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Anticarcinogenic Activity of Indole-3-carbinol Acid Products: Ultrasensitive Bioassay by Trout Embryo Microinjection[1]

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ABSTRACT

The relative contribution of indole-3-carbinol (I3C) and its acid condensation products to the anticarcinogenic activity of this crucifer phytochemical has been studied using trout embryo microinjection. I3C was treated with 0.07 N HCl to give a reaction mixture (RXM) comprising <0.5% parent compound and over 20 products, the most prevalent being the dimer 3,3′-diindolylmethane (I33′) and a related cyclic trimer (CT). RXM, I33′ or CT was injected into embryos with [^{3}H]aflatoxin B_1 (AFB_1) and total embryonic DNA was isolated 1, 3, or 10 days postinjection. Compared with controls given AFB_1 alone, I3C failed to inhibit carcinogen-DNA binding at any time point. In contrast I33′, CT, and RXM inhibited AFB_1-DNA binding by an average of 37, 51, and 65%, respectively. Coinjection of AFB_1 and 350 μM I3C, RXM, or I33′ into trout embryos reduced AFB_1-induced hepatocarcinogenesis after 1 year from 43.4% in positive controls to 36.0, 12.2 ($P < 0.05$), and 24.6% ($P < 0.05$), respectively. No tumor data were obtained in the AFB_1 plus CT group due to poor survival of the embryos posthatching. These results indicate that acid condensation products, not the parent compound, represent the anticarcinogenic species in trout and that their formation in the stomach is a likely prerequisite for I3C anticarcinogenesis.

INTRODUCTION

Cruciferous vegetables contain a variety of compounds that modulate the carcinogenic process (1–5). One such compound is I3C,[3] which is found naturally as a glucosinolate in vegetables such as cabbage, cauliflower, and broccoli (6). I3C and the related indole I33′ were reported over a decade ago to inhibit tumorigenesis in rodents exposed to polycyclic aromatic hydrocarbons (7). The anticarcinogenic properties of I3C have been confirmed in a number of more recent studies; these include inhibition of diethylnitrosamine-induced hepatocarcinogenesis in ACI/N rats, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung neoplasia in A/J mice, and AFB_1-induced hepatocarcinogenesis in trout (8–11).

It has been suggested that the mechanism of I3C anticarcinogenesis may be related to induction of cytochrome P-450 detoxification pathways (12–15). I3C was shown to induce hepatic ethoxyresorufin *O*-deethylase activities in rats when given p.o. but produced only marginal induction when administered by i.p. injection. However, treatment of I3C under simulated gastric conditions produced a RXM that was shown to induce ethoxyresorufin *O*-deethylase activities when administered either by p.o. gavage or i.p. injection (12). On the basis of these findings and the known sensitivity of I3C to low pH (16–18), Bradfield and Bjeldanes postulated that acid condensation products formed in the stomach may be responsible for the I3C inhibitory mechanism *in vivo* (12).

We previously examined a number of inhibitory mechanisms for I3C, namely, the induction of oxidation and conjugation enzymes, the scavenging of carcinogen electrophiles, and the inhibition of carcinogen activation (19). The results supported a role for I3C acid products as inhibitors of carcinogen activation; RXM, but not I3C, competitively inhibited the covalent binding of AFB_1 to DNA *in vitro* mediated by trout or rat liver microsomes (19). To extend this work, the present studies sought to evaluate the *in vivo* inhibitory activities of individual I3C acid-condensation products. Two such products, the dimer I33′ and a related cyclic trimer, were isolated in sufficient quantity from RXM to examine inhibition of AFB_1-DNA binding and hepatocarcinogenesis following coinjection of carcinogen and inhibitor into trout embryos. This model (20) was chosen because the small size at exposure permitted full tumor studies using μg doses of test compound. The results of this study demonstrate for the first time that I3C itself is not anticarcinogenic and provide further support for a role of acid condensation products in the anticarcinogenic mechanism of I3C.

MATERIALS AND METHODS

Chemicals. [*G*-^{3}H]AFB_1 (specific activity, 24 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA), and checked for purity by thin layer chromatography and UV spectrophotometry (19). I3C and other chemicals and reagents were from sources described previously (21). The RXM was generated by the addition of I3C to 0.07 N HCl as described by Bradfield and Bjeldanes (12). Following organic extraction, the RXM was dried under vacuum and stored under nitrogen in the dark at −20°C. For high-pressure liquid chromatography analysis and animal experiments, the RXM was dissolved in dimethyl sulfoxide:ethanol (1:1, v/v) immediately before use.

