Chemopreventive Effects of Hydroxymatairesinol on Uterine Carcinogenesis in Donryu Rats

SHIN-ICHI KATSUDA,*[,1] MIDORI YOSHIDA,† NIINA SAARINEN,‡ ANNIKA SMEDS,§ DAI NAKAE,† RISTO SANTTI,‡ AND AKIHIKO MAEKAWA†

Department of Biological Safety Research, Japan Food Research Laboratories, Tama-shi, Tokyo 206-0025, Japan; †Department of Pathology, Sasaki Institute, Chiyoda-ku, Tokyo 101-0062, Japan; ‡Department of Anatomy, Institute of Biomedicine, University of Turku, FIN-20520 Turku, Finland; and §Department of Organic Chemistry, Åbo Akademi University, Biskopsgatan 8, FIN-20500 Turku, Finland

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

Part of this study was supported by a grant-in-aid from the Ministry of Health, Labor, and Welfare of Japan.

[1] To whom requests for reprints should be addressed at Department of Biological Safety Research, Japan Food Research Laboratories, 6-11-10 Nagayama, Tama-shi, Tokyo 206-0025, Japan. E-mail: katudas@jfrl.or.jp

Received October 16, 2003.
Accepted February 5, 2004.

1535-3702/04/2295-0001$15.00

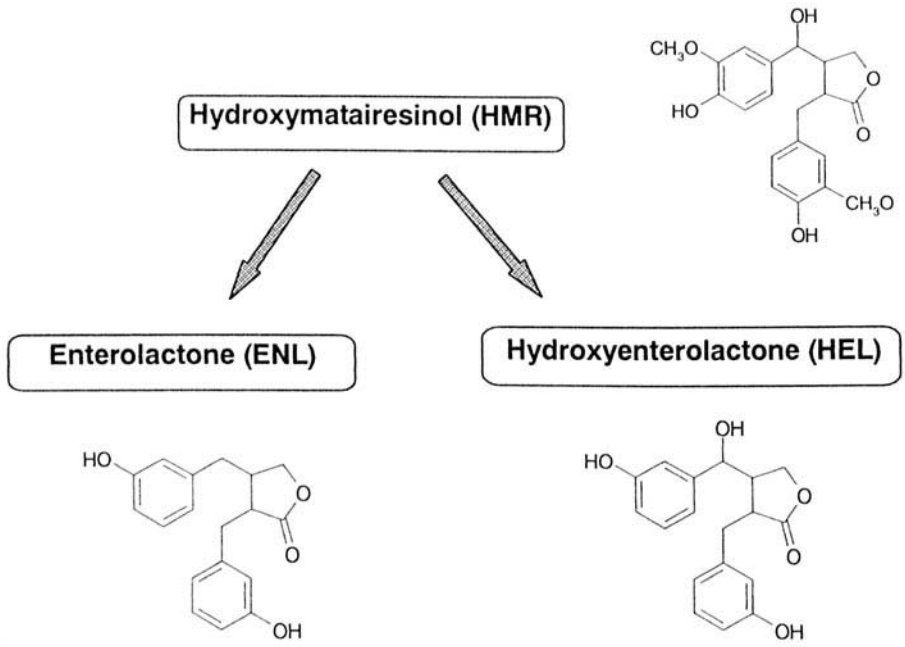

Figure 1. Chemical structure of hydroxymatairesinol (HMR) and schematic illustration of its putative metabolic pathway, which involve demethylation and dehydroxylation reactions catalyzed by intestinal bacteria.

