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Original article

CURCUMIN INHIBITS CARCINOGEN INDUCED C-HA-RAS AND C-FOS PROTO-ONCOGENES EXPRESSION, AND PROTEIN KINASE C ACTIVITY IN MOUSE SKIN

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Abstract We investigated the chemopreventive action of dietary curcumin in male Swiss albino mice 7 with 12-dimethylbenz(a) anthracene (DMBA)-initiated and 12, 0-tetradecanoylphorbol-13-acetate (TPA)-promoted skin tumor formation. At 6 weeks of age, the groups of animals were fed the control diet (modified AIN-76A) or one containing 0.2% or 1% curcumin. At 8 weeks of age, all animals, except those in the vehicle (acetone)-treated groups, received 100 μg of DMBA dissolved in 100 μl of acetone in a single application to the skin on their back. From 1 week after DMBA application, tumor promoter (2.5 μg of TPA dissolved in 100 μl of acetone) was applied to the same areas of the mouse skin twice a week for 26 weeks. All groups continued their respective dietary regimen until the termination of the experiment. The results showed that dietary administration at 0.2% or 1% curcumin significantly inhibited the number of tumors per mouse and the tumor volume. Curcumin in the diet did not hinder the animals. Overall results demonstrated the safety as well as the anti-carcinogenic effect of dietary curcumin in mice. Using the enhanced chemiluminescence Western blotting detection system (Amersham), we found a relative increase in the cellular oncogene of FBJ murine osteogenic sarcoma virus (c-fos) and cellular oncogene of Harvey rat sarcoma virus (c-Ha-ras) proteins in tumorous skin. This was compared with the non-tumorous skin isolated from the same mouse. Dot blot analysis of c-Ha-ras and c-fos RNA transcripts in the tumorous and non-tomorous skin was also determined. Both cellular oncogenes exhibited higher levels in tumorous rather than normal skin. The enhanced expression of ras and fos proto-oncogenes in skin tumors in DMBA and TPA-treated animals was decreased by dietary curcumin. Also, it was found that the curcumin inhibited protein kinase C (PKC) activities in mouse epidermal extracts in a dose dependent manner. **Chiang Mai Med Bull 2001;40(3):127-137.**

Curcumin is a yellow coloring matter isolated from the roots of *Curcuma longa* Linn, commonly called turmeric. It has been widely used in many Asian countries as a spice, to color cheese and butter, a cosmetic and, in some cases, medicinal preparations. Curcumin (diferuloylmethane), a phenolic compound, possesses antioxidant, free radical scavenger and anti–inflammatory properties.[1-5] Several works in epidemiology and animal model studies demonstrated that compounds, which possess antioxidant or anti-inflammatory properties, can inhibit carcinogenesis.[6-9] One of the classic models is the inhibition of 12-0-tetradecanoyl-

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phorbol-13-acetate (TPA)-induced tumor promotion in mouse skin. TPA is a strong promoter of chemically induced skin cancer. It has been shown that TPA-induced skin tumors have been inhibited by a topical application of curcumin.[10-11] Curcumin can inhibit the activity of cytochrome P450 and increase glutathione content in rat liver, which helps to explain theanticarcinogenic, antimutagenic and cytoprotective effects of curcumin.[12-13] Many reports have shown that curcumin inhibits a variety of biological TPA activities, which induces several biosynthetic processes, namely the induction of ornithine decarboxylase,[10] elevation or protein kinase C translocation[14] and the induction of cyclooxygenase and lipooxygenase.[11] Topical application of curcumin inhibits the TPA-induced c-fos, c-jun (cellular oncogene of avian sarcoma virus) and c-myc (cellular oncogene of avian myelocytomatosis virus) gene expression on mouse skin after 2 hours of TPA treatment.[15] In the present study, we investigated the modulating effect of dietary curcumin on DMBA and TPA-induced tumor formation and on the expression levels of c-Ha-ras and c-fos to provide an understanding of the molecular basis in the relationship between the dietary curcumin and transforming function of oncogenic ras and fos during multi-stage skin carcinogenesis. We also determined the effect of curcumin on TPA-induced elevation or the translocation of protein kinase C.

Materials and methods

Chemicals

DMBA and TPA were purchased from Sigma (St.Louis, MO, USA). Commercial curcumin (77% curcumin, 17% demethoxycurcumin, and 3% bisdemethoxy curcumin) was purchased from ICN Biomedical (Costa Mesa, CA, USA). Feed ingredients (casein, methionine, mineral and vitamin mixtures, cellulose and choline bitartate) were purchased from ICN Biomedical. All other chemicals were of analytical reagent grade.

Animals

Male Swiss albino mice, 5 weeks old, were purchased from the National Laboratory Animal Center, Thailand. All diet ingredients were stored at 4°C prior to the preparation of diets. The mice were quarantined for 7 days and had access to a modified AIN-76A diet. Following quarantine, all the mice were randomly distributed by weight into various groups and were housed individually under the controlled conditions of a 12-h light/12-h dark cycle at 22±2°C.

