Introduction

It is well established that the human diet contains both carcinogens and cancer chemopreventive agents (1–4). Dietary anticarcinogens and antimutagens have been identified using a range of end points, from a reduction in the frequency or multiplicity of tumors and intermediate biomarkers of cancer in vivo to inhibitory activity in short-term genotoxicity assays in vitro. The list of cancer chemopreventive agents includes both natural and synthetic derivatives of phytochemicals that occur in fruits and vegetables (1–4). Two that have received considerable attention during the past decade are chlorophyllin and indole-3-carbinol.

Chlorophyllin (CHL), a water-soluble salt of chlorophyll, acts as an anticarcinogen during the initiation phase of carcinogenesis (5) and is being evaluated by one of us (G.S.B.), in collaboration with Dr Tom Kensler and colleagues of Johns Hopkins University, against aflatoxin exposure in a clinical intervention biomarker trial in Daxin, China. During the post-initiation phase, 0.1% CHL (w/v) in the drinking water inhibited the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)- and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)-induced colonic aberrant crypts in the rat (6; R.H. Dashwood, unpublished results), but promoted 1,2-dimethylhydrazine (DMH)-induced colon tumor formation in the same species (7).

Indole-3-carbinol (I3C), a natural constituent of brassica vegetables that has been used in studies of estrogen metabolism in women (8), has both anticarcinogenic and tumor promoting activities in animal models depending upon the initiator, exposure protocol and species (see ref. 9 for a review). Inhibition of the formation of colonic aberrant crypt foci, which are putative neoplastic lesions and intermediate biomarkers of colon cancer (10,11), was observed in rats given 0.1% I3C in the diet before, during and after exposure to IQ (12). Potent inhibition of PhIP-induced colonic aberrant crypts also was observed in the rat when 0.1% dietary I3C was administered during the initiation phase, post-initiation phase or continuously throughout the entire study (6). However, 0.1% dietary I3C given before, during and after DMH treatment reportedly enhanced the formation of colon tumors in the rat (13). Collectively, these results suggested that the initiating agent, namely DMH or heterocyclic amine, might influence the response to a tumor modulator (CHL or I3C) administered for a prolonged period of time during the post-initiation phase. However, the dose–response relationships have not been adequately addressed, and the molecular mechanisms have yet to be studied in any detail.

Therefore, in the present study, we compared the post-initiation effects of CHL and I3C, administered at three different concentrations within the range of human exposure, in male F344 rats initiated with DMH or IQ. The results indicated that a low concentration of CHL increased the multiplicity of colon tumors in rats given DMH but had no effect on the colon tumors initiated by IQ, and that I3C inhibited the multiplicity and incidence of IQ-induced colon and liver tumors, respectively, but had no effect on the tumors initiated by DMH.
Materials and methods

Chemicals
I3C and CHL were purchased from Sigma Chemical Company (St Louis, MO). IQ was from Toronto Research Chemicals (Ontario, Canada) and DMH was from Aldrich Chemical Company (Milwaukee, WI).

Tumor bioassay
Male F344 rats, 4-5 weeks of age (103 ± 10 g body wt), were purchased from Taconic (Germantown, NY) and divided randomly into groups of 20–25 animals (2–3 rats per cage). Water and AIN-93G diet were provided ad libitum. During the first 5 weeks of a 1 year study, animals were given either DMH or IQ in order to induce tumors in the colon and other target organs (Figure 1). IQ was administered in the diet at a concentration of 0.03%, whereas DMH was injected once a week (20 mg/kg body wt, s.c.). Starting 1 week after the last carcinogen treatment until the end of the study, rats were given 0.1, 0.01 or 0.001% CHL (w/v) in the drinking water, or 0.1, 0.01 or 0.001% I3C in the diet. Final data for tumor incidence (%) and multiplicity (mean number of tumors/tumor-bearing tissue) are shown in Tables I and II.

