

Chemoprevention of Spontaneous Endometrial Cancer in Female Donryu Rats by Dietary Indole-3-carbinol¹

Toshihiro Kojima,² Takuji Tanaka, and Hideki Mori

First Department of Pathology, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu City 500, Japan

ABSTRACT

Indole-3-carbinol (I3C) present in cruciferous vegetables has been found to inhibit chemically induced neoplasms in forestomach, mammary gland, liver, and tongue in rodents. I3C is also known to induce estradiol 2-hydroxylase and reduce estrogenic activity. The current study was conducted to examine the possible inhibiting effect of I3C on spontaneous occurrence of endometrial adenocarcinoma in female Donryu rats. The high incidence of endometrial cancer in this strain of rats might be related to increased estrogen/progesterone ratio with aging. A total of 141 female Donryu rats were divided into four groups. Group 1 was given a basal diet alone throughout the study. Starting at 6 weeks of age, groups 2-4 were respectively given the diets containing 200, 500, and 1000 ppm I3C for the entire experimental period (660 days). At the termination of the experiment, the incidences of preneoplastic lesions and neoplasms in the endometrium were estimated. Also, estradiol 2-hydroxylation activity in the liver was assayed in rats fed I3C at these doses for 3 weeks. The incidences of endometrial adenocarcinoma in rats fed I3C [8 of 32 rats (25%) in group 2, 5 of 32 rats (16%) in group 3, and 5 of 35 rats (14%) in group 4] were respectively smaller than that in group 1 (12 of 32 rats, 38%). The incidence of uterine adenocarcinoma in group 4 was significantly lower than that in group 1 ($P < 0.05$). Dietary I3C also decreased the frequency of preneoplastic endometrial lesions (31% in groups 2-4 versus 44% in group 1). I3C exposure also inhibited the incidence of mammary fibroadenoma. Biochemical assay for estradiol 2-hydroxylation revealed that feeding of I3C significantly increased estradiol 2-hydroxylation (nmol/mg protein) compared to rats treated without I3C [0.34 ± 0.04 in group 2, 0.53 ± 0.13 in group 3, and 0.58 ± 0.11 in group 4 versus 0.28 ± 0.02 in group 1; $P < 0.02$, $P < 0.003$, and $P < 0.001$, respectively]. These results suggest that dietary I3C inhibits spontaneous occurrence of endometrial adenocarcinoma as well as preneoplastic lesions in Donryu rats. This chemopreventive effect of I3C may be due to its induction of estradiol 2-hydroxylation.

INTRODUCTION

It is well known that dietary factors influence the process of carcinogenesis. Several natural products in fruits and vegetables are reported to possess antimutagenic and anticarcinogenic properties (1-8). The cruciferous vegetables (e.g., cabbage, broccoli, brussels sprouts, cauliflower, etc.) contain indole derivatives, dithiolthiones, and isothiocyanates (9, 10) and these compounds have been shown to be anticarcinogenic in several rodent carcinogenesis models (11, 12). I3C,³ one of the indole derivatives, inhibits carcinogen-induced neoplasms of forestomach (13) and mammary gland (13) in mice or rats. Recently, we have demonstrated that I3C suppresses liver cell neoplasms induced by diethylnitrosamine (14) and tongue neoplasms induced by 4-nitroquinoline 1-oxide in rats (15). Such suppressing effects of I3C are considered to be due to alteration of drug-metabolizing enzymes including cytochrome P-450 and detoxifying enzymes (16-18).

In Japan, the age-adjusted mortality rate for endometrial cancer has been increasing for the last decade (19) and this tumor type is now about 20% of uterine cancer. Such an increase in the endometrial cancer patients is considered to be associated with progressive introduction of Western dietary habit (20). For the occurrence of endometrial cancer, fat intake and obesity lead to increased endogenous estrogen production exerting a promotional effect (21, 22). However, little is known about the modulation of carcinogenesis in the endometrium by micronutrients. This may be partly due to the lack of simple and reliable animal models for endometrial cancer (23). Recently, a high incidence of spontaneous endometrial adenocarcinoma as well as hyperplastic lesions in female Donryu rats was shown by Maekawa *et al.* (24) and this model appears to be a useful animal model for human endometrial cancer. Increasing estrogen/progesterone ratio with aging is suggested to be a causative factor for the high incidence of spontaneous endometrial adenocarcinoma of this strain (25).

