpora lutea in the ovary (29). In ovaries examined at 37 wk of age (termination of the experiment), zearalenone dose dependently decreased numbers of newly formed corpora lutea accompanied by large follicles. High frequency of anovulation was seen in the present zearalenone-treated animals. Infertility in rats significantly increases after daily oral administration of zearalenone at 10 mg/kg (30). Neonatal zearalenone exposure in mice results in

absence of uterine stromal glands and preneoplastic and/or neoplastic changes in the cervicovaginal epithelium (28). In the present study, uterus and vagina did not show zearalenone-related abnormality. Taken together, the present data indicate that zearalenone causes endocrine disruption, leading to anovulation in female rats.

Zearalenone lacks tumorigenic potential when fed to ≥28-day-old rats for >100 wk (31), and blood levels of zearalenone in human adults do not indicate a causal relationship between exposure and development of breast carcinoma (32). However, exposure of prepubertal children to zearalenone may pose a risk when the endogenenous estrogen level is low (33). Exposure to zearalenone at a physiological dose during a critical period of life can have irreversible effects on the female reproductive system. In conclusion, prepubertal exposure to zearalenone, at a dose comparable with that consumed by Americans, suppressed mammary carcinomas, but its profound effect on ovarian function in female rats indicates that consumption of zearalenone-containing food in the prepubertal period may have clinically significant consequences.

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Effect of Prenatal and Prepubertal Genistein Exposure on N-Methyl-N-nitrosourea-induced Mammary Tumorigenesis in Female Sprague-Dawley Rats

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Abstract. *Background: The effect of prenatal and prepubertal genistein exposure on the development of chemically-induced mammary carcinomas in rat was investigated. Materials and Methods: Genistein was subcutaneously (s.c.) injected daily, from gestational days 15 to 19, into pregnant Sprague-Dawley rats at 0, 1.5 or 30 mg/kg/day. Female offspring of mothers not exposed to genistein during pregnancy received daily s.c. injection, from prepubertal days 15 to 19, at a dose of 1.5 or 30 mg/kg/day. At 28 days of age, 6 female offspring from each group were sacrificed to observe the influence of genistein and the remaining rats were injected intraperitoneally with 50 mg/kg N-methyl-N-nitrosourea (MNU). Rats injected with MNU were sacrificed at 26 weeks of age or when their largest mammary tumor was ≥1 cm in size. Results: At the time when MNU was administered, the different groups had comparable mammary gland development; genistein-treated and -untreated rats had similar numbers of terminal end buds (TEBs) at the periphery of the mammary glandular tree. However, estrogen receptor α (ERα)- and progesterone receptor (PgR)-positive cells, p63-positive cells and proliferating cell nuclear antigen (PCNA)-labeling index were lower in genistein-exposed TEBs. Although tumor multiplicity and latency were not significant, prenatal or prepubertal genistein exposure, at low or high dosage, tended to suppress the incidence of mammary carcinomas ≥1 cm; suppression was significant in the prepubertal low-dose group. Conclusion: The majority of the mammary carcinomas in the present study were hormone-dependent. The present findings suggest that administration of genistein in the perinatal period has protective effects against MNU-induced mammary carcinoma in Sprague-Dawley rats, via reduction of levels of ERα- and/or PgR-positive cells (presumed progenitor cells of mammary carcinomas), p63-positive mammary progenitor/stem cells (involved in cell renewal) and PCNA-positive cells (necessary for cell proliferation).*

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It has been suggested that dietary factors are important in the development of breast cancer (1, 2). There is geographic variation in risk of breast cancer. High intake of dietary fat is believed to contribute to the high incidence of breast cancer in the West. However, the causative relationship between high dietary fat intake and high incidence of breast cancer is controversial (3, 4). Another dietary component suspected to be linked to breast cancer is soy and soy-based products. The low incidence of breast cancer in Asians can be attributed in part to the high intake of soy products, which contain large amounts of phytoestrogens (5-7). Phytoestrogens found in plants and plant products include the flavonoids, lignans and other nonsteroidal chemicals. Plant isoflavones, including genistein, are the major phytoestrogens found in soy. Genistein resembles steroidal estrogens in structure and mimics many of their actions (8, 9).

