Suppression of Altered Hepatic Foci Development by Curcumin in Wistar Rats

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Abstract: Curcumin, a yellow pigment of turmeric (Curcuma longa), is a commonly used spice and a coloring agent in foods, drugs, and cosmetics. Curcumin is known to possess chemopreventive properties in various animal tumor models. In the present study the effect of curcumin on the development of altered hepatic foci (AHF), by using a medium term liver bioassay, has been evaluated. AHF were analyzed by quantitative stereology using the Leica Qwin Image Analysis system from frozen liver sections stained for γ-glutamyl transferase, adenosine triphosphatase, glucose-6-phosphatase, alkaline phosphatase, and placental isozyme of glutathione S-transferase. A significant protection on diethylnitrosamine (DEN) initiated and 2-acetylaminofluorene (AAF) promoted AHF by curcumin was observed on these biological markers. The curcumin administration was found to restore the normal levels of the enzymes glutathione S-transferase and γ-glutamyl transferase in rat liver following DEN-AAF exposure. Similarly, a significant protection was provided by curcumin in the enzyme-deficient foci for the adenosine triphosphatase-, alkaline phosphatase-, and glucose-6-phosphatase-treated groups in comparison to the DEN-AAF-treated group. These results show that curcumin can effectively suppress the DEN-induced development of AHF in rat liver.

Introduction

Many human cancers that are widely prevalent today can be prevented through modifications in lifestyles of which diet appears to be an important agent (1). Several dietary constituents are known to modulate the process of carcinogenesis and prevent genotoxicity (2,3). Curcumin (diferuloylmethane), a major yellow pigment of turmeric obtained from powdered rhizomes of the plant Curcuma longa, is commonly used as a spice and coloring agent in foods, drugs, and cosmetics (4). Curcumin is well known for its pharmacological properties including antioxidant, antidiabetic, and anti-inflammatory (4–6). Curcumin blocks tumor initiation induced by benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene (7) and suppresses the phorbol ester-induced tumor promotion (8,9). In vivo curcumin suppresses carcinogenesis of skin (10,11) and forestomach (12,13) and duodenal (12) and colon carcinogenesis (14–16) in mice. The protective role of aqueous turmeric extract against direct acting carcinogens as well as benzo(a)pyrene-induced genotoxicity and carcinogenicity have also been documented. (17). Curcumin possesses antimutagenic potential in Salmonella typhimurium (18) and in Drosophila (19). Furthermore, it has also been found to protect cisplatin-induced clastogenesis by acting as free radical scavenger (20). Therefore, in the present study the role of curcumin on the development of altered hepatic foci (AHF) in rat liver bioassay has been studied to evaluate its antihepatocarcinogenic potential. To achieve this, medium-term liver bioassay developed for the rapid detection of carcinogenic/anticarcinogenic agents has been employed by using quantitative induction of placental isozyme of glutathione S-transferase (GST-P) and γ-glutamyl transpeptidase (GGT) foci and decrease in adenosine triphosphatase (ATPase), alkaline phosphatase (AlkPase), and glucose-6-phosphatase (G6Pase) foci in rat liver (21–23). Quantitation of these biological markers of cellular proliferation is the reliable marker for AHF and has been verified for the detection of carcinogenic/anticarcinogenic agents (24–26).

Materials and Methods

Chemicals

Curcumin (95.12% commercial grade) was obtained from Kancor Flavors and Extracts Ltd., India. Diethylnitrosamine (DEN), 2-acetylaminofluorene (AAF), γ-glutamyl-4-methoxy-β-naphthalamide, glycyglycine, Fast Blue BB salt, adenosine triphosphate, sodium-β-glycerophosphate, sodium diethylbarbiturate, glucose-6-phosphate monosodium salt, 3,3′-diaminobenzidine tetrahydrochloride were purchased from Sigma Chemical Company (USA). Anti-GST-P and antigoat IgG antibodies were procured from Biotrin (Ireland). The rest of the chemicals used in the study were of an analytical grade of purity and procured locally.