Diets and treatments

In all treatment groups, the mice were fed a modified AIN-76 diet (control diet). The control diet had the following composition (g%): 20 casein, 0.3 DL-methionine, 3.5 mineral, 1 vitamin, 5 cellulose, 5 corn oil, 32 corn starch, 33 sucrose, and 0.2 choline bitartrate. In curcumin treatment groups, the control diet was modified by the addition of 1% or 0.2% curcumin. The incorporation of curcumin into the diet was carried out with a V-blender after the curcumin had been premixed with a small quantity of diet in a food mixer ensure its uniform distribution. All feeds were pelleted to avoid stratification and to ensure uniform feed and curcumin intake in the treated animals. All control and experimental diets were prepared every 2 weeks in our laboratory and stored at –20°C before use. The mice had access to food and water at all times, and food bowls were replenished with a fresh diet three times weekly.

At 6 weeks of age, the animals were divided into six groups (20 mice/group) and fed the control diet (groups 1a and 2a) and an experimental one that contained 1% (groups 1b and 2b) or 0.2% (groups 1c and

2c) curcumin. At 8 weeks of age, three groups of animals (groups 1a, 1b and 1c) commenced carcinogen treatment, whereas, the other groups (groups 2a, 2b and 2c) were started on a vehicle (acetone solvent) chemical control. The animals were maintained on control or curcumin diets until the termination of the experiment. The backs of the mice were shaved with electric clippers 3 days before the first skin treatment and were, thereafter, shaved when neccessory for the duration of the experiment. Carcinogen or vehicle solvent was applied to the dorsal shaved area.

All of the animals in carcinogen treatment groups, which were subjected to tumor initiation and promotion, received 100 μg of 7, 12-dimethylbenz-[a]-anthracene (DMBA) dissolved in 100 μL of acetone in a single application to the skin on their back. From 1 week after DMBA application, tumor promoter (2.5 μg of 12-0-tetradecanoyllphorbol-13-acetate (TPA) dissolved in 100 μL of acetone) was applied to the same areas of the mouse skin twice a week for 26 weeks. The vehicle control groups received only 100 μL of acetone to the skin on their back. The mice were weighed at weekly intervals. Tumors that appeared on the mouse skin were counted and measured by a Pierre Vernier caliper weekly throughout week 26. The average tumor volume was calculated as (length) x (width) x (height) $\pi/6$. The data were analyzed by the Student's *t*-test and chi-square test.

Determination of c-fos and C-Ha-ras gene expression

After 26 weeks of TPA application, pooled tumors and non-tumorous mouse epidermis from each experimental group (control diet, and 1 and 0.2% curcumin diet) were collected by the protocol described by Huang *et al.*[16] The epidermis samples were stored at - 70°C they were until used for dot blot hybridization and western blot analysis. The c-fos and c-Ha-ras proteins were analyzed by enhanced chemiluminescence (ECL) Western blot analysis using specific anti-p62 fos and anti-p21 ras antibodies, respectively. The levels of c-fos and c-Ha-ras RNA were analyzed by dot-blot hybridization using a specific probe.

RNA extraction and dot blotting

Total RNA (5 μg) was extracted from frozen tissues and blotted onto membranes. Dot blotted filters were hybridized with digoxigenin (DIG) cDNA probes as described below.

Oncogene cDNA probes were obtained from the American Type Culture Collection (Rockville, MD). Preparation of plasmid DNA was performed by cesium chloride buoyant density centrifugation. The cDNA inserts (2.2 kb BamHI-EcoRI fragment of v-Ha-ras; 1.0 kb Pst I fragment of v-fos plasmid probe) were excised from pBR 322 and labelled with digoxigenin by a random-primed labelling method according to the instructions on the DIG-DNA labelling kit (Boehringer Mannheim). After digoxigenin labelling, the 2.2 kb ras and 1 kb fos were used as the specific probes for hybridization with RNA isolated from the mouse skin.

Hybridization and washing the nylon membranes (Zeta-probe blotting membranes, Bio-rad, Richmond, CA) were performed, as recommended by the supplier. After the hybridization and washing steps, the membranes were exposed to an Amersham X-ray film with intensifying screen at -70°C for 1 day. Relative intensity was determined by scanning densitometry.

Preparations of c-Ha ras and c-fos proteins

The pooled tumors and nontumorous mouse epidermis from each experimental group (control diet, and 1 and 0.2% curcumin diets) were washed with cold phosphate buffer saline and suspended in 1 ml of

buffer A (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 µg/mL aprotinin, and 10 µg/mL of leupeptin). The suspension was homogenized in a glass homogenizer with 30 strokes and centrifuged at 700 g for 5 min to remove unbroken cells and nuclei. The nuclear pellet was used for fos preparation. The supernatant was centrifuged further at 100,000 g for 30 min. The membrane pellet was resuspended in 1 ml of buffer B (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.6 mM PMSF and 1 mM sodium orthovanadate in PBS pH 7.4), rocked for 1 h, and then centrifuged at 100,000 g for 30 min. The supernatant was used as a membrane fraction for ras-p21 analysis and stored in aliquots at -80° C.

