

Preventive Effects of Indole-3-carbinol on Endometrial Carcinogenesis in Mice

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Abstract. [Object] The short- and long-term experiments were designed to determine the effects of indole-3-carbinol (I3C) on estrogen-related endometrial carcinogenesis in mice, associated with the expression of *c-fos* and *c-jun*.

[Methods] In the short-term assay (2 weeks), ovariectomized mice were examined for the expression of *c-fos* and *c-jun* mRNAs under estrogenic condition [5 ppm estradiol-17 β (E_2) in the diet]. In the long-term experiment (30 weeks), mice were administered of I3C (500 ppm) under estrogenic condition (5 ppm E_2 in the diet) after single exposure of a direct carcinogen, N-mehtyl-N-nitrosurea. The uteri were pathologically examined at the termination of the experiment.

[Results] In the short-term experiment, dietary I3C significantly reduced E_2 -stimulated expression of *c-fos* mRNA ($P < 0.05$). *c-Jun* mRNA showed a decreasing tendency by the I3C treatment. In the long-term experiment, administration of I3C in the diet reduced the incidence of MNU- and E_2 -induced endometrial atypical or simple hyperplasia ($P < 0.01$).

[Conclusion] Present results suggest that dietary exposure of I3C prevents estrogen-related endometrial tumorigenesis in mice, through the inhibition of estrogen-related *c-fos* and *c-jun* expression.

Key words: Indole-3-carbinol, Endometrial carcinogenesis, Prevention, *c-fos*, Mice

Introduction

Several natural products in fruits and vegetables are reported to possess anti-mutagenic and anti-carcinogenic properties [1-3]. Cruciferous vegetables, such as cabbage, broccoli, cauliflower, are known to contain indole derivatives, dithiolthiones and isothiocyanates, and shown to exert anti-carcinogenic potentials in several rodent carcinogenic models [4, 5]. Phytochemical indole-3-carbinol (I3C, Fig. 1), one of the indole derivatives, is reported to be anti-carcinogenic and anti-estrogenic. Dietary I3C functions as a potent inducer of 2-hydroxylation of estradiol in rodents [6] and humans [7], and increases the anti-proliferative metabolite 2-hydroxyestrone and decreases 16 α -hydroxyestrone. This effect on the estrogen metabolism may be related to

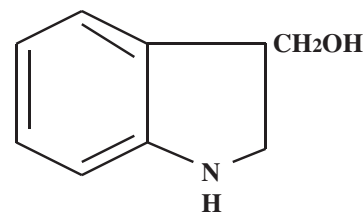


Fig. 1. Chemical structure of indole-3-carbinol

the prevention of I3C on the mammary [6,8], uterine cervical [9] and endometrial [10] tumorigenesis in rodent models.

The transient expression of the immediate early gene, *c-fos/jun*, is considered to be necessary for cellular proliferation and differentiation [11-13]. *c-fos/jun* mRNA in the uterine corpora of ovariectomized mice is overexpressed by the treatment of estrogens [14-16].

These circumstances prompted us to determine if I3C exerts inhibitory effects on mouse endometrial carcinogenesis induced by MNU and E_2 . Furthermore, the effects of I3C on the expression of *c-fos/jun* mRNAs were also investigated.

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Materials and methods

1) Animals and chemicals

Female ICR mice, 10 weeks of age, were purchased from Japan SLC Co. (Shizuoka). The basal diet (Oriental MF, Oriental Yeast Co., Tokyo) and filtered tap water were available ad libitum throughout the experiment. I3C, E₂ and MNU were purchased from Sigma Chem Co. (St. Louis, MO).

2) Experimental protocol for short-term effects of I3C

Female ICR mice, 12 weeks-of-age, were ovariectomized under general anesthesia with diethylether. Two weeks later, the ovariectomized mice were divided into four groups. Group 1 was given a diet containing 500 ppm I3C and 5 ppm E₂ (n=5); group 2 was given 5 ppm E₂ (n=5); group 3 was fed on a diet containing 5 ppm E₂ alone (n=6); group 4 served as a non-treatment control (n=5). After two weeks on the above diet, the mouse uteri were resected and cut in half longitudinally. One half was quickly frozen in liquid nitrogen for the following experiments, and the other was subjected to pathological examinations.

