treated on GD 12 were 10, 9, 21 and 11, respectively. Three females treated with 5 mg/kg/day on GD 6–15 died on GD 17 or 18. Decreases in locomotor activity, piloerection, tremor and/or wasting were observed at the late gestational period in dams treated with APNH at 2.5 and 5 mg/kg/day. The maternal toxicity and embryotoxic effects of APNH when applied on GD 6–15 or GD 12 are summarized in Tables 1 and 2. Body weight gain during GD 6–16 in the 5 mg/kg/day group was significantly decreased compared to that in the control group. There were no significant differences in body weight gain from GD 6–16 or terminal body weight (GD 18) minus uterus including contents between the groups treated with APNH at 2.5 mg/kg/day and less and the control group. At necropsy on ᄀ 18, ascites and oedema of pancreas were observed ııı 8 of 11 dams treated at 2.5 mg/kg/day on GD 6–15 and in all dams treated at 5 mg/kg/day on GD 6–15, respectively. In dams treated with APNH on GD 12, body weight gain from GD 12 to 18 was significantly decreased in the group treated at 10 mg/kg/day, whereas there was no significant difference in the terminal body weight minus uterus between the groups. Neither death nor adverse changes of general condition were observed, except for ascites and oedema of the pancreas in 5 of 11 dams treated with APNH at 20 mg/kg on GD 12.

Dams treated with APNH on GD 6–15 showed severe histopathological alterations in major internal organs. Histopathological changes in the liver, kidney and pancreas observed in dams treated with APNH at 2.5 or 5 mg/kg/day on GD 6–15 are shown in Figure 2. Hepatocytes observed in the 5 mg/kg/day group showed marked intracytoplasmic vacuolation. Vacuolar degeneration was also observed in the 2.5 mg/kg/day group, although the degree was not severe, and the dams treated with APNH at 1.25 mg/kg/day showed very slight degeneration. Necrotic hepatocytes scattered in the liver of dams in the 5 mg/kg/day group, and hepatocellular necrosis was also noted in dams of other APNH-treated groups to a lower extent. Extramedullary haematopoiesis was increased in the ≥2.5 mg/kg/day groups. Hypertrophy of Kupffer cells and slight proliferation of bile duct epithelium were noted in all APNH-treated groups. In the kidney, necrosis of proximal tubular epithelial cells was noted in the tubular lumen on dams treated with APNH at 5 mg/kg/day. Hyperplasia of the tubular epithelium was found in some animals in this group with or without tubular necrosis, and tubular cell hyperplasia consisted of dilated tubule lined by enlarged and eosinophilic epithelial cells. Marked oedema was noted in the interstitium of the pancreas in the groups treated with APNH at 2.5 mg/kg/day and above on GD 6–15, whereas no abnormalities were observed in the vessels. No histopathological changes in the liver or kidney were observed in the groups treated with APNH on GD 12. These results indicated that dams treated with APNH during foetal organogenesis (GD 6–15) showed alterations in the internal milieu including the uterus, and the environment of developing embryos was also adversely affected during organogenesis. Murakami *et al.* reported that norharman (1500 ppm) caused segmental coagulative necrosis of the tubular epithelium of the kidney when given orally to male rats for 28 days.[13]

ʾle 1 Effects of APNH on foetal development in mice when applied on GD 6–15 (mean±SE)

Dose (mg/kg/day)	*0*	*0.625*	*1.25*	*2.5*	*5*
Number of dams	10	9	9	11	19[a]
Body weight (g) gain from GD 6 to 16 of gestation	20.48±1.70	20.11±1.05	20.67±1.12	20.27±0.99	10.80±1.41**
Terminal body weight (GD 18) uterine contents (g)	38.46±1.05	39.37±0.75	36.00±0.55	38.60±1.15	ND
Number of implants/total number	13.1±0.21/129	13.5±0.24/122	12.5±0.16/105	14.5±0.41/166	14.4±0.35/232
Early embryonic death (%)/total number	4.41±1.57/5	9.74±4.46/11	4.71±3.71/3	3.26±1.45/5	69.14±11.82**/153
Late embryonic death (%)/total number	4.81±2.02/6	9.42±1.67/11	0.79±0.79/1	12.21±4.31*/21	23.67±9.53*/59
Number of live foetuses/total number	11.0±1.0/118	11.1±1.2/100	11.2±1.3/11	12.7±0.9/140	1.3±0.74**/20
Foetal body weight (g, female)	1.360±0.045	1.395±0.033	1.270±0.044*	1.099±0.024**	0.601±0.026**
Foetal body weight (g, male)	1.388±0.047	1.437±0.028	1.345±0.051*	1.127±0.023**	0.668±0.031*
External malformations (%)/total number	0	0	0.85±0.85/1[b]	4.23±0.98*/6[c]	0
Skeletal malformations (%)/total number	0	0	0.91±0.73/1[d]	0	0
Skeletal variations[e] (%)/total number	32.49±5.94/38	33.16±4.26/35	46.82±6.32/48	68.16±6.43**/97	97.73±2.27**/19
Number of ossified sacral and caudal vertebrae					
females	12.85±0.78	12.55±0.87	11.31±0.51*	9.03±0.31**	6.64±0.37**
males	13.78±0.59	13.76±0.42	12.01±0.46*	9.92±0.23**	7.01±0.28**

ND: Not determined because of high incidence of intrauterine embryonic death. *Significantly different from the control, $p<0.05$. **Significantly different from the control, $p<0.01$. [a]Three females died on GD 17 or 18. [b]One foetus showed dwarfism (a foetus weighing less than 70% of the average of the rest of the litter was classified as a dwarfism[14]). [c]Three foetuses showed cleft palate, two foetuses had cleft palate and dwarfism, and one foetus showed dwarfism. [d]One foetus showed fused ribs. [e]Lumbar ribs (rudimentary and extra ribs).

Table 2 Effects of APNH on foetal development in mice when applied on GD 12 (mean±SE)

Dose (mg/kg)	*0*	*5*	*10*	*20*
Number of dams	10	9	21	11
Body weight (g) gain from GD 12 to 18 of gestation	19.14±0.97	17.89±1.53	16.63±1.35*	ND
Terminal body weight (GD 18) uterine contents (g)	41.61±1.44	39.57±1.11	39.18±0.74	ND
Number of implants/total	13.9±0.28/138	13.4±0.16/135	13.7±0.11/273	14.0±0.31/154
Early embryonic death (%)/total number	6.55±0.44/8	4.66±2.73/6	4.54±1.14/12	3.26±1.45/5
Late embryonic death (%)/total number	3.57±2.02/2	2.80±2.81/3	3.09±1.43/8	12.21±4.31*/19
Number of live foetuses/total number	13.0±1.5/128	12.9±1.0/126	12.2±0.7/250	12.7±0.9/130
Foetal body weight (g, female)	1.370±0.055	1.307±0.047	1.100±0.031**	0.698±0.040**
Foetal body weight (g, male)	1.404±0.040	1.375±0.057	1.182±0.043**	0.741±0.047**
External malformations (%)/total number	0.67±0.67/1[a]	0	0.30±0.30/1[b]	0.63±0.63/1[c]

ND: Not determined because of high incidence of intrauterine embryonic death. *Significantly different from the control, $p<0.05$. **Significantly different from the control, $p<0.01$. [a]cleft palate. [b]exencephaly. [c]open eyelid.

Early embryonic death was significantly increased in the group treated with APNH at 5 mg/kg/day on GD 6–15, and late embryonic death including macerated foetuses was also significantly increased in the group treated with APNH at 2.5 or 5 mg/kg/day on GD 6–15, or in the group treated at 20 mg/kg/day on GD 12 in comparison with the controls. This resulted in a significant reduction in the number of live foetuses per litter in the group treated at 5 mg/kg/day on GD 6–15. The average foetal weight of both sexes per litter was dose-dependently decreased and significantly reduced in both males and females in the groups treated with APNH at 1.25, 2.5 and 5 mg/kg/day on GD 6–15, and at 10 and 20 mg/kg on GD 12 as compared to the controls. Foetal body weight in the group treated with APNH at 5 mg/kg/day on GD 6–15 and the group treated with APNH at 20 mg/kg on GD 12 was approximately 50% of the foetal weight in the control group. In summary, these results demonstrated that APNH has embryolethal effects and induces intrauterine growth retardation when applied orally at a maternally toxic dose.

The incidence of external malformations in foetuses in the group treated with APNH at 2.5 mg/kg/day on GD 6–15 was significantly increased as compared to the controls. One of 101 foetuses of dams treated with APNH at 1.25 mg/kg/day on GD 6–15 showed dwarfism (a foetus weighing less than 70% of the average of the rest of the litter was classified as a dwarfism[14]), 6 (from five litters) of 140 foetuses of dams treated with APNH at 2.5 mg/kg/day on GD 6–15 had cleft palate and 2 were accompanied by dwarfism.