The nuclear extract was prepared by the method of Dignam *et al.*[17] The protein contents in both nuclear and membrane fractions were determined with a Bio-Rad reagent.

Electrophoresis and Western blot

SDS-PAGE and Western transfers were carried out essentially by the methods of Laemmli[18] and Towbin *et al.*[19] Nuclear and membrane extractss corresponded to 25 µg of protein, were analyzed on a 12.5% polyacrylamide mini- slab gel containing SDS with an overlay of 4% polyacrylamide. Electrophoretically resolved proteins were electrotransferred onto a Hybond ECL membrane in a Trans-blot electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA, USA). The c-fos and c-Ha-ras proteins on the blots were analyzed by the ECL Western blot detection system (Amersham, New Territories, Hong Kong) using specific anti-p62 c-fos and anti-p21 c-Ha-ras anti-bodies, respectively (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Preparation of cytosolic and particulate fractions for PKC assay

Male Swiss Albino mice, 7–8 weeks of age, were divided into two groups. Group I (10 mice): received 2.5 µg of TPA dissolved in 100 µL of acetone to the shaved skin 30 min before termination of the experiment, and group II (10 mice): did not receive TPA. The animals were sacrificed by cervical dislocation. The skin was removed from the shaved area and placed, outer side up, on a strip of aluminum foil, incubated at 60°C for 30 sec and then plunged into liquid nitrogen.[16] The epidermal layer was scraped off with a blade and the epidermis was placed in a plastic centrifuge tube. All samples were kept in an ice bath throughout all procedures.

The preparations of cytosolic and particu late fractions from the mouse epidermis were performed by the method of Liu *et al.*[14] The PKC was partially purified by a DEAE-cellulose column. in solubilized membrane and cytosolic fractions of experimental mouse epidermis.

For the PKC activity assay, partial purification of the enzyme was performed immediately at 4°C by DEAE-cellulose chromatography (1 mL of DEAE-cellulose) using the protocol described by Liu *et al.*[14] The PKC activities in both fractions were assayed under standard conditions by using Promega's Pep Tag assay kit for the non-radioactive detection of PKC (Promega, Co.Ltd, Wisconsin, USA), except when various curcumin concentrations of 0, 15, 30, 60 µM were added in the reaction mixture.

Results

Effect of dietary curcumin on the tumorigenesis of DMBA and TPA

The effect of dietary curcumin on the tumorigenesis of DMBA and TPA was evaluated using the two-stage mouse skin model. Animals receiving a single topical application of DMBA followed by a 26

week promotion of TPA, developed 7.7±1.4 papillomas/ mouse when fed the control diet (Figure 1). The number of papillomas was significantly lower in the 1% curcumin diet group than in the control group (p < 0.05). Figure 2 shows the average volume of tumors per mouse in the curcumin diet group compared to the control group. The average volume was significantly lower in the 1% or 0.2% curcumin diet group than in the control group (p<0.01). No papillomas were observed in the groups that received the control diet or the diet supplemented with 1% or 0.2% curcumin, or the vehicle with no application of DMBA and TPA. There was no significant difference in body weight changes between the control and 1% or 0.2%

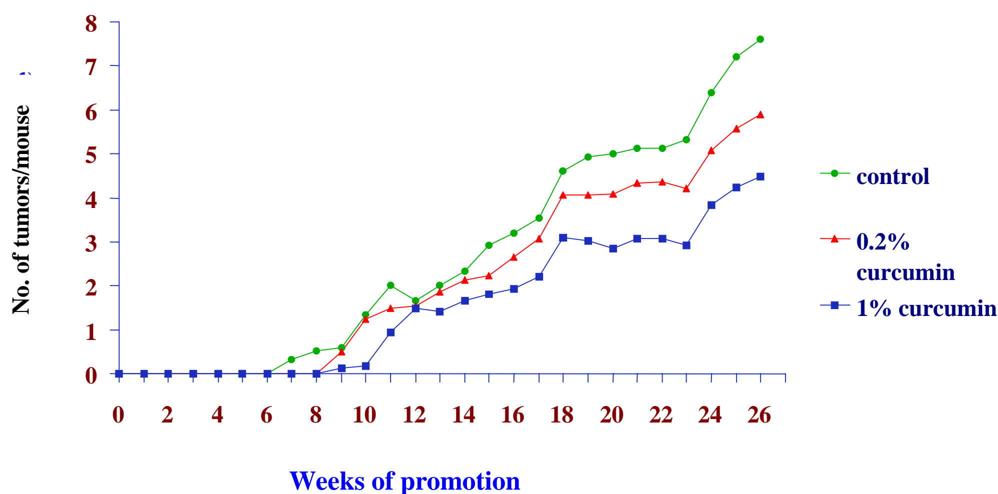

Fig. 1. Average number of tumors per mouse in Swiss mice treated with control, 0.2% and 1% curcumin diets. Each point represents the mean value (N=20 mice per group).

