Enhancement by indole-3-carbinol of liver and thyroid gland neoplastic development in a rat medium-term multiorgan carcinogenesis model

Dae Joong Kim,1,2,5 Beom Seok Han,2 Byeongwoo Ahn,2 Ryohi Hasegawa,3 Tomoyuki Shirai,3 Nobuyuki Ito4 and Hiroyuki Tsuda1

1Chemotherapy Division, National Cancer Centre Research Institute, 5–1–1 Tsukiji, Chuo-ku, Tokyo 104, Japan; 2Department of Pathology, National Institute of Safety Research, Nokbun-dong, Eunpyung-ku, Seoul 122–020, Korea; 3First Department of Pathology, Nagoya City University Medical School, and 4Nagoya City University, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467, Japan

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Introduction

Human foodstuffs contain many compounds that inhibit the carcinogenic process in experimental animals (1–3). Recently, many investigations have focused on the chemopreventive effects of naturally occurring compounds. Indole-3-carbinol (I3C*), a major indole metabolite in cruciferous vegetables (cabbages, broccoli, Brussels sprouts and cauliflowers) (2,4,5), has thus been found to inhibit the development of tumours in forestomach (2.6), glandular stomach (7), mammary gland (2.8,9), uterus (10), tongue (11), and liver (12,13) of rodents, as well as in the trout liver (14,15), when administered prior to or during carcinogen exposure by gavage or in the diet. However, dietary ‘anticarcinogens’ may exhibit adverse promoting activities in certain test protocols or in other organs. For example, exposure to I3C or cabbage during the post-initiation (promotion) stage was found to strongly enhance aflatoxin B1 (AFB1)-induced liver tumourigenesis in the rainbow trout (15–17), diethylnitrosamine (DEN)-induced liver tumourigenesis in newborn or young rats (13, 1, diethylhydrazine (DMH)-induced colon tumourigenesis in rats (18) and mice (19), and N-nitrosobis(2-oxopropyl)amine (BOP)-induced pancreas tumourigenesis in hamsters (20). It is, in fact, well established that a chemical may act as a tumour inhibitor in one organ and as a promoter in others.

While the rat medium-term liver bioassay model of 8 weeks duration can detect both promotion and inhibition potential (21,22), in order to determine the spectrum of modifying effects rat medium-term multiorgan carcinogenesis models of 20–36 weeks duration are more applicable (23–28).

To elucidate the influence of I3C in various organs, we therefore conducted a post-initiation study at the whole-body level using one of these models (23–28).

Materials and methods

Animals and chemicals

One-hundred 6-week-old male Sprague–Dawley (SD) rats were supplied by the National Institute of Safety Research, Seoul, Korea, and were housed in polycarbonate cages with hard wood chips in an air conditioned room (23 ± 2°C, 55 ± 10% RH) with a 12 h light/dark cycle. Diet (Jeil Sugar Co., Korea) and drinking water were available ad libitum. All animals were fasted for 24 h prior to death. DEN (CAS No. 55–18–5, N-0756), N-methyl-N-nitrosourea (NUN, CAS No. 684–93–5, Sigma N-4766), and I3C (CAS No. 700–06–1, I-7256) were purchased from Sigma Chemical Co. Ltd., USA, Dihydroxy-di-N-propylnitrosamine (DHPN; CAS No. 5369–64–6) was purchased from Nakarai Tesque, Inc., Japan. Anti-rat-pepsinogen I (pg 1) IgG was a generous gift from Dr Chie Furihata of The Department of Molecular Oncology, The Institute of Medical Science, The University of Tokyo, Japan.