Indoles derived from cruciferous vegetables especially I3C is known to induce hepatic estradiol 2-hydroxylase in both humans and rats (26). Dietary exposure of I3C has been described to reduce the incidence of spontaneous and estrogen-responsive mammary tumors in female C3H mice (27) and decrease cell proliferation of the *ras* oncogene-transfected mammary epithelial cell line (28). These findings led us to examine the possibility that I3C may suppress the occurrence of another estrogen-dependent neoplasm endometrial cancer by an increase in 2-hydroxylation. In the present study, the possible suppressing effect of I3C on spontaneous occurrence of endometrial adenocarcinoma in female Donryu rats was investigated. Furthermore, estradiol 2-hydroxylation in the liver was also assayed in order to examine such a mechanistic aspect if I3C could modify the spontaneous occurrence of endometrial cancers.

MATERIALS AND METHODS

Animals and Diet. Female Donryu rats, 4 week old, from Shizuoka Laboratory Animal Center (Shizuoka, Japan) were quarantined for 14 days and transferred to the holding room. Rats were housed 3 or 4 to a wire cage. The holding room was maintained at $23 \pm 2^\circ\text{C}$, $50 \pm 10\%$ humidity, and a 12-h light-dark cycle. Powdered CE-2 (CLEA Japan, Inc., Tokyo, Japan) was used as a basal diet throughout the experiment. The percentage composition of CE-2 was shown in our previous report (29). The care of animals conformed to the Institutional Animal Care and Use Committee of the Gifu University School of Medicine.

Chemical. I3C (>99% purity) was purchased from Aldrich Chemical Co., Milwaukee, WI.

Treatment of Animals. A total of 141 rats were divided into 4 groups as shown in Tables 1-4. Group 1 was given the basal diet alone. Groups 2, 3, and 4 were given the diets containing 200, 500 and 1000 ppm I3C, respectively, for 660 days, starting at 6 weeks of age. The diets containing I3C were prepared every 2 weeks and stored in a cold room at 4°C until used. The diets and tap water were freely available. Body weight and food intake were measured every week during the experiment. All rats were carefully observed and sacrificed under ether anesthesia by exsanguination from the aorta at 660 days after the start of the experiment.

Histology. Complete necropsies were performed on all animals. All organs were fixed in 10% buffered formalin. All tissues and gross lesions were processed for histology by the conventional methods and stained with hematoxylin and eosin for histological diagnosis. Preneoplastic lesions and neo-

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² To whom requests for reprints should be addressed.

³ The abbreviation used is: I3C, indole-3-carbinol.

plasms in endometrium were diagnosed according to the WHO criteria (30). The rats alive more than 365 days were counted as effective animals, since most endometrial tumors occur after 1 year of age (25).

Estradiol 2-Hydroxylation. 2-Hydroxylation activity in the liver (left lobe) of five rats from each group was assayed at 21 days after the start. A 10% (w/v) homogenate of the liver was prepared in 0.25 M sucrose and centrifuged at $8000 \times g$ for 15 min. A microsomal fraction was obtained from the supernatant by centrifuging at $105,000 \times g$ for 1 h and resuspending the pellet in sucrose (200 mg original tissue/ml) (31). The radiometric assay was performed with the use of 50 μ l of microsomes (~ 10 mg of protein/ml), ascorbic acid (2 mM) in 3.9 ml of 100 mM potassium phosphate buffer (pH 7.4), 1.0 mg of NADPH, and 50 μ l of ethanol containing 32 nM unlabeled estradiol and approximately 10^5 dpm of [3 H]estradiol. The reaction was initiated by supplement of NADPH and the mixture was shaken for 20 min in a water bath at 37°C. After incubation, the reaction vial was placed on ice and mixed with 1.0 ml of ice-cold water containing 2% charcoal for 10 min. The mixture was then centrifuged at $3000 \times g$ for 15 min. C-2 hydroxylation released 3 H₂O, which was collected for scintillation counting. The results were expressed as nM/mg/min (26, 32).

Statistical Analysis. Means of values from the measurements were compared using a two-tailed Student's *t* test. Frequencies of the tumors and preneoplastic lesions of each group were compared using test or Fisher's exact probability test.

RESULTS

General Observations. Rats in groups 2–4 tolerated p.o. administration of I3C well. The average body weight and liver weight in each group at the end of the experiment are shown in Table 1. The mean body weights in the groups treated with I3C were slightly lower than that of rats treated without I3C, although the differences were not significant. Mean food intakes/day in all groups were comparable (16.3 g in group 1, 16.5 g in group 2, 16.1 g in group 3, and 15.4 g in group 4). Total intakes of I3C/rat were 2.1, 5.3, and 10.9 g in groups 2, 3, and 4, respectively. The mean survival time in each group are shown in Table 2. The mean survival time of group 4 was slightly longer than that of group 1, but there were no significant differences between the two groups.

