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Inhibition of 12-*O*-Tetradecanoylphorbol-13-acetate-caused Tumor Promotion in 7,12-Dimethylbenz[*a*]anthracene-initiated SENCAR Mouse Skin by a Polyphenolic Fraction Isolated from Green Tea[1]

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ABSTRACT

Our laboratory has been studying cancer chemopreventive effects of polyphenolic fraction isolated from green tea (GTP). In prior studies we have shown that (*a*) GTP possesses antigenotoxic effects in various test systems; (*b*) topical application of GTP protects against UV radiation and chemical carcinogen-induced tumorigenesis in murine skin; and (*c*) feeding of GTP in drinking water p.o. to mice protects against carcinogen-induced forestomach and lung tumorigenesis. Recently, we showed that in a dose-dependent manner GTP inhibits tumor promoter-caused induction of epidermal ornithine decarboxylase activity in SENCAR mice (R. Agarwal *et al.*, Cancer Res., *52:* 3582–3588, 1992). In the present study, we assessed the effect of GTP on TPA-induced skin tumor promotion in 7,12-dimethylbenz(*a*)anthracene-initiated SENCAR mouse. Topical application of varying doses of GTP (1–24 mg) 30 min prior to that of each TPA application resulted in highly significant protection against skin tumor promotion in a dose-dependent manner. The animals pretreated with GTP showed substantially lower tumor body burden such as decrease in total number of tumors per group, number of tumors per animal, tumor volume per mouse, and average volume per tumor, as compared to the animals that did not receive GTP. Since TPA-induced epidermal cyclooxygenase and lipoxygenase activities and edema and hyperplasia are conventionally used markers of skin tumor promotion, we also assessed the effect of preapplication of GTP on these parameters. As quantitated by the formation of prostaglandin and hydroxy-eicosatetraenoic acid metabolites from, respectively, cyclooxygenase- and lipoxygenase-catalyzed metabolism of arachidonic acid, skin application of GTP to SENCAR mice resulted in significant inhibition of TPA-caused effects on these 2 enzymes. Prior application of GTP to mouse skin also resulted in 30–46% inhibition of TPA-induced epidermal edema and hyperplasia. The results of the present study suggest that GTP possesses anti-skin tumor-promoting effects, and that the mechanism of such effects may involve inhibition of tumor promoter-induced epidermal ornithine decarboxylase, cyclooxygenase and lipoxygenase activities, edema, and hyperplasia. Further studies are in progress to define which component present in GTP is responsible for its anti-skin tumor-promoting effects.

INTRODUCTION

Water extract of processed leaves and buds from the *Camellia sinensis* of the Theaceae family is, after water, the most widely consumed beverage popularly called either black tea or green tea, depending on processing (1). While black tea is more commonly consumed in Western countries including the United States, and green tea is more common in Asian countries, specifically in Japan, China, Korea, and India (1). The major difference between green tea and black tea is that the former is far richer in epicatechin derivatives (1), a group of polyphenolic compounds that has received considerable attention in recent years due to its diverse pharmacological activities including cancer chemopreventive effects (2, and references therein). Specific epicatechin derivatives identified either in polyphenolic fraction isolated from green tea (GTP[3]) or WEGT include (−)epigallocatechin, EGCG, (−)epicatechin, and (−)epicatechin-3-gallate (1); EGCG, however, constitutes the major component among them (1).

The epidemiological studies, though inconclusive, have suggested a protective as well as an enhancing effect of tea ingestion on human cancer risk (3). While Kinlen *et al.* (4) reported a positive correlation between tea consumption and stomach cancer, other studies have shown either a negative correlation (5) or no increase in the risk of stomach cancer with tea consumption (6). In addition, a case-control study by Kono *et al.* (7) has indicated that individuals consuming green tea tend to have a lower risk for gastric cancer. Epidemiological and physiological studies conducted in Shizuoka Prefecture in Japan by Oguni *et al.* (8) have reported a low mortality rate from stomach cancer, the leading cancer in Japan, in both male and female population groups with a high intake of green tea. Recently, the International Agency for Research on Cancer (3) suggested that "there is no evidence to implicate tea or tea component(s) as human carcinogen. However, based on recent studies, it is concluded that tea possesses potentially useful cancer chemopreventive agents" (2).

Our prior studies have shown that GTP and its constituent epicatechin derivatives interact with hepatic cytochrome P-450 and inhibit monooxygenase activities in skin and liver (9), and that they possess antimutagenic and antigenotoxic effects (10). Furthermore, studies from other laboratories have also shown the antimutagenic effect of WEGT in various mutagenicity test systems (11, 12). In a series of studies from our laboratory as well as by others, it has been shown that topical application or p.o. feeding in drinking water of GTP, WEGT, or EGCG protects against the UVB radiation-induced skin carcinogenesis in SKH-1 hairless mice (13, 14), and chemical carcinogen-induced development of neoplasia in skin, colon, forestomach, lung, duodenum, and esophagus in several animal tumor bioassay protocols (15–20). Preliminary reports have recently shown the anti-tumor-promoting effect of GTP (21) and EGCG (22) against, respectively, TPA- and teleocidin-caused tumor promotion in DMBA-initiated mouse skin. In a recent study (23), we have shown that skin application of GTP to SENCAR mice inhibits the skin tumor promoter-caused induction of epidermal

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[3] The abbreviations used are: GTP, polyphenolic fraction isolated from green tea; DMBA, 7,12-dimethylbenz[a]anthracene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; WEGT, water extract of green tea; EGCG, (−)epigallocatechin-3-gallate; ODC, ornithine decarboxylase; TLC, thin layer chromatography; HETE, hydroxyeicosatetraenoic acid.

ODC, and that EGCG, the major constituent present in GTP, is responsible for the majority of these effects (23).

In this paper, we describe the inhibitory effect of topical application of GTP on (*a*) TPA-caused tumor promotion in DMBA-initiated SENCAR mouse skin; and (*b*) TPA-induced *in vivo* cyclooxygenase and lipoxygenase activities, and edema and hyperplasia in the epidermis of SENCAR mice.

