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Biphasic Modifying Effect of Indole-3-carbinol on Diethylnitrosamine-induced Preneoplastic Glutathione S-Transferase Placental Form-positive Liver Cell Foci in Sprague-Dawley Rats

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The biphasic modifying effects of indole-3-carbinol (I3C), a naturally occurring constituent of edible cruciferous vegetables, on the development of glutathione S-transferase placental form (GST-P)-positive liver cell foci were investigated by using a medium-term liver bioassay system and a newborn rat hepatocarcinogenesis system. In Experiment 1, a total of 65 male Sprague-Dawley (SD) rats were divided into 5 groups. Animals were given a single intraperitoneal (i.p.) injection of 200 mg/kg diethylnitrosamine (DEN) dissolved in saline for groups 1, 2, and 3 or a single i.p. injection of saline for groups 4 and 5. Group 1 was given the diet containing 0.25% I3C for 2 weeks prior to DEN initiation and then basal diet for 8 weeks. Group 2 was given basal diet for 4 weeks prior to and after DEN initiation and then the diet containing 0.25% I3C for 6 weeks. The rats of group 3 were placed on basal diet during the experiment. Animals of groups 4 and 5 were treated in the same manner as those of groups 1 and 2 except for injection with saline instead of DEN solution. All rats were subjected to two-thirds partial hepatectomy at week 3 and were killed at week 8 after DEN or saline injection. In Experiment 2, a total of 45 female SD rats were dosed with DEN (100 mg/kg, i.p.) or saline at 24 h after birth. After weaning at week 3, the rats were fed diet containing 0.25% I3C for 9 weeks and then were killed at week 12. In Experiment 1, preinitiation exposure to 0.25% I3C caused a significant decrease in numbers of GST-P-positive liver cell foci ($P<0.05$), while postinitiation exposure to 0.25% I3C caused significant increases in both number (No./cm^2) and area (mm^2/cm^2) of GST-P-positive liver cell foci ($P<0.05$ or 0.01). In Experiment 2, the relative liver weight in the DEN+I3C group was significantly increased ($P<0.001$). The numbers and areas of GST-P-positive liver cell foci in the DEN+I3C group were significantly increased as compared to the values of the DEN-alone group ($P<0.001$). These results clearly demonstrated that I3C exerts a promoting effect on the postinitiation stage as well as an inhibitory effect on the preinitiation stage in the medium-term liver bioassay.

Key words: Biphasic effect — Indole-3-carbinol — Hepatocarcinogenesis — Glutathione S-transferase placental form — Diethylnitrosamine

For rapid detection of possible carcinogens, Ito *et al.* have established a medium-term liver bioassay method using preneoplastic GST-P[5]-positive liver cell foci as the endpoint marker lesion.[1–5] This system is useful for detecting not only carcinogenic or tumor-promoting agents but also tumor-inhibitory ones.[4,5] Newborn animals have been used for an effective hepatocarcinogenesis model by several investigators.[6–10] The advantages are a relatively short period to get results, use of smaller amounts of chemicals or drugs in the experiment and sensitive responses to the chemicals.

I3C is a naturally occurring constituent of edible cruciferous vegetables, such as broccoli, Brussels sprouts, cabbage, and cauliflower. It is a potent inducer of AHH and glutathione S-transferase.[11,12] I3C has been found to inhibit B[a]P-induced forestomach tumor in ICR/Ha mice,[13] DMBA-induced mammary tumor in SD rats,[13] B[a]P-induced pulmonary adenoma in mice[14] and AFB_1-induced hepatocarcinogenesis in rainbow trout when administered prior to or during carcinogen exposure.[15–17] Postinitiation exposure to I3C strongly enhanced AFB_1-induced tumorigenesis in rainbow trout liver[16–19] and DMH-induced colon tumorigenicity in