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A total of 50 male Wistar rats, 6 wk of age at the start of the study, were procured from ITRC animal colony and were fed a synthetic pellet basal diet and tap water ad lib. The animals were housed in stainless steel cages in an air-conditioned room at 25 ± 2°C and 55 ± 5% relative humidity with a 12/12-h light and dark period. The animals were randomly selected and divided into four groups each containing 12 animals.

The treatment schedule is shown in Fig. 1. Group I animals received the normal basal diet during the entire study period and served as negative controls. Group II was given DEN (200 ppm) as a sole source of drinking water for 5 days to initiate the process of hepatocarcinogenesis. After 15 days of recovery period, rats were given 2-acetylaminofluorene 0.05% in crushed diet for a period of 2 wk. Animals of Group III were given DEN and 2-acetylaminofluorene, as in Group II, and after 5 days of initiation received curcumin (200 mg/kg of body weight) by oral intubation for 5 days. Group IV animals served as controls in which only curcumin was given.

All surviving animals were killed at 8 wk after 12 h of fasting. Immediately after killing liver were excised out, cleaned, and weighed. Liver slices of approximately 3 mm thickness from right posterior, caudate, and right anterior were taken for immunohistochemical and cytochemical localization of biological markers, namely, GGT, GST-P, ATPase, AlkPase, and G6Pase.

**Immunohistochemical Localization of GST-P in Rat Liver**

For the demonstration of GST-P activity (22), 10-µ-thick fresh frozen tissue sections were fixed in 4% formal saline and the exogenous peroxidase activity was quenched by treating with methanol/hydrogen peroxide solution (100 ml/333 µl) for 1 h in the dark. The nonspecific binding was blocked with normal goat serum for 2 h. The sections were then incubated with anti-GST-P antibody (1:300 diluted) for 72 h at 4°C in a humid chamber. After two phosphate-buffered saline rinses, the sections were again incubated for 2 h with normal goat serum and then with horseradish peroxidase-conjugated antigoat IgG (1:20) for 6 h at 4°C. The color was developed in chromogen 3,3′-diaminobenzidine tetrahydrochloride for 30 min in darkness. The slides were dehydrated in ascending grades of alcohol and mounted in DPX.

**Cytochemical Localization of GGT in Rat Liver**

The activity of GGT in rat liver was demonstrated by the method of Rutenberg et al. (27). The 10-µ-thick cryostat sections were placed in chilled acetone for 1 h. The sections were incubated in a medium containing 5 mg of γ-glutamyl-4-methoxy-β-naphthylamide dissolved in 0.1 ml of dimethyl sulfoxide, 17.5 mg of glycylglycine, and 17.5 mg of Fast Blue BB salt in a 10-ml solution of 0.1 M Tris buffer (pH 7.4). After incubation, the slides were transferred in 1% copper sulfate solution, washed with distilled water, and counterstained with hematoxylin, air-dried, and mounted in glycerol gelatin.

**Cytochemical Localization of ATPase in Rat Liver**

For the cytochemical staining of ATPase as per protocol of Wiechestein and Meisel (28), sections were fixed in 0.5% glutaraldehyde and incubated in the medium containing 0.2 M Tris maleate buffer (pH 7.2), 2% lead nitrate, 0.1 M magnesium chloride, and 125 mg of adenosine triphosphate for 60 min at room temperature. After incubation, sections were washed in distilled water and developed in 7.5% ammonium sulfate solution for 60 s, followed by fixing in 5% glutaraldehyde and acetic acid solution for 5 min. The sections were again rinsed in distilled water, dehydrated in ascending grades of alcohol, cleared in xylene, and mounted in DPX. Brownish black deposits of lead sulfide mark the ATPase activity.

**Cytochemical Localization of AlkPase in Rat Liver**

For the localization of this enzyme activity, the 10-µ-thick fresh frozen sections were incubated according to the method of Gomori (29) in a freshly prepared medium containing 3% sodium β-glycerophosphate, 2% sodium diethyl barbiturate, 2% calcium chloride, and 5% magnesium sulfate for 45 min at 37°C in a humid chamber. After incubation, sections were washed in distilled water and developed in 5% ammonium sulfide solution for 60 s, followed by fixation in 5% glutaraldehyde and acetic acid solution for 5 min. The sections were air-dried and mounted in glycerol gelatin. The black deposits of cobalt sulfide indicated the site of activity.