Results

The results for groups initiated with DMH or IQ are summarized in Tables I and II, respectively. No significant effects of the CHL or I3C treatments were seen on the body weights of the animal throughout the study, except at the highest concentration of 0.1% I3C in rats initiated with IQ; at the time of sacrifice, average body weights (g) were 531 ± 38.9 and 489 ± 47.5 for IQ and I3C + 0.1%I3C groups, respectively (P < 0.05). None of the animals treated with vehicle and post-treated with CHL or I3C had tumors (not shown).

In rats initiated with DMH, tumors were found in the colon, small intestine and Zymbal’s gland, whereas IQ produced tumors in these organs plus the liver and skin, as reported previously (14–17). Examples of the tumors induced by IQ are shown in Figure 2. Tumors induced by DMH and IQ in the colon and small intestine were identified almost exclusively as adenocarcinomas, and the skin tumors and Zymbal’s gland tumors were squamous cell carcinomas. Liver tumors induced by IQ were primarily hepatocellular carcinomas, and occasionally adenomas or mixed hepatocellular and ductal carcinomas. The proportion of the latter tumor types was in the order of 10% in all IQ groups, and although the numbers of tumors did not permit statistical comparisons, none of the modulator treatments appeared to alter the overall frequency of carcinomas versus adenomas (data not shown).

In the present study, 87% of the rats given DMH alone had colon tumors, and each rat had on average 2.7 tumors per colon (Table I). Fifty-two percent and 17% of the DMH-induced animals also had tumors in the small intestine and Zymbal’s gland, respectively. The high incidence of colon tumors in this investigation, compared with only 10% reported before, might be due to the fact that a 20-week time point was used in the earlier study with DMH and CHL (7). In addition, the previous study used Purina chow diet (7), and we have reported recently that feeding chow versus AIN-76A or AIN-93G diets plays a significant role in determining the number of colonic aberrant crypts induced by IQ in the rat (18).

In the groups given DMH and post-treated with CHL or I3C, there was no obvious dose–response relationship for suppression or promotion of tumor incidence or multiplicity in the colon, small intestine and Zymbal’s gland (Table I). However, the lowest concentration of 0.001% CHL significantly increased the multiplicity of colon tumors, from a value of 2.70 ± 1.05 for DMH alone to 4.89 ± 2.3 tumors/tumor-bearing colon for DMH plus CHL (P < 0.05, mean ± SD). The finding that promotion occurred at the lowest concentration of CHL only is discussed further below.

In all of the groups given IQ, except the group administered the highest concentration of 0.1% I3C, the overall incidence of colon tumors was around 9–10% and the average multiplicity was 1.0–1.5 tumors/tumor-bearing colon (Table II). Thus, in contrast with the results with DMH, no tumor promotion was seen by CHL or I3C in the colon of rats induced with the heterocyclic amine. Indeed, in the group given IQ plus 0.1% I3C there was a complete absence of colon tumors, and the reduced multiplicity of colon tumors proved to be statistically significant compared with the group given IQ alone (1.5 ± 0.71 versus 0.0 ± 0.0 tumors/tumor-bearing colon, P < 0.05). No effect was seen for CHL or I3C on the incidence or multiplicity of IQ-induced tumors in the small intestine, Zymbal’s gland or skin (Table II). However, in the liver, which was the major target organ for IQ-induced tumorigenesis, the highest concentration of 0.1% CHL suppressed the incidence of tumors compared with the group given IQ alone (P < 0.05). Moreover, the dose–response for suppression by CHL in the liver was statistically significant: tumor incidence results were 73, 60, 50 and 36% for groups given IQ alone, IQ + 0.001% CHL, IQ + 0.01% CHL and IQ + 0.1% CHL, respectively (P < 0.05 for the trend). No significant effects were detected on liver tumor multiplicity in these groups.