Genistein has an estrogenic effect as an agonist for estrogen receptors (ERs) in cultured cells (10, 11), but acts as an antagonist at higher concentrations (12, 13). Sensitivity to estrogenic chemicals is greatest during the perinatal period. Maternal exposure to high doses of estrogen during pregnancy increases the risk of breast cancer among daughters (14, 15). Although several reports show that maternal exposure to genistein during pregnancy increases 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary tumorigenesis in female rat offspring (16, 17), another report shows that mammary tumorigenesis is not significantly affected by maternal exposure to genistein (18). In contrast, early pregnancy or early exposure to estrogen or estrogen-like chemicals reduces mammary cancer in women (19). Genistein administered neonatally (20) or prepubertally (21, 22) reduces the incidence of DMBA-induced mammary cancers; genistein administered to adult animals is only effective if they were exposed to genistein prepubertally (18). For protection to be

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achieved (decreased incidence of mammary tumorigenesis), genistein exposure must occur during the neonatal or prepubertal period; continuing exposure beyond the prepubertal period provides additional protection (23). The stage of mammary gland differentiation at the time of carcinogenic stimuli determines sensitivity of the mammary gland to neoplastic transformation. Thus, it appears that both the timing and dosage of genistein are critical. In rats, neonatal and prepubertal genistein enhance mammary gland differentiation, specifically cleavage of terminal end buds (TEBs) into alveolar buds (ABs) (20, 21). TEBs are more susceptible to chemically-induced carcinogenesis than ABs and it appears that ABs are not the target of carcinogens (24). The available evidence indicates that chemically-induced mammary carcinogenesis in rats is suppressed when the carcinogen is administered after induction of mammary gland differentiation by genistein. In addition, ERα levels in the mammary gland may affect the susceptibility of the mammary gland to carcinogens (16). Furthermore, the stage of estrous cycle at which a carcinogen is administered may affect formation of tumors (25, 26). In humans, the initial events of breast carcinogenesis can occur at any age. N-Methyl-N-nitrosourea (MNU), a direct-acting carcinogen, can induce mammary carcinomas in sexually immature female rats (27). In the present study, the effects of prenatal and prepubertal genistein exposure on mammary tumorigenesis were evaluated in female Sprague-Dawley rats injected with MNU before the onset of puberty (before differentiation of structures of the mammary glandular tree).

Materials and Methods

Animals. Sprague-Dawley CD-timed pregnant rats were purchased from Charles River Japan (Hino, Japan). To avoid exposure to environmental endocrine disrupters, all rats were housed individually in standard rat polyisopentene cages (TPX, Charles River, Yokohama, Japan) with sterilized white pine chips (White Flake, Charles River, Yokohama, Japan) as bedding. NIH-07 PLD (phytoestrogen low diet, Oriental Yeast, Chiba, Japan) was used as food for pregnant and lactating rats and weaned pups; NIH-07 PLD was used because it was determined to be free of phytoestrogens (28). Water was supplied in polycarbonate bottles with rubber stoppers (Charles River). Thus, the known environmental endocrine disrupters were eliminated. The animals were kept at a temperature of 22±2°C, with 60±10% humidity and a 12-hour light/dark cycle. Food and water were provided *ad libitum*.

Chemicals. Genistein (4',5,7-trihydroxyisoflavone) purified from soybean was purchased from Fujicco (Kobe, Japan). Purity was >99%, as determined by high-performance liquid chromatography (HPLC). The compound was supplied in powder form and was kept at 0°C in the dark. Immediately before use, genistein was dissolved in dimethylsulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan) and stored at 4°C. MNU was obtained from Nacalai Tesque and was kept at -20°C in the dark. Immediately before use, MNU was dissolved in physiological saline containing 0.05% acetic acid.