In the group treated with APNH at 5 mg/kg/day on GD 6–15, no malformed foetuses were found because of the small number of live foetuses (20 foetuses) observed resulting from the high incidence of intrauterine embryonic death (early embryonic death, 69%; late embryonic death, 24%). In the groups treated with APNH at a fairly high dose on GD 12, few external malformations were observed, and no foetuses showing cleft palate were found in the APNH-treated groups. One of 250 foetuses of dams treated wi APNH at 10 mg/kg on GD 12 were exencephalic an

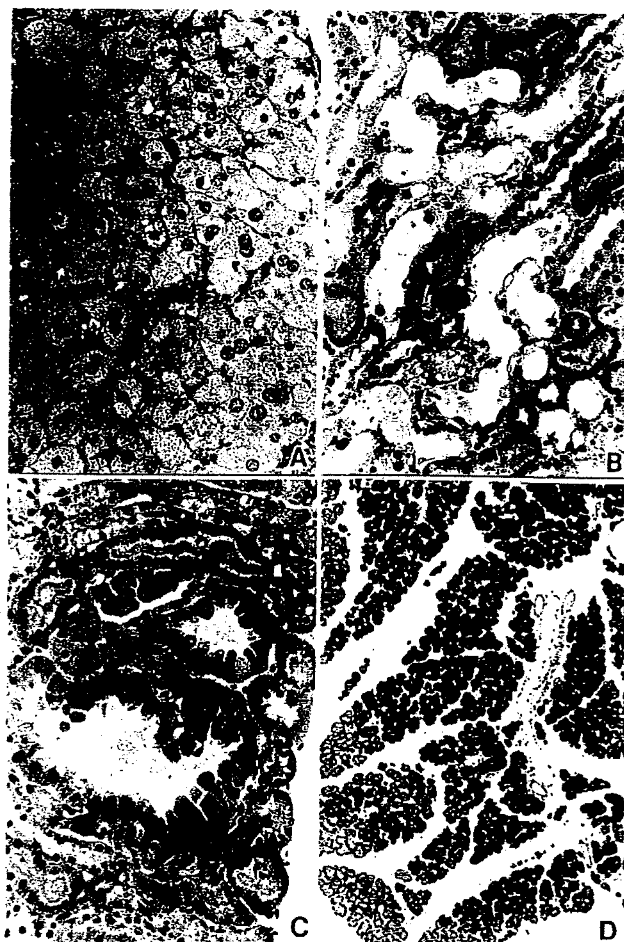

Figure 2 (A) Liver from a dam orally administered 5 mg/kg/day APNH on GD 6–15 showing severe vacuolar degeneration of hepatocytes. Necrotic hepatocytes were scattered. H&E, × 350. (B) Kidney from a dam orally administered 5 mg/kg/day APNH on GD 6–15 showing necrosis and desquamation of proximal tubular epithelial cells. H&E, × 350. (C) Kidney from a dam orally administered 5 mg/kg/day APNH on GD 6–15 showing hyperplasia of tubular epithelial cells. H&E, × 350. (D) Pancreas from a dam orally administered 2.5 mg/kg/day APNH on GD 6–15 showing oedema in the interstitium. H&E, × 88

1 of 130 foetuses of dams treated at 20 mg/kg had open eyelids. One of 128 foetuses in the control group showed cleft palate. There were no significant differences in the frequency of external malformations between the control group and the groups treated with APNH on GD 12. The critical period of induction of cleft palate is approximately embryonic day 12 or 13 in mice.[15] In the present study, cleft palate was not observed in foetuses of dams treated with APNH at 20 mg/kg on GD 12, whereas this defect was detected at a significantly increased frequency in foetuses of dams treated with APNH at 2.5 mg/kg/day on GD 6–15. Thus, APNH appears to have no potential to induce cleft palate by a direct teratogenic effect, but the maternal stress induced by this compound during palate morphogenesis resulted in a significant increase in the incidence of cleft palate in foetuses.

No skeletally malformed foetuses were found in any of the GD 6–15 APNH-treated groups or the controls, except for one foetus showing fused ribs in the 1.25 mg/kg/day group. However, the frequency of lumbar ribs, which was classified as a skeletal variation, was increased as dose increased, and significant differences were detected between the groups treated with APNH at 2.5 or 5 mg/kg/day on GD 6–15 and the controls. It is thus apparent that the incidence of skeletal variations can be increased by drug treatment during pregnancy, and that this increase may sometimes be correlated with embryotoxicity caused by the drug.[16] When an increase in lumbar ribs (either rudimentary or extra ribs) occurs during teratogenicity testing, it may be assumed that the maternal animal is being stressed sufficiently to express the developmental instability inherent in the species.[17] The present study suggested that the significant increase in the lumbar ribs observed in foetuses of dams treated with APNH on GD 6–15 was due to the marked stress on the dam induced by this agent during foetal organogenesis.

In conclusion, APNH applied orally during foetal organogenesis in mice induced cleft palate in term foetuses as well as early and late embryonic death, and severe foetal growth retardation. However, these embryotoxic effects may result from the severe stress on the maternal animals induced by this compound. More detailed animal studies are needed to assess the possible risk of effects on the neonates from exposure to APNH. Considerably more work, particularly with respect to establishing the likely level of human exposure to APNH by the dermal or inhalatory route, will be necessary in order to assess accurately the risk to humans resulting from exposure to APNH.

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Dietary indole-3-carbinol promotes endometrial adenocarcinoma development in rats initiated with *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine, with induction of cytochrome P450s in the liver and consequent modulation of estrogen metabolism

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Indole-3-carbinol (I3C), found in cruciferous vegetables, has been shown to suppress or promote carcinogenesis depending on various animal models. Regarding its preventive effects, I3C acts as an anti-estrogen and can induce apoptosis, but precise mechanisms remain to be determined. Since I3C induces cytochrome P450 enzymes in the liver, it affects hydroxylation of estrogens and might therefore be expected to influence endometrial adenocarcinoma development. The present study was performed to clarify the effects of I3C using a rat two-stage endometrial carcinogenesis model, focusing on induction of cytochrome P450s and other estrogen-metabolic enzymes in the liver. First, to determine the estrogenic or anti-estrogenic activity, an uterotropic assay was conducted using ovariectomized Donryu rats (experiment 1). Second, to elucidate the effects on endometrial carcinogenicity, female Donryu rats initiated with a single dose of *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine into a uterine horn were fed 0 or 500 p.p.m. I3C in diets for 12 months (experiment 2). In experiment 3, similarly initiated animals received 0 or 2000 p.p.m. I3C in their diet, or 1 μg/kg 17β-estradiol (E2) or 5 μg/kg 4-hydroxyestradiol (4HE) subcutaneously twice a week for 12 months. In the uterotrophic assay, neither 500 nor 2000 p.p.m. of I3C showed any estrogenic or anti-estrogenic activity. In the two uterine carcinogenicity studies, I3C and 4HE increased incidences of uterine adenocarcinomas and/or multiplicities of uterine proliferative lesions, E2-treatment being associated with a tendency for promotion. In the liver, I3C treatment consistently elevated estradiol 2- and 4-hydroxylase activities, in particular the latter, but without effects on estradiol 16α-hydoxylase activity. mRNAs for CYP 1A1, 1A2 and 1B1 were increased by I3C treatment, with translation confirmed immunohistochemically. These results suggest that induction of the CYP 1 family in the liver and sequential modulation of estrogen metabolism to increase 4HE might play a crucial role in promoting the effects of dietary I3C on endometrial adenocarcinoma development.

Abbreviations: CYP, cytochrome; E2, 17β-estradiol; ENNG, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine; 2HE, 2 hydroxyestradiol; 4HE, 4-hydroxyestradiol; I3C, indole-3-carbinol; PE, persistent estrus.

Introduction

Indole derivatives are contained in cruciferous vegetables such as cabbage, broccoli, brussels, sprout and cauliflower (1). Indole-3-carbinol (I3C) is known to be an anti-estrogenic (2–4) or apoptosis-inducing compound (5), and has shown anticarcinogenic activity in a number of animal studies such as DMBA-induced rat mammary tumorigenesis (6) and spontaneous rat uterine adenocarcinoma development (7). I3C also has chemopreventive activity against benzo[*a*]pyrene-induced mouse forestomach carcinogenicity (8). It is neither cytotoxic, nor mutagenic *in vitro* (9,10), and thus I3C is a promising candidate for a chemopreventive agent against various tumors, especially estrogen-related examples. However, the compound has been documented to promote development of colon proliferative lesions in an animal model (11), and in a multi-organ rat model both inhibition and promotion were apparent, depending on the organ (12).

As for a hypothesized mechanism of chemopreventive effects of I3C, an anti-estrogenic and/or apoptosis-inducing effect have been widely accepted. In addition, the compound induces hepatic cytochrome P450s (CYPs) such as 1A1 and/or 1A2 (13–15), and increased activity of some phase I drug-metabolizing enzymes, including the CYP 1 family, can protect in some instances by increasing the rate of oxidation to less toxic metabolites (16–19). Recently I3C treatment was reported to also induce CYP 1B1 in the liver and/or other organs (20,21). In most animal species, it is well established that estradiol is metabolized by microsomal P450s in the liver and other organs/tissues, and that these enzymes therefore have the ability to modulate its effects (22–24).

In rats, CYP 1A2, 2B1/2B2 and 3A catalyze 2- or 4-hydroxylation of estradiol, mainly in the liver (24,25). In addition, evidence has recently been presented that CYP 1B1 is a major enzyme catalyzing 17β-estradiol (E2) to 4-hydroxyestradiol (4HE) (26). In the rat liver, E2 is metabolized by estradiol 2- and 4-hydroxylases into two types of catechol estrogens, 2-hydroxyestradiol (2HE) and 4HE, respectively. 2-Hydroxylation of estradiol is the dominant pathway for catechol estrogen formation (22,24), and 2HE can bind to the classical estrogen receptors, but with a markedly reduced binding affinity. This metabolite possesses much weaker hormonal potential than the parent hormone (27,28), and is not a carcinogenic agent (7,24,29). In contrast, 4HE, produced only in small amounts in the liver compared with 2HE, is hormonally active and can stimulate uterine growth by strong binding to estrogen receptors when injected into animals (24,26, 29–31). In addition, this catechol estrogen causes tumor development in the kidney in hamsters (23), and also has been implicated in uterine and mammary tumor development in human beings (32,33).

Much attention has been paid to modulation of estrogen metabolism by chemicals such as phenobarbital, dexamethasone, 3-methylcholanthrene and environmental pollutants via

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induction of cytochrome P450 enzymes, especially of the CYP 1 family, in the liver or other organs (26,34,35). Thus, it is hypothesized that chemicals exerting no estrogenic activity themselves but inducing CYP 1 might also modify estrogen-dependent tumor development. However, solid evidence in animal models is limited, although Kojima *et al.* (7) reported previously that dietary I3C inhibited spontaneous uterine adenocarcinoma development by increasing estradiol 2-hydroxylation activity.

Cancers of the uterine corpus, most of them being histologically endometrial adenocarcinomas, have recently been increasing in many countries of the economically developed world. The tumor development is strongly related to estrogen statement in women. In rats, spontaneous endometrial adenocarcinomas are generally very rare but Maekawa and his co-workers have described high incidences of such lesions with morphological and biological similarities to human tumors in aged Donryu rats, and shown that this is due to an age-related ovarian hormonal imbalance resulting in an increase of the serum estrogen/progesterone ratio (36–38). In addition, they have established a two-stage uterine carcinogenesis model using this rat strain to detect promotive or preventive effects of test-chemicals (39–42). The present study was conducted to clarify effects of I3C on uterine carcinogenesis using this rat model, focusing on modulation of estrogen-metabolic enzymes in the liver. In addition, estrogenic or anti-estrogenic activity of I3C on ovariectomized rat uteri was also investigated.