Chromatography. The high-pressure liquid chromatography conditions used to isolate RXM components were similar to those reported previously (19) except that the flow rate was 4 ml/min using a preparative C_{18} column (μBondapak; 7.8-mm inside diameter x 30 cm). The RXM contained over 20 major UV-absorbing peaks but only a minor peak (<0.5%) corresponding with the parent compound. The first major UV-absorbing peak ("peak 1" in Ref. 19) was identified by electron impact mass spectrometry as I33′: *m/z* 246(100,M^+), 130(80), 117(32). Rechromatography gave a single peak with retention time (Rt) 14.48 min which coeluted with authentic I33′ used in previous studies (19, 22). Peak 2 corresponded with the previously reported (16) symmetrical cyclic trimer CT: *m/z* 387(100,M^+), 271(40), 257(60), 158(18), 146(38), 130(63), 117(18), 116(18). The compound isolated from the RXM gave a single peak upon rechromatography (retention time, 20.19 min) and coeluted with authentic CT isolated previously from RXM (16).

Animals. Shasta rainbow trout were used in all studies and were exposed during the embryo stage of development. Details of the rearing conditions, diets, and animal care have been published elsewhere (23, 24). Embryos were maintained at a temperature of 9–10°C with continuous aeration and were given injections with test compounds 25 days postfertilization. The average egg weight at the time of injection was ~100 mg. During this stage of development the embryo is visible within each egg, and test compounds can be readily administered into the yolk sac.

DNA Binding Studies. Positive controls were injected with 1 μl of a solution containing [^{3}H]AFB_1 (10 ng/egg; 0.168 μCi/egg) in test vehicle (dimethyl sulfoxide:ethanol, 1:1, v/v). Test groups were coinjected with

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[3] The abbreviations used are: I3C, indole-3-carbinol; I33′, 3,3′-diindolylmethane; CT, 5,6,11,12,17,18-hexahydrocyclononal[1,2-*b*:4,5-*b*′:7,8-*b*″]triindole; RXM, reaction mixture; AFB_1, aflatoxin B_1.

[^{3}H]AFB_1 plus 5 μg/egg I3C, 8.6 μg/egg I33′, or 13.5 μg/egg CT or RXM, *i.e.*, a final concentration of 350 μM based on an average egg volume of 0.1 ml. These levels were chosen to approximate the concentration of I3C derivatives present in the livers of trout fed an anticarcinogenic dose (2000 ppm) of parent I3C (22). One, 3, and 10 days after injection, triplicate groups of nine eggs from each treatment group were removed from the holding cups. By careful visual inspection, only embryos that were clearly viable inside the egg were used for DNA binding analysis. Nonviable embryos can be detected by an opaque appearance of the egg or by the lack of movement of the embryo within.

Each embryo was dissected from the egg and the yolk sac was removed using a scalpel. Embryos were placed in 2-ml Corning cryogenic tubes and, after addition of 0.25 ml of buffer (1% sodium dodecyl sulfate in 0.1 M NaCl-10 mM Tris-1 mM EDTA, pH 8.0), the samples were subjected to three cycles of freeze-thawing in liquid nitrogen and water. Samples were treated with RNase for 5 h at 37°C (10 units/ml RNase A plus 200 units/ml RNase T_1) prior to overnight incubation with proteinase K (Sigma protease type XI; 1.4 units/ml). After addition of an equal volume of Tris-saturated phenol and a mixing by inversion, each tube was left to stand for 15 min before centrifuging (5 min at 100 × *g*). The aqueous layer was transferred to a new tube and extracted with 3 volumes chloroform. After the chloroform layer was discarded, the extraction was repeated using chloroform:isoamyl alcohol (24:1). DNA (~300 μg/group) was recovered by transferring the aqueous layer to 2.5 volumes isopropyl alcohol and centrifuging for 5 min at 100 × *g*. Each DNA pellet was washed with ethanol and dried with nitrogen before dissolving in Tris buffer and determining the specific activity (21). The present study examined total AFB_1-DNA binding levels since previous work showed only quantitative changes in DNA adduct profiles after trout were given AFB_1 and I3C in their diets (21). Due to the small size of the embryos at the time of injection, it was not practical to isolate the livers alone and to obtain sufficient DNA for analysis of AFB_1 binding. Therefore, by design, these experiments tested the feasibility of using total embryonic DNA as a surrogate for target tissue DNA binding.