3) Reverse transcriptase-PCR (RT-PCR)

Total RNA was isolated from the frozen tissues by a guanidium thiocyanate-phenol-chloroform extraction method [19]. Total RNA (3 µg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RTase, 200 units, Gibco BRL, Gaithersburg, MO) in 20 µM Tris-HCl buffer (PH 8.4) with 50 µM KCl, 2.5 µM MgCl₂, 0.1 µg/ml bovine serum albumin, 10 µM dithiothreitol, and 0.5 µM deoxynucleotides to generate cDNAs, using random hexamers (50 ng, Gibco BRL) at 37°C for 60 min. RT reaction was carried out at 94°C for 5 min to inactivate MMLV-RTase. For c-fos or c-jun mRNA expression, treatment included 40 cycles of PCR consisting of 1 min at 94°C for denaturation, 1 min at 55°C for annealing and 1 min at 72°C for extension. The reactions were carried out in reverse transcribed cDNA and 0.1 mM specific primers described below, using an IWAKI thermal sequencer TSR-300 (IWAKI Glass, Tokyo) with Vent DNA polymerase (New England Biolabs, Beverly, MA) in 20 µM Tris-HCl buffer (PH 8.8) with 10 µM KCl, 10 µM (NH₄)₂SO₄, 2 µM MgSO₄, 0.1% Triton X-100, and 0.15 µM dextran sulfate. Twenty cycles of PCR for glyceraldehyde-3-phos-

phate dehydrogenase (GAPDH, a house-keeping gene) mRNA (995 bp) as an internal standard were performed at the same time.

The following oligodeoxynucleotides were synthesized as specific primers in PCR according to the published information (cDNA for c-fos [20], c-jun [21] and GAPDH [22]: sense for c-fos, 5'-CTTACGCCAGAGCGGGAATG-3'; anti-sense for c-fos, 5'-AAGCCTCAGGCAGACCTCCA-3'; sense for c-jun, 5'-AGCGTGTCTGGCT-3'; anti-sense for c-jun, 5'-CTGGGAAGCGTGTCTGGCT-3'; sense for GAPDH, 5'-TGAAGGTCGGTGTGAACGGATTTGG-3'; anti-sense for GAPDH, 5'-CTCCTTGGAGGCCATGTAGGCCAT-3'.

4) Semi-quantitative analysis of I3C mRNA expression in the mice uterine corpus by PCR products

PCR products were applied on 1.5% agarose gel electrophoresis at 50-100 V. The quantification of the products was carried out with Bio image (Nihon Millipore Corp., Tokyo). The intensity of specific bands was standardized with that of GAPDH mRNA.

5) Experimental protocol for long-term effects of I3C

A total of 90 female ICR mice, 10 weeks of age, underwent laparotomy under general anesthesia with diethylether. MNU solution (total volume: 0.1 ml) at a dose of 1 mg/100 g body weight was injected into the left uterine tube and normal saline into the right. One week after the exposure to MNU, the animals were divided into the following four experimental groups. Group 1 (20 mice) was given a diet containing 500 ppm I3C and 5 ppm E₂. Group 2 (24 mice) was treated with 5 ppm E₂ alone. Group 3 (20 mice) were fed with a diet containing 500 ppm I3C. The dose of E₂ was the same as in the short-term assay, and the above diets were given throughout the experiment. Group 4 (26 mice) served as a control group. Thirty weeks after the start of the experiment, all major organs, especially the reproductive organs, were grossly inspected. The uterus, ovaries, vagina, and other lesions suspected of being hyperplastic or neoplastic were cut in half. After being fixed in 10% formaline, tissues were sectioned at 3 µm and stained with hematoxyline and eosin.

6) Histology of the uterine lesions

Uterine endometrial lesions were divided into 4 types ac-

according to the WHO criteria [23]; a) endometrial hyperplasia, simple; b) endometrial hyperplasia, complex; c) atypical endometrial hyperplasia; d) adenocarcinoma.

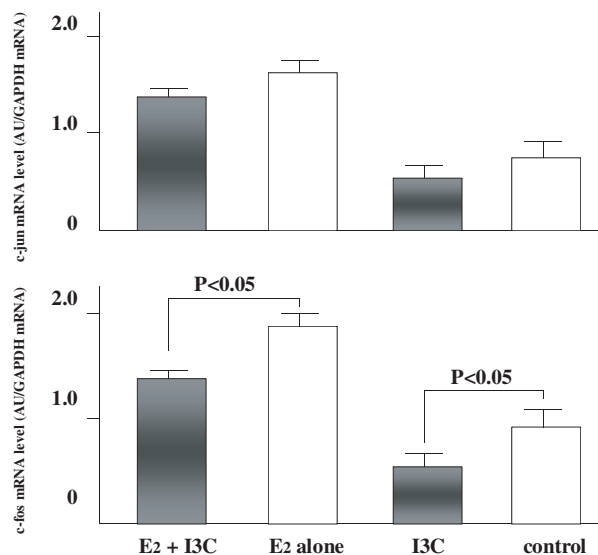


Fig. 2. Expressions of c-fos mRNA treated with E₂ plus I3C, E₂ alone, I3C alone and the control. I3C treatment significantly reduced the expressions of c-fos (P<0.05). Each column shows the mean and each bar expresses S.D. Data are calculated from each triplicated samples of the treated animals in each group. Expressions of c-jun mRNA treated with E₂ plus I3C, E₂ alone, I3C alone and the control. I3C treatment showed a decreased tendency of the expressions of c-jun. Each column shows the mean and each bar expresses S.D. Data are calculated from each triplicated samples of the treated animals in each group.

7) Statistical analysis

Statistical analysis was done according to the χ^2 test or student's t test.