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,

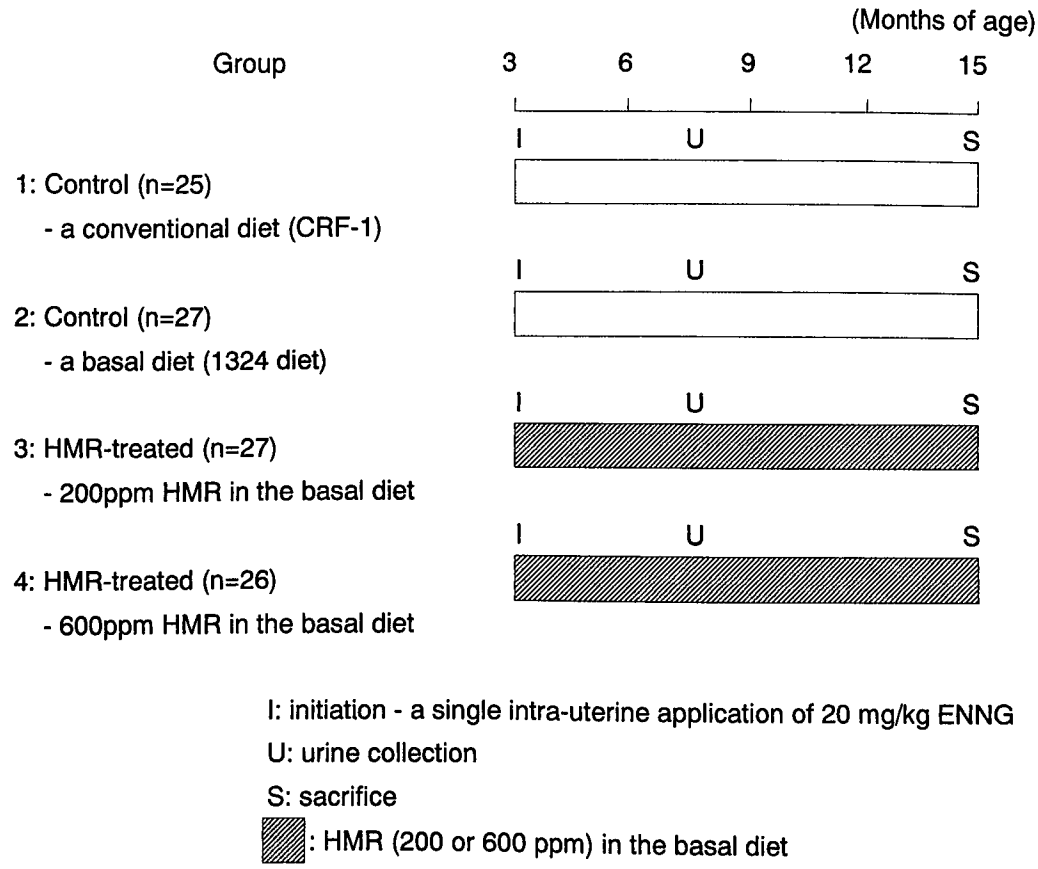

Figure 2. Experimental design for examination of the effects of hydroxymatairesinol (HMR) on rat uterine carcinogenesis. Rats were initiated with *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) and then fed a conventional diet (CRF-1; Group 1), a basal diet (1324; Group 2), or HMR (200 and 600 ppm;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

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 ($P < 0.001$).

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.

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	2	8	6	9
		(0)	(8)	(32)	(24)	(36)
Control—basal diet (1324 diet alone)	27	2	2	8	7	8
		(7)	(7)	(30)	(26)	(30)
200 ppm HMR in the basal diet	27	3	5	9	7	3*
		(11)	(19)	(33)	(26)	(11)
600 ppm HMR in the basal diet	26	1	4	12	5	4*
		(4)	(15)	(46)	(19)	(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

