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SHORT COMMUNICATION

Curcumin-containing diet inhibits diethylnitrosamine-induced murine hepatocarcinogenesis

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Curcumin has been widely used as a spice and coloring agent in foods. Recently, curcumin was found to possess chemopreventive effects against skin cancer, forestomach cancer, colon cancer and oral cancer in mice. Clinical trials of curcumin for prevention of human cancers are currently ongoing. In this study, we examine the chemopreventive effect of curcumin on murine hepatocarcinogenesis. C3H/HeN mice were injected i.p. with *N*-diethylnitrosamine (DEN) at the age of 5 weeks. The curcumin group started eating 0.2% curcumin-containing diet 4 days before DEN injection until death. The mice were then serially killed at the scheduled times to examine the development of hepatocellular carcinoma (HCC) and changes in intermediate biological markers. At the age of 42 weeks, the curcumin group, as compared with the control group (DEN alone), had an 81% reduction in multiplicity (0.5 versus 2.57) and a 62% reduction in incidence (38 versus 100%) of development of HCC. A series of intermediate biological markers were examined by western blot. While hepatic tissues obtained from the DEN-treated mice showed a remarkable increase in the levels of p21^{ras}, PCNA and CDC2 proteins, eating a curcumin-containing diet reversed the levels to normal values. These results indicate that curcumin effectively inhibits DEN-induced hepatocarcinogenesis in the mouse. The underlying mechanisms of the phenomenon and the feasibility of using curcumin in the chemoprevention of human HCC should be further explored.

Curcumin (diferuloyl methane) is a plant phenolic compound present in large quantities in the root of the plant *Curcuma longa.* It has been widely used as a spice and coloring agent in food. Recently, curcumin has been considered a potentially important chemopreventive agent against cancer (1). Animal studies have demonstrated that curcumin inhibits carcinogenesis in various tissues, including skin (2,3), colorectal (4,5), oral (6), forestomach (5,7) and mammary cancers (8,9). The inhibition of tumor formation by curcumin has been attributed to its anti-initiation (5,10) and anti-promotion (3,5) effects. The anti-initiation effect may result from its ability to inhibit the formation of DNA damage (10–13), while the anti-promotion effect may be mediated through anti-proliferation or apoptosis-promotion of the initiated cells (14–17). Curcumin has been repeatedly shown to inhibit the formation of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin cancer in mice. Since the strong tumor promotion effect of TPA is mediated through protein kinase C (PKC) activation, we and others have addressed this question and demonstrated that curcumin suppresses several pivotal steps in the TPA-activated PKC signal transduction pathway (18,19). In this regard, curcumin has been demonstrated to decrease the levels of c-Jun transcript (20) and the binding of c-Jun/AP-1 protein to the TPA-responsive element (21–23). Recently we completed a phase I clinical trial of curcumin. No treatment-related toxicity was observed up to an oral dose of 8000 mg/day for 3 months (data not shown). In addition, curcumin has many pharmacological properties of interest, including anti-inflammation, anti-thrombosis, hypoglycemic and hypocholesterolemic effects. The non-toxic nature of curcumin, as well as its multiple beneficial clinical effects, has made it one of the most attractive compounds to be explored for chemoprevention of cancers. In this communication we report the protective effect of curcumin on *N*-diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC) formation in the mouse.

Male C3H/HeN mice, one of the most hepatocarcinogenesis-susceptible inbred strains, were used in this study. They were purchased from the National Cheng-Kung University (Tainan, Taiwan) and the National Science Council of Taiwan and maintained in the Laboratory Animal Center of the National Taiwan University College of Medicine and fed with the assigned diets and water *ad libitum*. Tumor initiation was achieved by a single i.p. injection of DEN (20 μg/g body wt) at the age of 5 weeks. Curcumin (purity 99.3%) was manufactured by a chemical method (Yung-Shin Pharmaceutical Co. Taichung, Taiwan). Mice in the curcumin groups were fed basal diet (Purina rodent diet 5001) containing 0.2% curcumin starting 4 days before DEN injection until death. This curcumin dose was chosen based on: (i) most published animal studies having used between 0.05 and 2% dietary curcumin; (ii) our phase I clinical trial indicating that no adverse effects were found up to an oral dose of 8000 mg/day (data not shown). A 0.2% curcumin diet is equivalent or close to this dose. At death, the body weights and liver weights were measured and hepatic tumors on the surface were enumerated. Parts of the fresh liver tissues were preserved at –135°C for later biochemical and histochemical staining and western blot analysis. All mice were killed by decapitation.

