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Dietary grape seed proanthocyanidins inhibit 12-*O*-tetradecanoyl phorbol-13-acetate-caused skin tumor promotion in 7,12-dimethylbenz[*a*]anthracene-initiated mouse skin, which is associated with the inhibition of inflammatory responses

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Grape seed proanthocyanidins (GSPs) possess anticarcinogenic activities. Here, we assessed the effects of dietary GSPs on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin tumor promotion in 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated mouse skin. Administration of dietary GSPs (0.2 and 0.5%, wt/wt) supplemented with control AIN76A diet resulted in significant inhibition of TPA-induced skin tumor promotion in C3H/HeN mice. The mice treated with GSPs developed a significantly lower tumor burden in terms of the percentage of mice with tumors ($P < 0.05$), total number of tumors per group ($P < 0.01$, $n = 20$) and total tumor volume per tumor-bearing mouse ($P < 0.01$–0.001) as compared with the mice that received the control diet. GSPs also delayed the malignant progression of papillomas into carcinomas. As TPA-induced inflammatory responses are used routinely as markers of skin tumor promotion, we assessed the effect of GSPs on biomarkers of TPA-induced inflammation. Immunohistochemical analysis and western blotting revealed that GSPs significantly inhibited expression of cyclooxygenase-2 (COX-2), prostaglandin E_2 (PGE_2) and markers of proliferation (proliferating cell nuclear antigen and cyclin D1) in both the DMBA-initiated/TPA-promoted mouse skin and skin tumors. In short-term experiments in which the mouse skin was treated with acute or multiple TPA applications, we found that dietary GSPs inhibited TPA-induced edema, hyperplasia, leukocytes infiltration, myeloperoxidase, COX-2 expression and PGE_2 production in the mouse skin. The inhibitory effect of GSPs was also observed against other structurally different skin tumor promoter-induced inflammation in the skin. Together, our results show that dietary GSPs inhibit chemical carcinogenesis in mouse skin and that the inhibition of skin tumorigenesis by GSPs is associated with the inhibition of inflammatory responses caused by tumor promoters.

Introduction

The incidence of skin cancer is equivalent to the incidence of malignancies in all other organs combined (1), and thus represents a major, and growing, public health problem. The continuing increase in life expectancy, the depletion of the ozone layer that allows more solar ultraviolet (UV) radiation to reach at the surface of the Earth, together with changing dietary habits and lifestyle appear to be contributing factors for the increasing risk of skin cancer. In addition to the morbidity and mortality associated with this disease, it is a major burden on the health care system as it has been estimated that the cost of treating non-melanoma and melanoma skin cancers in the USA is US$ 2.9 billion annually (www.cancer.org/statistics). Effective chemopreventive and chemotherapeutic agents and strategies to address this disease are being sought and one such strategy, the use of botanicals, is becoming increasingly popular in USA as a means to protect against skin disorders, including skin cancers.

Grapes (*Vitis vinifera*) are consumed worldwide. They are rich in polyphenols, with 60–70% of grape polyphenols being found in the seeds, which are available as by-products of the industrial production of grape juice and wine. The seeds contain a larger fraction of proanthocyanidins, which are primarily composed of dimers, trimers and oligomers of monomeric catechins or epicatechins (2–4). These grape seed proanthocyanidins (GSPs) have been shown to have antioxidant (5,6), anti-mutagenic, anti-inflammatory and anticarcinogenic (7,8) properties. GSPs induce cytotoxic effects in various cancer cell lines (9–11) with no apparent adverse biological effects on normal cells, i.e. human epidermal keratinocytes (5,12). We have shown previously that supplementation of the diet with GSPs inhibits UV radiation-induced skin carcinogenesis in SKH-1 hairless mice (8), and long-term feeding of a GSPs-supplemented diet did not result in any apparent signs of toxicity in mice (8,9).

The development of skin cancer is a multistage process that includes initiation, promotion and progression in experimental animal models and possibly in human cancer includes induction and propagation (13). During the early tumor promotion stage of multistage carcinogenesis, the process is reversible but the initiation stage is irreversible and presumably unavoidable because of continuing exposure to carcinogenic chemicals and physical agents (13). A commonly used two-stage model of skin cancer involves initiation by treatment of the skin with 7,12-dimethylbenz(*a*)anthracene (DMBA) followed by promotion through treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

In this study, we determined the effect of dietary GSPs on TPA-promoted skin tumor development in DMBA-initiated mouse skin and then examined the chemopreventive mechanism of GSPs in this model. To our knowledge, this is the first study showing that dietary GSPs exert an antitumor promoting effect in TPA-promoted skin tumor development in DMBA-initiated mouse skin. We further show using this two-stage skin cancer model that the inhibition of TPA-caused skin tumor promotion by dietary GSPs is associated with the inhibition of TPA-induced inflammatory responses.

