ABSTRACT

Indole-3-carbinol (I3C), a secondary metabolite from cruciferous vegetables, inhibits aflatoxin B1 (AFB1) hepatocarcinogenesis in trout (Bailey et al., J. Natl. Cancer Inst., 78: 931–934, 1987) and rats (Selivonchick et al., unpublished results) when given prior to and with carcinogen but promotes carcinogenesis in both species when given continuously following AFB1 initiation. Since human I3C intake may not be continuous, and the promotional stimulation may be reversible, we have assessed I3C promotion using delayed and discontinuous exposure protocols. Following initiation with AFB1, I3C was fed to trout for varying periods of time, with varying lengths of delay after initiation and continuous or intermittent patterns of I3C treatment. Promotional enhancement of tumor incidence by I3C was found to be significant when I3C treatment was delayed for several weeks or months after the initial AFB1 challenge. Promotion also was found to increase with length of exposure to I3C treatment and to be decreased but still evident when I3C was given in alternating months or weeks, or twice per week only. These results do not support the idea that promotional stimulation in hepatocarcinogenesis is a reversible phenomenon.

To quantify I3C promotional potency in terms of its dietary concentration, a series of AFB1 tumor dose-response curves was established, each with a different level of I3C fed continuously following AFB1 initiation. The resultant tumor dose-response curves, plotted as log log percentage of incidence versus log AFB1 dose, were displaced parallel toward lower AFB1, 50% tumor take (TD50) values with increasing I3C concentration. The level of I3C that halves the AFB1 dose for 50% tumor incidence was calculated to be approximately 1000 ppm I3C, fed continuously, with no substantial threshold for promotion. By comparison, I3C, when fed before and with AFB1, shows a 50% inhibitory value (I3C concentration that doubles the dose of AFB1 for 50% tumor incidence) in trout of 1400 ppm I3C (Dashwood et al., Carcinogenesis (Lond.), 10: 175–181, 1989). Thus the potential for I3C as a dietary additive to promote prior hepatic initiation events when fed continuously is approximately as great as its potential to inhibit concurrent AFB1 initiation.

INTRODUCTION

The human diet contains many compounds that inhibit the carcinogenic process in experimental animals (1–3). However, the protection afforded by such compounds and their potential for human chemoprevention remain difficult to ascertain. In many studies, unreasonably high doses are required to elicit an inhibitory effect. Moreover, in some test protocols, dietary “anticarcinogens” may exhibit promotional activity. For example, I3C, a naturally occurring compound from cruciferous vegetables which inhibits AFB1, carcinogenesis in rainbow trout when given prior to and during carcinogen exposure (4, 5) but which also promotes it in the same species when given continuously after AFB1 initiation (6). Similar effects have been observed using rats. Since human exposure to such agents may be unavoidable, it is important to provide detailed quantitative potency information for these opposing activities (promotion versus inhibition) in order to assess risk versus benefit. Because promotion of liver foci has been shown to be reversible (7), it is also important to determine tumor promotional activity under conditions which mimic submaximal patterns of human exposure, including shorter-term, delayed, or intermittent consumption following initiation. To address these issues, several tumor studies were established in trout which were pretreated with AFB1 and subsequently given I3C either immediately or with various periods of delay and which were fed either continuously or for various lengths of time. There is also little quantitative information on the degree to which inhibition and promotion would alter population response to a given carcinogen exposure. It should be stressed that the range or magnitude of possible modulation cannot be derived by the usual comparisons of simple tumor incidences between treated and control populations. A more reliable approach is to determine the effect of varying modulator dose on the TD50 value of carcinogen dose-response curves (4). This report describes preliminary tumor dose-response studies assessing the concentration dependence of I3C promotional potency for comparison with data reported previously on the inhibitory potency of this “ambivalent modulator” in trout (4, 8). Preliminary results from this study appeared in a recent review (9).

MATERIALS AND METHODS

Chemicals. AFB1 was purchased from Calbiochem Inc. (San Diego, CA) and checked for purity by UV spectrometry and thin-layer chromatography. I3C was from Aldrich Chemical Company (Milwaukee, WI) and used without further purification. All other chemicals and reagents were of the purest grade available and were from sources described previously (5).