MATERIALS AND METHODS

Chemicals. TPA was purchased from Sigma Chemical Co. (St. Louis, MO). DMBA was purchased from Aldrich Chemical Co. (Milwaukee, WI). Arachidonic acid, 5-(*S*)-HETE, 8-(*S*)-HETE, 12-(*S*)-HETE, 15-(*S*)-HETE, PGE_2, $PGF_{2\alpha}$, and PGD_2 were purchased from Biomol Research Laboratories, Inc. (Plymouth, PA). [1-^{14}C]Arachidonic acid (52 mCi/mmol) was from New England Nuclear (Boston, MA). All other chemicals obtained were the purest form commercially available. GTP from green tea leaves (a product of Korea; distributed by Hanni, Inc., Los Angeles, CA) was prepared by the method described recently (23). As described in detail recently (23), the reverse-phase high pressure liquid chromatography analysis of GTP showed that it was mainly composed of epicatechin derivatives such as (−)epicatechin, (−)epigallocatechin, EGCG, and (−)epicatechin-3-gallate.

Skin Tumorigenesis Studies. Female SENCAR mice (6–8 weeks old) obtained from National Cancer Institute-Frederick Cancer Research Facility, Bethesda, MD, were utilized in DMBA-TPA-induced 2-stage skin tumorigenesis protocol (15), and shaved using electric clippers. Mice with hair cycle in resting phase were used for tumor studies, and divided into several groups of 20 animals each. In experiment 1, the animals were initiated by a single topical application of 10 nmol of DMBA in 0.2 ml of acetone on the dorsal shaved skin, and 1 week later promoted with twice-weekly applications of 3.2 nmol of TPA in 0.2 ml of acetone. To assess the anti-skin tumor-promoting effect of GTP, various doses of GTP in 0.2 ml of acetone were applied topically 30 min prior to that of each TPA application. This treatment regimen was based on our recent study in which it was shown that topical application of GTP 30 min prior to that of TPA produces a maximum inhibitory effect against TPA-induced epidermal ODC activity (23). Six treatment groups in experiment 1 were composed of: group 1, DMBA + TPA; group 2, DMBA + (1 mg GTP + TPA); group 3, DMBA + (3 mg GTP + TPA); group 4, DMBA + (6 mg GTP + TPA); group 5, DMBA + (12 mg GTP + TPA); and group 6, DMBA + (24 mg GTP + TPA). Similarly, in experiment 2, the animals were initiated by a single topical application of 40 nmol of DMBA in 0.2 ml of acetone on the dorsal shaved skin, and 1 week later promoted with twice-weekly applications of 4 nmol of TPA in 0.2 ml of acetone. To assess the anti-skin tumor-promoting effect of GTP, various doses of GTP in 0.2 ml of acetone were applied topically 30 min prior to that of each TPA application. Four treatment groups in experiment 2 were composed of: group 1, DMBA + TPA; group 2, DMBA + (6 mg GTP + TPA); group 3, DMBA + (12 mg GTP + TPA); and group 4, DMBA + (24 mg GTP + TPA). Treatment of TPA or various doses of GTP + TPA in experiments 1 and 2 was repeated twice weekly up to the termination of the experiments at 22 weeks. One group of animals was treated with 0.2 ml of acetone alone and served as negative control to assess spontaneous tumor induction. To test whether GTP itself possesses tumor-promoting effects, a group of animals was initiated with the highest dose of DMBA (40 nmol) and 1 week later treated with the highest dose of GTP (24 mg) up to the end of the experiment. Animals in all the groups were watched for food and water consumption, as well as any apparent signs of toxicity such as weight loss or mortality during the entire period of the study. Skin tumor formation was recorded weekly, and tumors greater than 1 mm in diameter were included in the cumulative total if they persisted for 2 weeks or more. Latent periods for the onset of tumor in various groups were computed by the method of Shimkin and Andervont (24), as described earlier (25). The tumors were diagonosed histologically at the termination of the experiment.

Treatment of Animals for Short-Term *in Vivo* Studies. GTP and TPA treatment protocol of experiment 1 was followed for these studies. For each study, female SENCAR mice, shaved on the dorsal side of the skin, were divided into 3 groups of 8 each, and treated topically on the shaved area with either acetone (0.2 ml), TPA (3.2 nmol in 0.2 ml acetone), or GTP (6 mg in 0.2 ml acetone, a dose that produced significant protection against TPA-caused skin tumor promotion; see "Results") followed 30 min later with TPA (3.2 nmol in 0.2 ml acetone). Animals in all the groups were sacrificed 24 h after the last treatment, and skin was used either for determination of cyclooxygenase and lipoxygenase activities, or for assessment of edema and hyperplasia.

***In Vivo* Epidermal Cyclooxygenase and Lipoxygenase Activities.** Epidermis was separated from the whole skin by scraping with a scalpel blade and homogenized in 0.1 M Tris-HCl buffer, pH 7.2, using a Polytron tissue homogenizer and 100,000 × *g* supernatant, and microsomal fractions were prepared as described earlier (9).

Epidermal microsomal cyclooxygenase activity was determined by the method of Lysz and Needleman (26) with slight modifications as described by Huang *et al.* (27). In brief, 150 μl of reaction mixture contained 12 μM [^{14}C]arachidonic acid (400,000 dpm), 1 mM epinephrine, 1 mM glutathione, and 10 μg epidermal microsomal protein in 50 mM potassium phosphate buffer, pH 7.4. After incubation at 37°C for 15 min, the reaction was terminated by the addition of 50 μl of 0.2 M HCl. The prostaglandin metabolites of arachidonic acid were extracted from the incubation mixture with 0.5 ml of ethyl acetate 3 times. The combined extract was evaporated to dryness, redissolved in chloroform/methanol/acetic acid/water (113/10/1.25/1.0, v/v/v/v), and subjected to thin layer chromatography on a precoated TLC plastic sheet SILICA GEL 60 (20 × 20 cm, layer thickness 0.2 mm; MC/B Manufacturing Chemists, Inc., Cincinnati, OH). TLC plates were developed with the same solvent system and exposed in an iodine chamber for 5 min to visualize the prostaglandin standards. The metabolites of [^{14}C]arachidonic acid corresponding to PGE_2, $PGF_{2\alpha}$, and PGD_2 were detected by their comigration with authentic standards. Those areas of TLC corresponding to metabolites were cut, and the radioactivity was determined by counting the samples in a Packard Tri-Carb 460 CD liquid scintillation counter equipped with automatic external standardization.