Materials and methods

Animals and housing conditions

236 female Crj:Donryu rats at 8 weeks of age were purchased from Charles River Japan (Kanagawa, Japan). The animals were maintained in an air-conditioned animal room under constant conditions of 24 ± 2°C and 55 ± 10% humidity with a 12-h light/dark cycle (light, 08:00–20:00; dark, 20:00–08:00), housed three or four to a cage. Commercial powder diet (CRF-1, Oriental Yeast, Kanagawa, Japan) and drinking water were available *ad libitum* for the acclimatizing period. Animal care and use followed the NIH Guide for the Care and Use of Laboratory Animals.

Chemicals

I3C, 4HE, 2HE and 16α-hyroxyestradiol (16αHE) were purchased from Sigma-Aldrich (MO), *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) from Nacalai Tesque (Kyoto, Japan), and E2 and dimethylsulfoxide (DMSO) from Wako Pure Chemicals (Osaka, Japan).

Selection of dosing of I3C

2000 p.p.m. of I3C in diet has been reported as an effective dose in a multi-organ tumorigenesis model in rats (12) and 500 p.p.m. is known to induce cytochrome P450s in the rat liver (20).

Uterotrophic assay (experiment 1)

To assess estrogenic or anti-estrogenic activity of I3C, 38 female Donryu rats were ovariectomized under ether anaesthesia at 9 weeks of age, and starting 2 weeks thereafter were assigned to nine groups receiving: only ovariectomy (controls); daily s.c. treatment of E2 at a dose of 1 μg/kg; 4HE at a dose of 5 μg/kg; 2HE at a dose of 5 μg/kg; 16αHE at dose of 1 μg/kg: daily administration of 500 or 2000 p.p.m.-I3C in basal diets (I3C500 or I3C2000, respectively); or daily s.c. treatment of 1 mg/kg E2 plus I3C500 or I3C2000 for 2 weeks. After 2 weeks treatment, all animals were killed and the uteri were weighed. The uteri and livers were fixed in 10% neutral-buffered formaldehyde solution, routinely processed, sectioned and stained with hematoxylin and eosin. The uteri were measured for the height of the luminal epithelium with an image analyzer, IPAP-Win (Sumika-techno Service Co., Osaka, Japan).

Uterine carcinogenesis (experiments 2 and 3)

To clarify the effects of I3C on rat uterine endometrial adenocarcinoma development, female rats were treated with a single dose of 20 mg/kg ENNG into a unilateral uterine horn via the vagina using a stainless catheter at 11 weeks of age. This is known to exert no carcinogenic effects except in the uteri (39). After the initiation, in experiment 2, 30 animals were fed dietary I3C500 up to 15 months of age (for 12 months), and compared with 24 control rats fed powder basal diet without I3C. At 15 months of age, all surviving animals were necropsied (experiment 2).

For experiment 3 to elucidate sequential changes regarding the effects of I3C on uterine carcinogenesis and hepatic metabolism of E2, 144 females were allocated to the following four groups after the ENNG initiation: control (39 females); dietary I3C2000 (39 females); and twice weekly s.c. treatment with 1 μg/kg E2 (E2, 30 females) or 5 μg/kg 4HE (4HE, 36 females). At 6, 9 and 12 months of age, four to nine animals per group were examined, and all survivors were terminated at 15 months of age. After macroscopic examination, the reproductive system and related organs, including the ovaries, uteri and vagina, endocrine system organs and any macroscopical abnormalities, were fixed in 80% cold ethanol solution (uteri), or 10% neutral-buffered formaldehyde solution (other organs). These tissues and/or organs fixed were routinely processed for histopathological examination.

In both experiments, the upper, middle and lower parts of each uterine horn and the cervix were cut into three pieces in cross-section to evaluate uterine proliferative lesions, classified into three degrees of atypical hyperplasia (slight, moderate or severe) and adenocarcinomas, according to the criteria described previously (37,38). Briefly, slight hyperplasia was used when the numbers of glands with no or slight cellular atypia were increased within the endometrium. Moderate hyperplasia referred to increased numbers of glands with slightly to moderately atypical cells in focal and/or diffuse areas of the endometrium. Severe hyperplasia was composed of irregular proliferations of atypical glands in diffuse area of the endometrium. Adenocarcinomas were diagnosed on the basis of invasion of tumor cells into the muscularis. In addition, adenocarcinomas were subdivided into well, moderately and poorly differentiated types, and also classified as to the degree of invasion: limited to the uterus, invading into the serosa and/or surrounding adnexae, and with distant metastasis, in accordance with the simplified FIGO histopathological grades for human uterine cancers (43). Animals found dead or killed when moribund were also examined in the same manner. Throughout the two experiments, body weights were measured at regular intervals and clinical signs were checked daily for all animals.

Estrous cyclicity

Vaginal cytology was observed in all animals to investigate estrous cyclicity throughout the study (experiments 2 and 3).

Preparation of livers

At 6, 9, 12 and 15 months of age in experiment 3, right and median lobes of selected livers of each group were frozen in liquid nitrogen for analysis of enzyme activities related to estrogen metabolism (6, 9, 12 and 15 months of age) or mRNA expression of cytochrome P450s by reverse transcription PCR (RT–PCR) (15 months of age), and stored at −80°C until use.

mRNA expression of cytochrome P450 enzymes in the liver

Small pieces of the liver (~200 mg) were obtained from three control and four I3C2000-treated animals in experiment 3 at 15 months of age. The samples were homogenized in 4 ml RLT buffer, mixed with 40 μl β-mercaptoethanol, and RNA was isolated using an RNeasy Midi extraction Kit (QIAGEN, Germany) and stored at −80°C until RT–PCR analysis.

RT–PCR and PCR primers of cytochrome P450 1A1, 1A2, 1B1 and GAPDH mRNA transcription in the present study were done as reported previously (26,44,45). Aliquots (500 ng) of total liver RNA were used for the RT–PCR. The primers were synthesized and purified by Takara Bio (Shiga, Japan). Levels of cytochrome P450s mRNA expression relative to GAPDH mRNA expression were calculated as ratios using an image analyzer (NIH image, Bethesda, MD).

Immunohistochemical distribution of cytochrome P450 enzymes in the liver

Cytochrome P450 protein amounts in the liver were examined immunohistochemically using paraffin-embedded sections from animals in experiments 2 and 3. After blocking endogenous peroxidase by incubation with hydrogen peroxidase (3%, v/v) in methanol, deparaffinized liver sections were incubated with anti-rat CYP 1A1, 1A2, 2B1 or 3A2 (Daiichi Pure Chemicals, Tokyo, Japan), diluted 1:100 in Tris-buffered solution (Takara Bio) with 1% skim milk at 37°C for 1 h. After the incubation, the sections were exposed to secondary antibodies and linked with streptavidin peroxidase using a DAKO LSAB+ kit (DAKO cytomation, CA). Binding was visualized by incubating sections with 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemicals), and counterstaining with hematoxylin for histopathological examination. Immunohistochemical distribution of CYP 1B1 could not be examined in the present study, because no anti-rat CYP 1B1 antibody was available for immunohistochemistry using formalin-fixed and paraffin-embedded sections.

Enzyme activities related to estrogen metabolism in the liver

Estradiol 2- and 4-hydroxylase and 16α-hydroxylase activities in liver (median lobe) samples obtained from four or five rats in the control-, I3C2000-, E2- or 4HE-treated groups at 6, 9, 12 and 15 (except 16α-hydroxylase activity) months of age in experiment 3 were determined by SRL (Tokyo, Japan), as for previous reports (7,22).

Statistical analysis

Values for incidences including data of uterine proliferative lesions and estrous cyclicity were analyzed statistically using the Fisher's exact probability test. Other data were analyzed using ANOVA, and post hoc comparisons between the treated and control groups were made with the Dunnett's *t*-test. P values $<$ 0.05 were considered to be statistically significant. In the uterotrophic assay, the uterine weights and heights in treated groups were compared with those in the control (only ovarietomized rats) and positive control (E2-treated) groups.

Results

Estrogenic or anti-estrogenic activities of I3C (experiment 1)

Uterine weights and heights of the luminal epithelium are shown in Figure 1. Neither dose of I3C affected parameter in ovariectomized rats, with or without E2 replacement. The uterine weights and heights with 5 μg/kg 4HE treatment were comparable with those with 1 μg/kg E2 treatment, while 16αHE and 2HE treatments had much lower and no estrogenic activity, respectively.

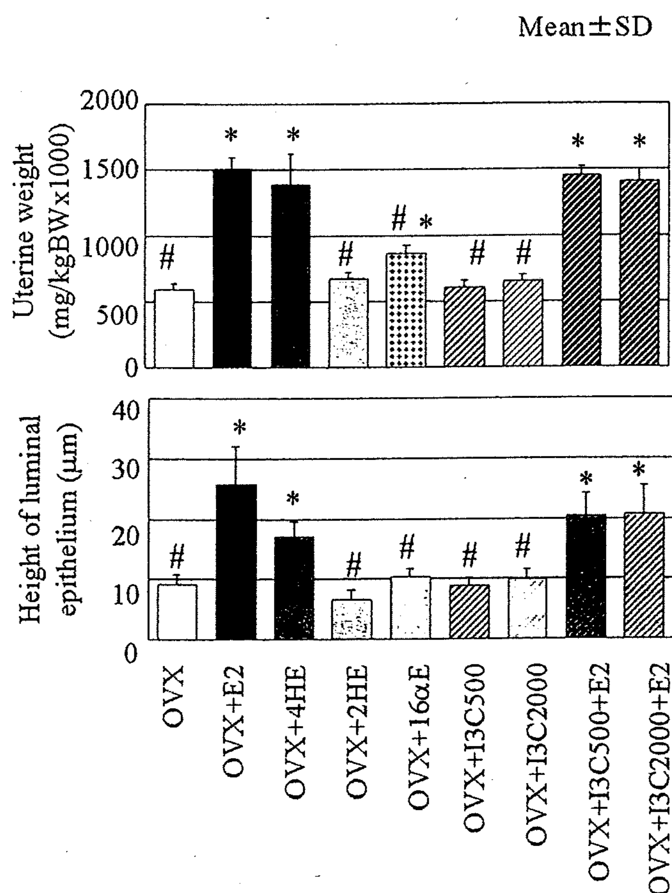

Fig. 1. Relative uterine weights and heights of uterine luminal epithelium in experiment 1. *P or #P refer to significant differences from the control (ovariectomy only) and E2-treated groups, respectively, at 1% or below.