Experimental procedures. For prenatal treatment, the pregnant rats were divided into 3 groups: group 1 (5 rats), injected with vehicle (DMSO) only; group 2 (6 rats), injected with 1.5 mg/kg/day genistein; group 3 (6 rats), injected with 30 mg/kg/day genistein. From gestational days 15 to 19, daily subcutaneous (*s.c.*) injections of genistein or vehicle were administered to these rats. Group 1 produced 26 female offspring; group 2 produced 30 female offspring; and group 3 produced 30 female offspring. For prepubertal treatment, 60 female offspring of mothers not exposed to genistein received daily *s.c.* injection of genistein from 15 to 19 days of age, at a dose of 1.5 mg/kg/day (group 4; 30 females) or 30 mg/kg/day (group 5; 30 females). In Asian populations with low rates of breast cancer, the average consumption of genistein is 20 to 80 mg/day, or 0.4 to 1.5 mg/kg/day for a 55-kg woman (29). Thus, genistein at a dose of 1.5 mg/kg is comparable to an average daily intake in these Asian countries. After the rats were weaned at 21 days of age, puberty onset and sexual maturation were determined by examining vaginal opening daily, and body weight was recorded weekly. At 28 days of age, 6 randomly-selected rats from each group were sacrificed to assess the effect of genistein, and a single intraperitoneal injection of 50 mg/kg MNU was administered to the remaining rats in each group. The animals were palpated once every week to detect the presence, location and size of mammary tumors. The rats were euthanized at 26 weeks of age or when their largest tumor was ≥1.0 cm in diameter. From 10 to 14 weeks of age, estrous cyclicity was monitored by examining vaginal smears. Estrous cycles were classified as normal (4- or 5-day), 3-day or 6-day, and percent time spent in each phase of the cycle was calculated. Animal care and treatments were conducted in accordance with guidelines and protocols approved by the Animal Experimentation Committee of Kansai Medical University, Japan.

Growth evaluation of mammary glands. Cervical, thoracic and inguinal mammary glands from one side of rats sacrificed at 28 days of age were processed to produce sections stained with hematoxylin and eosin (HE), and the corresponding glands from the other side were processed to produce whole-mount preparations stained with hematoxylin. These preparations were evaluated using established criteria (24). Uterus and ovaries were weighed and then processed to produce HE sections.

Mammary carcinogenesis. At the time of autopsy, all visible mammary tumors were dissected, fixed in 10% neutral buffered formalin, and stained with HE. In addition, apparently normal mammary glands were dissected and processed to produce routine histologic preparations, to detect microscopic tumors. We determined the percentage of rats with mammary tumors ≥1 cm (tumor incidence), the number of tumors (all sizes) per rat (tumor multiplicity), and the interval between MNU injection and harvest of mammary tumors ≥1cm (tumor latency). The histological criteria for identification of mammary tumors were based on those of Russo *et al.* (30).

Immunohistochemistry. The formalin-fixed paraffin-embedded slides from left inguinal mammary glands of 28-day-old rats and the largest mammary carcinomas from rats bearing mammary tumors ≥1 cm were processed for immunohistochemical evaluation of the number of ERα- and progesterone receptor (PgR)-positive cells, the number of p63-positive cells and the level of proliferating cell nuclear antigen (PCNA). Antibodies to ERα (6F11, Novocastra, Newcastle upon Tyne, UK), PgR