Abbreviations: DEN, *N*-diethylnitrosamine; HCC, hepatocellular carcinoma; PCNA, proliferating cell nuclear antigen; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

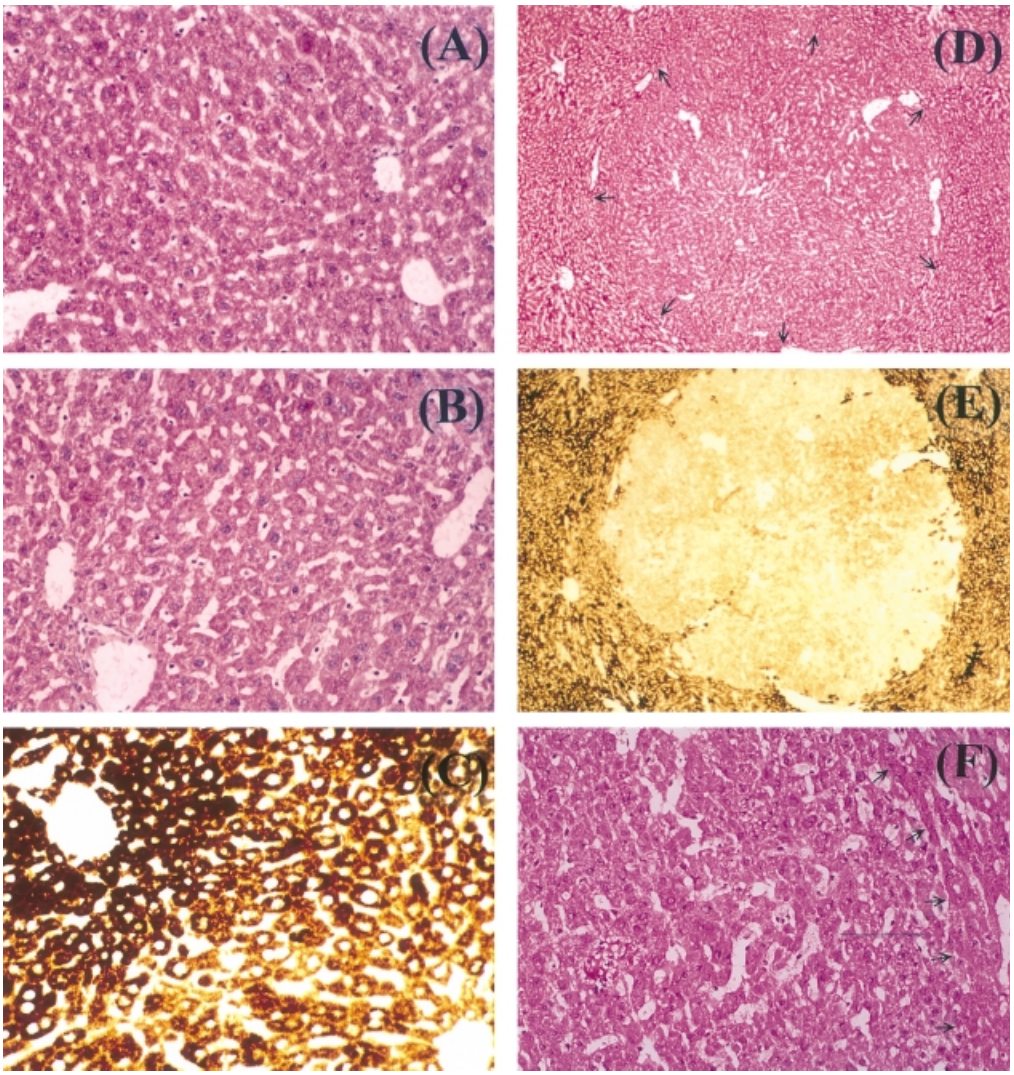

Fig. 1. DEN-induced hepatocarcinogenesis. Cryostat sections of tissues of the DEN-treated mice were examined by HE staining (**A**, **B**, **D** and **F**) and glucose 6-phosphatase biochemical staining (**C** and **E**). (A and B) Livers at 20 and 26 weeks of ages, respectively; (D, E and F) at 42 weeks of age. (C) Positive staining for glucose 6-phosphatase activity of a normal liver. (D and E) Two consecutive sections of the same nodule, at 40× magnification; (A–C and F) at 200× magnification. Note that while the tumor tissues are very weak in glucose 6-phosphatase activity, the surrounding normal tissues were strongly stained (E). The arrows in (D) and (F) indicate the border between normal liver and the HCC tissue. Most animals from each group were examined histochemically and each section shown was representative of each group. According to histology data, all the tumors examined were found to be HCC.