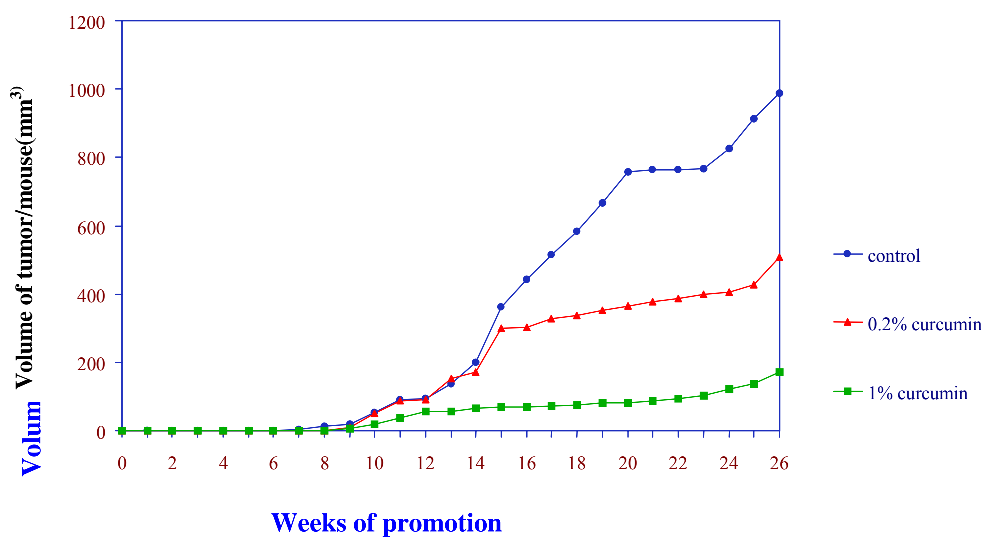

Fig. 2. Average volume of tumors per mouse in Swiss mice treated with control, 0.2% and 1% curcumin diets. Each point represents the mean value (N=20 mice per group).

curcumin treated groups, as observed by the Student's *t*-test ($p>0.05$, data not shown).

Differential expression of ras-p21 and fos-p62

Fig. 3a and 3c demonstrate the representative Western blot examples analysis of the membrane-bound ras-p21 and nuclear fos-p62 protein. The samples exhibited detectable levels of c-Ha-ras with anti ras-21 mouse monoclonal antibody and levels of c-fos anti fos-62 rabbit polyclonal antibody. Regardless of dietary regimen, very low (background) levels of ras-p21 and fos-p62 were detected in acetone treated animals (data not shown). The mice receiving a single topical application of DMBA, followed by a 26 week promotion of TPA, developed increasingly higher levels of skin ras-p21 and fos-p62 expression in all dietary groups. Skin tumors exhibited higher levels of ras-p21 and fos-p62 than the normal skin. The enhanced expression of the ras-p21 and fos-p62 in skin tumors was decreased by curcumin diets of 1% and 0.2%, as compared with ras-p21 or fos-p62 in the mouse counterparts on a control diet ($p<0.05$). Fig. 3b and 3d demonstrate the representative dot blot example analysis of c-Ha-ras (3b) and c-fos (3d) RNA transcripts in tumorous and non-tumorous skin from the mice receiving a single topical application of DMBA, followed by a 26 week promotion of TPA. Both cellular oncogenes exhibited higher levels in tumorous rather than normal skin. The enhanced expression of the c-Ha-ras and c-fos in skin tumors was decreased by curcumin diets of 1% and 0.2%, as compared with the ras or fos in the mouse counterparts on the control diet ($p<0.05$). Ethidium bromide staining demonstrated that equivalent amounts of RNA were loaded per lane (data not shown).

Table 1 summarizes the densitometric analysis of differential expression of ras-p21 and fos-p62 in the tumorous and non-tumorous skin of DMBA and TPA-treated animals that were fed the control, 1% or 0.2% curcumin diets.

Effect of curcumin on PKC activity

As shown in Fig. 4, the exposure of mice which received 2.5 µg of TPA dissolved in 100 µl of acetone to the shaved skin for 30 min, caused a redistribution of PKC activity from the cytosol to the membrane. The in vitro effect of curcumin on the partially purified PKC cytosol and membrane preparation from the TPA-treated mice (30 min) is illustrated in Fig. 5. Curcumin inhibited PKC activities in both cytosol and particulate fraction doses, dependently.