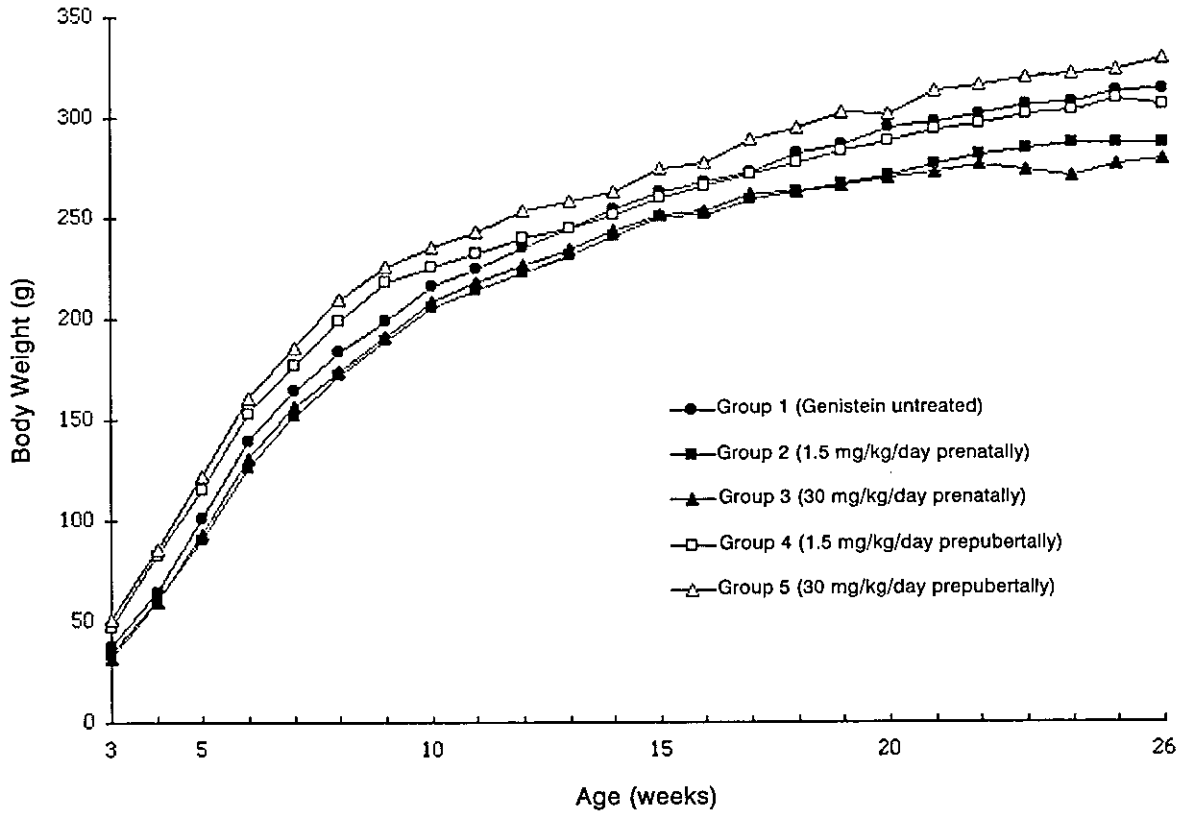

Figure 1. *Body weight gain in genistein-treated and -untreated Sprague-Dawley rats. Compared to controls, the body weight of prepubertally genistein-treated rats was greater and the body weight of prenatally genistein-treated rats was lower.*

(PR10A9, Immunotech, Marseille, France), p63 (4A4, NeoMarkers, Fremont, CA, USA) and PCNA (PC10, Novocastra) were used. The assay was performed using the labeled streptavidin-biotin (LSAB) method and an LSAB staining kit (DAKO, Carpinteria, CA, USA), in accordance with the manufacturer's instructions. Antigenicity was retrieved by treatment in a microwave oven (31). TEBs (terminal ductal structures with a diameter >100 μm and 3 to 6 epithelial layers) were evaluated. TEBs are considered to be the target of neoplastic transformation in carcinogen-treated rats. More than 1000 cells from more than 5 different TEBs and 5 different areas per cancer section, respectively, were counted, and the resulting data was used to calculate numbers of ERα- and/or PgR-positive cells, numbers of p63-positive cells and PCNA-labeling index. Mammary carcinomas containing more than 80% ERα- and/or PgR-positive cells were considered hormone-dependent (31). Photographs were obtained using a Fujix HC-2000 color digital camera and an Olympus AX-80 microscope.