**Cytochemical Localization of G6Pase in Rat Liver**

The staining of G6Pase was performed by the method of Wiechestein and Meisel (28). Briefly, slides of cryostat sec-

![Figure 1. Treatment schedule for the assessment of altered hepatic foci (AHF) suppressing potential of curcumin in Wistar rats.](image)

- **Group I**: Normal basal diet
- **Group II**: DEN (200 ppm) in drinking water for 5 days
- **Group III**: DEN and 2-acetylaminofluorene, followed by curcumin (200 mg/kg of body weight) orally for 5 days
- **Group IV**: Curcumin only

Groups II and III were further subjected to 2-acetylaminofluorene (2-AAF) for 2 weeks.
tions were fixed in 0.5% glutaraldehyde and incubated in a mixture containing 0.2 M Tris maleate buffer (pH 6.5), 2% lead nitrate solution, and 7.5 mg of glucose-6-phosphate for 30 min at 37°C followed by incubation in 7.5% yellow ammonium sulfide for 60 s. Sections were then placed in 5% glutaraldehyde and acetic acid solution for 5 min, rinsed with distilled water, dehydrated in ascending grades of alcohol, cleared in xylene, and mounted in DPX. Brownish black deposits of lead sulfide indicate the site of G6Pase activity.

Quantitative Stereology

The AHF were quantified with a Leica Image Analysis system using Leica software Qwin 500. The negative and positive AHF were captured with a microscope attached to a CCD camera (JVC) and the images were transferred to computer. The image analysis was performed for each slide in triplicate with at least six fields in each slide. The foci were scored only if their diameter was >0.20 mm. Statistical determinations were made by using analysis of variance and $P < 0.05$ was considered significant.

Results

No significant change was observed in average body weights following curcumin administration. However, significant weight loss over control was observed in the DEN-AAF-treated group (Table 1). Similarly, absolute and relative liver weight was found to decrease in the case of Group II in comparison to the rest of the groups (Groups I, II, and IV; Table 1). No marked differences were observed in water or diet consumption between any of the groups.

Quantitation of cytochemically stained slides for ATPase, AlkPase, and G6Pase showed about 0.73 ± 0.1, 0.64 ± 0.11, and 0.53 ± 0.06 area (mm$^2$/cm$^2$) occupied in control rats. The treatment of DEN-AAF inhibited these levels to 0.25 ± 0.08, 0.26 ± 0.13, and 0.35 ± 0.08. Curcumin administration along with DEN and AAF restores the activity of ATPase, AlkPase, and G6Pase enzymes to 0.69 ± 0.09, 0.59 ± 0.12, and 0.44 ± 0.09 mm$^2$/cm$^2$, respectively, in comparison to Group III (Figs. 2 and 3).

Similarly, analyses of the area positive for GGT and GST-P foci revealed significant induction in their activity following DEN-AAF treatment (Group II) compared with

Table 1. Effect of Curcumin on Liver and Body Weights of Rats$^{a,b}$

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Body Weight (g)</th>
<th>Liver Weight (g)</th>
<th>Relative Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Untreated</td>
<td>233 ± 11</td>
<td>7.75 ± 0.52</td>
<td>3.33 ± 0.15</td>
</tr>
<tr>
<td>II</td>
<td>DEN + 2AAF</td>
<td>206 ± 8*</td>
<td>6.13 ± 0.65*</td>
<td>2.98 ± 0.17</td>
</tr>
<tr>
<td>III</td>
<td>DEN + CUR + 2AAF</td>
<td>225 ± 10#</td>
<td>7.26 ± 0.47#</td>
<td>3.23 ± 0.5</td>
</tr>
<tr>
<td>IV</td>
<td>CUR</td>
<td>228 ± 11</td>
<td>7.53 ± 0.5</td>
<td>3.3 ± 0.14</td>
</tr>
</tbody>
</table>

$a$: All data are mean ± SE of 10 animals. DEN, diethylnitrosamine; 2AAF, 2-acetylaminofluorene; CUR, curcumin.