Discussion

The immediate aim of this work was to study the inhibitory action of curcumin on mouse skin tumorigenesis, and the long-range goal of investigations into human skin carcinogenesis. We hope to contribute to an understanding of the action mechanism of curcumin *in vivo* and *in vitro* at the molecular level. Results of this experiment in mice demonstrate that dietary curcumin at concentration levels of 0.2% or 1% caused a decrease in tumor incidence as well as a distinct decrease in tumor volume. There was no evidence of the deleterious effect of curcumin in the mice. Growth rates in the animals on a control diet plus curcumin was no different from those in animals on a control diet only.

One of the purposes in the present study was to investigate the modulating effect of dietary curcumin on the expression levels of ras-p21 and fos-p62 to provide an understanding of the molecular basis in the relationship between the dietary curcumin and the transforming function of oncogenic ras and fos during multi-stage skin carcinogenesis. Results of this experiment demonstrate that 0.2% or 1% dietary curcumin inhibits the DMBA- and TPA-

induced expression of ras and fos oncogenes and suppresses skin tumor development.

Previously, data from our laboratory demonstrated that animals fed high amounts

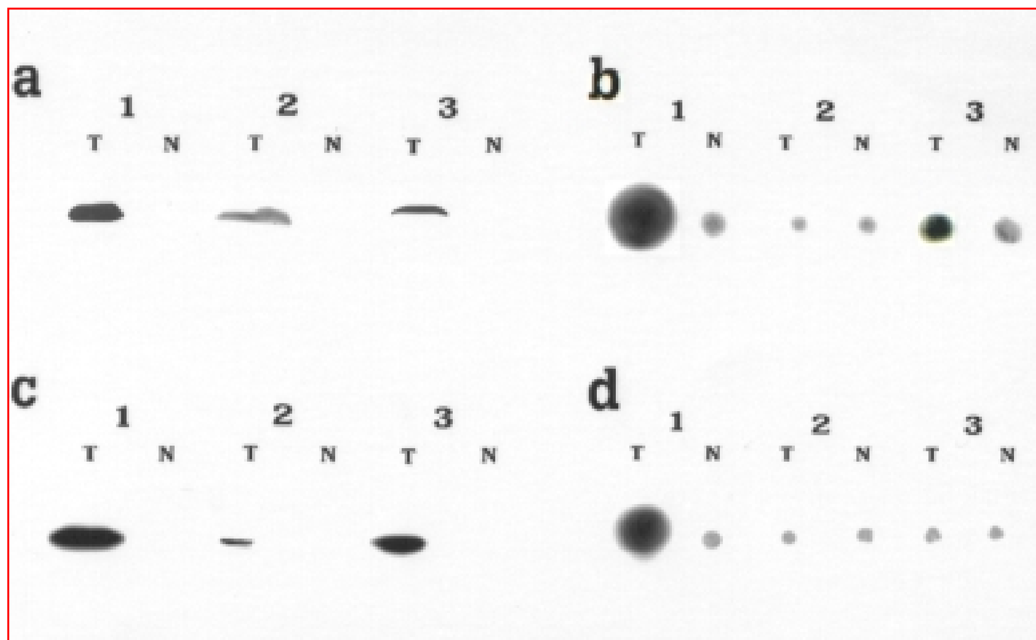

Fig. 3. Analysis of ras p-21 (a,b) and fos p-62 (c,d) gene expression.
a; Western blot analysis of c-Ha-ras using anti ras-21 mouse monoclonal antibody, b; Dot blot hybridization of c-Ha-ras RNA using specific c-Ha-ras DNA probe (EcoRI and BamHI digests of ras-pBR322, 2.2 kb), c; Western blot analysis of c-fos using anti fos-62 rabbit polyclonal antibody, d; Dot blot hybridization of c-fos RNA using specific c-fos DNA probe (PstI digest of fos-pBR322, 1.0 kb), Lane 1; Control diet, Lane 2; 1% Curcumin diet, Lane 3; 0.2% Curcumin diet, T;Tumorous skin, N; Non-tumorous skin