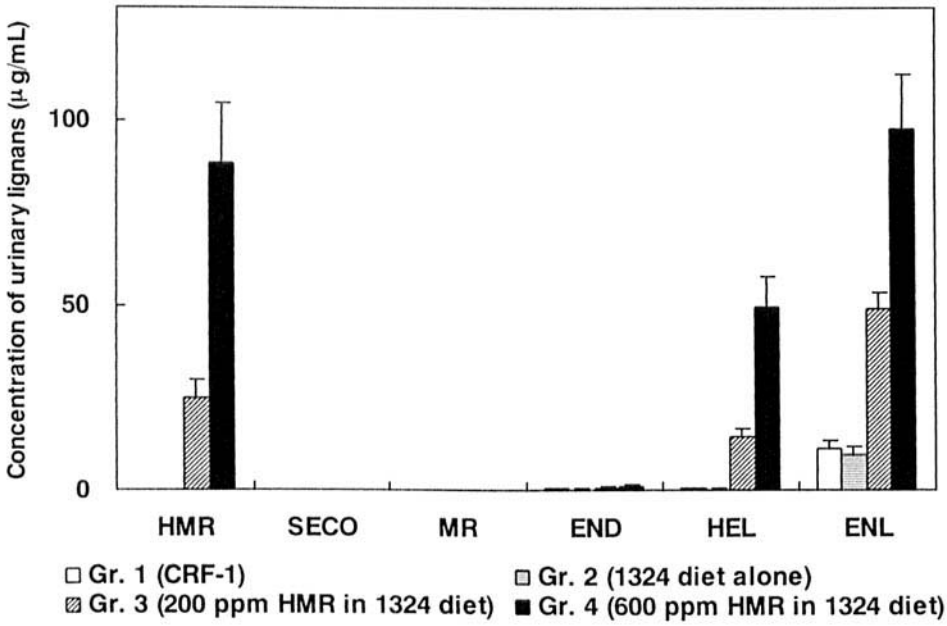

Figure 3. Urinary lignans at 8 months of age. Hydroxymatairesinol (HMR), secoisolariciresinol (SECO), mateiresinol (MR), enterodiol (END), hydroxyenterolactone (HEL), and enterolactone (ENL) were analyzed by HPLC-MS-MS. Values are mean ± SEM (n = 6).

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.

We thank Hiromi Ichihara for her expert technical assistance with the histopathological preparations, Michiyo Tosa and Ryotaro Ebisui for analysis of isoflavones of diets, and Dr. Hidetaka Sato for expertise in statistical analysis. We would also like to thank Christer Eckerman for HMR extraction, and Teija Toivonen and Pauliina Penttinen for urine sample preparation. Finally, we thank Katariina Kauppila and Tadaaki Toyoshima at the Embassy of Finland in Japan.