$b$: Statistical significance is as follows: *, significantly different from Group I; #, significantly different from Group III.

Figure 2. Effect of curcumin on altered hepatic foci in Wistar rats. ATPase, adenosine triphosphatase; AlkPase, alkaline phosphatase; G6Pase, glucose-6-phosphatase; GGT, $\gamma$-glutamyl transpeptidase; GST-P, glutathione S-transferase.
Figure 3. Effect of curcumin on histochemical staining of marker enzymes for altered hepatic foci (b, d, h, and j) over positive controls (a, c, g, and i). ATPase, adenosine triphosphatase; AlkPase, alkaline phosphatase; G6Pase, glucose-6-phosphatase.
the untreated group (Group I; Fig. 2). Curcumin when given along with DEN-AAF brought back the area occupied by GGT and GST-P, respectively, from 0.69 ± 0.09 and 0.32 ± 0.05 to 0.49 ± 0.09 and 0.205 ± 0.03 mm²/cm², which were levels close to the control levels, i.e., 0.36 ± 0.06 and 0.2 ± 0.06 (Figs. 2 and 3). However, no significant difference in the area positive for GGT and GST-P foci was observed in Group IV, where animals received curcumin only, suggesting a non-toxic nature of curcumin at the tested dose levels.

**Discussion**

Recently, considerable attention has been paid to the modulation of carcinogenesis by dietary constituents (2). Curcumin, a major component of turmeric and a seasoning commonly used in Indian food, is one of the most attractive compounds to be explored for chemoprevention of cancers because of its nontoxic nature and multiple beneficial clinical effects (4). Curcumin is known to inhibit a multitude of chemically induced carcinogenesis in various experimental tumor models (10,12,16). But its effect on hepatocarcinogenesis has not been thoroughly investigated. Soni et al. (30) reported a protective effect of turmeric and curcumin on aflatoxin-induced hepatic preneoplastic focus formation in rats. In another study, dietary curcumin has been shown to effectively inhibit DEN-induced hepatocellular carcinoma formation and to modulate alterations in the levels of several representative cellular markers including p21^{ras}, PCNA, and CdC2 in mouse (31). The present study further demonstrates the antipreneoplastic potential of curcumin on DEN-induced formation of AHF in rat liver. A significant de-
crease in the area positive for GST-P and GGT foci was observed with curcumin compared with the DEN- and 2AAF-treated group (Figs. 2 and 3). Similarly, curcumin administration significantly potentiates the development of ATPase-, AlkPase-, and G6Pase-positive foci compared to the group treated with DEN and AAF. Earlier, curcumin with cisplatin administration was also shown to modulate certain tumor marker indices, namely, aminotranferase, GGT, and AlkPase in experimental fibrosarcoma (32).

The suppressing effect of curcumin on the development of AHF may result either from its ability to scavenge free radicals thereby acting as a potent antioxidant (33,34), or by inhibiting genetic damage through the modulation of phase I and phase II enzymes (7,35). The antimutagenic potential of curcumin may also account for its protective effect on AHF formation. Curcumin is known to be antimutagenic in micronucleus assay, Salmonella typhimurium assay, and in recombinant mutation assay in Drosophila (18,19). Recently, previous work from our laboratory has also shown the antimutagenic potential of curcumin on chromosomal aberrations in Wistar rats at the same dose level as tested in the present investigation (36). The anticarcinogenic and antimutagenic potential of curcumin has been reported at comparatively high doses, i.e., up to 0.5% in diet or 500 mg/kg of body weight administered orally (37,38). This may be attributed to its avid metabolism and poor absorption. The systemic bioavailability of curcumin is reported to be low and its pharmacological activities may be mediated in part by its metabolites (38). Hydroxylated derivatives of curcumin are also known to be antimutagenic and its p-hydroxy group has been shown to be essential for its chemopreventive effect (33,39). Therefore, the inhibitory effect of curcumin on the formation of AHF is related to its antioxidant and antimutagenic activities. The present study thus demonstrates the protective role of curcumin on the development of DEN-induced AHF in rat medium-term liver bioassay, suggesting the antihepatocarcinogenic potential of curcumin.

Acknowledgments and Notes

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