Tumor Studies. Each egg (90–100/group) was injected with 1 ng of AFB_1 alone or AFB_1 plus 350 μM I3C, I33′, CT, or RXM. After hatching, trout were fed a semipurified diet (Oregon test diet) for the remainder of the study, and necropsy examinations were performed after 1 year as described previously (23). All livers were fixed in Bouin's solution and hand-sliced to detect internal tumors and determine tumor multiplicity. At least one tumor from each tumor-bearing liver was processed by routine methods for histopathological evaluation. Classification of tumors was by criteria described by Hendricks *et al.* (25). In the final analysis, there were only a few of the grossly detected tumors that were not observed histologically (14 of 67, 8 of 50, 2 of 15, and 0 of 9 in the AFB_1, AFB_1 plus I3C, AFB_1 plus RXM, and AFB_1 plus I33′ groups, respectively). No tumor data were obtained from the AFB_1 plus CT group since most of the embryos did not survive beyond 1 month posthatching.

RESULTS

Results from the AFB_1-DNA binding studies are presented in Fig. 1. DNA binding levels generally peaked 3 days after injection of AFB_1 or AFB_1 plus inhibitor and then declined significantly by day 10 (all groups, $P < 0.05$ by analysis of variance, *versus* results from days 1 and 3). This trend was most obvious in the positive control groups (Fig. 1, □), where the results obtained on days 1, 3, and 10 were 0.85 ± 0.34 (SD), 1.16 ± 0.37, and 0.49 ± 0.02 pmol AFB_1/mg DNA, respectively. Since trout appear to lack significant excision-repair activity for removing DNA adducts (21), the lower overall DNA-binding levels observed on day 10 probably reflect dilution caused by rapid growth of the embryos immediately prior to hatching and loss of adducts by spontaneous depurination.

At all three time points, the order of inhibition by the test compounds was I3C < I33′ < CT < RXM (Fig. 1). Although I3C showed slight inhibition of mean AFB_1-DNA-binding levels, this was not statistically significant and may be due to the slow formation of I33′ when I3C is put into solution. In contrast, RXM and the isolated products I33′ and CT exhibited significant inhibitory activity 3 and 10 days postinjection (*$P < 0.05$ by analysis of variance; Fig. 1).

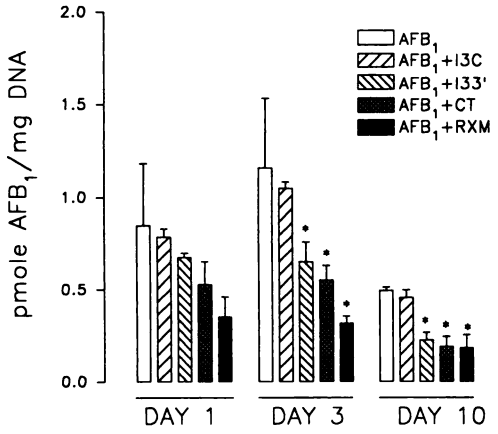

Fig. 1. Inhibition of AFB_1-DNA binding in trout embryos following microinjection of I3C acid condensation products. Trout embryos were exposed to [^{3}H]AFB_1 (10 ng; 0.168 μCi), or [^{3}H]AFB_1 plus 350 μM test compound. DNA was extracted from triplicate groups of nine embryos 1, 3, and 10 days postinjection. Data are given as means ± SD (*bars*); for each time point, *columns* with an *asterisk* are significantly different from controls by analysis of variance (*$P < 0.05$). Each group at day 10 was significantly lower than corresponding groups on days 1 and 3 ($P < 0.05$, analysis of variance). The percentage of inhibition was calculated for each test compound

$$\% \text{ of inhibition} = 1 - \frac{\text{DNA binding (test group)}}{\text{DNA binding (AFB}_1\text{ group)}} \times 100$$

Inhibition values were: 8.5% ± 1.1% for I3C, 37.0% ± 13.7% for I33′, 50.5% ± 12.0% for CT, and 64.7% ± 7.2% for RXM; means ± SD after combining results from the three time points.