Incidence of Neoplasms and Preneoplastic Lesions. In this study, tumors occurred mainly in the endometrium and mammary gland. Uterine tumors were located in the uterine horn near the uterine cervix. Histologically, they were mostly well or moderately differentiated types. The incidence of endometrial adenocarcinoma in each group is indicated in Table 3. The incidence of endometrial adenocarcinoma in rats of group 1 was 38% (12 of 32 rats). In rats fed I3C-containing diets, the frequencies of uterine cancer were 25% (8 of 32 rats) in group 2, 16% (5 of 32 rats) in group 3, and 14% (5 of 35 rats) in group 4. Statistical analysis on the incidence of endometrial adenocarcinoma revealed a significant difference between groups 1 and 4. ($P < 0.05$). Metastasis of uterine adenocarcinoma was found of groups 1–3 (group 1, 3 of 12; group 2, 2 of 8; and group 3, 2 of 5). However, no metastases were observed in rats bearing uterine cancer in group 4. Besides the endometrial malignant neoplasms, a high incidence of preneoplastic lesions was found in rats in all groups. In group 1, 14 of 32 rats (44%) had endometrial hyperplasia. As indicated in Table 3, exposure to I3C suppressed the frequency of these

Table 1 Effect of indole-3-carbinol on body and liver weights at the end of the study

Group	Treatment	Effective no. of rats	Body wt (g)	Liver wt (g)
1	No treatment	32	322 \pm 101 ^a	15.6 \pm 5.7
2	200 ppm I3C	32	299 \pm 81	13.7 \pm 4.8
3	500 ppm I3C	32	280 \pm 66	15.4 \pm 4.4
4	1000 ppm I3C	35	294 \pm 89	14.4 \pm 4.5

^a Mean \pm SD.

Table 2 Mean survival time in each group

Group	Treatment	Effective no. of rats	Survival time (days)
1	No treatment	32	593 \pm 89 ^a
2	200 ppm I3C	32	578 \pm 83
3	500 ppm I3C	32	595 \pm 70
4	1000 ppm I3C	35	612 \pm 76

^a Mean \pm SD.

hyperplastic lesions (31% in groups 2–4), although statistical differences among the groups were not present.

Other than endometrial tumors, spontaneous mammary tumors were developed in all groups, and they were histologically fibroadenomas. The incidences of such neoplasms in rats fed I3C (38% in group 2, 31% in group 3, and 43% in group 4) were lower than that in group 1 (56%). However, no significant differences were obtained among the groups.

Estradiol 2-Hydroxylation Activity. The results of estradiol 2-hydroxylation assay are indicated in Table 4. Estradiol 2-hydroxylation concentration in rats fed I3C was 0.34 ± 0.04 nM/mg/min in group 2, 0.53 ± 0.13 in group 3, or 0.58 ± 0.11 in group 4. These values were significantly greater than that of group 1 (0.28 ± 0.02); $P < 0.02$, $P < 0.003$, or $P < 0.001$.

DISCUSSION

The results in the present study demonstrated that p.o. administration of I3C inhibited the spontaneous occurrence of endometrial adenocarcinoma in female Donryu rats. Moreover, exposure of I3C suppressed the occurrence of endometrial hyperplasia, which is considered to be a precursor lesion for endometrial cancer (33).

Previously, I3C was reported to inhibit benzo(a)pyrene-induced forestomach in female ICR/Ha mice, 7,12-dimethylbenz(a)anthracene-induced mammary neoplasms in female Sprague-Dawley rats (13), and aflatoxin B₁-induced hepatic tumorigenesis in rainbow trout when administered prior to or during the carcinogen exposure (34), although there is a report that postinitiation exposure of I3C enhanced aflatoxin B₁-induced hepatocarcinogenesis in rainbow trout (35). We have recently reported that feeding I3C during the initiation phase of diethylnitrosamine-induced hepatocarcinogenesis could inhibit the development of liver cell foci and neoplasms in rats (14). A more recent study by us revealed that dietary exposure of I3C during the initiation or postinitiation phase suppressed tongue carcinogenesis by 4-nitroquinoline 1-oxide in male rats (15). These inhibitory effects of I3C on chemical carcinogenesis might be explained by alterations in the metabolism of carcinogens (mixed-function oxidase enzyme systems including cytochrome P-450), antioxidation (36), DNA adduct formation (37), or detoxification enzymes [glutathione S-transferase, epoxide hydratase (16–18), and UDP glucuronyltransferase (38)]. The results in the present study confirmed these earlier reports and furnished the evidences that I3C could inhibit spontaneous tumor development as well as chemically induced neoplasms.