Discussion

To our knowledge, this is the most detailed dose–response analysis to date for CHL and I3C in the rat using a post-initiation exposure protocol and comparing two different initiating agents. In previous studies, short-term treatment of rats with PhIP, followed by post-initiation exposure to a high-fat diet, caused the rapid induction of colonic aberrant crypts or mammary tumors (19,20); however, this is the first paper to describe the induction of tumors in the rat using a short,
adducts and overall carcinogen bioavailability in vivo during the initiation phase and to reduce hepatic IQ tumorigenesis. CHL has been shown to complex with IQ. There was evidence for dose-related suppression of liver tumors in rats treated as suppressing agents during the post-initiation phase. However, in the present investigation, rats given IQ and post-treated with CHL showed no inhibition in the colon, but, unexpectedly, there was evidence for dose-related suppression of liver tumors. In previous studies of IQ- and PhlP-induced colonic aberrant crypts (6, 12), CHL and I3C were shown to be effective inhibitors in the rat colon when given at the time of carcinogen exposure, and they were more or less equally potent when tested as suppressing agents during the post-initiation phase. In the present investigation, rats given IQ and post-treated with CHL showed no inhibition in the colon, but, unexpectedly, there was evidence for dose-related suppression of liver tumors. CHL has been shown to complex with IQ during the initiation phase and to reduce hepatic IQ-DNA adducts and overall carcinogen bioavailability in vivo (22–25).

However, the 1-week period between the last dose of IQ and the first treatment with CHL (Figure 1) should have allowed for most of the carcinogen to be metabolized and excreted via the urine and feces (26–28). The complexing of CHL with residual amounts of IQ or its metabolites cannot be completely ruled out, but this is unlikely to represent a major inhibitory mechanism during the post-initiation phase. Although it is generally assumed that an oral dose of CHL is largely retained within the GI tract of the rat (29), suppression of IQ-induced liver tumorigenesis in the present study points to systemic mechanisms, and suggests at least some degree of CHL uptake. There is currently no information available on the mechanisms by which CHL might protect in the liver when administered post-initiation, but the results are noteworthy in view of the intervention trial currently under way in Daxin, China (see Introduction). Assuming that uptake also occurs in people ingesting an oral dose, CHL might operate as a suppressing agent in individuals exposed unavoidably to hepatocarcinogens in the diet (30, 31), as well as protecting via molecular complex formation or other blocking mechanisms (5).

In previous studies of IQ- and PhlP-induced colonic aberrant crypts (6, 12), CHL and I3C were shown to be effective inhibitors in the rat colon when given at the time of carcinogen exposure, and they were more or less equally potent when tested as suppressing agents during the post-initiation phase. In the present investigation, rats given IQ and post-treated with CHL showed no inhibition in the colon, but, unexpectedly, there was evidence for dose-related suppression of liver tumorigenesis. CHL has been shown to complex with IQ during the initiation phase and to reduce hepatic IQ-DNA adducts and overall carcinogen bioavailability in vivo (22–25).

Table I. Final tumor incidence and multiplicity data for DMH groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Colon</th>
<th>Small intestine</th>
<th>Zymbal’s gland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Incidence</td>
<td>Multiplicity</td>
<td>Incidence</td>
</tr>
<tr>
<td>DMH</td>
<td>23</td>
<td>87</td>
<td>2.70</td>
<td>52</td>
</tr>
<tr>
<td>DMH + 0.001% CHL</td>
<td>20</td>
<td>90</td>
<td>4.89</td>
<td>30</td>
</tr>
<tr>
<td>DMH + 0.01% CHL</td>
<td>20</td>
<td>100</td>
<td>3.25</td>
<td>55</td>
</tr>
<tr>
<td>DMH + 0.1% CHL</td>
<td>20</td>
<td>85</td>
<td>3.53</td>
<td>25</td>
</tr>
<tr>
<td>DMH + 0.001% I3C</td>
<td>20</td>
<td>100</td>
<td>3.75</td>
<td>30</td>
</tr>
<tr>
<td>DMH + 0.01% I3C</td>
<td>20</td>
<td>90</td>
<td>3.11</td>
<td>25</td>
</tr>
<tr>
<td>DMH + 0.1% I3C</td>
<td>20</td>
<td>90</td>
<td>2.50</td>
<td>45</td>
</tr>
</tbody>
</table>