Treatments

One-hundred male SD rats were randomly divided into three groups (Figure 1). Animals of groups 1 and 3 were then given diet containing 0.25% I3C from week 4 until week 24, followed by a return to basal diet for 28 weeks, and subgroups were killed at weeks 24 and 52. I3C caused significant increases in both number (no./cm2) and area (mm2/cm2) of glutathione S-transferase plasmid form (GST-P)-positive liver cell foci assessed at week 24 of the experiment (P < 0.01, 0.001). The incidence of hepatocellular adenomas in the DMD and I3C group at week 52 showed a tendency for elevation as compared to the DMD alone group, but this was not statistically significant. The thyroid gland tumour incidences in the DMD and I3C groups were significantly increased compared with the DMD alone group values at week 52 (P < 0.01). In conclusion, I3C enhanced liver and thyroid gland neoplastic development when given during the promotion stage in the present rat medium-term multiorgan carcinogenesis model.

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Treatments

One-hundred male SD rats were randomly divided into three groups (Figure 1). Animals of groups 1 and 2 were sequentially treated with DEN (100 mg/kg b.w., a single i.p. injection, at the commencement of the experiment), MNU (20 mg/kg b.w., i.p., in citrate-buffered solution pH 6.0, four times at days 5, 8, 11 and 14), and DHPN (0.1% in the drinking water for 2 weeks during weeks 1 and 3) (DMD treatment). Non-initiation controls (group 3) were given diet containing no carcinogen from week 4, subgroups being killed under ether anaesthesia at weeks 24 and 52 of the experiment.
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Fig. 1. Experimental protocol for the multiorgan carcinogenesis bioassay (DMD model). Animals were sequentially treated with DEN (100 mg/kg b. wt., i.p., single dose), MNU (20 mg/kg b. wt., i.p., four times, on days 5, 8, 11 and 14) and DHPN (0.1% in the drinking water, during weeks 1 and 3). Animals of groups 1 and 3 were given diet containing 0.25% I3C (shaded areas) for 20 weeks after DMD treatment and were given basal diet (clear areas) for 28 weeks. Survivors were killed at weeks 24 and 52.

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<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>no. of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMD → I3C 20 × 2</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>DMD alone 20 × 2</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>I3C 10 × 2</td>
<td>3</td>
</tr>
</tbody>
</table>
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Fig. 2. Number and area of GST-P-positive liver cell foci in SD rats treated with DMD with or without subsequent I3C administration. Values are for weeks 24 and 52. DMD and I3C group (shaded areas); DMD alone group (clear areas). **Significantly different from the DMD alone group at P < 0.01 and P < 0.001, respectively.

Results

Body and liver weights

At week 24 of the experiment, the body weights in the DMD plus I3C group was significantly decreased as compared with the DMD alone group value (P < 0.05) and the relative liver weight was significantly increased (P < 0.001). However, no intergroup variation in the body or relative liver weights was evident at week 52 of the experiment after the return to basal diet (Table I).

Quantitative values for GST-P-positive liver cell foci

Quantitative values for GST-P-positive liver cell foci per cm² are shown in Figure 2. The average number (no./cm²) and area (mm²/cm²) of GST-P-positive liver cell foci in the DMD plus I3C group as well as the Dₘₐₓ of GST-P-positive liver cell foci were significantly increased as compared to the respective DMD alone group values at week 24 of the experiment (P < 0.01, 0.001 and 0.05, respectively). At week 52 of the experiment, these DMD plus I3C group values for GST-P-positive liver cell foci still showed tendencies for increase, but these values were not significant.

PAPG in pyloric mucosa

The numbers of PAPG in pyloric mucosa in each group showed tendencies for decrease, but these PAPG in pyloric mucosa did not significantly different between groups at week 52 of the experiment (data not shown).

Incidence of tumours

Incidences of neoplastic lesions are shown in Table II. The combined incidence of hepatocellular adenomas or carcinomas in the DMD plus I3C group at week 52 was significantly higher than in the same group at week 24 of the experiment (38 vs. 5%, P < 0.05) with a tendency for increase as compared with DMD alone group (38 vs. 18%). However this was not statistically significant. At week 52 the combined incidence of follicular cell adenomas and adenocarcinomas of the thyroid gland was also significantly increased in the DMD plus I3C group with a preponderance of solid and poorly differentiated follicular cell adenocarcinomas (P < 0.01) (Table II). Colloid follicles of the thyroid gland appeared normal. The numbers of neoplastic lesions of other organs in each group did not significantly differ. Rats in both the DMD treated groups developed alveolar hyperplasias and adenomas and/or adenocarcinomas of the lung but no effect of I3C was evident. Pathological changes were also observed in the adrenal gland (an adenocarcinoma) and kidney (renal cell adenomas and a nephroblastoma).
Table I. Body and relative liver weights for SD rats sequentially treated with DMD followed by I3C at weeks 24 and 52