Body weights, clinical signs, survival curves and estrous cyclicity (experiments 2 and 3)

Body weights were depressed by I3C treatment with both doses (data not shown). During experiments 2 and 3, no treatment-related clinical signs were observed and survival curves in all treated groups were comparable with those of the relevant control groups (data not shown). In both experiments, I3C and E2 treatment did not increase persistent estrus (PE) status up to 15 months of age, while subcutaneous treatment of 4HE in experiment 3 significantly increased PE status after 5 months of age (Figure 2).

Effects of I3C on uterine carcinogenesis (experiments 2 and 3)

Incidences of uterine proliferative lesions and data for their multiplicity are shown in Table I. In experiment 2, the incidence of adenocarcinomas in the group treated with I3C500 was significantly elevated compared with the control group. In experiment 3, the incidence of adenocarcinomas was significantly increased in the 4HE group, compared with that of the control group. I3C2000 and E2 treatments also increased the incidences (44 and 50%, respectively) as compared with the control value (22%), but not significantly. Multiplicities of the uterine proliferative lesions were significantly increased by both I3C and 4HE treatments, whereas only a tendency for increase was evident with E2. Histologically, almost all uterine adenocarcinomas were of well-differentiated type, and morphological or biological malignancy was not influenced by the I3C treatment. In sequential observation of uterine tumor development, slight atypical hyperplasias had already appeared in the 4HE- and E2-treated groups at 6 months of age when no proliferative lesions were detected in controls. Development profiles for uterine proliferative lesions for I3C-treated and control animals were comparable up to 12 months of age.

Pathological examination of other organs

At all examined times in experiment 3, the relative liver weights were consistently elevated in the I3C2000 treated group (data no shown). Microscopically, centrilobular hypertrophy of hepatocytes was observed in all I3C-treated groups of experiments 1, 2 and 3. Most ovaries in all groups were atrophic with small cystic atretic follicles and lacking corpus lutea at termination of experiments 2 and 3. In these two experiments, various non-neoplastic and neoplastic lesions were observed in representative organs and other endocrine tissues; however, all lesions were similar to those detected

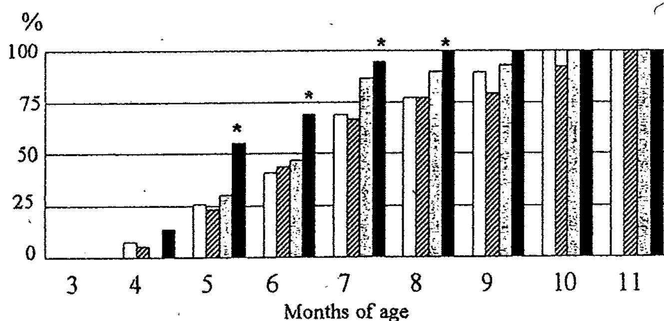

Fig. 2. Percentage incidences of animals showing PE from vaginal cytology in experiment 3. White, stripe, gray and black columns indicate the control, I3C2000-, E2- and 4HE-treated groups, respectively. *P refers to significant differences from control incidences at 5% or below.

Table I. Incidence of uterine proliferative lesions[a] and their multiplicities in experiments 2 and 3

	No. of rats with no abnormalities	Hyperplasia			Adenocarcinoma	Multiplicities[b]
		Slight	Moderate	Severe		
Experiment 2						
15 months of age						
Control ($n = 24$)	4	2	5	7	6	1.04 ± 0.62
I3C500 ($n = 30$)	1	2	3	7	17*	1.50 ± 0.63*
Experiment 3						
15 months of age						
Control ($n = 18$)	2	2	7	3	4	1.17 ± 0.62
I3C2000 ($n = 18$)	1	2	5	2	8	1.78 ± 0.73**
E2 ($n = 16$)	0	3	2	3	8	1.50 ± 0.52
4HE ($n = 16$)	0	0	5	1	10*	1.69 ± 0.60**

[a]Uterine proliferating lesions include slight to severe atypical hyperplasia and adenocarcinomas, these criteria referred to Nagaoka *et al.* (37,38).
[b]Multiplicities are calculated average number of uterine proliferative lesion per rats, and indicated mean ± SD.
Values in parentheses show the number of rats examined.
*,**Significantly different from relevant control group at $P < 0.05$ and $P < 0.01$, respectively.

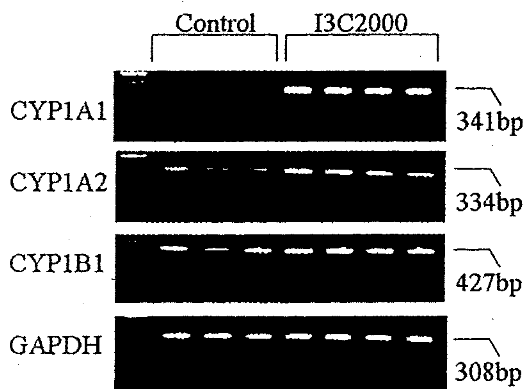

Fig. 3. mRNA expression for CYPs 1A1, 1A2, 1B1 and GAPDH in the livers of control and I3C2000-treated groups at 15 months of age in experiment 3.

spontaneously in this rat strain (36), and there were no differences in these lesions among the groups. Necropsy of animals found dead or killed when moribund also did not reveal any treatment-related changes.

mRNA expression of cytochrome P450s in the liver

Findings for mRNA expression of CYP 1A1, 1A2 and 1B1 in the liver of experiment 3 are demonstrated in Figures 3 and 4. In the control group, CYP 1A1 was not detectable. I3C treatment significantly increased CYP 1A1, 1A2 and 1B1 mRNA expression compared with the control group, with induction of 1A1 expression being the most prominent.

Immunohistochemical staining of cytochrome P450s

CYP 1A1 and 1A2 were clearly demonstrable in the hepatocytes of centrilobular areas in all I3C-treated groups in experiments 2 and 3 up to 12 months of age, while very weak expression of 1A2 was observed in relevant controls (Figure 5). At 15 months of age, 1A1 expression in the I3C-treated group was similar to that at the other examined times, whereas 1A2 expression was too varied to detect any differences from relevant controls in experiments 2 and 3. Results for other CYPs such as 2B1 or 3A2 were comparable among the livers in the control and treated groups up to 15 months of age.

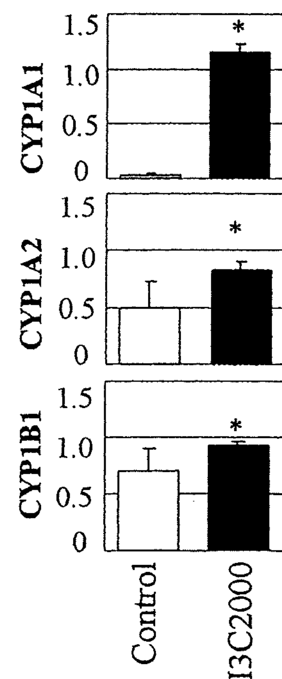

Fig. 4. Levels of expression of CYPs 1A1, 1A2 and 1B1 mRNAs relative to GAPDH mRNA in the liver, as for Figure 3. The intensities of P450s are relative to GAPDH mRNA levels (calculated as 1.0).

Enzyme activities related to estrogen metabolism in the liver

Estadiol 2-, 4- and 16α-hydroxylase activities in the liver (experiment 3) are shown in Table II. The estradiol 2-hydroxylase activities in the I3C-, E2- and 4HE-treated groups showed increasing trends compared with the control group at most of the examined points. However, there were no significant differences among them due to great variation except 15 months of age, when a significant increase was increased by I3C treatment. The 4-hydroxylase activities demonstrated significant increases in the I3C- and 4HE-treated groups at 9 and 15 months of age, or tendencies for increase in all treated groups at all examined times, except the 4HE-treated group at 12 months of age. At all examined points, 16α-hydroxylase activities showed neither significant differences nor any tendency for change with the treatments.

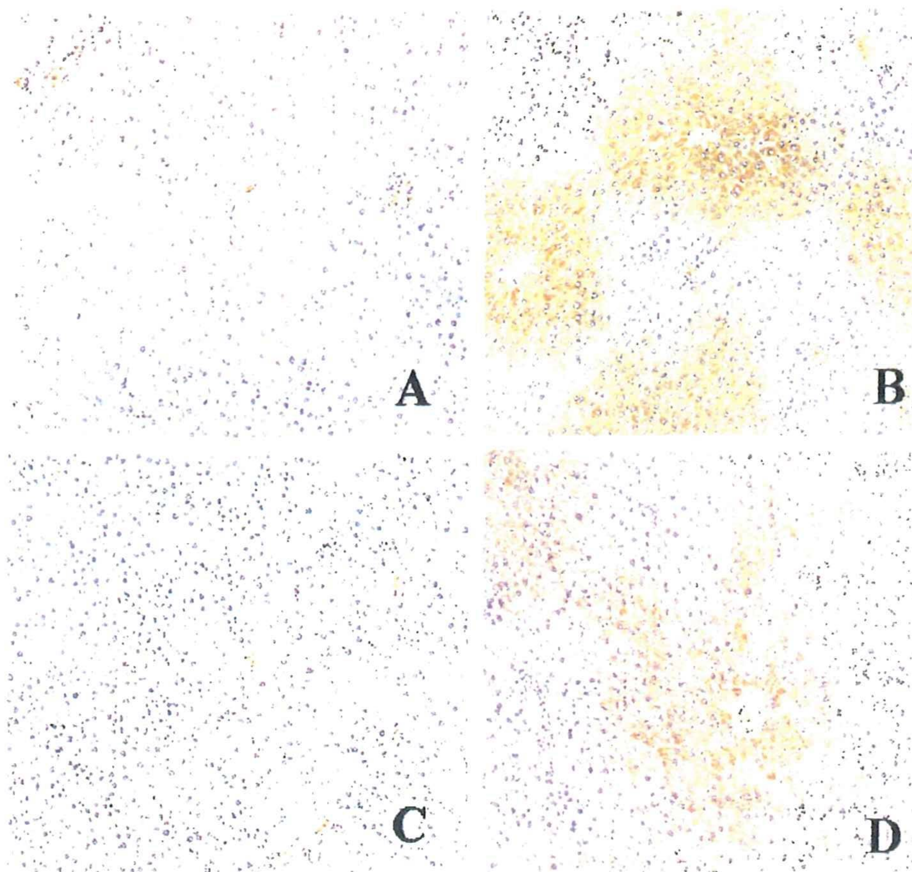

Fig. 5. Immunohistochemical staining of CYP 1A1 and 1A2 in the livers of animals at 15 months of age in experiment 3. (**A** and **B**) CYP 1A1 expression in control and I3C2000-treated animals. (**C** and **D**) CYP 1A2 expression in control and I3C2000-treated animals. Hematoxylin was used for counterstaining.