1. Adlercreutz H, Mazur W. Phyto-oestrogens and Western diseases. Ann Med 29:95–120, 1997.
2. Hirose K, Tajima K, Hamajima N, Inoue M, Takezaki T, Kuroishi T, Yoshida M, Tokudome S. A large-scale, hospital-based case-control study of risk factors of breast cancer according to menopausal status. Jpn J Cancer Res 86:146–54, 1995.
3. Wu AH, Wan P, Hankin J, Tseng CC, Yu MC, Pike MC. Adolescent and adult soy intake and risk of breast cancer in Asian-Americans. Carcinogenesis 23:1491–1496, 2002.
4. Wu AH, Ziegler RG, Nomura AM, West DW, Kolonel LN, Horn-Ross PL, Hoover RN, Pike MC. Soy intake and risk of breast cancer in Asians and Asian Americans. Am J Clin Nutr 68:1437S–1443S, 1998.
5. Dai Q, Franke AA, Jin F, Shu XO, Hebert JR, Custer LJ, Cheng J, Gao YT, Zheng W. Urinary excretion of phytoestrogens and risk of breast cancer among Chinese women in Shanghai. Cancer Epidemiol Biomarkers Prev 11:815–821, 2002.
6. Chen J, Tan KP, Ward WE, Thompson LU. Exposure to flaxseed or its purified lignan during suckling inhibits chemically induced rat mammary tumorigenesis. Exp Biol Med 228:951–958, 2003.
7. Goodman MT, Wilkens LR, Hankin JH, Lyu LC, Wu AH, Kolonel LN. Association of soy and fiber consumption with the risk of endometrial cancer. Am J Epidemiol 146:294–306, 1997.
8. Saarinen NM, Wärri A, Mäkelä SI, Eckerman C, Reunanen M, Ahotupa M, Salmi SM, Franke AA, Kangas L, Santti R. Hydroxymatairesinol, a novel enterolactone precursor with antitumor properties from coniferous tree (*Picea abies*). Nutr Cancer 36:207–216, 2000.
9. Saarinen NM, Wärri A, Mäkelä SI, Eckerman C, Reunanen M, Ahotupa M, Salmi SM, Franke AA, Kangas L, Santti RS. Uptake and metabolism of hydroxymatairesinol in relation to its anticarcinogenicity in DMBA-induced rat mammary carcinoma model. Nutr Cancer 41:82–90, 2001.
10. Ingram D, Sanders K, Kolybaba M, Lopez D. Case-control study of phyto-oestrogens and breast cancer. Lancet 350:990–994, 1997.
11. Pietinen P, Stumpf K, Mannisto S, Kataja V, Uusitupa M, Adlercreutz H. Serum enterolactone and risk of breast cancer: a case-control study in eastern Finland. Cancer Epidemiol Biomarkers Prev 10:339–344, 2001.
12. Hulten K, Winkvist A, Lenner P, Johansson R, Adlercreutz H, Hallmans G. An incident case-referent study on plasma enterolactone and breast cancer risk. Eur J Nutr 41:168–176, 2002.
13. Kilkkinen A, Virtamo J, Vartiainen E, Sankila R, Virtanen MJ, Adlercreutz H, Pietinen P. Serum enterolactone concentration is not associated with breast cancer risk in a nested case-control study. Int J Cancer 108:277–280, 2004.
14. Adlercreutz H. Phytoestrogens and breast cancer. Steroid Biochem Mol Biol 83:113–118, 2002.
15. Maekawa A, Onodera H, Tanigawa H, Furuta K, Matsuoka C, Ogiu T, Hayashi Y. Spontaneous neoplastic and non-neoplastic lesions in aging Donryu rats. Jpn J Cancer Res (Gann) 77:882–890, 1986.
16. Nagaoka T, Onodera H, Matsushima Y, Todate A, Shibutani M, Ogasawara H, Maekawa A. Spontaneous uterine adenocarcinomas in aged rats and their relation to endocrine imbalance. J Cancer Res Clin Oncol 116:623–628, 1990.
17. Nagaoka T, Takeuchi M, Onodera H, Matsushima Y, Ando-Lu J, Maekawa A. Sequential observation of spontaneous endometrial adenocarcinoma development in Donryu rats. Toxicol. Pathol 22, 261–269, 1994.
18. Nagaoka T, Takegawa K, Takeuchi M, Maekawa A. Effects of reproduction on spontaneous development of endometrial adenocarcinomas and mammary tumors in Donryu rats. Jpn J Cancer Res 91:375–382, 2000.
19. Ando-Lu J, Takahashi M, Imai S, Ishihara R, Kitamura T, Iijima T, Takano S, Nishiyama K, Suzuki K, Maekawa A. High-yield induction of endometrial adenocarcinomas in Donryu rats by a single intra-uterine administration of *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine. Jpn J Cancer Res 85:789–793, 1994.