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of rats</th>
<th>Body wt (g)</th>
<th>Relative liver wt (g%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 weeks</td>
<td>52 weeks</td>
</tr>
<tr>
<td>DMD→I3C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20</td>
<td>14</td>
<td>159.6 ± 11.6</td>
</tr>
<tr>
<td>DMD alone</td>
<td>19</td>
<td>16</td>
<td>158.8 ± 8.85</td>
</tr>
<tr>
<td>I3C</td>
<td>10</td>
<td>10</td>
<td>160.0 ± 9.96</td>
</tr>
</tbody>
</table>

<sup>a</sup>DMD represents ‘DEN+MNU+DHPN’ treatment.<sup>b</sup>I3C was given at 0.25% in the diet for 20 weeks.

*<sup></sup>, **<sup></sup>, ***<sup></sup>Significantly different from the DMD alone group at P<0.05 and 0.001, respectively.

Table II. Histological findings at weeks 24 and 52 of the experiment in rats treated with DMD and/or I3C

<table>
<thead>
<tr>
<th>Organ/lesions</th>
<th>Treatment</th>
<th>24 weeks</th>
<th>52 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DMD→I3C&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DMD alone</td>
</tr>
<tr>
<td></td>
<td>(n = 20)</td>
<td>(n = 19)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocellular adenoma (HA)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hepatocellular carcinoma (HCC)</td>
<td>1 (5)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HAV/ HCC</td>
<td>1 (5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thyroid gland</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular cell hyperplasia</td>
<td>1 (5)</td>
<td>1 (5)</td>
<td>0</td>
</tr>
<tr>
<td>Follicular cell adenoma</td>
<td>1 (5)</td>
<td>1 (5)</td>
<td>0</td>
</tr>
<tr>
<td>Follicular cell adenocarcinoma</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adenoma/ adenocarcinoma</td>
<td>1 (5)</td>
<td>1 (5)</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alveolar hyperplasia</td>
<td>13 (65)</td>
<td>16 (84)</td>
<td>0</td>
</tr>
<tr>
<td>Adenoma</td>
<td>1 (5)</td>
<td>2 (10)</td>
<td>0</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>1 (5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adenoma/ adenocarcinoma</td>
<td>2 (10)</td>
<td>2 (10)</td>
<td>0</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
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<tr>
<td>Renal cell adenoma</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nephroblastoma</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Testis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interstitial cell adenoma</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Two moribund animals in the DMD+I3C group are included.<sup>b</sup>One moribund animal in the DMD alone group is included.
<sup>c</sup>Percentages in parentheses.
*<sup></sup>, **<sup></sup>, ***<sup></sup>Significantly different from the DMD alone group at P<0.05 and 0.01, respectively.
†, ††, ††† Significantly different from those of same group at week 24 at P<0.05, 0.01 and 0.001, respectively.

Discussion

The results of the present study demonstrate clearly enhancing effects of I3C on liver and thyroid gland neoplastic development in the post-initiation (promotion) stage of the present rat medium-term multiorgan carcinogenesis model (DMD treatment). The liver results are in line with our previous findings in a rat medium-term bioassay (13).