Animal Experiments. Rainbow trout [Oncorhynchus mykiss, formerly Salmo gairdneri (10)] were reared at the Oregon State University Food Toxicology/Carcinogenesis Laboratory. Details of the rearing conditions, diets, and animal care have been published elsewhere (5, 11). All animals were reared at 12.5 ± 1°C (SD). In all experiments, trout were exposed as fry to static solutions of AFB1 for 30 min and then returned to holding tanks and fed OTD (control) diet or OTD containing I3C. In Experiment 1, duplicate tanks of 100 fry were exposed to 12.5 ppb AFB1, followed by OTD or I3C diet given immediately or at various times after terminating AFB1 exposure (1, 3, and 6 months) and fed for various lengths of time (9, 6, and 3 months, or on alternating months). As an extension to Experiment 1, triplicate tanks of 100 fry were exposed to 50 ppb AFB1 and fed I3C in alternating weeks or on Mondays and Fridays of each week only. Experiment 2 was designed to provide information on the promotional potency of I3C by constructing a series of tumor dose-response curves. Triplicate tanks of 100 fry were exposed to a range of AFB1 concentrations and then fed diets containing 0, 750, 1500, or 2000 ppm I3C. All experiments were...
terminated after a total of 36 weeks, and necropsy examinations were performed as described previously (5). Incidences were based on grossly observed liver tumors present 36 weeks after AFB1 exposure. Classification into benign or malignant and hepatocellular or mixed hepatocellular-cholangiocellular categories was by criteria established previously (11, 12). Hepatocarcinogenesis in rainbow trout and other species of fish progresses through enzyme and/or tinctorially altered preneoplastic focal stages comparable at least in part to those observed in mammals (13, 14). However, these early detectable stages were not used in the present study.

RESULTS

Timing of I3C Postinitiation Treatment

Duration of I3C Treatment. Control trout exposed for 30 min to 12.5 ppb AFB1 and subsequently given control diet (OTD) for the entire study period of 36 weeks had an average tumor incidence of 19.1% (Fig. 1, Group a). By comparison, trout exposed to the same AFB1 concentration but fed 2000 ppm I3C for 12, 24, or 36 weeks had, respectively, average tumor incidences of 27.3, 41.7, or 53.9% (Fig. 1, Groups b–d). A linear relationship (correlation coefficient, r² = 0.99) was observed when these data were plotted as percentage of tumor incidence versus weeks that I3C was fed (Fig. 2). Thus, it is clear that the extent of promotion increased regularly with length of I3C treatment.

Influence of Delayed I3C Treatment. To investigate whether I3C postinitiation treatment is required immediately after AFB1 exposure in order to promote, various “lag phases” were assessed between the time of initiation and administration of I3C. Following AFB1 exposure, trout were given OTD for 4, 12, or 24 weeks and then switched to I3C diet for the remainder of the experiment. As shown in Fig. 1, a delay of 4 or 12 weeks (Fig. 1, Groups e and f) gave equal or greater promotion than the no-delay group (Fig. 1, Group d), even though the total period of I3C treatment was reduced. A 3-month I3C treatment beginning at week 24 (Fig. 1, Group g) was at least as effective as the 3-month treatment starting at week 0 (Fig. 1, Group b), and a 12-week delay provided significantly greater promotion (Fig. 1, Group h). Collectively, these data show that I3C is capable of promoting AFB1 liver tumors if given several weeks or months after the initial carcinogen challenge.

Effect of Intermittent I3C Exposure. Additional comparisons were conducted to investigate the frequency of I3C exposure required to elicit a promotional effect. Initially, trout were exposed to AFB1 and then given OTD and I3C diets for alternating 3-month (Fig. 1, Group h) or 1-month (Fig. 1, Group i) periods; the corresponding tumor incidences were 43.6 and 35.0%, versus 19.1% for the controls (Fig. 1a, Group a). To further titrate the alternating OTD and I3C treatments to model discontinuous human exposures, 50 ppb AFB1 were administered and I3C was given to trout either in alternating weeks or on Mondays and Thursdays of each week only. The corresponding tumor incidences were 63.9 and 62.0% versus 40.0% for OTD-fed control trout in this study (Fig. 1b). These results demonstrate that daily I3C exposure was not a prerequisite for promotion. The data also suggest that a period of at least 12 weeks of 2000 ppm I3C consumption, administered continuously or fractionated over a longer period, was required before promotion was significant under the conditions of this study. By comparison, previous studies in trout showed that dietary I3C pretreatment for only 8 days was sufficient for I3C inhibitory activity (15).