Table II. Enzyme activity related to estrogen metabolism in the liver (experiment 3)

	Enzyme activities related to estrogen metabolism (pmol/min/mg protein)		
	Estradiol 2-Hydroxylase	Estradiol 4-Hydroxylase	Estradiol 16α-Hydroxylase
6 months of age			
Control (5)	66.76 ± 23.50	2.97 ± 1.18	2.88 ± 1.00
I3C2000 (5)	139.72 ± 83.72	5.24 ± 2.39	2.65 ± 1.31
E2 (3)	104.88 ± 23.44	5.46 ± 2.40	3.34 ± 0.59
4HE (4)	108.14 ± 5.84	5.66 ± 0.58	4.37 ± 0.90
9 months of age			
Control (5)	61.82 ± 29.35	1.78 ± 0.73	1.30 ± 0.21
I3C2000 (5)	88.30 ± 25.79	4.19 ± 1.63*	1.75 ± 0.62
E2 (4)	92.72 ± 24.47	3.44 ± 1.66	2.18 ± 0.34
4HE (3)	110.22 ± 31.36	4.60 ± 1.54*	1.99 ± 0.85
12 months of age			
Control (4)	78.05 ± 29.45	3.52 ± 2.52	1.24 ± 0.36
I3C2000 (4)	149.80 ± 51.88	5.14 ± 2.52	1.05 ± 0.13
E2 (4)	86.37 ± 64.90	5.06 ± 3.16	1.47 ± 0.64
4HE (4)	78.57 ± 8.63	3.20 ± 0.49	1.42 ± 0.64
15 months of age			
Control (4)	75.08 ± 15.23	3.94 ± 1.18	NE
I3C2000 (4)	205.32 ± 51.92**	7.14 ± 1.36**	NE
E2 (4)	67.79 ± 10.55	3.01 ± 0.87	NE
4HE (4)	108.16 ± 7.32	5.97 ± 0.75*	NE

Values in parentheses mean number of rats examined. Values mean average ± SD. NE, not examined.
*Significantly different from control group, $P < 0.05$.
**Significantly different from control group, $P < 0.01$.

Discussion

In the present study using rat uterine cancer model, dietary treatment with I3C clearly demonstrated promoting effects on endometrial adenocarcinoma development. I3C can act both as an inhibitor and promoter of carcinogenesis, and our data are in line with the promoting results observed earlier with several animal carcinogenesis models (11,12). As for a cause of the complex effects, I3C is unstable under the acid condition and a number of acid-catalyzed metabolites such as 3,3′-diindolylmethane and indolcarbazole are produced in the gut (46). The acid condensation product has shown to be a potent aryl hydrocarbon receptor agonist, providing anti-estrogenic and antitumorigenic activity (47). In the present study, the activity of each acid-catalyzed metabolite of I3C to the rat uteri was not investigated; however, the dietary treatment with I3C at doses of 500 or 2000 p.p.m. did not show any estrogenic- or anti-estrogenic activity in the rat uteri, indicating that the promoting effect did not result from direct binding of I3C to estrogen receptor α in the rat uteri as estrogenic or anti-estrogenic agents.

I3C is widely accepted to induce CYPs 1A1, 1A2 and/or 1B1 in the liver and other organs (13–15,20,21). In rats, CYPs 1A1 and 1A2 catalyze mainly E2 into 2HE, the dominant product of catechol estrogen with weak hormonal potency and no carcinogenic effects (7,22,24,25,31), by hepatic 2-hydroxylation of estradiol (24,25), whereas CYP 1B1 is a major catalyzing enzyme of E2 to 4HE, a strongly carcinogenic and toxic metabolite (23,26,32,33,48).

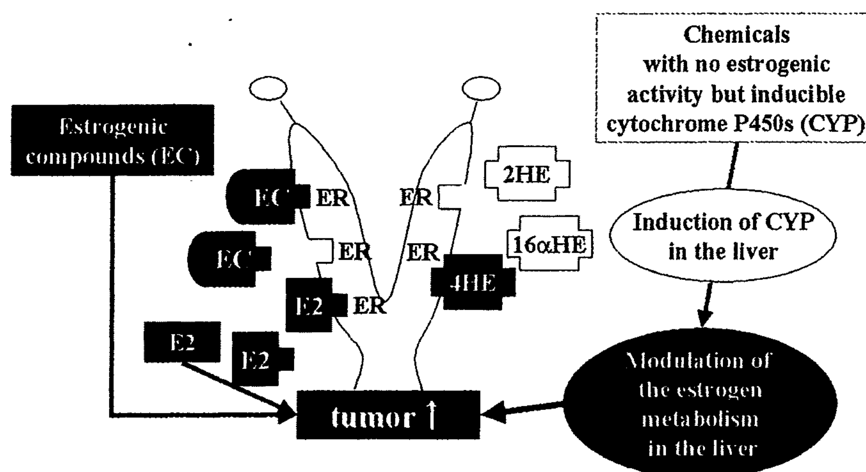

Fig. 6. Hypothesized mechanisms of promoting effects of chemicals with or without estrogenic activity on uterine carcinogenesis in rats. 16αHE, 16α-hydroxyestradiol; ER, estrogen receptor α. Black blocks indicate promotion of uterine carcinogenesis, while white blocks indicate weak or no promoting activity.

In the present study, dietary I3C treatment increased the induction of CYPs 1A1, 1A2 and 1B1 enzymes in the liver at either the mRNA level or its producing proteins. In the assays of estradiol hydroxylase activities in the liver, dietary I3C increased both 2- and 4-hydroxylase activities, in particular the latter. These results strongly suggest that the induction of the CYP 1 family by I3C is linked to modulation of E2 metabolism. In this study we could not determine which enzyme in the CYP 1 family was most effective in this regard. The present finding that 4HE treatment increased uterine adenocarcinoma development provides the evidence that it possesses stronger carcinogenic effects on rat uterus than E2, whereas uterotrophic activity of 4HE was weaker, in line with previous reports (23,32,33,48).

Endometrial adenocarcinoma development is strongly related to estrogen exposure in women and the Donryu rat features endocrinological similarities to the human case, ovarian hormonal imbalance leading to elevation of the serum estrogen/progesterone ratio, manifested as atrophic ovary with small polycystic atretic follicles and lack of corpora lutea and a long-term PE status as indicated by vaginal cytology (36–38). Using the two-stage uterine carcinogenesis model in this rat strain (39), continuous stimulation by estrogens or estrogenic compounds, which directly bind to estrogen receptor in the uteri or induction of early occurrence of the PE status enhanced uterine carcinogenesis (49,50).

In the present study, dietary I3C enhanced uterine carcinogenesis without affecting estrous cyclicity or showing estrogenic activity in the uteri. Induction of CYPs 1A1, 1A2 and 1B1 in the liver by dietary I3C and sequential modulation of estrogen metabolism therefore should be nominated as crucial to the promoting effects. The modulation, in particular the continuous increase of 4HE level, by I3C treatment might be important as part of the hypothesized pathway described schematically in Figure 6.

A number of chemicals and environmental pollutants induce CYP 1 family enzymes in the liver or other organs (26,34,51,52). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin or diesel exhaust is reported to up-regulate CYP 1B1 (45,51–54). In most mammalian species, the main estradiol metabolites were generated by hepatic microsomal P450s in the liver and other tissues (22,23) and the functions and regulation of CYPs 1A1 and 1A2 appear to be highly conserved (55). Therefore, animal data concerning modulation of estrogen metabolism via induction of CYP 1 family may provide useful information for human risk assessment, although further investigations are required to detail their exact significance.

The precise reason for the discrepancy between the promotion observed here and the previous report that dietary I3C inhibited spontaneous uterine tumor development in Donryu rats (7) could not be determined. The differences might be due to the dietary doses applied, or resultant variation in the ratios of E2 to 2HE and 4HE, especially the latter, in addition to the difference in the uteri with or without initiation of ENNG. Several reports proposed that the ratio of 2HE/4HE formation was important as a marker of estrogen-dependent tumor development (26,33).