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[5] Abbreviations used are: GST-P, glutathione S-transferase placental form; I3C, indole-3-carbinol; AHH, aryl hydrocarbon hydroxylase; B[a]P, benzo[*a*]pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; AFB_1, aflatoxin B_1; DMH, 1,2-dimethylhydrazine; DEN, diethylnitrosamine; NDMA, N-nitrosodiethylamine; PH, two-thirds partial hepatectomy; PB, phenobarbital; MNU, N-methylnitrosourea; DBN, N,N-dibutylnitrosamine.

rats.[20] I3C has an inhibitory effect on the development of hepatic nodules and the induction of GST-P-positive liver cell foci in a rat multi-organ carcinogenesis model[21] and on DEN-induced hepatocarcinogenesis when administered concurrently with the carcinogen.[22] Chinese cabbage extract also exerts an inhibitory effect on the development of GST-P-positive liver cell foci induced by DEN when administered with the carcinogen.[23] I3C also exerts a promoting effect[16-19] or an inhibitory effect[15-17] depending on the administration stage (preinitiation or postinitiation stage) in an AFB_1-induced hepatocarcinogenesis model in rainbow trout. I3C thus exerts different modifying effects in the liver of rainbow trout[15-19] or rats[21-23] according to the experimental carcinogenesis model employed.

To evaluate the biphasic modifying effects of I3C on hepatocarcinogenesis, we used medium-term liver bioassay. In this study we analyzed the modifying effects of I3C on the induction of GST-P-positive liver cell foci in rats when administered prior to or after DEN initiation.

MATERIALS AND METHODS

Animals and chemicals A total of 65 male Sprague-Dawley rats, 6 weeks old, born and reared under specific pathogen-free conditions (National Institute of Safety Research, Korea), for Experiment 1 and a total of 45 female newborn Sprague-Dawley rats for Experiment 2 were housed in polycarbonate cages with hard wood chips in an air-conditioned room (23±2°C, 55±10% humidity) with a 12 h light/dark cycle. Diet (Jeil Sugar Co., Korea) and drinking water were given *ad libitum*. All animals were fasted for 24 h prior to being killed. DEN (CAS No. 55-18-5, N-0756) and I3C (CAS No. 700-06-1, I-7256) were obtained from Sigma Chemical Co. Ltd., USA. Diet containing 0.25% I3C was prepared. DEN was dissolved in saline for i.p. injection. Anti-GST-P IgG was a generous gift from Professor Kiyomi Sato of the Second Department of Biochemistry, Hirosaki University School of Medicine, Japan.

Experimental design

Experiment 1 Sixty-five rats were divided into 5 groups. All animals were given a single i.p. injection of 200 mg/kg DEN solution for groups 1, 2, and 3 or a single i.p. injection of saline for groups 4 and 5. Group 1 was given the diet containing 0.25% I3C for 2 weeks prior to DEN initiation and then basal diet for 8 weeks. Group 2 was given basal diet for 4 weeks prior to and after DEN initiation and then the diet containing 0.25% I3C for 6 weeks. The rats of group 3 were placed on basal diet during the experiment. Animals of groups 4 and 5 were treated in the same manner as groups 1 and 2 except for injection with saline instead of DEN solution (Fig. 1). All rats were subjected to PH at week 3 and were killed at week 8 after DEN or saline. PH was performed by a procedure similar to that described by Higgins and Anderson for rats.[1, 2, 5, 24] Body weights were measured weekly.

Experiment 2 Forty-five newborn SD rats were divided into 3 groups. All rats were initially given a single i.p. injection of 100 mg/kg body weight DEN solution or saline using a microsyringe (100 μl, Hamilton, USA) at one day after birth. After being weaned at week 3, the rats were given diets containing 0.25% I3C or basal diet (control) for 9 weeks. All animals were killed at week 12. The body weights were measured during the experiment (Fig. 2).