20. Katsuda S, Yoshida M, Kuroda H, Ando J, Takahashi M, Kurokawa Y, Watanabe G, Taya K, Maekawa A. Uterine adenocarcinoma in *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine-treated rats with high-dose exposure to *p-tert*-octylphenol during adulthood. Jpn J Cancer Res 93:117–124, 2002.
21. Maekawa A, Takahashi M, Ando J, Yoshida M. Uterine carcinogenesis by chemicals/hormones in rodents. J Toxicol Pathol 12:1-11, 1999.
22. Yoshida M, Katsuda S, Tanimoto T, Asai S, Nakae D, Kurokawa Y, Taya K, Maekawa A. Induction of different types of uterine adenocarcinomas in Donryu rats due to neonatal exposure to high-dose *p-t*-octylphenol for different periods. Carcinogenesis 23:1745–1750, 2002.
23. Kudou S, Fleury Y, Welti D, Magnolato D, Uchida T, Kitamura K, Okubo K. Malnyl isoflavone glycosides in soybean seeds (*Glycine max* Merrill). Agric Biol Chem 55:2227–2233, 1991.
24. Ekman R Analysis of lignans in Norway spruce by combined gas chromatography-mass spectrometry. Holzforschung 30:79–85, 1976.
25. Ekman R. Distribution of ligans in Norway spruce. Acta Acad Aboensis Ser B 39:1–6, 1979.
26. Saarinen NM, Smeds A, Mäkelä SI, Ämmälä J, Hakala K, Pihlava J-M, Ryhänen E-L, Sjöholm R, Santti R. Structural determinants of plant lignans for the formation of enterolactone *in vivo*. J Chormatogr B 777:311–319, 2002.
27. Shimizu K, Hirose N, Hirotsu C, Adachi E. A classification of circadian blood pressure profiles using a cumulative chi-square technique. Scand J Clin Lab Invest 60:703–712, 2000.
28. Tou JCL, Thompson LU. Exposure to flaxseed or its lignan component during different development stages influences rat mammary gland structure. Carcinogenesis 20:1831–1835, 1999.
29. Saarinen NM, Houvinen R, Wärri A, Mäkelä SI, Valentín-Blasini L, Sjöholm R, Ämmälä J, Lehtilä R, Eckerman C, Collan YU, Santti RS. Enterolactone inhibits the growth of 7,12-dimethylbenz(*a*)anthracene-induced mammary carcinomas in the rat. Mol Cancer Ther 1:869–876, 2002.
30. Axelson M, Sjövall J, Gustafsson BE, Setchell KD. Origin of lignans in mammals and identification of a precursor from plants. Nature 298:659–660, 1982.
31. Heinonen S, Nurmi T, Liukkonen K, Poutanen K, Wahala K, Deyama T, Nishibe S, Adlercreutz H. In vitro metabolism of plant lignans: new precursors of mammalian lignans enterolactone and enterodiol. J Agric Food Chem 49:3178–3186, 2001.
32. Lingeman CH. Hormones and hormonomimetic compounds in the etiology of cancer. Recent Results Cancer Res 66:1–48, 1979.
33. Hilliard G.D, Norris HJ. Pathologic effects of oral contraceptives. Recent Results Cancer Res 66:49–71, 1979.
34. Zeil HK. Estrogen's role in endometrial cancer. Obstet Gynecol 60:509–515, 1982.
35. Fox H. Endometrial carcinogenesis and its relation to oestrogen. Pathol Res Pract 179:13–19, 1984.
36. Wang L-Q. Mammalian phytoestrogens: enterodiol and enterolactone. J Chromatogr B 777:289–309, 2002.
37. Tou JCL, Chen J, Thompson LU. Flaxseed and its lignan precursor, secoisolariciresinol diglycoside, affect pregnancy outcome and reproductive development in rats. J Nutr 128:1861–1868, 1998.
38. Orcheson LJ, Rickard SE, Seidl MM, Thompson LU. Flaxseed and its

mammalian lignan precursor cause a lengthening or cessation of estrous cycling in rats. Cancer Lett 125:69–76, 1998.

39. Kitamura T, Nishimura S, Sasahara K, Yoshida M, Ando J, Takahashi M, Shirai T, Maekawa A. Transplacental administration of diethylstilbestrol (DES) causes lesions in female reproductive organs of Donryu rats, including endometrial neoplasia. Cancer Lett 141:219–228, 1999.
40. Lian Z, Niwa K, Tagami K, Hashimoto M, Gao J, Yokoyama Y, Mori H, Tamaya T. Preventive effects of isoflavones, genistein and daizein, on estradiol-17?-related endometrial carcinogenesis in mice. Jpn J Cancer Res 92:726734, 2001.
41. Campbell DR, Kurzer MS. Flavonoid inhibition aromatase enzyme activity in human preadipocytes. J Steroid Biochem Mol Biol 46:381–388, 1993.