Statistical analysis. All data were expressed as mean±SEM. Tumor incidence was analyzed by Mantel-Cox Log-rank test. The estrous cycle and hormone-dependency were analyzed for independence using the Chi-square test. For all other data, after assurance of homogeneity of variance, analysis was performed using the non-repeated measure ANOVA parametric test or Kruskal-Wallis non-parametric test. If the p value of these pre-tests was <0.05, *post-hoc* analysis was performed using Fisher's protected least significant difference test or Bonferroni/Dunn's test. Differences between groups were considered significant if the p value was <0.05.

Results

General remarks. Prenatal or prepubertal genistein exposure did not cause direct toxic effects to the animals, as determined from body weight gain (Figure 1). At 28 days of age (time at MNU administration), compared with untreated controls (group 1), rats exposed to genistein prenatally had lower body weight (groups 2 and 3), whereas rats treated with genistein prepubertally had greater body weight (groups 4 and 5) (Table I). All genistein-treated rats had lower relative uterine-ovarian weight than untreated controls; no apparent histological differences were detected. Vaginal opening (puberty onset) was observed from 32 to 40 days of age. Prepubertal 30 mg/kg genistein accelerated vaginal opening, compared with untreated controls (Figure 2), but timing of vaginal opening was not affected in other genistein-treated groups. Vaginal smears obtained from 10 to 14 weeks of age indicated that all