Table I. Inhibition of DEN-induced hepatoma formation by curcumin

Group	*n*	DEN/curcumin	Body wt (g)	Liver wt (g)	Liver wt (%)	Multiplicity[a]	Incidence[b] (%)
A	3	–/–	28.82 ± 4.45	1.33 ± 0.25	4.76 ± 0.16	0	0
B	7	+/–	31.73 ± 3.90	1.87 ± 0.34	5.84 ± 0.62	2.57 ± 2.07	100
C	3	–/+	27.72 ± 2.08	1.24 ± 0.10	4.74 ± 0.37	0	0
D	8	+/+	30.47 ± 3.70	1.59 ± 0.21	5.23 ± 0.45	0.50 ± 0.76[c]	38[d]

Mice were killed and examined at the age of 42 weeks. Numbers are expressed as averages ± SEM.
[a]Average HCC numbers/mouse; tumor size (diameter) = 0.5–1.5 mm.
[b]Percent mice with tumors.
[c]Significantly different from group B, $P = 0.01$.
[d]Significantly different from group B, $P = 0.005$.

Enzyme biochemical staining of cellular glucose 6-phosphatase was performed as previously described (24). Briefly, slides of cryostat sections were incubated in a buffer containing 0.05% glucose 6-phosphate and 0.18% lead nitrate, pH 6.7, for 5–10 min at 37°C. The slides were then rinsed with distilled water and placed in 0.5% yellow ammonium sulfide for 2 min, rinsed again with distilled water and finally mounted. Cells with enzyme activity stained black (color of the precipitated lead sulfide). Methods for analysis of cellular proteins by western blot were as described previously (25). Briefly, tissues were homogenized in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1% NP-40, 1 mM Na_3VO_4, 1 mM

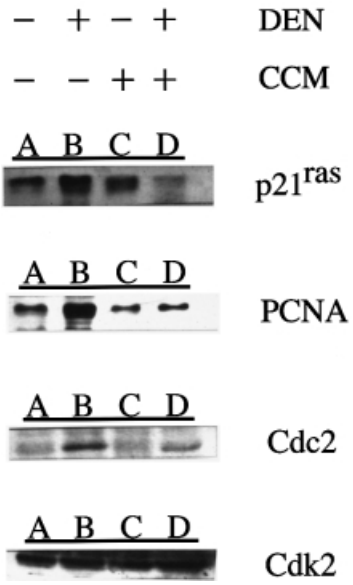

Fig. 2. Western blot analysis of $p21^{ras}$, PCNA, CDC2 and CDK2. Snap frozen liver tissues, at the age of 34 weeks, were homogenized and total protein extracts were prepared as described. The four proteins were analyzed with respective specific monoclonal antibodies. A, B, C and D are as in Table I. Several animals from each group were examined and all samples gave similar results. These blots are representative of each group.

phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and leupeptin, pH 7.4). The homogenates were centrifuged at 4°C to remove debris. Lysate equivalent to 50 μg total protein was subjected to 12% SDS–PAGE, transferred onto nitrocellulose paper and probed with anti-$p21^{ras}$, anti-proliferating cell nuclear antigen (PCNA), anti-CDC2 and anti-CDK2 antibodies (Santa Cruz Biotechnology). Detection was performed with the ECL chemiluminescence kit (Amersham).

Upon killing at or before the age of 26 weeks (Figure 1A and B), no hepatic lesions were observed in any of the four groups. All the DEN-induced hepatic nodules observed at the age of 42 weeks fulfilled the histological criteria of HCC (Figure 1D–F). These tumors were readily observed by glucose 6-phosphatase biochemical staining. While the normal tissues of the liver were strongly stained, the HCC tissues were stained very weakly (Figure 1E). Decreased activity of this enzyme is one of the markers for both preneoplastic and carcinoma lesions. The HCC nodules were randomly distributed in the livers of both the DEN and DEN/curcumin groups. No indication of preferential protection for different areas of the liver was observed.