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Chemopreventive Effects of Hydroxymatairesinol on Uterine Carcinogenesis in Donryu Rats

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Hydroxymatairesinol (HMR), obtained from the heartwood of spruce (*Picea abies*), has been demonstrated to exert chemopreventive effects on the development of mammary tumors in rats. To examine the influence of HMR on uterine carcinogenesis, adult Donryu rats were initiated with a single intrauterine treatment of *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine (ENNG) at 11 weeks of age and fed thereafter 0, 200, or 600 ppm HMR mixed in the soy-containing diet until 15 months of age. Incidences of uterine adenocarcinoma in both 200 and 600 ppm HMR-dosed groups were significantly reduced to 11% and 15%, respectively, less than 50% of 0 ppm, at the end of the experiment ($P < 0.05$). A delay in the start of persistent estrus by HMR was observed at 8 months of age compared with controls given carcinogen alone. From urinalysis, HMR was metabolized mainly to enterolactone and hydroxyenterolactone. These findings suggest that HMR or its metabolites exert chemopreventive effects in the rat ENNG-uterine carcinogenesis model. Exp Biol Med 229:417–424, 2004

Key words: hydroxymatairesinol; rat; endometrial adenocarcinoma; chemoprevention

Various natural and man-made substances possessing possible adverse influences, such as induction or promotion of cancer development, are present in our contemporary environment. Likewise, cancer-preventive potential has been found in both natural and synthetic substances. In Asian countries, the risk of acquiring steroid hormone–dependent cancers, for example in the breast and prostate, appears to be relatively low compared with that of Western countries (1). This may be because of dietary factors, such as lower consumption of fruits, vegetables, and legumes—particularly soy—in the West compared with Asia. In epidemiological studies, soy or soy food intake may protect against breast cancer (2–4). Isoflavonoids primarily found in soybeans may have a strong influence (5). Similarly, both epidemiological (5) and experimental evidence (6) have shown that lignans, which humans ingest mostly from a fiber-rich diet, may also reduce the risk of breast cancer. In a case-control study in the San Francisco Bay Area (7), lignan and isoflavone were also found to be associated with a low risk of endometrial cancer.

Large amounts of lignans are present in coniferous trees. Hydroxymatairesinol (HMR; Fig. 1) is one example obtained from the heartwood of Norway spruce (*Picea abies*). Because HMR exists mainly in an unconjugated free form, it can be isolated by simple extraction without hydrolysis (8). Anticarcinogenic properties of HMR against 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced rat mammary adenocarcinomas have already been demonstrated (8, 9). Hydroxymatairesinol is metabolized to enterolactone (ENL) (8), a lignan produced by intestinal bacteria from plant lignan precursors in fiber-rich diets. In epidemiological studies, high serum and urine ENL concentrations have been linked to a low risk of breast cancer (10, 11), but controversial results have also been obtained (12, 13). A cause-effect relationship between high ENL concentration and influenced disease risk remains to be demonstrated. It is not known whether ENL is biologically active as an anticarcinogenic agent or merely a marker for healthful, fiber-rich diets in general (14).

We have documented that the Donryu rat has a high

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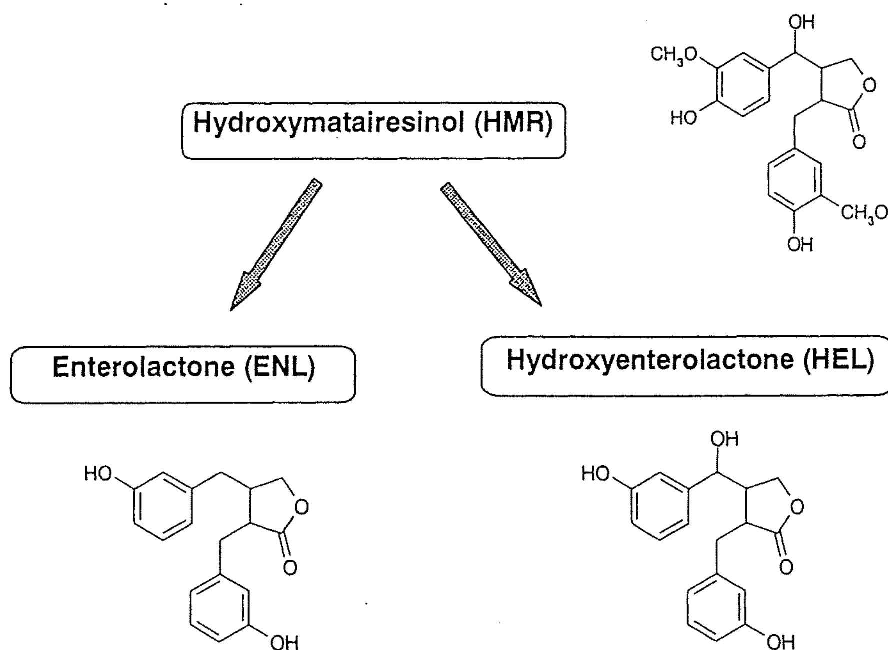

Figure 1. Chemical structure of hydroxymatairesinol (HMR) and schematic illustration of its putative metabolic pathway, which involve demethylation and dehydroxylation reactions catalyzed by intestinal bacteria.

incidence of spontaneous development of endometrial adenocarcinoma, which is associated with hormonal imbalance, and is characterized by an age-dependent increase in the estrogen to progesterone (E2/P) ratio (15–17). The incidence of spontaneous endometrial adenocarcinoma in this rat strain tends to decrease in the reproducing animal, compared with the nulliparous case, the suppression being associated with changes in the hormonal milieu (18). These results indicate that the Donryu rat might be a valuable animal model for the study of endometrial adenocarcinoma linked to endogenous estrogens in humans. The incidence of such tumors in this rat strain is elevated after a single intrauterine administration of *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine (ENNG). A two-stage rat uterine carcinogenesis model has been shown to be very useful for detection of the tumor-promotive effects of various agents, including endocrine-disrupting chemicals (EDCs; Refs. 19, 20). This animal system can be used successfully for studies of tumor-chemopreventive effects of long-term exposure to various compounds during adulthood, with normalization or suppression of cell proliferation in the uterus or indirect effects such as perturbation of endocrine regulation (21).

To clarify the chemopreventive effects of HMR on uterine carcinogenesis, we performed an experiment using the carcinogenesis model in Donryu rats. Types and characteristics of uterine adenocarcinoma in the rat vary, depending on the age of exposure to exogenous compounds (20, 22). Hydroxymatairesinol was dosed to adult rats after precise estrous cycles were established at 11 weeks of age and continued until 15 months of age. In all our past studies of endometrial adenocarcinoma using the Donryu rat model, the soy-containing conventional diet, CRF-1, was supplied but its composition was not analyzed. The possibility that phytoestrogens such as isoflavones in a diet also influence tumor development should be considered. On the other hand, the soy-containing 1324 diet has been widely used as a basal diet in European countries. Accordingly, two controls, each supplied with diets of differing phytochemical components, were designed into the present study. First, isoflavone contents of both diets were analyzed by high-performance liquid chromatography (HPLC), according to previously described methods (23). Average contents of total isoflavones were 471 and 257 ppm in the basal (1324) and conventional (CRF-1) diets, respectively.

Materials and Methods

Animals and Housing Conditions. Female Crj:Donryu rats were obtained from Charles River Japan Inc. (Kanagawa, Japan). They were housed in plastic cages and kept in an air-conditioned animal room under constant conditions of 23° ± 2°C and 50% ± 20% humidity with a 12:12-hr light:dark cycle and were maintained on a soy-containing conventional diet, CRF-1 (Oriental Yeast Inc., Tokyo, Japan) and tap water *ad libitum*. Animal care and use followed the NIH Guide for the Care and Use of Laboratory Animals.

Experimental Design. Hydroxymatairesinol was isolated from the heartwood of Norway spruce (*Picea abies*) by using a method previously described (9, 24, 25). The purity of HMR was determined to be 96.6% using the GC-MS method. The 105 rats were divided into 4 groups of 25 to 27 animals each. Hydroxymatairesinol was mixed according to the weight of a pure HMR extract in a nonpurified, soy-containing 1324 diet (Altromin GmbH,

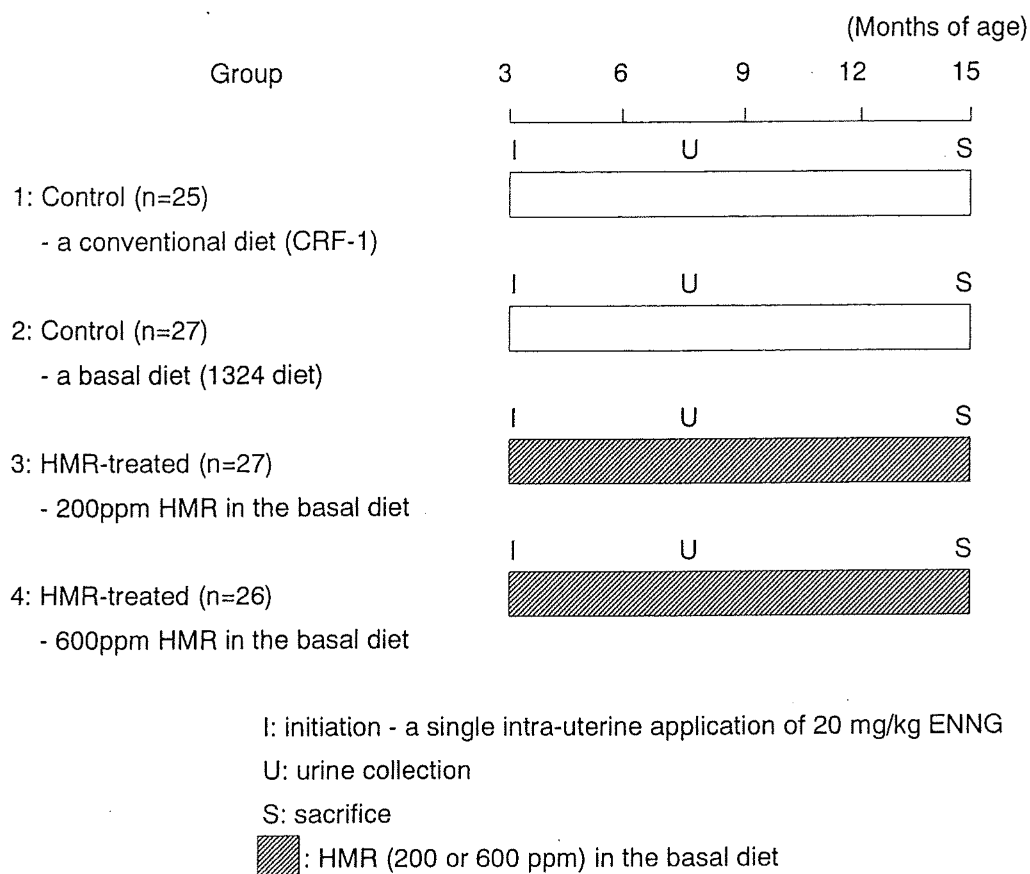

Figure 2. Experimental design for examination of the effects of hydroxymatairesinol (HMR) on rat uterine carcinogenesis. Rats were initiated with *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) and then fed a conventional diet (CRF-1; Group 1), a basal diet (1324; Group 2), or HMR (200 and 600 ppm; Groups 3 and 4) in the basal diet.