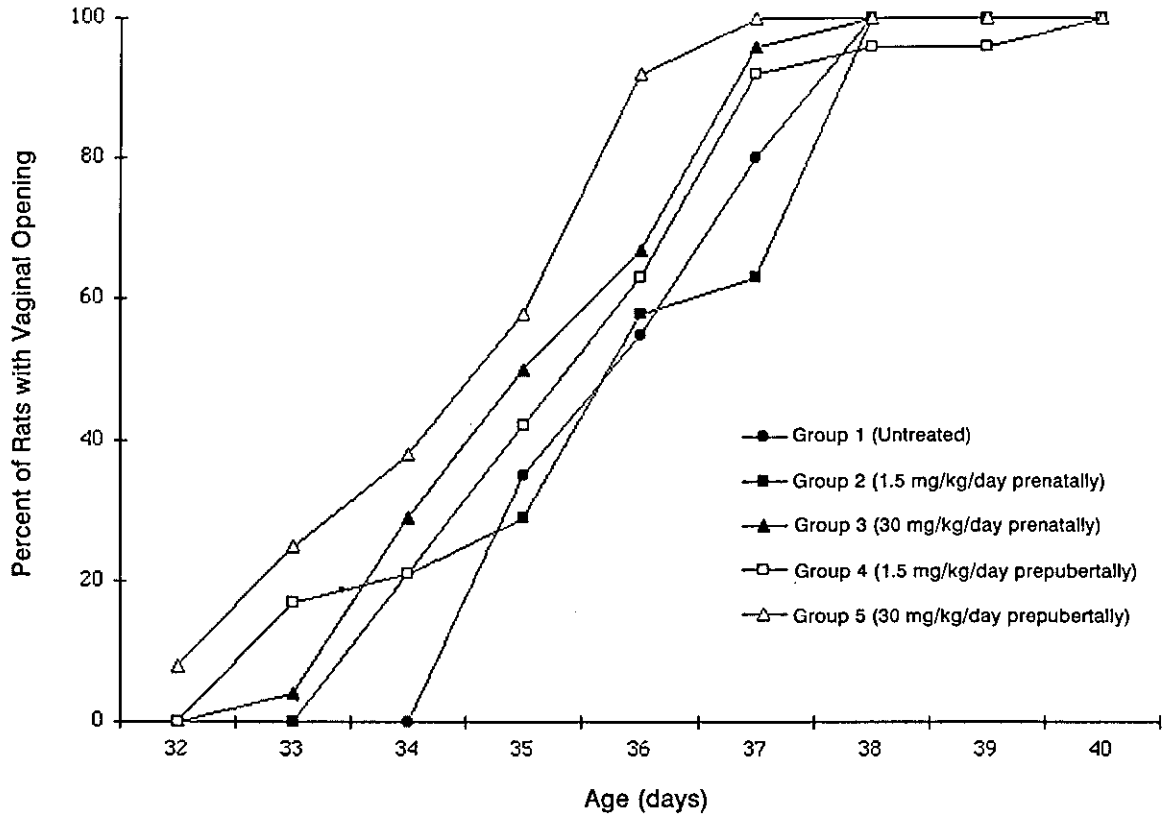

Figure 2. *Vaginal opening in genistein-treated and -untreated rats. The group treated prepubertally with a high dose of genistein exhibited earlier vaginal opening than untreated controls ($p<0.01$).*

Table I. *Effect of genistein on body weight and relative uterine-ovarian weight in female Sprague-Dawley rats at 28 days of age.*

Group	Treatment	Body weight (g)	Relative uterine-ovarian weight (mg/100g BW)
1	Untreated	65.5 ± 2.8	239 ± 13
2	1.5 mg/kg/day prenatally	57.3 ± 1.4*	184 ± 10*
3	30 mg/kg/day prenatally	59.2 ± 2.3*	163 ± 12**
4	1.5 mg/kg/day prepubertally	83.8 ± 2.3**	191 ± 14*
5	30 mg/kg/day prepubertally	82.2 ± 2.5**	213 ± 27

Each group consists of 6 rats. Values represent mean ± SEM.
* $p<0.05$ and ** $p<0.01$ *versus* group 1.

rats were cycling. However, although all untreated controls had a normal (4- to 5-day) cycle, 3-day and 6-day cycles were observed in genistein-treated groups (Figure 3). Prepubertal genistein treatment resulted in a prolonged estrus phase and a shortened diestrus phase (Table II).

Mammary gland development and tumor induction. At 28 days of age (time at MNU administration), all groups had comparable mammary gland development; similar numbers of TEBs were observed at the periphery of the mammary glands (Figure 4). Also at 28 days of age, genistein treatment significantly decreased the number of ERα- and/or PgR-positive cells and p63-positive cells, and lowered the PCNA-labeling index, compared with untreated controls (Table III). During the course of the present study, there were 2 unscheduled deaths due to weight loss (1 each in groups 3 and 5); these rats were omitted from the carcinogenic study. Histologically, almost all of the induced mammary tumors were adenocarcinomas. Four tubular adenomas were detected: one in group 1, one in group 3, and two in group 5. However, only tumors classified as adenocarcinomas were included in data analysis (Table IV). Genistein-treated rats had fewer mammary carcinomas ≥1 cm (tumor incidence) than genistein-untreated controls (Figure 5). Prepubertal administration of 1.5 mg/kg genistein (group 4) suppressed mammary cancer