Epidermal cytosolic lipoxygenase activity was determined by the method of Fischer *et al.* (28) with slight modifications. In brief, 150 μl reaction mixture contained [^{14}C]arachidonic acid (5 nmol, 460,000 dpm), 2 mM calcium chloride, and 400 μg epidermal 100,000 × *g* cytosolic protein in 100 mM Tris-HCl, pH 7.2. After incubation for 30 min at 37°C, the reaction was terminated by the addition of 10 μl of 0.2 M HCl. The HETE metabolites of arachidonic acid were extracted from the incubation mixture with 0.5 ml of ethyl acetate 3 times. The combined extract was evaporated to dryness, redissolved in acetic acid/isopropanol/*n*-hexane (0.1/4/96, v/v/v), and subjected to normal phase high-pressure liquid chromatography utilizing a 3.9 × 300 mm μ-PORASIL column (Waters Associates). The solvent system used was same as described to dissolve the residues, and was run isocratic at the flow rate of 0.7 ml/min. The column eluate was monitored at 236 nm using a Shimadzu Variable length detector, and 0.1-ml fractions were collected and radioactivity was determined in each fraction, and the radioactive profile was compared with the 236 nm UV absorption profile of authentic HETEs subjected to high pressure liquid chromatography under the identical conditions.

Edema and Hyperplasia. To assess the extent of edema in TPA-induced SENCAR mouse skin, and the inhibitory effect of preapplication of GTP on TPA-induced edema, 1-cm-diameter punches of skin were removed, made free of fat pads, and quickly weighed. After drying for 24 h at 50°C, the skin punches were reweighed and the loss of water content was determined. The difference in the amount of water gain between acetone control and TPA represented the extent of edema induced by TPA, whereas that between acetone control and GTP + TPA represented the inhibitory effect of GTP. For the hyperplasia study, skin was removed, fixed in 10% formalin, and embedded in paraffin. Vertical sections (5 μm) were cut, mounted on a glass slide, and stained with hematoxylin and eosin. For each section of the skin, the thickness of the epidermis from the basal layer to stratum corneum

was measured at 5 equidistant interfollicular sites utilizing an Olympus light microscope (Palo Alto, CA) equipped with an ocular micrometer.

Statistical Analysis. In tumorigenesis experiments, the statistical significance of difference between TPA and GTP + TPA groups was evaluated by the Wilcoxon rank sum test and χ^2 analysis, whereas the Student's t test was used in biochemical studies.

RESULTS

Anti-Skin Tumor-promoting Effects of GTP. As shown by data in Figures 1 and 2, topical application of GTP prior to that of TPA in DMBA-initiated SENCAR mouse skin resulted in dose-dependent inhibition against skin tumorigenesis. In experiment 1, where comparatively smaller doses of DMBA and TPA were used to induce the skin tumors, topical application of 1 to 24 mg GTP 30 min prior to that of TPA resulted in a dose-dependent inhibition against the skin tumorigenesis. This occurred when tumor data were considered as percent of mice with tumors (Fig. 1*B*), total number of tumors per group (Fig. 1*C*), and number of tumors per animal (Fig. 1*D*). Although the time of the appearance of the first tumor in the cases of 1, 3, 6, and 12 mg GTP-treated groups was delayed by 1 week as compared to the non-GTP-treated group, a 3-week delay of the onset of first tumor appearance was observed in the 24 mg GTP-treated group ($P < 0.001$, χ^2 test). At the termination of the experiment at 22 weeks, compared to 100% animals with skin tumors in the non-GTP-treated group, 95, 85, 75, 60, and 35% of the animals exhibited skin neoplasms, respectively, in the 1, 3, 6, 12, and 24 mg GTP-treated groups of animals. The tumor incidence data at the doses of 1 and 3 mg GTP were not significant from control group (χ^2 test). However, at the 6-, 12-, and 24-mg doses of GTP, they were highly significant ($P < 0.01$ or 0.001, χ^2 test). Similarly, when these data were considered as cumulative number of tumors per group or number of tumors per mouse, beginning with the tumor appearance up to the termination of the experiment, 6- to 24-mg doses of GTP produced significant protection against skin tumorigenesis ($P < 0.001$, Wilcoxon rank sum test). At the termination of the experiment, compared to 140 total neoplasms in the non-GTP-treated groups of animals, 97, 68, 60, 50, and 23 tumors/group, respectively, in 1, 3, 6, 12, and 24 mg GTP-treated groups were observed. Compared to the non-GTP-treated group, such a decrease in total number of tumors in the GTP-treated group corresponded to 31, 51, 57, 64, and 84% inhibition, respectively. In addition, when tumor multiplicity data were evaluated using the Cochran-Mantel-Haenzel statistic test, a linear trend of decrease in tumor incidence was evident with increasing doses of GTP ($P < 0.001$).