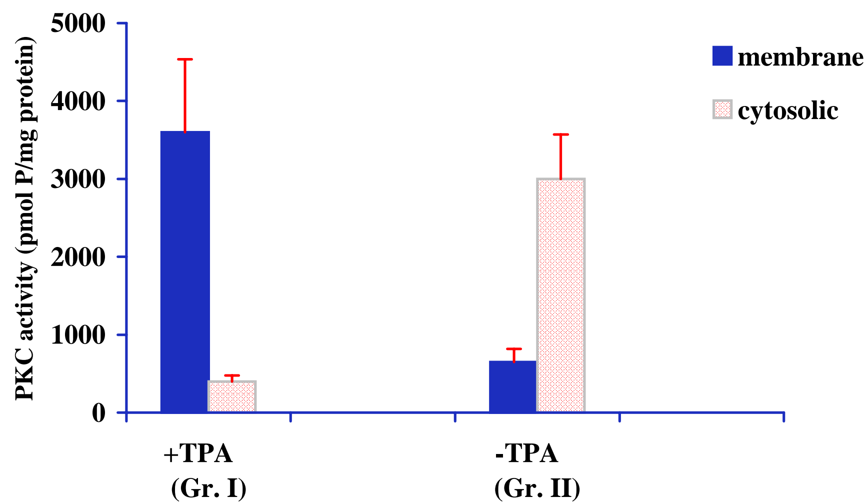

Fig. 4. Effect of TPA on the distribution of PKC activity in mouse skin. Each point represents the mean ±SE (Number of PKC assays = 3).

Table 1. Effect of dietary curcumin on expression levels of c-Ha-ras (p21) and c-fos (p62) in the skin of DMBA and TPA treated animals (N = 20).

Condition	Ras (Arbitary unit)				Fos (Arbitary unit)			
	RNA		Protein		RNA		Protein	
	T	N	T	N	T	N	T	N
Control	9.4±1.5[a] (100%)[b]	3.2±1.2 (34.2%)	8.74±1.7 (100%)	0.17±0.1 (1.94%)	2.0±0.4 (100%)	1.36±0.4 (68.3%)	5.8±0.8 (100%)	0.8±0.5 (13.8%)
1%Curcumin	0.47±0.2 (4.99%)	0.14±0.1 (1.5%)	2.73±1.1 (31.2%)	0.07±0.05 (0.8%)	0.06±0.04 (3.02%)	0.06±0.05 (3.02%)	2.0±0.6 (34.5%)	0 (0%)
0.2%Curcumin	0.62±0.2 (6.6%)	0.20±0.1 (2.12%)	5.30±2.2 (60.6%)	0.15±0.05 (1.72%)	0.48±0.2 (24.1%)	0.11±0.07 (5.53%)	3.6±0.9 (62.1%)	0.9±0.15 (15.5%)

T; Tumorous skin, N; Non-tumorous skin, [a]; Results are expressed as arbitary unit; values are mean ± SD. [b];Values in parentheses relatively represent percentage of ras or fos gene expression. The levels of ras and fos RNAs and proteins in the control diet are represented as 100%.

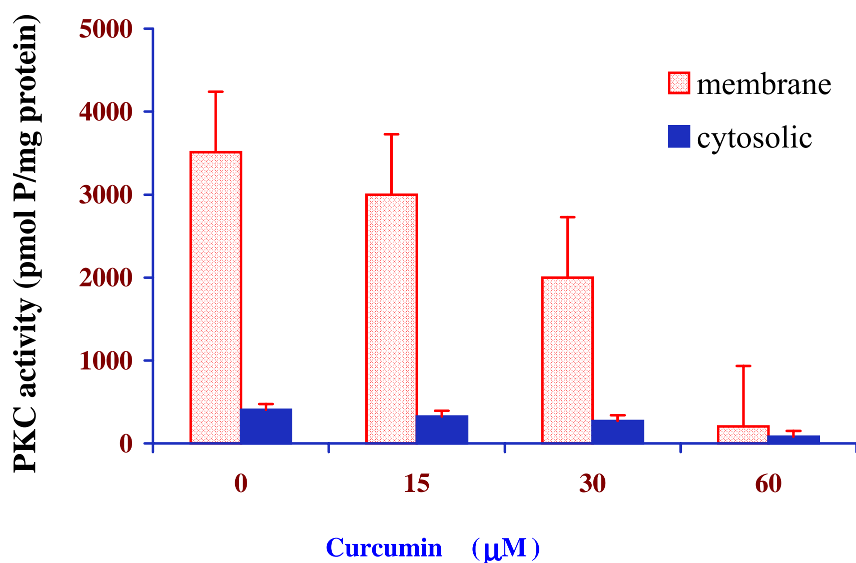

Fig. 5. Effect of curcumin on TPA induced PKC activity. Each point represents the mean ± SE (Number of PKC assays = 3)

of dietary curcumin (1%) had a significantly lower multiplicity of TPA-induced skin papillomas compared with their counterparts ingesting a control diet.[20] Therefore, in the present study, we demonstrated the inhibition of tumor development at a lower concentration of curcumin (0.2%) to minimize the possibility of overconsumption. Our previous data (not shown here) have shown that at higher concentration of curcumin (2%) did not show as good an inhibitory effect as the lower concentration (1%). This may be due to the second effect of curcumin, which may not be of benefit for the prevention of carcinogenesis. Our results are in agreement with other reports on the inhibitory action of turmeric or curcumin on carcinogenesis in different experimental designs. Azuine and Bhide demonstrated that Swiss mice with multiple doses of DMBA applied on their back for eight weeks, and fed a 2% turmeric diet,