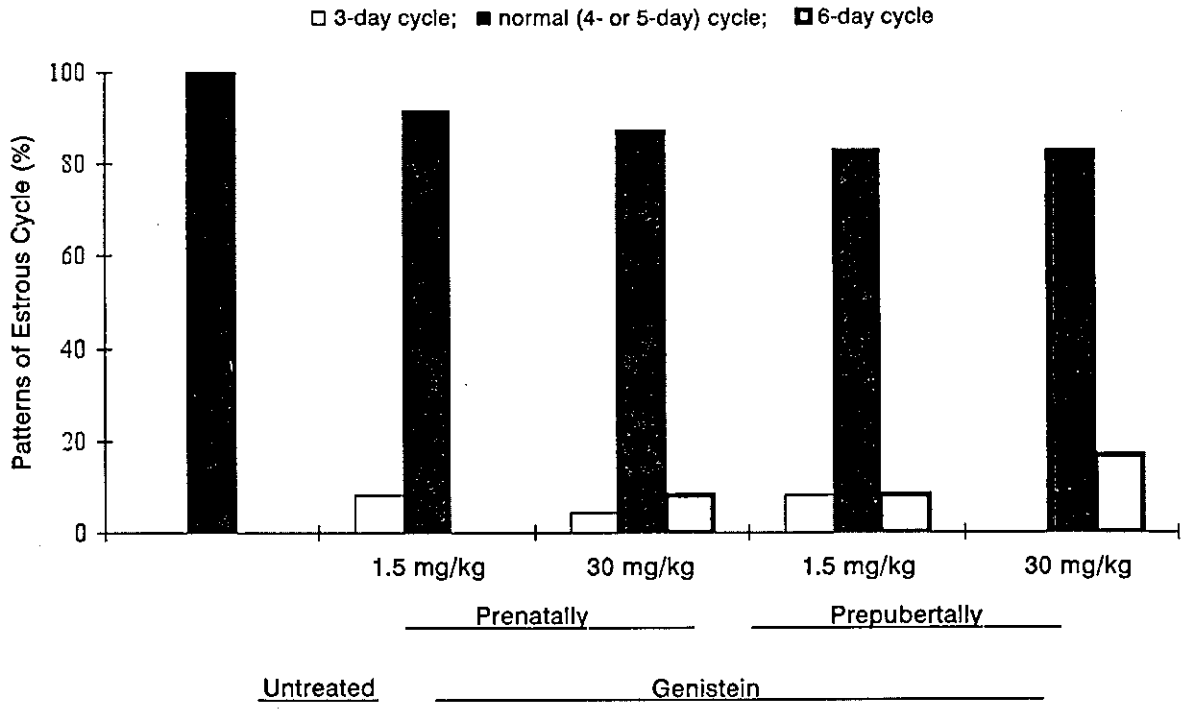

Figure 3. *Effect of genistein treatment on estrous cycle in female Sprague-Dawley rats. All rats cycled and >80% of genistein-treated rats had a normal (4- or 5-day) cycle.*

Table II. *Estrous cycle in genistein-treated and -untreated Sprague-Dawley rats.*

Group	Treatment	No. of rats	% of time spent in each phase of cycle			
			Proestrus	Estrus	Metestrus	Diestrus
1	Untreated	20	15.9 ± 1.5	27.1 ± 0.9	16.9 ± 1.4	40.1 ± 1.5
2	1.5 mg/kg/day prenatally	24	17.5 ± 1.7	27.0 ± 1.0	18.1 ± 0.8	37.4 ± 1.6
3	30 mg/kg/day prenatally	24	14.1 ± 1.5	29.0 ± 1.1	19.3 ± 1.0	37.6 ± 1.9
4	1.5 mg/kg/day prepubertally	24	16.6 ± 1.5	32.6 ± 1.2*	18.4 ± 1.5	32.4 ± 1.6*
5	30 mg/kg/day prepubertally	24	12.7 ± 1.4	36.4 ± 1.1*	19.5 ± 1.5	31.4 ± 1.1*

Examined from 10 to 14 weeks of age.
Values represent mean ± SEM. *$p<0.05$ *versus* group 1.

significantly, compared with untreated controls ($p<0.05$). A non-significant tendency toward inhibition was observed in other genistein-treated groups (groups 2, 3 and 5). The number of tumors per rat (tumor multiplicity) was low in groups 2, 4 and 5, and tumor latency was slightly longer in all genistein-treated groups; these differences were not statistically significant. In all groups, ≥91% of MNU-induced carcinomas were hormone-dependent (Table V).

Discussion

Genistein administered to female Sprague-Dawley rats prenatally or prepubertally can affect body weight gain. In previous studies using female Sprague-Dawley rats, prenatal genistein exposure reduced body weight, whereas prepubertal exposure did not affect body weight (16, 22). In the present study, prenatal genistein reduced body weight and prepubertal genistein increased body weight. The doses