Lage, Germany), at doses of 200 and 600 ppm (Groups 3 and 4). The two control groups were provided with a conventional diet (CRF-1; Group 1) or the basal diet (1324; Group 2) alone (Fig. 2). Crude protein, crude fat, crude fiber, ash, moisture, nitrogen-free extract, and metabolizable energy of the conventional diet were 22.4%, 5.7%, 3.1%, 6.6%, 7.8%, 54.5%, and 3590 kcal/kg, respectively. Those of the basal diet were 19.0%, 4.0%, 6.0%, 7.0%, 13.5%, 50.5%, and 2050 kcal/kg, respectively.

Duration of Treatment. Just after carcinogen treatment, the feeding of each diet including HMR was started at 11 weeks of age and continued until 15 months of age.

Chemical Carcinogen Treatment. At 11 weeks of age, a single dose of 20 mg/kg *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG), purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and dissolved in polyethylene glycol, was introduced into one of the uterine horns of the rats, using a stainless catheter via the vagina.

Observation and Laboratory Examinations. Each animal was weighed weekly during the first 3 months of treatment and at least once a month thereafter. The amounts of feed supplied were measured, and HMR-intake per animal was calculated from food consumption. Vaginal smears were checked at 4, 5, 6, 8, and 12 months of age for confirmation of the estrous cycle stage. Persistent estrus was determined based on continued estrus for at least 4 days. At termination, all animals were weighed and sacrificed. Reproductive tract tissues and the other organs were quickly removed and fixed in 10% neutral buffered formalin, then routinely processed for histopathological examination. Each uterus was cut into about 12 slices in cross-section, and proliferative endometrial lesions were classified into three degrees of hyperplasia (slight, +; moderate, ++; severe, +++) and adenocarcinoma using the categories for rat uterine proliferative lesions reported previously (8, 10).

Urinary Lignan Analysis. In the previous studies (18), age-related persistent estrus followed by anovulation in Donryu rats was started at 5 months of age, and its incidence was markedly increased until 8 months. Hence, urine was collected at 8 months of age when many animals were thought to be subjected to the hormonal imbalance. Six rats selected at random from each group were placed in metabolic cages, and urine was collected for 24 hours in glass jars containing 120 µl of 0.56 *M* ascorbic acid and 120 µl of 0.15 *M* sodium azide as preservatives. Urine samples

Table 1. Mean Food Consumption of the Experimental Groups and Mean Daily Intakes of Hydroxymatairesinol (HMR) in the Dosed Groups[a]

Group	Consumption (g/kg/day)	HMR intake (mg/kg/day)
Control—conventional diet (CRF-1)	45.6±1.1	—
Control—basal diet (1324 diet alone)	53.1±1.9**	—
200 ppm HMR in the basal diet	55.2±1.4**	11.0±0.3
600 ppm HMR in the basal diet	54.5±1.9**	32.7±1.1***

[a] Values represent the mean ± SEM. Mean food consumption in Group 1 was significantly lower than that in Groups 2, 3, and 4 (**, $P < 0.01$). Mean HMR-intakes are significantly different (***, $P < 0.001$) between Groups 3 and 4.

Table 2. Delay of Persistent Estrus by Hydroxymatairesinol (HMR) Dosing[a]

Group	Mean week of age of persistent estrus start
Control—conventional diet (CRF-1)	30.3±1.2
Control—basal diet (1324 diet alone)	32.1±1.3
200 ppm HMR in basal diet	35.4±1.6*
600 ppm HMR in basal diet	35.3±1.6*

[a] Values represent the mean ±SEM, n = 25, 27, 27, and 26 rats of each group, respectively. Means of Group 3 and 4 are significantly different (*$P < 0.05$) from Groups 1 and 2.

were stored at −70°C until analysis of lignans such as HMR, secoisolariciresinol (SECO), mateiresinol (MR), enterodiol (END), hydroxyenterolactone (HEL), and ENL was made. Aliquots of 0.5 ml of thawed urine samples were mixed with 1.0 ml of 0.15 *M* sodium acetate buffer (pH 4.0) and 15 µl of *Helix pomatia* enzyme mixture. For hydrolysis of lignan conjugates, the samples were incubated at 37°C overnight. The hydrolyzed samples were extracted using Sep-Pak tC18 columns (Waters Associates, Milford, MA) conditioned with 2.0 ml of 0.15 *M* sodium acetate buffer. The urine samples to which 2.5 µg of the internal standard flavone had been added were loaded into columns and washed with 0.15 *M* acetate buffer, then the polyphenolic fraction was eluted with 2.0 ml methanol. The samples were gently evaporated to dryness under nitrogen flow in a water bath at 45°C, dissolved in 5.0 ml methanol, and then an aliquot of 0.1 ml was diluted with 0.9 ml of 0.1% acetic acid. The final flavone concentration was 50 ng/ml. A variety of lignans were analyzed by HPLC-MS-MS using a PE Sciex API3000 triple quadrupole mass spectrometer equipped with a Turbo ion spray ionization source (electrospray ionization). Detailed methods have been described in a previous paper (26).

Statistical Analysis. Data on tumor incidence were statistically analyzed using the cumulative chi-square test (27). If significance was detected, differences between groups were confirmed by chi-square test. Other data were analyzed using the Student's *t* test for comparison between two groups and one-way analysis of variance (ANOVA) for multiple groups. *Post hoc* multiple comparisons were performed by Tukey's test when numbers of data were equal or Scheffe's test in other cases. A *P* value less than 0.05 was considered to be statistically significant.

Results

Food Consumption and Daily Intake of HMR in the Experimental Groups. Data for food consumption and intake of HMR in the experimental groups are summarized in Table 1. In the control group fed conventional diet (Group 1), food consumption was lower than those in Groups 2, 3, or 4 ($P < 0.01$). There were no significant differences in food consumption among the other 3 experimental groups. Mean daily intakes of HMR were 11.0 and 32.7 mg/kg/day in the 200- and 600-ppm dosed groups, respectively, and they differed statistically ($P < 0.001$).

Effects of HMR on Start of Persistent Estrus. At 4 months of age (about 1 month after dosing started), the estrous cycle stage could be easily identified using vaginal smears, a precise 4-day cycle being evident in all groups. In the control groups supplied with the conventional (Group 1) or basal (Group 2) diet, no significant difference of the beginning of persistent estrus, characterized by vaginal smears exhibiting nucleated epithelial or cornified cells, was detected. Mean ages of persistent estrus start were 30.3 and 32.1 weeks in Groups 1 and 2, respectively. However, in HMR-dosed groups (Groups 3 and 4), persistent estrus was significantly lengthened for 3 to 5 weeks ($P < 0.05$), as shown in Table 2. Almost all animals in the experimental groups were in persistent estrus until 12 months of age.

Uterine Proliferative Lesions and Other Histopathologic Findings. During the experimental period, no animals in any of the groups died. No significant changes in relative weights of the uterus and ovaries were detected at termination at 15 months of age (data not shown). A comparison of development of uterine proliferative lesions in controls and HMR-dosed rats is given in Table 3. Almost all animals had endometrial hyperplasia or adenocarcinoma. Total incidences of endometrial hyperplasia were 64%, 63%, 78%, and 80% in Groups 1–4, respectively, no shifting the burden from late stage lesions to early stage being obvious. Most hyperplasias were focal proliferations of uterine glands with apparent duct structures in the stroma of the endometrium. The characteristics and incidences of endometrial hyperplasia did not differ among the groups. Endometrial adenocarcinoma was significantly decreased by the HMR treatments ($P < 0.05$). Incidences of adenocarcinoma in 200- and 600-ppm, HMR-dosed groups were reduced to 11% and 15%, respectively, compared with those

Table 3. Numbers and Incidences of Uterine Endometrial Proliferative Lesions at 15 Months of Age in the Four Experimental Groups[a]

Group	N	Hyperplasia				Adenocarcinoma
		−	+	++	+++	
Control—conventional diet (CRF-1)	25	0 (0)	2 (8)	8 (32)	6 (24)	9 (36)
Control—basal diet (1324 diet alone)	27	2 (7)	2 (7)	8 (30)	7 (26)	8 (30)
200 ppm HMR in the basal diet	27	3 (11)	5 (19)	9 (33)	7 (26)	3* (11)
600 ppm HMR in the basal diet	26	1 (4)	4 (15)	12 (46)	5 (19)	4* (15)

[a] Values in parentheses are incidences (%). Significantly different from Groups 1 and 2 (* $P < 0.05$). These data show that hydroxymatairesinol (HMR) significantly reduced the number of endometrial adenocarcinomas versus controls in Groups 1 and 2 given no HMR.

of the two control groups (conventional and basal diet), being 36% and 30%, respectively. The adenocarcinomas were well-differentiated, invading the serosa of the corpora uteri, glandular structures being obvious.

Ovarian atrophy or cyst formation and lack of any corpora lutea were observed in almost all animals. Proliferation of ovarian interstitial cells was also evident. Various neoplastic and nonneoplastic lesions were also found in other organs and tissues, but no differences were apparent among the groups.

Urinary Lignans. Urinary concentrations of lignans such as HMR, SECO, MR, END, HEL, and ENL at 8 months of age are shown in Figure 3. Those in the control group supplied with conventional diet (Group 1) were comparable to those with the basal diet (Group 2), and HMR was undetectable in either control group. In the 200- and 600-ppm groups, however, urinary concentrations of HMR were dose-dependently increased at 25 and 88 μg/ml, respectively, and similar elevation was evident for ENL and HEL concentrations. Urinary concentrations of SECO, MR, and END were low or undetectable.