developed less tumors than those fed on a standard diet.[21] Curcumin has already been linked to lower rates of forestomach, duodenal and colon cancer. Administration of 0.5%-2% curcumin in a diet decreased the number and size of benzopyrene-induced forestomach tumors in A/J mice, N-ethyl-N'-nitrosoguanidine-induced duodenal tumorigenesis and azoxymethane induced colon tumorigenesis in CF-1 mice.[22] A 1% turmeric diet suppressed benz(a) pyrene-induced forestomach tumors and spontaneous mammary tumors in mice.[23] Dietary curcumin at a concentration of 500 ppm (equivalent to 0.05%) inhibited 4-nitroquinoline 1-oxide induced oral carcinogenesis in F344 rats.[24]

Intensive studies on the action of curcumin in various biological systems have indicated that this compound has an inhibitory effect in both tumor initiation and promotion pathways. Our results are the first report to demonstrate that dietary curcumin significantly inhibits DMBA- and TPA-induced ras and fos gene expressions in mouse skin. Kakar *et al.*[15] demonstrated that a topical application of curcumin on mouse skin inhibited the TPA induced expression of c-fos, c-jun and c-myc oncogene.

We also demonstrated that TPA (2.5 μg) applied to shaved mouse skin for 30 min caused a redistribution of PKC activity from the cytosol to the membrane. Curcumin inhibited TPA-induced PKC activities in both cytosol and particulate fractions of mouse skin at an *in vitro* dose, dependently. This result is in agreement with other reports on the inhibitory action of curcumin on TPA-induced PKC activites in NIH 3T3 cells.[14] The inhibitors of PKC may block the phosphorylation induced by TPA-type tumor promoters (such as TPA, teleocidin and aplysiatoxin) through inhibiting the PKC activity, and also block TPA-type tumor promoter-induced biological activities and tumor promotion.[25-28] Curcumin may block a certain point on the signal transduction pathway leading to the fos and ras oncogene expression. Our finding that curcumin inhibited TPA-induced fos gene expression as well as TPA-induced PKC activities in mouse skin provides some insight into the possible action mechanism of curcumin. Although the role of ras in malignancy is not clear, it is well established that the association of ras-p21 to the inner surface of the plasma membrane is an absolute requirement for triggering ras oncogenicity.[29] Curcumin might be involved in the modulation of postranslational modification (such as farnesylation) and membrane association of ras-p21 as a plausible molecular mechanism for the promotion of skin tumorigenesis.[30]

In conclusion, our results agree with other findings that the ingestion of dietary curcumin could prevent chemical carcinogenesis in mice, and probably humans, without eliciting direct toxicity to the host. Because curcumin is non-toxic, non-carcinogenic, and already a part of dietary habit, eating curcumin could be encouraged to help offset exposure to sunlight and other agents thought to initiate or promote cancer in humans. The underlying mechanisms of the phenomenon and feasibility of using curcumin in the chemoprevention of human cancer should be explored further.

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References

1. Tonnesen HH. Chemistry of curcumin and curcuminnoids. In : Phenolic Compounds in Food and their Effect on Health, Vol. 1: Analysis, Occurrence and Chemistry. ACS Symposium Series No. 506. Edited by Ho CT, Lee CY and