Table II. Final tumor incidence and multiplicity data for IQ groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Colon</th>
<th>Small intestine</th>
<th>Zymbal’s gland</th>
<th>Liver</th>
<th>Skin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Incidence</td>
<td>Multiplicity</td>
<td>Incidence</td>
<td>Multiplicity</td>
<td>Incidence</td>
</tr>
<tr>
<td>IQ</td>
<td>22</td>
<td>9</td>
<td>1.50</td>
<td>0</td>
<td>0.00</td>
<td>23</td>
</tr>
<tr>
<td>IQ + 0.001% CHL</td>
<td>20</td>
<td>10</td>
<td>1.00</td>
<td>5</td>
<td>1.00</td>
<td>15</td>
</tr>
<tr>
<td>IQ + 0.01% CHL</td>
<td>20</td>
<td>10</td>
<td>1.50</td>
<td>10</td>
<td>1.00</td>
<td>25</td>
</tr>
<tr>
<td>IQ + 0.1% CHL</td>
<td>22</td>
<td>9</td>
<td>1.00</td>
<td>0</td>
<td>0.00</td>
<td>14</td>
</tr>
<tr>
<td>IQ + 0.001% I3C</td>
<td>20</td>
<td>10</td>
<td>1.00</td>
<td>0</td>
<td>0.00</td>
<td>10</td>
</tr>
<tr>
<td>IQ + 0.01% I3C</td>
<td>20</td>
<td>10</td>
<td>1.50</td>
<td>15</td>
<td>1.67</td>
<td>30</td>
</tr>
<tr>
<td>IQ + 0.1% I3C</td>
<td>20</td>
<td>0</td>
<td>0.00</td>
<td>10</td>
<td>1.00</td>
<td>15</td>
</tr>
</tbody>
</table>

Colon tumor incidence data represent the number of rats with colon tumors/total rats in the group, multiplied by 100 to give percent values (%), and these were based on the number of animals shown in column 2. Some of the rats died early in the study, prior to I3C or CHL dosing, and were not included in the final analyses. Colon tumor multiplicity indicates the number of colon tumors per animal, independent of whether or not the animal had tumors in other target organs. Similarly, liver tumor multiplicity indicates the number of liver tumors per liver tumor-bearing animal, independent of whether or not the animal had tumors in other target organs. For clarity, only the mean values are given for tumor multiplicity in the table; however, when the results were significantly different from controls, the data were presented in the text (mean ± SD). Numbers shown in bold are significantly different from the corresponding value, in that column, for the carcinogen alone (P < 0.05, using ANOVA followed by pairwise comparisons using Fisher’s LSD model), and the bracketed data in Table II contain results for livers that were significant with respect to the dose-response trend (*P < 0.05). None of the vehicle control groups in this study had tumors in the colon or other organs examined (not shown). For details on the histopathological findings on the tumor type(s) in each target organ, see text.

5-week dietary exposure protocol for IQ. Based on the original carcinogenicity bioassay that defined IQ as a carcinogen in the F344 rat, in which IQ was given continuously for over 1 year at a dietary level of 0.03% (16), the present exposure protocol was successful at inducing tumors in all of the major target organs. The 73% liver tumor incidence in this study (Table II) compares favorably with the 68% liver tumor incidence reported previously for IQ (16, 21). However, the incidence of tumors in other target organs was lower than expected, including tumors of the colon, the target organ of principle interest at the outset of this investigation. Whereas the original report found a colon tumor incidence of 63% (16), the present study detected only a 9% incidence of IQ-induced colon tumors.