Biophasic modifying effects of I3C on hepatocarcinogenesis were earlier demonstrated in terms of AFB1-induced liver tumours in the rainbow trout (14–17). Exposure during the initiation stage reduced the yield of hepatocellular carcinomas, while post-initiation administration caused a significant increase (14,15). Dietary exposure to I3C or cabbage of rodents also enhanced the development of colon tumours after DMH treatment (18,19) although inhibition of aberrant crypt formation by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) was observed for both initiation and promotion stages (31). The mechanisms responsible for the observed promotion effects of I3C (13,15–19) remain to be clarified. However, in most of the studies, high levels of I3C were administered daily over long periods and this would be expected to result in induction of aryl hydrocarbon hydroxylase (AH) activity (4,5), as well as glutathione S-transferase (GST) (32–35), glucuronyl transferase (GT) (33), and DT-diaphorase (DTD) (33). Although this property of I3C might normally be expected to inhibit carcinogen action (2,4,35), the same type of pleiotropic response has been observed for phenobarbital (PB)-type enzyme inducers resulting in promotion/enhancement depending on the period of administration of I3C on the liver (36–38). I3C has been shown to induce hepatic cytochrome P450 (CYP) 2B1/2, as well as CYP 1A1 and 1A2 (33).

In contrast with our present results and the data from our earlier study (13), Jang et al. (39) reported that dietary intake of 0.5% I3C significantly decreased the development of hyperplastic nodules and GST-P-positive liver cell foci after sequential treatment with DEN, MNU, and N,N-dibutylnitrosamine (DBN). The reason for this discrepancy is unclear since the strains of rats, dose, duration and kinds of carcinogens applied were basically the same.
Dietary supplements of 0.1% I3C and 0.12% sinigrin, major constituents of cruciferous vegetables, were found to exert an inhibitory effect on DEN-induced hepatocarcinogenesis in ACI/N rats when given prior to and during carcinogen exposure (12). Ethanol extracts from Chinese cabbages were also reported to inhibit the development of GST-P-positive liver cell foci in newborn SD rats after initiation with DEN (40). This, however, could have been due to other components such as sinigrin, benzyl isothiocyanate (BITC) and benzyl thiocyanate (BTC) (41). BITC and BTC are considered effective in the pre-initiation, as well as the post-initiation (promotion) stages (42), with effects on the inactivation or detoxification of the carcinogen. Thus, they both inhibit unscheduled DNA synthesis (UDS) and replicative DNA synthesis (RDS) in rat hepatocytes in response to DEN exposure (43). However, I3C increased liver tumour induction in F344 rats by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone associated with augmented 7-methylguanidine adduct formation in liver DNA while sinigrin only affected the latter (44). Thus, there is no simple explanation for inhibition and promotion of hepatocarcinogenesis by members of the *Brassica* family.

The mechanisms of I3C promotion of thyroid tumourigenesis are also unclear, but might be related to its effects on the liver. Several PB-type inducers of hepatic microsomal enzymes (including CYP2B1/2) are known to enhance follicular cell tumour development initiated by a variety of carcinogens in rats (45–50). The cumulative effect of PB-type inducers on various drug metabolizing activities and on liver mass leads to increased metabolic clearance of thyroid hormones, resulting in a hyperplasogenic influence through the thyroid stimulating hormone (TSH) feedback loop (47–51). Dietary exposure of rats to cooked Brussels sprouts for only 2 days can change the metabolic activity of CYP2B1/2, which is the predominant phase I form in the small intestine and liver (33). A number of glucosinolate hydrolysate products have been shown to be goitrigenic, the most potent of which, 5-vinylindole-2-thione (derived from progoitrin, IV) is potentially present in large amounts in Brussels sprouts (52–54). Chronic feeding trials of materials containing glucosinolates have shown these to be dose-dependently linked to lesions in the liver, kidney and pancreas, haemorrhage and death (55,56). Pancreatic cancer development in the hamster model was also markedly increased in animals fed cabbages and a high fat diet (20).

We did not observe gross or microscopic lesions in organs other than the liver, thyroid gland, lung, kidney, and adrenal gland in our experiment. It should be borne in mind that I3C reduced the life-span of the rats compared with the DMD alone group, perhaps due to the presence of liver or thyroid gland tumour masses (Table II). From the available results, we conclude that I3C is capable of causing toxicity and enhancing tumourigenesis in the liver and thyroid gland of experimental animals. Therefore, the efficacy and risk potential of cruciferous vegetables deserves further careful attention.

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