In experiment 2, when higher doses of DMBA and TPA were used to induce skin tumors, similar inhibitory effects of prior application of GTP to that of TPA on the percent of mice with tumors (Fig. 2*B*), cumulative number of tumors per group (Fig. 2*C*), and number of tumors per mouse (Fig. 2*D*) were observed. These inhibitory effects were also dependent on the dose of GTP. Compared to non-GTP-treated animals, in which the first tumor appeared at 4 weeks on test, preapplication of 6, 12, and 24 mg GTP resulted in, respectively 3, 4, and 5 weeks' delay on the onset of first tumor. Similarly, at the end of the experiment, at 22 weeks, 10, 20, and 30% inhibition in percent of mice with tumors was observed with 6, 12, and 24 mg preapplication of GTP, respectively. The tumor incidence data at the doses of 6 and 12 mg GTP were not significant from control group (χ^2 test). However, at the dose of 24 mg GTP, they were highly significant ($P < 0.001$, χ^2 test). When these data were considered as cumulative number of tumors per group, or number of tumors per mouse, these doses of GTP showed significant protection. As compared to non-GTP-treated groups of animals, in 6, 12, and 24 mg GTP-treated

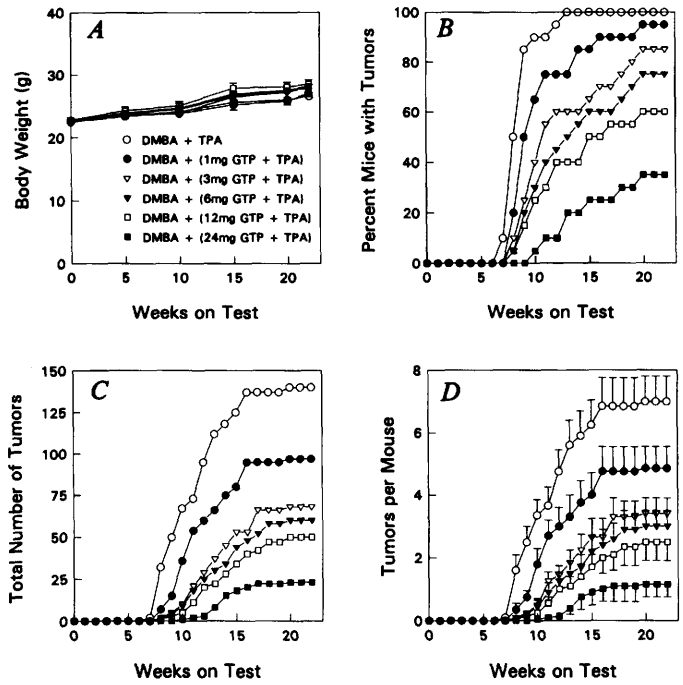

Fig. 1. Dose-dependent inhibitory effect of topical application of GTP on TPA-caused skin tumor promotion in DMBA-initiated SENCAR mice. The doses of DMBA and TPA used were 10 and 3.2 nmol, respectively. The body weight (g) per animal (*A*), percent mice with tumors (*B*), total number of tumors (*C*), and number of tumors per mouse were plotted as a function of the number of weeks on test. Other details are provided in "Materials and Methods."

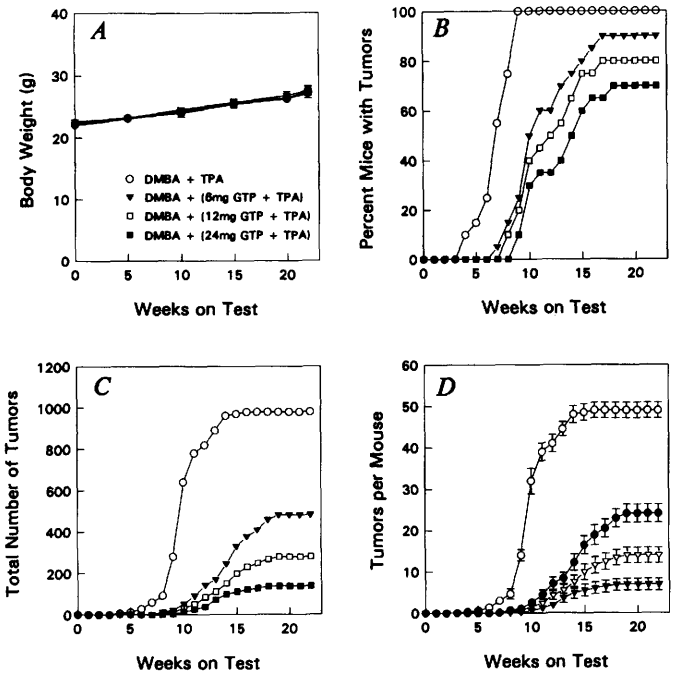

Fig. 2. Dose-dependent inhibitory effect of topical application of GTP on TPA-caused skin tumor promotion in DMBA-initiated SENCAR mice. The doses of DMBA and TPA used were 40 and 4 nmol, respectively. The body weight (g) per animal (*A*), percent mice with tumors (*B*), total number of tumors (*C*), and number of tumors per mouse were plotted as a function of the number of weeks on test. Other details are provided in "Materials and Methods."

groups of animals, 51, 71, and 86% inhibition ($P < 0.001$, Wilcoxon rank sum test) in total number of tumors was observed.

When the data of experiments 1 and 2 were considered in terms of total tumor volume, tumor volume per mouse, and average volume of each tumor, compared to the non-GTP-treated group of animals, preapplication of varying doses of GTP (6 to 24 mg) in both experiments 1 and 2 resulted in statistically significant reduction ($P < 0.001$, Wilcoxon rank sum test) in all the 3 parameters (Table 1).

In experiments 1 and 2, preapplication of varying doses of GTP did not produce any significant change in weight gain profile when compared to the non-GTP-treated control group of animals (Figs. 1*A* and 2*A*). The mean water consumption per animal per day in varying doses of GTP-treated groups of animals was also found to be the same when compared with non-GTP-treated group of animals (data not shown). These observations suggested that topical application of GTP up to 24 mg twice weekly, the highest dose used, does not produce any apparent toxicity during the entire period of the experiments. Moreover, animals initiated with DMBA and promoted twice weekly with 24 mg GTP were devoid of any tumors up to the termination of the experiment at 22 weeks (data not shown), further suggesting that GTP by itself is not a tumor promoter. Histological analysis indicated that all the tumors in non-GTP-treated and GTP-treated groups of animals in both the experiments 1 and 2 were papillomas (data not shown).