Immunohistochemical staining for GST-P The animals were killed under ether anesthesia and the livers were removed and weighed. Three lobes of the liver were cut

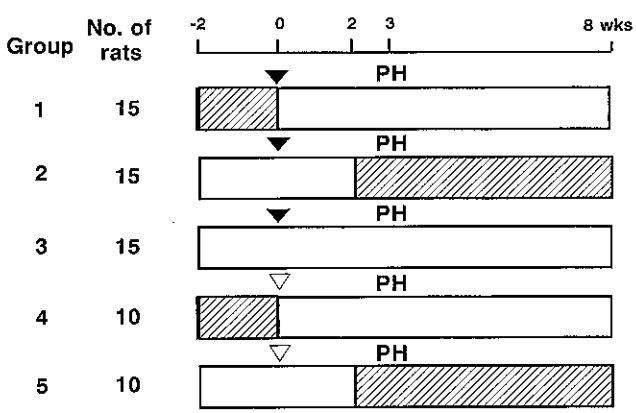

Fig. 1. Experimental design 1 for medium-term liver bioassay. Group 1, I3C+DEN; group 2, DEN+I3C; group 3, DEN alone; group 4, I3C+saline; group 5, saline+I3C. All rats were subjected to PH at week 3. DEN (▼): 200 mg/kg, i.p.; saline (▽): 0.9% sodium chloride, 5 ml/kg body weight, i.p.; PH: 2/3 partial hepatectomy; ▨: 0.25% I3C in diet for 2 or 6 weeks.

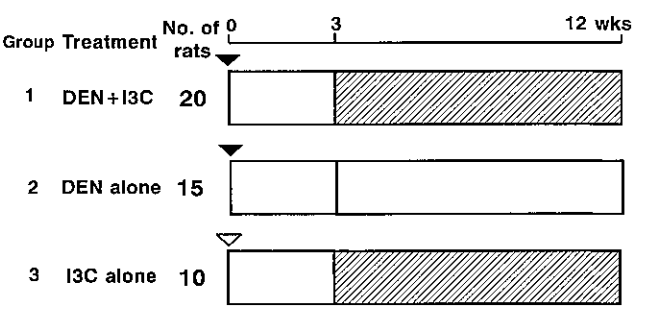

Fig. 2. Experimental design 2 for newborn rat hepatocarcinogenesis model. DEN (▼): diethylnitrosamine, 100 mg/kg, i.p.; saline (▽): 5 ml/kg, i.p.; ▨: 0.25% I3C in diet for 9 weeks after being weaned at week 3.

into 2–3 mm thick slices with a razor blade and the slices were fixed in cold acetone for immunohistochemical staining of GST-P. The acetone-fixed liver tissues were processed for embedding in Paraplast and sectioned at 4 μm. The slices were routinely passed through xylene and a graded alcohol series and then treated sequentially with normal goat serum, rabbit anti-GST-P (1:5,000) IgG, affinity-purified biotin-labeled goat anti-rabbit IgG and avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, PK-6101, Vector Lab. Inc., USA). Diaminobenzidine was used as a chromogen to demonstrate the site of peroxidase binding. The sections were counterstained with hematoxylin for microscopic examination. As a negative control for the specificity of anti GST-P IgG, preimmune rabbit serum was used instead of antiserum.

Quantitative analysis The numbers and areas of GST-P-positive liver cell foci larger than 0.1 mm in diameter were measured with an IBAS automatic image analysis system (Kontron Co. Ltd., Germany). The statistical analysis was carried out using the two-tailed Student's *t* test.

RESULTS

Experiment 1

Body weight and relative liver weight: Final body weight and relative liver weight are shown in Table I. The body weight changes were not significant between the DEN+I3C and DEN-alone groups. However, the relative liver weight with respect to final body weight in the DEN+I3C group was significantly increased compared with that of the DEN-alone group ($P<0.001$).

Quantitative values of GST-P-positive liver cell foci: The results on GST-P-positive liver cell foci are shown in Table II. Preinitiation exposure to I3C resulted in a significant decrease in the number of GST-P-positive liver cell foci compared with that of the DEN-alone group ($P<0.05$), but the difference in area of foci was not significant. On the other hand, postinitiation exposure to I3C caused significant increases in both number and area of GST-P-positive liver cell foci ($P<0.05$ or 0.01).