The effects of curcumin on body weight, liver weight, liver tumor multiplicity and liver tumor incidence are shown in Table I. At the age of 42 weeks, the most prominent effect of curcumin was inhibition of HCC multiplicity and incidence by 81% (2.57 versus 0.5, $P = 0.01$) and 62% (100 versus 38%, $P = 0.005$), respectively. The body weights were not affected by curcumin (compare group A with C and group B with D).

When proteins of the liver tissues were examined at the age of 34 weeks by western blot analysis (Figure 2), DEN groups showed a remarkable increase in the levels of $p21^{ras}$, PCNA and CDC2 (compare B with A). Curcumin effectively reversed the levels of all of these three proteins back to normal (lane D). Curcumin alone did not possess significant effects on these proteins (lane C). The CDK2 levels remained unchanged in all mice.

HCC is one of the major malignant tumors in humans and causes more than 250 000 deaths annually world wide. It is especially prevalent in sub-Saharan areas of Africa and in Southeastern Asia, where HCC is closely associated with aflatoxin exposure and chronic viral hepatitis. Since the prognosis of HCC is extremely poor, effective measures of chemoprevention represent an important hope for people living in these areas. While curcumin has been shown to prevent tumors of many organs, its effects on hepatocarcinogenicity have not been addressed until recently. Soni *et al*. (26) reported a protective effect of curcumin on aflatoxin-induced hepatic preneoplastic focus formation in rats, shedding light on the feasibility of using curcumin in the prevention of human hepatocarcinogenesis. In this study, we have further demonstrated that curcumin effectively inhibits DEN-induced HCC formation in the mouse. Alterations in the levels of several representative cellular markers, including $p21^{ras}$, PCNA and CDC2, indicate the beneficial biological effect of curcumin. $p21^{ras}$ is a proto-oncogene activated during carcinogenesis in various organs, especially in the lung and colon (27–29). PCNA is a biomarker of cell proliferation (30). Increased levels were observed in both preneoplastic and tumor cells. CDC2 (31) is required in normal progression through the G_2/M phase of the cell cycle. Decreased expression of these proteins indicates growth inhibition and may lead to cell cycle arrest and/or apoptosis, which in turn may attenuate the development of cancer. These curcumin-induced cellular changes were in accord with the criteria for an effective chemopreventive agent. It seems that curcumin differentially, and maybe beneficially, inhibits only those targets that showed elevated levels in HCC, such as $p21^{ras}$, PCNA and CDC2, but not those that do not show differences in tumor tissues, such as CDK2. Although CDC2 (G_2/M transition) and CDK2 (G_1/S transition) are both crucial in cell cycle progression, their role in hepatocarcinogenesis and response to curcumin intervention are apparently different. The biological significance of this observation awaits further investigation. Many other cellular targets of curcumin have been reported, for example, the inflammatory reaction (32), AP-1, PKC (18; unpublished data), ornithine decarboxylase, lipoxygenase, cyclooxygenase, free-radical scavenging activity (33), i-NOS (32), tumor necrosis factor-α and nuclear factor κB (34–36; unpublished data), the cellular detoxification system (7,37) and the apoptosis-related machinery (38,39). Among these, the anti-inflammatory activity of curcumin is particularly noteworthy. In one of our animal experiments, curcumin dramatically suppressed a severe hepatic inflammatory reaction induced by high dose DEN in the rat (unpublished data). Previous reports have suggested that curcumin inhibits both the initiation and promotion stages of tumor formation (3,5,10,40). For curcumin to act on these two stages, an ideal target may be the inflammatory reaction. During liver inflammation, the activities of phase I and phase II metabolizing enzymes are changed. For example, the aflatoxin B1- and DEN-activating P450 isozyme CYP2A6 is markedly increased (41), which may play a role in inflammation-related tumor initiation. Inflammation also activates many proliferation factors and cytokines (42,43), which are involved in tumor promotion. Therefore, the potent anti-inflammatory effect of curcumin may play a crucial role in its chemopreventive activity. If curcumin has inhibitory effects on both stages of HCC development, chemopreventive activity should be observed when it is given to mice (i) along with the DEN

treatment (during the initiation stage) or (ii) after the DEN treatment until the end of the experiment (during the post-initiation stage). This issue should be further addressed.

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