Tumor Dose-Response Studies

A preliminary study was conducted to quantify the potency of I3C promotion as a function of its dietary concentration. To achieve this, a series of AFB1 dose-response curves was produced, each receiving a different level (0, 750, 1500, 2000 ppm) 13C incorporated into a control diet. Data are average values from duplicate (A) or triplicate (B) tanks, each containing approximately 100 trout at necropsy; variances ranged from 0.389 to 13.774. *, P < 0.05; **, P < 0.01 versus control groups receiving OTD only.

![Fig. 1. Timing of I3C postinitiation treatment. Effect of lag phase, intermittent exposure, and length of I3C treatment. All groups receiving I3C were fed 2000 ppm I3C incorporated into a control diet. Data are average values from duplicate (A) or triplicate (B) tanks, each containing approximately 100 trout at necropsy; variances ranged from 0.389 to 13.774. *, P < 0.05; **, P < 0.01 versus control groups receiving OTD only.](image-url)

![Fig. 2. Effect of duration of 13C postinitiation exposure on AFB1 tumor incidence. Results were derived from data presented in Fig. 1 (Groups a–d). Points describe a straight line over the tumor incidence range 20–60%; bars, SD; r² = 0.99.](image-url)
As shown recently (4), modulator potency can be quantified based on the extent to which a given modulator dose offsets the carcinogen dose-response curve. First, carcinogen TD50 values are determined from the logit percentage of tumor incidence versus log carcinogen dose plots, for each modulator dose investigated. In the case of I3C anticarcinogenesis protocols, percentage of inhibition was defined as

\[ \text{% of inhibition} = 100 \left(1 - \frac{\text{TD}x_{target}}{\text{TD}x}\right) \]

where \( x \) was the tumor incidence being compared (e.g., 50%), \( \text{TD}x \) was the dose of AFB1 needed to give that tumor incidence in the 0 ppm (control) group, and \( \text{TD}x_{target} \) was the AFB1 dose required to produce that incidence in the group receiving \( i \) ppm I3C.

Similarly, the percentage of promotion can be calculated for the data in Fig. 3 by reversing the numerator and denominator functions in the above equation, thereby reflecting horizontal displacements toward lower carcinogen dose relative to the control curve. Thus

\[ \% \text{ of promotion} = 100 \left(1 - \frac{\text{TD}x}{\text{TD}x_{target}}\right) \]

The promotional potency of I3C (percentage of promotion versus I3C dose) is shown in Fig. 4b. For comparison we also show the analogous curve of percentage of inhibition versus I3C dose from our previous study (4). Using the inhibition protocol (Fig. 4a), the AFB1 tumor incidence was reduced by 95% at 4000 ppm I3C, while the dose producing 50% inhibition was 1400 ppm I3C. Although more limited, the data reported here for I3C promotion enable determination of the 50% promotion. Interpolating from Fig. 4b, the concentration of I3C producing 50% promotion was 1000 ppm, while the maximum promotional response in these studies was 75% at the I3C dose of 1500 ppm.

DISCUSSION

Liver Tumors as an End Point in Promotion Studies. Enzyme-altered foci in rat liver have been shown to relate linearly to initiator dose (16) and to display a threshold for promotion in number by phenobarbital (17). However, their reversibility or stability upon withdrawal of promoter appears to depend on the carrier diet chosen in some protocols (7, 17). The use of liver foci as a promotional end point is further complicated by the demonstration of agents that promote foci but not tumors and agents that promote tumors but not foci (18). The present study uses true neoplasms, not foci, to examine the influence of promoter withdrawal, dietary concentration, and dose fractionation on liver cancer.