Discussion

In the present study, HMR, obtained from the heartwood of spruce (*Picea abies*), demonstrated an inhibitory effect on the development of uterine adenocarcinoma in Donryu rats initiated by ENNG, in line with the experimental evidence of inhibition of the growth of DMBA-induced rat mammary tumors published earlier (8, 9). Secoisolariciresinol diglycoside (SDG), isolated from flaxseed, is metabolized to both END and ENL, and has shown chemopreventive properties in the DMBA-induced mammary-tumor model (28). ENL potently inhibits the growth of DMBA-induced mammary carcinoma in the rat (29). Until now, however, there has been no report of anticarcinogenic effects of lignans on female genital tracts, including rodent uteri. The present results provide the first support for the hypothesis that long-term exposure to HMR might similarly result in a chemopreventive effect on rat uterine carcinogenesis.

Plant lignans are metabolized by the mammalian gut microflora mainly to ENL and END, called mammalian lignans (30). The urinary lignans of HMR-dosed animals were mostly HMR, HEL, and ENL in the present study; the increase was 63- and 210-fold for HEL and 5- and 10-fold for ENL in urine 24 hours after feeding 200- and 600-ppm HMR-containing diets, respectively. Previously, it was reported that ENL excretion in urine was elevated 1.5- to 9-fold after single oral dosing of HMR at 3–50 mg/kg (8). Oral administration of HMR to Sprague-Dawley rats resulted in doubled excretion of ENL, with a single gavage at 25 mg/kg (26). Daily dosage of HMR in the present study was 11.0 and 32.7 mg/kg, respectively; thus urinary ENL concentrations after a single administration in the present study were comparable to those in the previous reports. In a recent *in vitro* study, metabolites of HMR generated by human intestinal microflora were characterized as ENL and HEL (31), strongly suggesting that HMR might be transformed into these two forms in the mammalian gut (Fig. 1).

Estrogens are well established as important etiological agents for uterine carcinogenesis in humans (32–35). Although exact roles remain to be detailed, tumor-promoting effects involving up-regulation of cell proliferation have long been suggested. Recently, natural compounds having antiestrogenic activity were proven to have chemopreventive effects against estrogen-dependent carcinoma development (1). Competition for estrogen receptor-binding (36) and inhibition of aromatase activity (29) are plausible explanations for chemopreventive effects of compounds such as flavonoids and lignans. Lignans and endogenous estrogens have structural similarities, suggesting possible estrogen-like or antiestrogen-like activity. Secoisolariciresinol diglycoside feeding to rats during pregnancy and lactation has been found to increase the uterine weights of offspring at weaning, but not at later stages (37). It causes irregular estrous cycling and/or persistent estrus in adult, normal-cycling rats (38). HMR, however, exerted no significant estrogen-like or antiestrogenic effects on the immature rat uterine growth test (8). There were also no

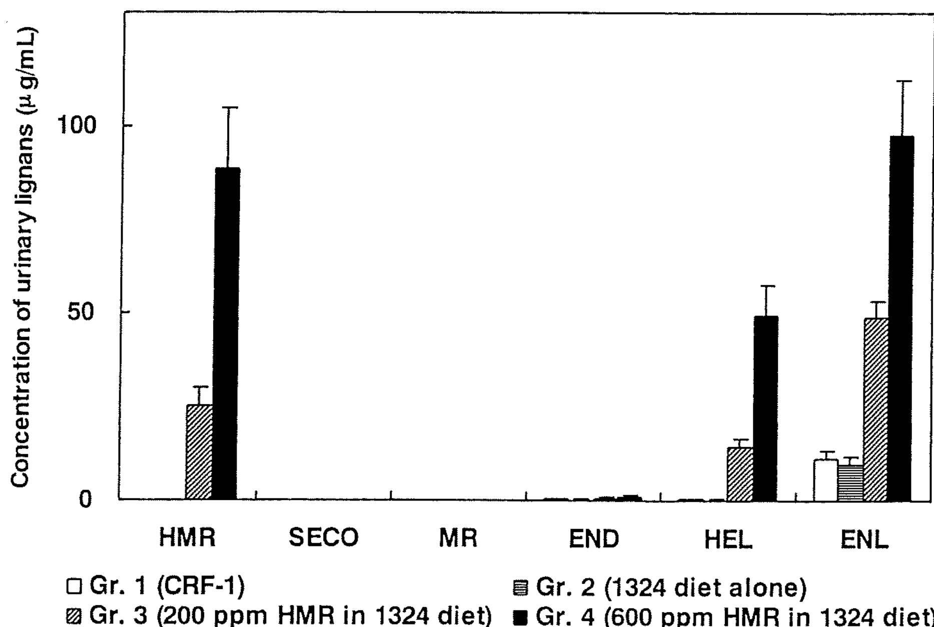

Figure 3. Urinary lignans at 8 months of age. Hydroxymatairesinol (HMR), secoisolariciresinol (SECO), mateiresinol (MR), enterodiol (END), hydroxyenterolactone (HEL), and enterolactone (ENL) were analyzed by HPLC-MS-MS. Values are mean ± SEM (n = 6).

effects on uterine weights, ovarian weights, or estrous cycle-period in the present experiment.

We have documented that the Donryu rat is a high-incidence strain for spontaneous development of endometrial adenocarcinoma and is associated with a hormonal imbalance characterized by early development of persistent estrus (15–17). Previously, it was reported that disorders or suppression of the estrous cycle, appearing very early in rats exposed prenatally to DES, might be associated with neoplastic development (39). It has been shown that *p-tert*-octylphenol, known as an EDC and having estrogenic activity, causes early occurring, persistent estrus with exposure for the first 5 days after puberty, although no abnormalities in growth and development of the reproductive organs could be found up to maturation; finally, development of uterine adenocarcinoma was accelerated (22). Although the pathogenesis of uterine tumor development by these compounds remains to be elucidated, a hormonal disorder characterized by early development of persistent estrus and increase of the E2/P ratio is exclusively involved (21). In the present study, delay in starting persistent estrus because of HMR dosing was significant. Persistent estrus results from anovulation, which is effected by change in action of various hormones such as LH-RH, LH, and estrogen. Recently, ENL was demonstrated to act as a weak aromatase inhibitor *in vitro* and to reduce the relative uterine weights of DMBA-treated, nonovariectomized rats (29). Aromatase inhibitors and antiestrogenic pharmaceuticals can reduce estrogen levels, followed by elevation of FSH and growth of ovarian follicles. The mechanisms underlying the delay of persistent estrus with HMR dosing is unclear, but ENL, a major metabolite, could be responsible through its action on aromatase.

In the present experiment, the 1324 diet was selected as a basal diet, instead of the conventional CRF-1 diet for Donryu rats (21), but both were included as controls, the CRF-1 group for historical background data in the rat strain and the 1324 diet group for HMR dosing. No significant differences in tumor development, start of persistent estrus, or other parameters were evident between the two control groups. There is evidence that subcutaneous injections of genistein and daidzein have an inhibitory effect on endometrial carcinogenesis in *N*-methyl-*N*-nitrosourea and E2-treated mice (40). The fact that the tumor incidences in both controls were comparable despite different contents of isoflavones suggests that these isoflavones are unlikely to have an inhibitory effect on cancer development in the present study. Difference in route of exposure, dose and/or inhibitory effect on the aromatase activity of these isoflavones (41) might be responsible for the discrepancy. Tumor incidence in the conventional-diet group was relatively low compared with that of the previous data (21). Although the reason is unclear, the design of the experiment appears appropriate for investigation of the chemopreventive effects on uterine carcinogenesis, given the positive influence detected.

In conclusion, long-term administration of HMR can reduce the development of uterine adenocarcinoma in Donryu rats, suggesting that its indirect modulation of hormonal regulation and its effect on estrogen production create an unfavorable milieu for tumor growth. To test this hypothesis, further examination of the detailed mechanisms of HMR's cancer-chemopreventive activity will be required.

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—Original—

Maternal Exposure to Low Doses of Bisphenol A Has No Effects on Development of Female Reproductive Tract and Uterine Carcinogenesis in Donryu Rats

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Abstract. Effects of maternal exposure to low doses of bisphenol A (BPA), including those comparable with human exposure levels, on growth and development of the female reproductive system and uterine carcinogenesis in Donryu rats were investigated. Dams were administered BPA (0, 0.006 and 6 mg/kg/day) daily by gavage from gestation day 2 up to the day before weaning (postnatal day 21 at offspring). The serum levels of BPA were significantly elevated in the dams receiving 6 mg/kg/day, however, BPA levels in the milk of dams, and those in the serum and liver of offspring were similar between control and treated groups. The treatment did not exert any influences on uterine development including weight, gland genesis and estrogen receptor α expression, vaginal opening and gonadotropin secretion in the female offspring up to puberty. After maturation, no effects were evident with regard to estrous cyclicity in female offspring treated with BPA. In addition, the treatment had no effects on age-related morphological changes of the reproductive and endocrine organs and uterine carcinogenesis until 15 months of age. The results demonstrate that maternal exposure to BPA at levels comparable to human exposure did not have any effects on the female reproductive system of offspring in rats. In addition, BPA was also found in the serum, milk and liver of control dams and pups, and low levels of BPA were detected in drinking water and pellet diet. The present study showed that the experimental animals were also exposed to environmental BPA in the animal room.

Key words: Bisphenol A, Low doses, Female reproductive system, Toxicity, Uterine carcinogenesis

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Bisphenol A (BPA), volume chemical used in the manufacture of polycarbonate plastics, has been found in canned foods packed in lacquer subjects, containers and composite dental sealants [1]. BPA is reported to be an endocrine disrupting chemical (EDC) with weak estrogenic activity in both *in vitro* and *in vivo* systems [2], binding to both estrogen receptor (ER)α and ERβ with low affinity and causing reporter gene transactivation *in vitro* [3, 4]. Uterotropic effects of BPA at high doses (3 daily oral applications of 400, 600 or 800 mg/kg/day) were reported in immature female rats [5], and significant increases in the luminal epithelial height and the thickness of both stromal and myometrial layers of the uterus were also observed in ovariectomized mice injected 0.8–8 mg/day of BPA for 4 days [6]. A study conducted by the National Toxicology Program (NTP) in the USA demonstrated that maternal exposure to high doses of BPA, at 0.5 or 1.0% in feed (approximately daily intakes of 875 and 1750 mg/kg/day), reduced the number of live pups per litter and litters per pair in

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