Huang MT. Washington, DC: American Chemical Society, 1992. p.143-53.
2. Srimal RC, Dhawan BN. Pharmacology of diferuloylmethane (curcumin), a non-steroidal antiinflammatory agent. J Pharm Pharmacol 1973;25:447-52.
3. Satoskar RR, Shah SJ, Shenoy SG. Evaluation of antiinflammatory property of curcumin (diferuloylmethane) in patients with postoperative inflammation. Int J Clin Pharmacol Ther Toxicol 1986;24:651-4.
4. Sharm OP. Antioxidant activity of curcumin and related compounds. Biochem Pharmacol 1976; 25:1811-2.
5. Ruby AJ, Kuttan G, Dinesh BK, Rajasekharan KN, Kuttan R. Anti-tumor and antioxidant activity of natural curcuminoids. Cancer Lett 1995;94:79-83.
6. Viaje A, Slage TJ, Wigler M, Weinstein IB. Effects of antiinflammatory agents on mouse skin tumor promotion, epidermal DNA synthesis, phorbol ester-induced cellular proliferation, and production of plasminogen activator. Cancer Res 1977;37:1530-6.
7. Kozumbo WJ, Seed JL, Kensler T. Inhibition by 2(3)-tert- butyl-4-hydroxyanisole and other antioxidants of epidermal ornithine decarboxylase activity induced by 12-O-tetradecanoylphorbol-13-acetate. Cancer Res 1983;43:2555-9.
8. Nakadate T, Yamamoto S, Aizu E, Kato R. Effects of flavonoids and antioxidants on 12-O-tetradecanoly-phorbol-13-acetate caused epidermal ornithine decarboxylase induction and tumor promotion in relation to tolipooxygenase inhibition by these compounds. Gann 1984;75:214-22.
9. Smart RC, Huang HT, Han ZT, Kaplan MC, Focella A, Conney AH. Inhibition of 12-O-tetradecanoylphorbol-13-acetate induction of ornithine decarboxylase activity, DNA synthesis, and tumor promotion in mouse skin by ascorbic acid and ascorbyl palmitate. Cancer Res 1987;47:6633-8.
10. Huang MT, Smart RC, Wong GQ, Conney AH. Inhibitory effect of curcumin, chlorogenic acid, caffeic acid, and ferulic acid on tumor promotion in mouse skin by 12-O-tetradecanoylphorbol-13-acetate. Cancer Res 1988;48:5941-6.
11. Huang MT, Lysz T, Ferraro T, Abidi TF, Laskin JD,Conney AH. Inhibitory effects of curcumin on in vitro lipoxygenase and cyclooxygenase activities in mouse epidermis. Cancer Res 1991;51:813-9.
12. Azuine MA, Bhide SV. Chemopreventive effect of turmeric against stomach and skin tumors induced by chemical carcinogens in Swiss mice. Nutr Cancer 1992;17:77-83.
13. Nagabhushan M, Bhide SV. Curcumin as an inhibitor of cancer. J Am Coll Nutr 1992;11:192-8.
14. Liu JY, Lin SJ, Lin JK. Inhibitory effects of curcumin on protein kinase C activity induced by 12-O-tetradecanoylphorbol-13 acetate in NIH 3T3 cells. Carcinogenesis 1993;14:857-61.
15. Kakar SS, Roy D. Curcumin inhibits TPA induced expression of c-fos, c-jun and c-myc proto-oncogenes messenger RNAs in mouse skin. Cancer Lett 1994;87:85-9.
16. Huang MT, Lysz T, Ferraro T, Abidi TF, Laskin JD, Conney AH. Inhibitory effect of curcumin on in vitro lipooxygenase and cyclooxygenase activities in mouse epidermis. Cancer Res 1991; 51:813-9.
17. Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Research 1983;11:1475-89.
18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 1970;227:680-5.
19. Towbin H , Stachelin T, Gordon J. Electrophoresis transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedures and some applications. Proc Natl Acad Sci USA 1979;76: 4350-4.
20. Limtrakul P, Lipigorngoson S, Namwong O, Apisariyakul A, Dunn FW. Inhibitory effect of dietary curcumin on skin carcinogenesis in mice. Cancer Lett 1997;116:197-203.
21. Azuine MA, Bhide SV. Chemopreventive effect of turmeric against stomach and skin tumors induced by chemical carcinogens in Swiss mice. Nutr Cancer 1992;17:77-83.
22. Huang MT, Lou YR, Ma W, Newmark HL, Reuhl KR, Conney AH. Inhibitory effects of dietary curcumin on forestomach, duodenal, and colon carcinogenesis in mice. Cancer Res 1994; 54:5341-7.
23. Nagabhushan M, Bhide SV. Antimutagenicity and anticarcinogenicity of turmeric (Curcuma longa). J Nutr Growth Cancer1987;4:83-9.
24. Tanaka T, Makita H, Ohnishi M, et al. Chemoprevention of 4-nitroquinolene 1-oxide-induced oral carcinogenesis by dietary curcumin and hesperidin: comparison with the protective effect of β-carotene. Cancer Res 1994;54:4653-9.
25. Fujiki H, Sugimura T. New classes of tumor promoters: teleocidin aplysiatoxin, and palytoxin. In: Klein, G, Weinhouse S. editors. Advances in Cancer Research. London: Academic Press; 1987. p.223-64.

26. Fujiki H, Suganuma M, Sugimura T. Significance of new environmental tumor promoters. Environ. Carcinogen Rev 1989;7:1-51.
27. Wise BC, Glass DB, Kibler RF, Kuo JF. Phospholipid-sensitive Ca^{2+}-dependent protein kinase in heart. II. Substrate specificity and inhibition by various agents. J Biol Chem 1982; 257:8489-95.
28. Nakadate T, Yamamoto S, Aizu E, Kato R. Inhibition of 12-O-tetradecanoyl-phorbol-13-acetate-induced tumor promotion and epidermal ornithine decarboxylase activity in mouse skin by palmitoylcarnitine. Cancer Res 1986;46:1589-93.
29. Der CJ, Cox AD. Isoprenoid modification and plasma membrane associations: critical factors for ras oncogenicity. Cancer Cells 1991;3:331-40.
30. Kohl NE, Wilson FR, Mosser SD, et al. Farnesyl-transferase inhibitors block the growth of ras-dependent tumors in nude mice. Proc Natl Acad Sci USA 1994;91:9141-5.