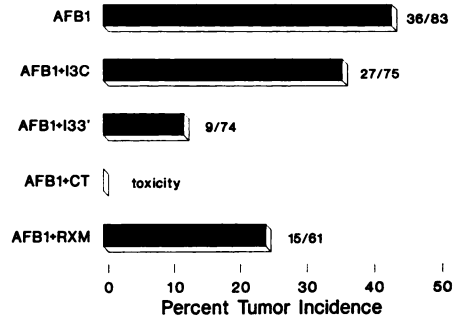

Fig. 2. Inhibition of AFB_1-induced hepatocarcinogenesis in trout by I3C acid products. Trout embryos were given injections of 1 ng AFB_1 (positive controls) or AFB_1 plus 350 μM I3C, I33′, CT, or RXM. The tumor incidence was determined in each group after 1 year; data represent the number of animals with tumors per total number animals at the time of sacrifice. Tumor multiplicity data paralleled the incidence figures, as described in the text. Histopathological examination revealed primarily mixed cell and hepatocellular carcinomas.

Fig. 2 presents results from the tumor studies. Injection of 1 ng AFB_1 into trout embryos was an effective dosing regimen for producing liver tumors, giving a final tumor incidence of 43.4% after 1 year. Coinjection of AFB_1 with I3C, I33′, or RXM gave corresponding tumor incidences of 36.0, 12.2, and 24.6%, respectively. Results from the χ^2 test showed that the AFB_1/RXM group was significantly less ($P < 0.01$) than the AFB_1-only group, while the AFB_1/I33′ group was significantly less than all other groups ($P < 0.05$), excluding the AFB_1/CT group. Tumor multiplicity results paralleled the incidence figures; AFB_1 alone produced 1.86 tumors/tumor-bearing liver, while I3C, I33′, and RXM reduced the number of tumors/liver to 1.56, 1.00, and 1.4, respectively.

Histopathology of the AFB_1-positive group revealed 66.7% of the tumors were mixed-cell hepatocholangiocellular carcinomas, 27.8% were hepatocellular carcinomas, and 2.8% were each of cholangiomas and cholangiocellular carcinomas. These relative percentages of tumor type were consistent in the I3C, I33′, and RXM inhibitor groups.

Since the tumors were selected for histology randomly and only a few were not observed, we would not expect the percentages to change if all the tumors had been classified.

DISCUSSION

We have taken advantage of certain features of the trout embryo exposure model to study the inhibitory activity of I3C acid condensation products *in vivo*. The small size of the trout embryos was important in the present investigation because test chemicals were available in limited supply. For each inhibitor treatment, <3 mg of test compound were required for the ~100 animals used in the DNA binding and tumor studies. To our knowledge, this is the most sensitive tumor bioassay described for use in anticarcinogenesis studies. A second advantage of embryo exposure is that test compounds can be absorbed slowly and directly from the yolk sac without direct exposure to acid conditions in the stomach. Advantages and limitations of this model have been discussed by Bailey *et al.* (26).

Previous studies have shown that I3C and I33′ inhibit tumor formation in the forestomach and mammary gland of rats given benzo(*a*)pyrene and dimethylbenzanthracene (7). The present studies extend the observations of Wattenberg and Loub (7) by demonstrating that I33′, CT, and RXM inhibit AFB_1-DNA binding in trout embryos, while I3C was essentially ineffective. The results from the DNA-binding experiments are in general agreement with data obtained from the tumor studies, in which I3C failed to exhibit significant inhibitory activity, while I33′ and RXM were effective blocking agents. This would support the use of total embryonic DNA binding as a surrogate for AFB_1-DNA binding in trout liver, but further studies are required to determine the limits of such an approach with AFB_1 and with other carcinogens and target organs.

In the tumor studies, treatment with AFB_1 plus 350 μM CT proved to be toxic to embryos, and there were essentially no survivors after 1-month posthatch. The basis for this toxicity has yet to be investigated but may be important in the previously observed toxicity to trout given prolonged dietary doses ≥2000 ppm I3C (21). It is possible that the total amount (μg) of inhibitor may be a factor, since embryo microinjections were given on an equivalent molar basis; thus, embryos given CT would receive approximately 3 times the weight of test compound compared with embryos given I3C.

The results reported here indicate clearly that acid products, not the parent compound, represent the anticarcinogenic species in trout and imply that their formation at low pH in the stomach is an essential component of I3C anticarcinogenesis by dietary exposure protocols. I33′ has been detected as one of the major species in the livers of fingerling trout fed I3C in their diet (22). Based on the results from the present study, I33′ may be assumed to contribute in an important way to the anticarcinogenic activity of dietary I3C, administered either prior to or during carcinogen exposure (11). It remains to be determined whether I33′ also contributes to the promotional or enhancing activity that has been reported for I3C using certain exposure protocols in trout (27, 28) and rats (29).

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