Experiment 2

Body weight and relative liver weight: Final body weight and relative liver weight are shown in Table III. The body weight was significantly decreased in the group treated with I3C compared with the DEN-alone group ($P<0.05$). The relative liver weight with respect to final body weight in the DEN+I3C group was significantly increased compared with that of the DEN-alone group ($P<0.001$).

Quantitative values of GST-P-positive liver cell foci: The results on GST-P-positive liver cell foci are shown in Table IV. The number (No./cm^2) and the area (mm^2/

Table I. Mean Body and Relative Liver Weights

Group	Treatments	No. of rats	Mean body weight (g)		Relative liver weight (g%)
			initial (−2 wk)	final (8 wk)	
1	I3C+DEN	15	160.7±26.4[a)]	383.5±26.5**	2.27±0.16
2	DEN+I3C	15	153.1±21.5	372.1±35.4**	2.98±0.42***
3	DEN alone	11	178.1±10.3	415.2±23.6	2.28±0.26
4	I3C+Saline	8	173.6±11.6	414.0±37.2	2.45±0.20
5	Saline+I3C	7	168.9±26.9	415.7±39.9	2.89±0.25***

a) Values represent mean±SD.
** and ***: Significantly different from the DEN-alone group at $P<0.01$ and 0.001.

Table II. Values of GST-P-positive Liver Cell Foci

Group	Treatments	No. of rats	GST-P$^+$ liver cell foci		D_{max}[a)]
			No./cm^2	mm^2/cm^2	
1	I3C+DEN	15	4.76±3.86[b)] *	0.23±0.20	0.20±0.04
2	DEN+I3C	15	19.50±10.4*	0.79±0.30**	0.27±0.03
3	DEN alone	11	9.50±6.24	0.29±0.21	0.26±0.04
4	I3C+Saline	8	0.11±0.30	0.00±0.01	0.04±0.11
5	Saline+I3C	7	0.05±0.14	0.00±0.00	0.03±0.09

a) Maximum diameter (mm).
b) Values represent mean±SD.
* and **: Significantly different from the DEN-alone group at $P<0.05$ and 0.01. Foci more than 0.1 mm in diameter were quantified.

Table III. Body Weight Change and Relative Liver Weight with Respect to Final Body Weight

Group	Treatments	Body weight (g)			Relative liver weight (g%)
		4	8	12 (wk)	
1	DEN+I3C	82.5±7.3	186.6±12.9	205.1±15.0*	4.09±0.26***
2	DEN alone	83.7±7.1	191.1±13.0	216.3±15.6	2.99±0.23
3	I3C	76.3±9.3	191.3±18.7	208.5±19.8	3.67±0.49

Values represent mean±SD.
* and ***: Significantly different from DEN-alone group at $P<0.05$ and 0.001.

Table IV. Quantitative Values of GST-P-positive Liver Cell Foci

Group	Treatments	No. of rats	GST-P$^+$ liver cell foci		D_{max} (mm)
			No./cm^2	mm^2/cm^2	
1	DEN+I3C	18	3.71±2.59***	0.43±0.28***	0.48±0.07***
2	DEN alone	15	1.17±1.02	0.07±0.07	0.31±0.11
3	I3C	10	0	0	0

Values represent mean±SD. D_{max} represents maximum diameter (mm). Foci more than 0.1 mm in diameter were quantified.
***: Significantly different from the values of the DEN-alone group at $P<0.001$.

cm^2) of GST-P-positive liver cell foci of the DEN+I3C group were significantly increased as compared to the values of the DEN-alone group ($P<0.001$), and the value of D_{max} of GST-P-positive liver cell foci was significantly increased ($P<0.001$).

DISCUSSION

The results of the present study clearly demonstrate that I3C exerted a promoting effect on the postinitiation stage in both experiments, as well as an inhibitory effect on the preinitiation stage in the medium-term liver bioassay.