Results

1) Short-term experiment

The levels of c-fos mRNA expression are shown in Fig. 2. I3C treatment significantly reduced the E₂-induced expression of c-fos (P<0.05). The levels of c-jun mRNA expression are also shown in Fig. 2. I3C treatment generated a decreasing tendency, but the effect was not significant.

2) Long-term experiment

Two mice in group 1 and three in group 3 died within 15 weeks, yet no pathological abnormalities other than pneumonia were found. The remaining animals survived until the termination of the experiment and were enrolled as effective animals (group 1, 18; group 2, 17; group 3, 25; and group 4, 25). Histological properties of endometrial hyperplasias and adenocarcinoma in the present study were basically as the same as in our previous report [14,16-18]. All adenocarcinomas seen in the endometria were well- or moderately-differentiated. The incidence of the preneoplastic and neoplastic lesions of the left uterine endometria is summarized in Fig. 3. The incidence of atypical hyperplasia

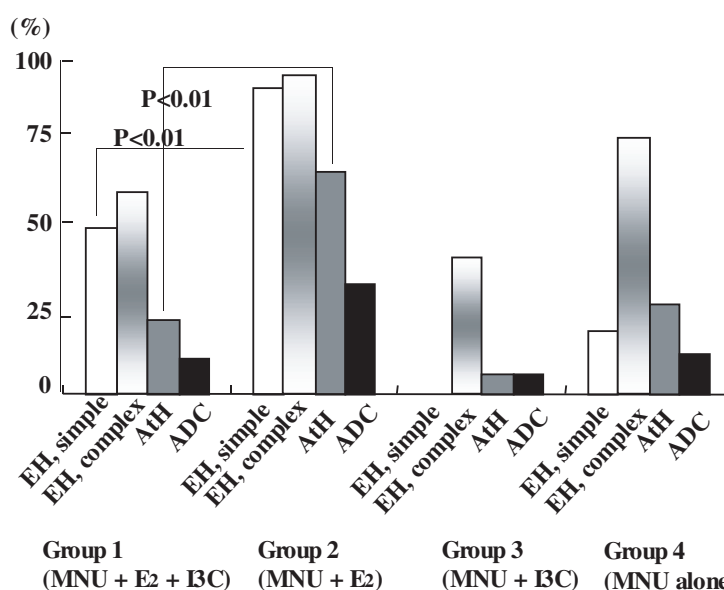


Fig. 3. Incidence of the preneoplastic and neoplastic endometrial lesions of the treated (left) side with MNU. I3C treatment significantly reduced the incidences of atypical hyperplasia and endometrial hyperplasia, simple compared with the group 2 (P<0.01). Treatment of E₂ increased the incidence of (pre)neoplastic endometrial lesions (groups 1 versus 3; 2 versus 4).

(4/18, 22%) on the left side of the uterine corpus of group 1 was significantly lower than that of group 2 (16/25, 67%, $P < 0.01$). The incidence of endometrial hyperplasia, simple (9/18, 50%) was also significantly lower than that of group 2 (22/25, 92%, $P < 0.01$). The incidences of adenocarcinoma and endometrial hyperplasia, complex on the left side of the uterine corpus in group 1 tended to be lower than those of group 2, although the differences were not significant. The incidences of endometrial hyperplasias and adenocarcinoma of the left side of the uterine corpus in group 3 were also rather lower than those of group 4, but the differences were not significant.

Discussion

In our previous experiment, dietary I3C had a suppressive effect on E_2 -related endometrial tumorigenesis in mice, possibly through suppression of estrogen-induced *c-fos/jun* expression [15,16]. The incidence of endometrial hyperplasia, simple, being considered to be affected by estrogens in this study, was more prominently decreased by I3C compared with our previous reports [16–18]. I3C is suggested to possess an anti-estrogenic activity more potently than other agents, such as *Glycyrrhizae radix*, *Juzen-taiho-to* and *toremifene*, which were examined by us previously.

The chemopreventive effects of I3C on estrogen-related endometrial tumorigenesis thus probably relate to the anti-estrogenic function of indole derivative. Acid condensation products of I3C are known to be ligands for the aryl hydrocarbon receptor [24]. This interaction is suggested to be the reason why I3C alters expression of some cytochrome P-450 that regulates the estrogen metabolism [25,26]. I3C is also reported to increase 2-hydroxyestrone [26]. Both of I3C and 2-hydroxyestrone are found to function as an anti-estrogen, and compete with E_2 for the estrogen receptor [27]. Other possible anti-cancer effects of I3C are assumed to relate to G_1 -arrest [28] or apoptosis [29, 30].

Results in this study suggest that dietary I3C acts as a chemopreventive agent on the estrogen-related endometrial tumorigenesis. This indole compound, I3C is already regarded as a promising agent for therapy as well as prevention against laryngeal papillomatosis [31,32]. The attractiveness of I3C is the possibility that a diet enriched with cruciferous vegetables or a supplementation of I3C

could be effective for prevention of human endometrial carcinoma.

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