***In Vivo* Inhibitory Effects of GTP on TPA-induced Epidermal Cyclooxygenase and Lipoxygenase Activities.** The effect of preapplication of GTP on single or multiple doses of TPA-caused induction of epidermal cyclooxygenase activity is shown in Table 2. As quantitated by the formation of PGE_2, $PGF_{2\alpha}$, and PGD_2, application of TPA to the SENCAR mouse skin resulted in significant induction in epidermal cyclooxygenase activity that was also dependent on the number of TPA treatments (single or multiple). However, the application of GTP prior to that of TPA resulted in significant inhibition of TPA-induced epidermal cyclooxygenase activity. As shown by data in Table 2, the effect of GTP in the first set of experiments utilizing a single application of TPA, or GTP + TPA, was not significant. However, in a second and third set of experiments utilizing, respectively, 2 and 3 applications of TPA or GTP + TPA at an interval of 3 days, significant inhibition was observed, as evidenced by the quantitative analysis of prostaglandin metabolite formation. Compared to TPA alone, in the second set, GTP application showed 37, 45, and 35% inhibition in, respectively, PGE_2, $PGF_{2\alpha}$, and PGD_2 formation, whereas in the third set, respectively, 78, 52, and 34% inhibition was observed (Table 2).

Table 1 *Dose-dependent inhibitory effect of topical application of GTP on tumor size (mm^3) during TPA-caused skin tumor promotion in DMBA-initiated SENCAR mice*

Treatments[a]	Total tumor vol.	Tumor vol./ mouse	Av. vol./ tumor
Experiment 1			
DMBA + TPA	13,680	684 ± 135[b]	98 ± 19
DMBA + (1 mg GTP + TPA)	7,062	353 ± 52[c]	73 ± 10[c]
DMBA + (3 mg GTP + TPA)	4,273	214 ± 37[d]	63 ± 15[e]
DMBA + (6 mg GTP + TPA)	3,120	156 ± 29[f]	52 ± 10[e]
DMBA + (12 mg GTP + TPA)	1,569	78 ± 17[f]	31 ± 8[f]
DMBA + (24 mg GTP + TPA)	315	16 ± 7[f]	14 ± 5[f]
Experiment 2			
DMBA + TPA	93,884	4,694 ± 436	96 ± 19
DMBA + (6 mg GTP + TPA)	30,511	1,525 ± 298[f]	63 ± 10[e]
DMBA + (12 mg GTP + TPA)	10,730	536 ± 112[f]	38 ± 6[f]
DMBA + (24 mg GTP + TPA)	2,680	134 ± 24[f]	19 ± 5[f]

[a] In experiment 1, the doses of DMBA and TPA used per animal were, respectively, 10 and 3.2 nmol dissolved in 0.2 ml acetone. In experiment 2, the doses of DMBA and TPA were, respectively, 40 and 4 nmol in 0.2 ml acetone/animal. Other details are provided in "Materials and Methods."

[b] Mean ± SE obtained from 20 animals in each group at the termination of the experiments at 22 weeks.

[c] Not significant *versus* TPA alone, $P < 0.24$.

[d] Significant *versus* TPA alone, P 0.025.

[e] Significant *versus* TPA alone, $P < 0.01$.

[f] Highly significant *versus* TPA alone, P 0.001.

Table 2 In vivo *inhibitory effect of topically applied GTP on TPA-induced epidermal cyclooxygenase activity*[a]

Treatments[b]	Metabolite formation (pmol/15 min/mg protein)		
	PGE_2	$PGF_{2\alpha}$	PGD_2
Acetone	1199 ± 39[c]	738 ± 75	698 ± 52
GTP	1168 ± 32	719 ± 69	702 ± 62
TPA	3465 ± 211	1454 ± 71	1075 ± 56
GTP + TPA	3075 ± 166[d]	737 ± 46[e]	974 ± 47[d]
TPA (2×)	4548 ± 269	1506 ± 117	1178 ± 85
GTP + TPA (2×)	2848 ± 195[e]	829 ± 55[e]	766 ± 65[f]
TPA (3×)	5448 ± 289	1797 ± 93	1392 ± 67
GTP + TPA (3×)	1209 ± 110[e]	857 ± 42[e]	918 ± 55[e]

[a] Enzyme activity was determined in epidermal microsomes utilizing the thin layer chromatography procedure as described in "Materials and Methods."

[b] In the first set of experiments, animals were treated only once with 0.2 ml acetone, 6 mg GTP in 0.2 ml acetone, 3.2 nmol TPA in 0.2 ml acetone, or 6 mg GTP in 0.2 ml acetone followed 30 min later by 3.2 nmol TPA in 0.2 ml acetone. In the second set of experiments, a similar treatment protocol was used as in the first set, but was repeated twice during the 3-day period. In the third set of experiments, a similar treatment protocol was used as in the first set, but was repeated 3 times during the 3-day period. Twenty-four h after the last TPA treatment, animals were killed, and skin was used for *in vivo* studies as described in "Materials and Methods."

[c] Mean ± SE of 4 individual values, and the epidermis from 2 animals was pooled for each determination.

[d] Not significant *versus* TPA alone, $P < 0.1$.

[e] Highly significant *versus* TPA alone, $P < 0.0005$.

[f] Significant *versus* TPA alone, $P < 0.005$.

The effect of preapplication of GTP on single or multiple doses of TPA-caused induction of epidermal lipoxygenase activity is shown in Table 3. As quantitated by the formation of 12/15-HETE, 8-HETE, and 5-HETE, application of TPA to the SENCAR mouse skin resulted in significant induction in 8- and 5-lipoxygenase activity in epidermis, which was also dependent on the number of TPA treatments (single or multiple). The application of GTP prior to that of TPA resulted in significant inhibition of TPA-induced epidermal lipoxygenase activity. As shown by data in Table 3, in the first, second, and third set of experiments utilizing, respectively, 1, 2, and 3 applications of TPA or GTP + TPA at an interval of 3 days, significant inhibition was observed, as evidenced by the quantitative analysis of different HETE metabolite formation from arachidonic acid. Compared to TPA alone, in the first set, GTP application showed 18, 24, and 39% inhibition in, respectively, 12/15-HETE, 8-HETE, and 5-HETE formation. In the second set of experiments, respectively, 25, 44, and 36% inhibition was observed, whereas in the third set, the inhibition was 43, 48, and 51%, respectively (Table 3). Application of GTP alone at the dose of 6 mg did not produce any change in epidermal cyclooxygenase and lipoxygenase activities when compared to acetone-treated control animals (Tables 2 and 3).