Timing of Postinitiation Treatment. To provide data on the effects of various postinitiation treatments, including those which attempt to mimic human I3C exposures, a series of tumor studies has been established in trout pretreated with AFB1 and subsequently given I3C: (a) for various lengths of time (12, 24, and 36 weeks); (b) after various lag phases (4, 12, and 24 weeks following AFB1 initiation); and (c) for selected intermittent periods of time (alternating 3-month, 1-month, and 1-week periods or Mondays and Thursdays each week). Promotion was found to increase with duration of exposure, to persist or even increase with delayed onset, and to be reduced but still evident when treatment was given in alternating months or weeks or twice per week only. Thus, significant promotion by high dietary I3C levels required prolonged consumption (at least 12 weeks) but still occurred even if the consumption pattern was intermittent and if I3C was first administered several weeks or months after the initial carcinogen challenge. These results do not support the idea that promotional stimulation in hepatocarcinogenesis is an entirely reversible phenomenon. The fractionation experiments suggest that the effects of I3C could be cumulative in this organ.

These conclusions are based on one I3C dietary concentration (2000 ppm) and an observation period of 36 weeks. From Fig. 4, concentrations of I3C less than 2000 ppm should give proportionately less promotional stimulation. The effect of an observation period longer than 36 weeks is not immediately apparent but could be minor, since tumor development would likely increase in the positive controls (AFB1 only) as well as the I3C-promoted groups.

Promotional versus Inhibitory Potency. The rationale for constructing several tumor dose-response curves to assess inhibi-
tory or promotional potency was discussed in detail in a recent publication (4). In brief, tumor dose-response curves plotted on log-linear coordinates are sigmoidal quantal data. Thus, comparisons between control and promoter or inhibitor groups in the usual single carcinogen dose experiment can be misleading because the ratio for control/treated incidences will vary depending on the carcinogen dose selected. Logit incidence ratios would be a more valid comparison, but only if it is established that the modulator produces parallel offset tumor dose-response curves. Rather than performing the usual vertical comparisons, a more reliable approach is to construct several tumor dose-response curves and to compare horizontally across the various groups. For example, one could determine the TD_{50} for each curve (4). From these comparisons, percentage of promotion or percentage of inhibition may be calculated from the tumor dose index (see "Results") and plotted as a function of I3C dose, as shown in Fig. 4.

The data presented in Fig. 4 indicate that, using the experimental protocols described in these studies, the inhibitory and promotional potencies of I3C were similar at I3C doses <1500 ppm. This is reflected in the shape of the dose-response curves in the dose range 0–1500 ppm I3C and in the 50% inhibition and promotion values of 1400 and 1000 ppm, respectively. At higher I3C doses, some discrepancy was observed due to toxicity in the promotion study (Fig. 4b). This probably reflects the fact that trout received a total I3C exposure of only 6 weeks in the inhibition studies (4, 8) compared with 9 months of postinitiation treatment in the promotion study and that the inhibition studies involved older animals.

These observations highlight an important point, i.e., that the I3C inhibitory and promotional potencies are highly dependent upon the exposure protocols used. By design, the promotional potency studies were aimed in part at evaluating a possible worst-case scenario for I3C, i.e., continuous postinitiation exposure ranging up to high dietary concentrations. It is unlikely that human exposure would occur according to this dosing schedule. However, results from Experiment 1 indicate that intermittent I3C exposure is sufficient for promotion, and it would be predicted that I3C doses within the human range (0–1000 ppm) if given only twice per week may still lead to some degree of promotion of cells initiated from prior carcinogen exposure. Collectively, the results from the present study suggest approximate equivalence of the I3C promotion and inhibition activities in trout when fed continuously. This could be taken to imply no overall benefit from chronic human exposure to I3C in terms of cancer chemoprevention. However, until I3C promotional mechanisms and the susceptibility of human liver to promotion are known, the relevance of dietary I3C as a promoter of human liver cancer is uncertain. It is also well to remember that complex food mixtures contain many anticarcinogenic agents, including those which might counteract the promotional effects of I3C without diminishing its inhibitory potency.

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Promotion of Aflatoxin B₁ Carcinogenesis by the Natural Tumor Modulator Indole-3-carbinol: Influence of Dose, Duration, and Intermittent Exposure on Indole-3-carbinol Promotional Potency

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