The biphasic modifying effects of I3C on hepatocarcinogenesis have been well demonstrated in AFB_1-induced liver tumor of rainbow trout.[15,16] In this model, preinitiation exposure to I3C reduced AFB_1-initiated hepatocellular carcinomas.[15,16] These results are in accordance with those of the present study. When administered for 2 weeks prior to DEN exposure, I3C inhibited the development of GST-P-positive liver cell foci in SD rats (Experiment 1). Though the mechanism of the inhibitory effect of I3C on the preinitiation stage of the DEN-induced hepatocarcinogenesis is not known, it has been shown that dietary indoles are capable of inhibiting tumor formation in experimental animals.[13,16,17,21,22] I3C is a good inducer of AHH and glutathione S-transferase.[11,12] Wattenberg and Loub[13] suggested that this property of I3C may be related to the mechanism of inhibitory effect of I3C on carcinogenesis. On the other hand, Shertzer[25] has demonstrated significant reductions in B[a]P and NDMA binding to DNA by I3C both *in vitro* and *in vivo* in mice without concomitant AHH induction. He proposed that the *in vivo* administration of I3C may protect hepatic macromolecules against covalent binding of the metabolites of B[a]P and NDMA, which are indirect-acting carcinogens like DEN. Dashwood *et al.*[26] also suggested that the inhibition of AFB_1-induced hepatocarcinogenesis in rainbow trout by I3C is associated with attenuated AFB_1-DNA binding in the liver. Inhibition of microsome-activated AFB_1 binding to DNA by acid reaction products of I3C rather than by I3C itself may be significant in I3C inhibition of hepatocarcinogenesis in trout and other species.[27]

The present results also show that I3C possesses a promoting effect on the induction of GST-P-positive liver cell foci when administered after DEN exposure. Postinitiation exposure to I3C strongly enhanced the incidence of AFB_1-induced hepatocellular carcinomas in rainbow trout.[16-19] Furthermore, dietary I3C enhanced colon tumorigenicity induced by DMH in rats when administered in the diet for the duration of the experiment.[20] The mechanisms responsible for the promoting effect of I3C on the hepatocarcinogenesis are unknown.

PB is also a biphasic modulator of hepatocarcinogenesis, acting as an inhibitor at the preinitiation stage and as a promoter at the postinitiation stage.[6,28] PB-treated liver tissue, whether normal or preneoplastic, shows increased activity of several drug-metabolizing enzymes and cell proliferation.[29] PB has been found to increase the number and size of foci, as well as to enhance their phenotypic deviation from normal hepato-

cytes.[30, 31] PB treatment was also found to increase the liver weight and to reduce the incidence of apoptosis. Thus, prolongation of the life-span of altered hepatocytes appears to be important for tumor promotion by PB. There was no evidence of genotoxicity of I3C in the *Salmonella* and CHO systems.[32] Thus, I3C may be the same kind of liver tumor promoter as PB, possibly inducing adaptive response in the liver.

Jang *et al.*[21] reported that dietary intake of 0.5% I3C had an inhibitory effect on the development of hepatic nodules and GST-P-positive liver cell foci after sequential treatment with DEN, MNU, and DBN, while Chinese cabbage had an inhibitory effect on the development of GST-P-positive liver cell foci induced by DEN.[23] To clarify the modifying effects of I3C on hepatocarcinogenesis, we conducted a dose-response study of I3C in the medium-term liver bioassay system. Our results show that dietary intake of 0.1, 0.25, and 0.5% I3C had a promoting effect on the postinitiation stage in the same model (data were not shown). The highest dietary dose group showed decreased body weight compared with other dose groups. The contradictory results may be due to some differences in the experimental model or the presence of various components in the Chinese cabbage extract which was used.

Biphasic modifying effects of I3C as a promoter at the postinitiation stage but as an inhibitor at the preinitiation stage were demonstrated in the medium-term liver bioassay system (Experiment 1). I3C has a promoting effect at the postinitiation stage in the newborn rat hepatocarcinogenesis model using preneoplastic GST-P-positive liver cell foci as the endpoint marker lesion (Experiment 2). Further studies on the relationship between cell proliferation and mixed-function oxidase activity, and multi-organ carcinogenesis studies are needed to clarify the multiple effects of I3C on hepatocarcinogenesis.

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