***In Vivo* Inhibitory Effects of GTP on TPA-induced Epidermal Edema and Hyperplasia.** The effect of preapplication of GTP on single or multiple doses of TPA-caused induction of epidermal edema is shown in Table 4. As determined by the weight of a 1-cm-diameter punch of skin, application of TPA to SENCAR mouse skin showed a significant increase in

Table 3 In vivo *inhibitory effect of topically applied GTP on TPA-induced epidermal lipoxygenase activity*[a]

Treatments[b]	Metabolite formation (pmol/30 min/mg protein)		
	12/15-HETE	8-HETE	5-HETE
Acetone	1314 ± 92[c]	112 ± 15	138 ± 22
GTP	1297 ± 95	115 ± 12	135 ± 26
TPA	897 ± 83	530 ± 36	156 ± 10
GTP + TPA	737 ± 96[d]	405 ± 20[e]	95 ± 8[f]
TPA (2×)	906 ± 78	608 ± 45	181 ± 11
GTP + TPA (2×)	680 ± 55[g]	343 ± 25[f]	115 ± 7[f]
TPA (3×)	972 ± 80	668 ± 45	309 ± 20
GTP + TPA (3×)	557 ± 42[f]	347 ± 30[f]	152 ± 15[f]

[a] Enzyme activity was determined in epidermal cytosols utilizing the normal phase high-pressure liquid chromatography procedure as described in "Materials and Methods."
[b] The details of treatments were same as described in Table 2.
[c] Mean ± SE of 4 individual values, and the epidermis from 2 animals was pooled for each determination.
[d] Not significant *versus* TPA alone, $P < 0.1$.
[e] Significant *versus* TPA alone, $P < 0.01$.
[f] Highly significant *versus* TPA alone, $P < 0.0005$.
[g] Significant *versus* TPA alone, $P < 0.025$.

Table 4 In vivo *inhibitory effect of topically applied GTP on TPA-induced epidermal edema*[a]

Treatments[b]	Skin punch wt (mg)	% inhibition against edema
Acetone	21.5 ± 1.2[c]	
GTP	21.1 ± 1.2	
TPA	31.1 ± 1.1	
GTP + TPA	28.2 ± 1.3[d]	32
TPA (2×)	34.5 ± 1.4	
GTP + TPA (2×)	29.6 ± 1.6[e]	38
TPA (3×)	43.8 ± 1.2	
GTP + TPA (3×)	33.1 ± 1.5[f]	46

[a] Epidermal edema was determined by weighing the 1-cm-diameter punch of treated skin as described in "Materials and Methods."
[b] The details of treatments were the same as described in Table 2.
[c] Mean ± SE of four individual values, and the epidermis from 2 animals was pooled for each determination.
[d] Significant *versus* TPA alone, $P < 0.05$.
[e] Significant *versus TPA alone*, $P < 0.025$.
[f] Highly significant *versus* TPA alone, $P < 0.0005$.

the epidermal edema that was dependent on the number of TPA applications (Table 4). The application of GTP 30 min prior to that of TPA, however, showed significant inhibition of TPA-induced epidermal edema. As shown by data in Table 4, in the first, second, and third set of experiments utilizing, respectively, 1, 2, and 3 applications of TPA at an interval of 3 days, prior application of GTP before each TPA application resulted in 32, 38, and 46% inhibition in epidermal edema.

The effect of preapplication of GTP on TPA-caused induction of epidermal hyperplasia is shown in Fig. 3. To determine the induction in mean epidermal thickness by TPA, and inhibition by GTP, 5 epidermal thickness measurements were obtained at equidistant points along the length of the epidermis from dermoepidermal junction to the top of stratum corneum, and all 5 values were averaged and reported as mean epidermal thickness in μm. Similarly, cell layers were also counted from dermoepidermal junction to the bottom of the stratum corneum to determine the mean vertical thickness of cell layers in epidermis. As shown in Fig. 3, in the second set of experiments, 2 applications of TPA at an interval of 3 days followed 24 h later by killing of animals (Fig. 3, *middle*) resulted in a significant increase in mean epidermal thickness (74 μm) and mean vertical thickness of epidermal cell layers (7.6) when compared to the skin of acetone-treated animals (2.5 μm and 2.8, respectively; Fig. 3, *top*). The preapplication of GTP, however, prior to that of each TPA treatment, resulted in significant inhibition (>65%) in the induction of these parameters in epidermis by TPA (42 μm and 4.4, respectively; Fig. 3, *bottom*). Application of TPA also resulted in mixed cell inflammation in dermis that was comprised of mostly neutrophils with some mononuclear cells admixed (Fig. 3, *middle*); this effect of TPA in dermis was also inhibited in a profound manner by preapplication of GTP