In previous studies of IQ- and PhlP-induced colonic aberrant crypts (6, 12), CHL and I3C were shown to be effective inhibitors in the rat colon when given at the time of carcinogen exposure, and they were more or less equally potent when tested as suppressing agents during the post-initiation phase. In the present investigation, rats given IQ and post-treated with CHL showed no inhibition in the colon, but, unexpectedly, there was evidence for dose-related suppression of liver tumorigenesis. CHL has been shown to complex with IQ during the initiation phase and to reduce hepatic IQ-DNA adducts and overall carcinogen bioavailability in vivo (22–25).
since 87% of the rats given DMH alone had colon tumors (Table I). Nonetheless, the lowest concentration of 0.001% CHL increased significantly the multiplicity of colon tumors induced by DMH, without promoting the colon tumors induced by IQ. These findings with CHL raise two important issues. First, the results suggest that the choice of initiating agent might, in some cases, influence the response to a modular given post-initiation. In other words, it may be possible for the same modulator to act as a tumor promoter against one initiating agent but to have no effect, or potentially to operate as a suppressing agent, against other carcinogens. This concept is well established for compounds that are effective during the time of initiation, when changes in carcinogen metabolizing enzymes can facilitate the detoxification of one class of carcinogen but augment the metabolic activation of others (32). However, it has received less attention during the post-initiation phase, largely due to the fact that, unlike the case of 'blocking agents', the mechanisms are often poorly defined for suppressing agents (2). As an additional level of complication, humans most likely receive a lifetime of exposure to endogenous and exogenous carcinogens, with modulators acting on previous as well as concurrent initiation events. For these
reasons, we are cautious about extrapolating the present data with CHL and I3C to the situation of people consuming chlorophyll- or indole-rich foods.

Second, the results reported here and elsewhere (7) suggest that different mechanisms operate in the colon according to the concentration and duration of CHL exposure. Recent studies with butyrate, a short-chain fatty acid that protects against colon cancer in the rat (33), provided evidence for cell cycle arrest in colon cancer cells at low concentrations, but at higher concentrations caused the induction of apoptosis (34). We have reported on the loss of apoptosis during IQ-induced colon tumor formation in the rat (15), as evidenced by increased expression of anti-apoptotic Bcl-2, decreased levels of pro-apoptotic Bax and loss of terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL). Moreover, a recent investigation of TUNEL and bromodeoxyuridine labeling indices in the rat colon showed that cell proliferation was increased following post-initiation treatment with 0.001% CHL, with no corresponding changes in TUNEL, whereas higher CHL concentrations augmented both of these endpoints, such that the net balance between cell proliferation and apoptosis was unaffected (35). We have also shown that DMH-induced colon tumors from the present study, in particular those from the group promoted by 0.001% CHL, contained a unique spectrum of β-catenin mutations compared with the tumors from rats given DMH alone (36). The results strongly implicated the Apc/β-catenin pathway, and indicated that during the post-initiation phase of carcinogenesis, CHL and I3C might alter the expression of β-catenin/Tcf/Lef target genes. We are now conducting further studies in this direction, in light of the important ‘gatekeeping’ function of the APC/β-catenin pathway in human colon cancer (37).

In summary, the present investigation examined the post-initiation effects of CHL and I3C in rats given DMH or IQ. Suppression of IQ-induced colon tumor multiplicity was significant at the highest concentration of 0.1% I3C, which is in general accordance with previous studies using heterocyclic amine-induced colonic aberrant crypts as the end-point (6,12). However, there was no significant effect of I3C on DMH-induced colon tumors, in contrast with a previous study showing enhancement of colon tumorigenesis when I3C was given before, during and after DMH exposure (13). In rats given CHL, significant, dose-related suppression of IQ-induced liver tumorigenesis was observed, but at the lowest concentration tested, this compound increased the multiplicity of DMH-induced colon tumors while having no promotional effect on the colon tumors induced by IQ.

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References
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