Estradiol metabolism including the key pathways of 16 α - and 2-hydroxylation is P-450 dependent (39). Administration of I3C p.o. is suggested to affect estradiol metabolism. In fact, induction of hepatic estradiol 2-hydroxylase by I3C was reported in rats and humans (26). In general, any estrogen metabolized by the 2-hydroxylase pathway would no longer be estrogenic activity, although the metabolite 2-hydroxyestrone by 2-hydroxylase has a weak estrogenic activity in the breast and endometrium (40). Several reports have indicated an antiestrogenic activity on MCF-7 human breast cancer cells (41). The results of 2-hydroxylation assay in the liver in this study suggest that increased estradiol 2-hydroxylation is induced by dietary I3C. Therefore, prevention of the effect of I3C on spontaneous endometrial

Table 3 Incidences of hyperplasia and neoplasm of the endometrium

Group	Treatment	Effective no. of rats	No. of rats with hyperplasia (%)				No. of rats with ADC (%)
			Total	CGH ^a	AdH	AtH	
1	No treatment	32	14 (44)	1	4	9	12 (38)
2	200 ppm I3C	32	10 (31)	4	2	4	8 (25)
3	500 ppm I3C	32	10 (31)	3	3	4	5 (16)
4	1000 ppm I3C	35	11 (31)	3	2	6	5 (14) ^b

^a CGH, cystic glandular hyperplasia; AdH, adenomatous hyperplasia; AtH, atypical hyperplasia; ADC, adenocarcinoma.

^b Significantly different from group 1 by Fisher's exact probability test: $P < 0.05$.

Table 4 Effects of indole-3-carbinol on estradiol 2-hydroxylation

Group	Treatment	Av. food intake (g/day)	Liver/body wt (%)	Estradiol 2-hydroxylation (nm/mg/min)
1	No treatment	30.1	5.12 ± 0.32 ^a	0.28 ± 0.02
2	200 ppm I3C	30.7	5.22 ± 0.24	0.34 ± 0.04 ^b
3	500 ppm I3C	29.3	5.67 ± 0.29	0.53 ± 0.13 ^c
4	1000 ppm I3C	30.3	5.98 ± 0.25	0.58 ± 0.11 ^d

^a Mean ± SD.

^{b-d} Significantly different from group 1 by Student's *t* test.

^b $P < 0.02$.

^c $P < 0.003$.

^d $P < 0.001$.

cancer may be through an increase of estradiol 2-hydroxylase. Epidemiologically, a low fat diet and smoking contribute a lower risk of mammary and endometrial cancers (42–44); this may be due to increased levels of estradiol 2-hydroxylase caused by low fat intake (45) and smoking habit (46). On the other hand, a high 16 α -hydroxylase activity is reported in patients with breast cancer (47) and a close relationship between the extent of tumor incidence and 16 α -hydroxylation of estradiol is also shown in C3H mice carrying exogenous mouse mammary tumor virus (48). Furthermore, 7,12-dimethylbenz-(a)anthracene treatment is suggested to up-regulate the 16 α /C2 hydroxylation ratio in mouse mammary epithelial cells (49). These findings imply that activation of 2-hydroxylase lowers estrogen activity and protects against estrogen-dependent cancers such as breast and endometrial cancers. Dietary exposure of I3C to female C3H mice has been reported to reduce occurrence of spontaneous mammary tumor (27). In the current study, feeding of I3C also decreased the development of mammary tumors, but such inhibition was not significant.

At present, there are a few animal models for endometrial carcinogenesis (23, 50–52) and these need complicated procedures for induction of uterine cancer (23). Moreover, the incidence of induced tumors was relatively low. Therefore, modifying effects of specific chemicals on uterine carcinogenesis in rodents have not yet been studied well (53, 54). The rat model for uterine cancer used in the present study provides a relatively high incidence of endometrial cancer and their precursor lesions (24, 25). The estrogen/progesterone ratio is also reported to increase with aging and is suggested to be responsible for the development of endometrial adenocarcinoma in females of this strain (25). These findings are similar to those in human endometrial cancer. Accordingly, this may be a useful animal model for modifying effects of chemicals on endometrial cancer as shown in the present study.

In conclusion, dietary administration of I3C clearly inhibited the development of spontaneous endometrial adenocarcinoma in Donryu rats by activating estradiol 2-hydroxylation in the liver. Since almost all hormones currently used for the therapy for uterine cancer are regarded as nongenotoxic carcinogens (55), a naturally occurring substance I3C which may be more safe, is considered to be a possible chemopreventive agent for human endometrial cancers, especially those associated with endometrial hyperplasia (56). Further studies on potential risk and other mechanistic aspects of I3C are necessary for its long-term use as a chemopreventive agent for endometrial cancer,

since a change in the estrogen metabolic pathway from 16 α -hydroxylation to 2-hydroxylation forms catechol estrogen being a risk factor for breast cancer (57) and producing quinones and free radicals (58, 59) which may exert genotoxic effects (60).

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