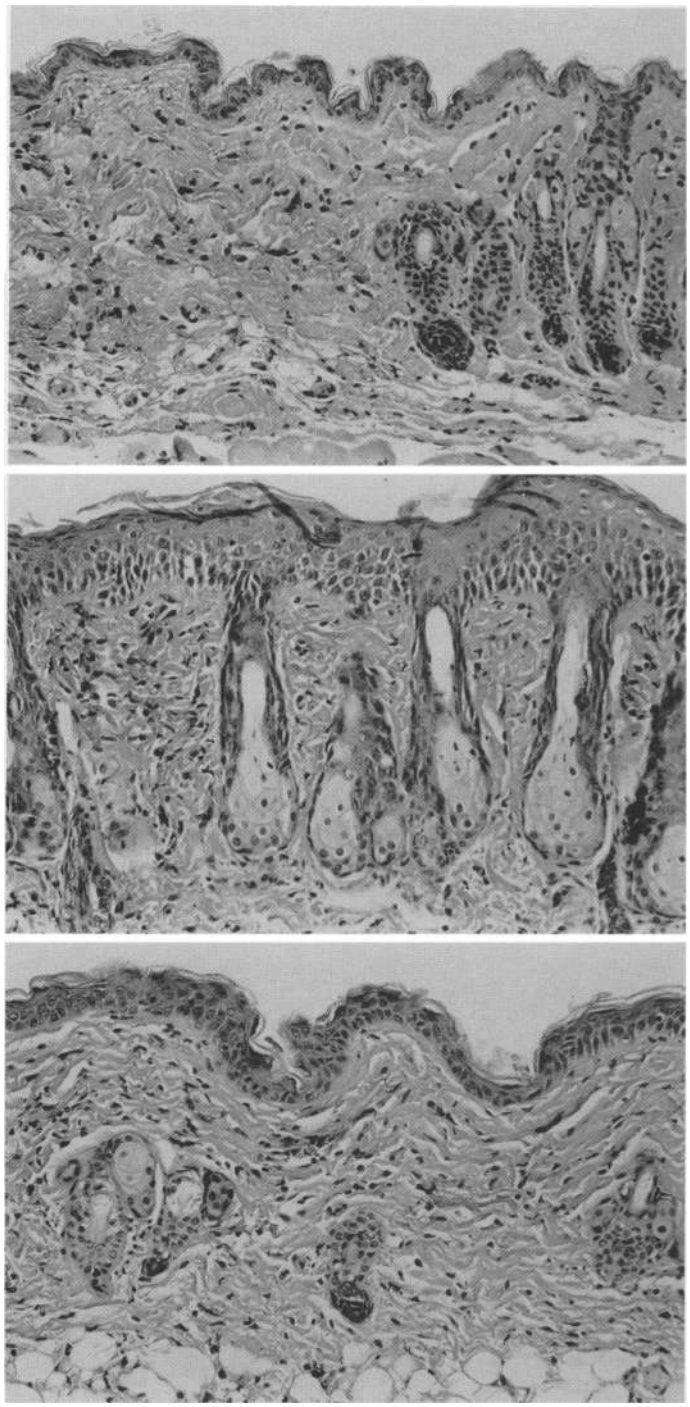

Fig. 3. Inhibitory effects of skin application of GTP on TPA-induced epidermal hyperplasia in SENCAR mouse. The data shown here are those obtained in the second set of experiments (see "Materials and Methods" and "Results"). *Top*, acetone alone; *middle*, 2 applications of TPA alone in acetone at an interval of 3 days; *bottom*, GTP applied 30 min prior to each application of TPA in acetone. Other details are provided in "Materials and Methods." Sections were photographed using a × 80 objective.

(Fig. 3, *bottom*). Similar effects were obtained for the induction of epidermal hyperplasia by TPA and inhibition by GTP in first and third set of experiments (data not shown). GTP alone, however, did not induce any epidermal edema or hyperplasia in these experiments, since the mean epidermal thickness (26 μm) and mean vertical thickness of epidermal cell layers (2.7 were comparable to that observed with acetone-treated animals (data not shown).

DISCUSSION

Since the treatment of cancer is often ineffective once the tumor is diagnosed, in recent years considerable efforts have been made to develop agents for "chemoprevention" that would prevent cancers from developing in the first place (29). Cancer chemoprevention, therefore, has become an important emerging area of research that, in addition to providing a practical approach to identifying potentially useful inhibitors of cancer development, also affords opportunities to study the mechanisms of carcinogenesis (30, 31). It is appreciated that extrinsic factors such as environmental pollutants and lifestyle play major roles in the development of most human cancers (32). Our food contains not only mutagens and carcinogens, but also a variety of chemicals that are antimutagenic and block carcinogenesis in animal tumor bioassay systems (32). For these reasons, changes in dietary habits with the routine intake of more cancer chemopreventive agents, specifically those that have shown preventive effects in a wide range of animal tumor bioassay systems, may offer a potential alternative approach for cancer prevention. Utilizing experimental animal models, it has been shown that cancer can be prevented by administration of a variety of chemical compounds, some of which are naturally occurring and others, synthetic (30). At present, 25 classes of chemicals with anticarcinogenic effects in animal tumor bioassay systems have been described (30). Among these, polyphenols from plant sources are a class of compounds that have been studied extensively as cancer chemopreventive agents (33). Several studies from our laboratory as well as by other investigators have shown the chemopreventive effects of a wide range of naturally occurring polyphenols against tumorigenicity in various organs utilizing different animal tumor bioassay protocols (33–35, and references therein), and have defined possible mechanisms of the chemopreventive effects (33–35). One such class of compounds that has received considerable attention in recent years are the polyphenols present in green tea (2, and references therein). From a series of collective studies reported from our laboratory as well as by others (13–23), it seems reasonable to assume that green tea possesses an interesting and potentially important group of compounds with anticarcinogenic effects against a wide range of carcinogens in several animal tumor bioassay systems.

To access the cancer chemopreventive effects of potential agents, several animal tumor bioassay systems are available (30), however, because of several reasons (35), multistage mouse skin tumorigenesis represents an ideal test system for such studies. While the effect of topical application or oral feeding in drinking water of GTP against chemical carcinogen-induced tumor initiation and complete carcinogenesis in murine skin is well studied (15), only preliminary reports exist in the literature showing the anti-skin tumor-promoting effect of GTP and EGCG against, respectively, TPA and teleocidin-caused tumor promotion (21, 22). The present study, however, describes in detail under 2 different dose protocols of initiation-promotion, the dose-dependent effects of GTP on tumor promotion, and correlates these effects of GTP with the possible mechanisms of TPA-caused tumor promotion in murine skin.

The results in Figs. 1 and 2 show the protective effects of skin application of GTP on TPA-caused tumor promotion in DMBA-initiated SENCAR mouse skin. In experiments 1 and 2, although the doses of DMBA and TPA to induce skin tumors were significantly different, the preapplication of GTP to that of TPA showed dose-dependent protective effects. Except in the case of the percentages of mice with tumors, where 6-, 12-, and 24-mg doses of GTP showed more protection in experiment 1 compared to experiment 2 (Figs. 1 and 2), when the tumor data were considered as total number of tumors or tumors per mouse (Figs. 1 and 2) and tumor volume per mouse or average volume per tumor (Table 1), no significant difference in the protection by GTP at these doses was evident in between experiments 1 and 2 (Wilcoxon rank sum test). Such an observation suggests that these doses of GTP possess saturating protective effects against tumor promotion even when the dose of tumor promoter is higher (experiment 2).

The topical application of phorbol ester TPA to mouse skin or its treatment in certain epidermal cells is known to result in a number of biochemical alterations, changes in cellular functions, and histological changes leading to skin tumor promotion (35–37). Among these, the ones that best correlate with skin tumor-promoting effects include induction of ODC activity, epidermal hyperplasia, inflammation, and increase in the number of dark basal keratinocytes (35, 36). It is, however, difficult to establish which of these effects or many other effects of phorbol ester tumor promoters are obligatory or sufficient components of the tumor promotion process. The induction of inflammation in skin mediated by TPA is believed to be governed by cyclooxygenase- and lipoxygenase-catalyzed metabolites of arachidonic acid, specifically PGs and HETEs, respectively (35, 37). Such an assumption is strongly supported by the studies showing that inhibitors of various parts of these metabolic pathways inhibit skin tumor promotion (34, 37–39, and references therein). These inhibitory studies support the involvement of arachidonic acid metabolism pathways in skin tumor promotion (34, 37–39).

Cyclooxygenases, the microsomal enzymes, play an important role in cutaneous inflammation, cell proliferation, and skin tumor promotion (37, 39, 40, 41, and references therein). Arachidonic acid, released predominantly from skin phosphatidylcholine by the activation of skin phospholipase A_2 due to a variety of mechanical, chemical, or hormonal stimuli, undergoes oxidative metabolism via cyclooxygenase pathway resulting in the formation of PGs such as PGE_2, $PGF_{2\alpha}$, and PGD_2 (42, 43, and references therein). In the skin, several studies have shown the involvement of PGs in inflammatory processes (44, 45), epidermal growth in response to wound repair (46), and in proliferative skin disease psoriasis (47, 48). While PGs are apparently not involved in cell proliferation in normal epidermis, they do play an important role in the induction of hyperplasia induced by TPA (43, 49). In addition to dominant inflammatory effects, TPA application also produces epidermal hyperproliferation, including ODC induction (50), associated with epidermal hyperplasia (51).

Since, in addition to these effects, TPA finally promotes the development of tumors in initiated skin (35, 36), the role of cyclooxygenase pathway in tumor promotion is being studied in several laboratories (41). Using CD-1 and NMRI mice, it was

reported that prior application of indomethacin, a cyclooxygenase inhibitor, to that of TPA results in a dose-dependent inhibition of tumor development in the skin (43, 52); on the contrary, an inverse effect was observed in SENCAR mice, in which indomethacin was found to induce the tumor development (53). Additional studies to examine this apparent discrepancy showed that in the case of SENCAR mice, lipoxygenase catalyzed metabolism of arachidonic acid, its role in tumor promotion was a predominant pathway (54), and the treatment of SENCAR mouse keratinocytes with indomethacin results in increased generation of 12-hydroperoxyeicosatetraenoic acid (54). The results of this study suggested that while indomethacin inhibits the cyclooxygenase pathway, the lipoxygenase pathway starts working in a more profound manner. In addition to these studies, several laboratories have shown the role of cyclooxygenase pathway in skin tumor promotion utilizing various inhibitors of cyclooxygenase, and investigating the role of different prostaglandin treatments on skin tumor promotion (41). While the results of the present study show the inhibitory effects of GTP against TPA-caused induction of epidermal cyclooxygenase activity in SENCAR mouse (Table 2), they also correlate them with the inhibitory effect of GTP against TPA-caused induction of epidermal edema (Table 4) and hyperplasia (Fig. 3).

Arachidonic acid is released from membrane phospholipids by phospholipase activity in the skin by a number of stimuli, phorbol ester tumor promoters being one of them (55). Depending on their nature, different lipoxygenases catalyze the metabolism of arachidonic acid, resulting in the formation of HETEs (37, 40, 41, 56–58). Lipoxygenase-catalyzed metabolites of arachidonic acid have been considered to play an important role in many physiological and pathophysiological events including inflammation and growth regulation; the presence of these metabolites in the mammalian epidermis has been reported by several laboratories (40, 41, 55, 59–61). While 12-lipoxygenase is characterized predominantly in normal murine epidermis (62, 63) along with small amounts of 5-, 8-, 12-, and 15-lipoxygenases (28, 64), treatment of mammalian skin or cell-free extract with TPA results in the induction of 8-lipoxygenase, identified by the generation of 8-HETE (28, 65).

As observed in the present study, single or multiple topical applications of TPA on SENCAR mouse skin resulted in the highly significant induction of 8-lipoxygenase activity (Table 3). The results of several literature studies (65, and references therein) as well as of our present findings, suggest that 8-lipoxygenase-catalyzed arachidonic acid metabolites, specifically 8-HETE, play an important role in skin tumor promotion at least in the case of phorbol ester type skin tumor promoters (65). In the present study, skin application of GTP also showed significant inhibition against TPA-caused induction of epidermal lipoxygenase. These data suggest that, unlike indomethacin, with which inhibition of cyclooxygenase results in the more profound role of lipoxygenase pathway in tumor promotion (54), with GTP, overall inhibition results against TPA-caused induction in the metabolism of arachidonic acid by cyclooxygenase and lipoxygenase pathways. Such an inhibitory effect of GTP may be responsible for the TPA-caused induction of epidermal ODC, as reported from this laboratory in a recent study (23), and epidermal edema and hyperplasia, as observed in the present study. Finally, these inhibitory effects of GTP may collectively be responsible for its protective effects against TPA-caused tumor promotion in murine skin observed in the present study.

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