Abbreviations: COX-2, cyclooxygenase-2; DMBA, 7,12-dimethylbenz[*a*]anthracene; GSP, grape seed proanthocyanidin; MPO, myeloperoxidase; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PG, prostaglandin; PGE_2, prostaglandin E_2; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; UV, ultraviolet.

Materials and methods

Animals, antibodies and reagents

The female C3H/HeN mice (6–7 weeks old) used in these studies were purchased from Charles River Laboratory (Wilmington, MA). All mice were maintained under standard conditions of a 12 h dark/12 h light cycle, a temperature of 24 ± 2°C and relative humidity of 50 ± 10%. The mice were fed either a standard AIN76A control diet with or without GSPs (0.2 or 0.5%, wt/wt) and water *ad libitum*. The animal protocol used in this study was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Immunostaining-specific cyclooxygenase-2 (COX-2) antibody and a kit for prostaglandin E_2 (PGE_2) analysis were obtained from Cayman Chemicals (Ann Arbor, MI). The antibodies used to detect proliferating cell nuclear antigen (PCNA) and cyclin D1 and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). TPA, mezerein, benzoylperoxide and anthralin were purchased from Sigma Chemical Co. (St Louis, MO) as were the trypsin, DNase and all other chemicals of analytical grade.

Dietary administration of GSPs

We routinely receive GSPs from the Kikkoman Corporation (Tokyo, Japan) for our research. Quality control of the GSPs is maintained by the company and it has been established that GSPs are stable for at least 2 years when refrigerated at 4°C. The GSPs product contains ~89% proanthocyanidins, with dimers

(6.6%), trimers (5.0%), tetramers (2.9%) and oligomers (74.8%), as described earlier (6,8). Experimental diets containing GSPs (0.2 and 0.5%, wt/wt) are prepared in pellet form in the AIN76A powdered control diet by TestDiet® (Richmond, IN) for our research using the GSPs that we provide for this purpose. In carcinogenesis studies, the GSP-supplemented diet was provided to the mice starting 1 week after initiation with DMBA or at the start of TPA application and continued until the termination of the experiment.

Skin tumorigenesis protocol: DMBA-initiated and TPA-promoted two-stage skin tumor protocol

The dorsal skin area of the female C3H/HeN mice was shaved with electric clippers and depilated skin lotion was applied for 2–3 min. The area was then washed with water. Sixty mice received a single topical application of 400 nmol of DMBA in 0.2 ml of acetone (tumor initiation). One week later, the mice were treated topically with TPA (10 nmol per mouse per 100 µl acetone) (tumor promotion) and this treatment was repeated twice weekly throughout the course of the experiment. The 60 mice that were treated with DMBA were randomly allocated into three treatment groups with 20 mice in each group. All groups were treated with TPA. Mice in group 1 were fed an unsupplemented standard AIN76A diet; mice in group 2 received the standard AIN76A diet supplemented with GSPs (0.2%, wt/wt) and mice in group 3 received the standard AIN76A diet supplemented with GSPs (0.5%, wt/wt). The selection of the concentrations of GSPs in the diet was based on our prior studies in which dietary administration of GSPs as a supplement to an AIN76A diet inhibited UVB-induced oxidative stress (6) as well as UVB-induced skin tumor development in mice (8). Two groups of control mice were used. One group of mice ($n = 10$) was treated with vehicle (0.2 ml acetone) alone twice a week and served as a negative control to assess spontaneous tumor induction. A second group of mice ($n = 10$) was initiated with the DMBA (400 nmol per mouse per 0.2 ml acetone) and 1 week later were provided a diet supplemented with GSPs (0.5%, wt/wt) until the termination of the experiment, as described for groups 1–3. At the termination of the experiment, tumor samples and tumor-uninvolved skin samples were collected for the analysis of various biomarkers of interest as described below.

Short-term in vivo analysis of chemopreventive effects

To determine the chemopreventive mechanism of GSPs on TPA-induced tumor promotion activities, we also conducted short-term *in vivo* experiments. In these experiments, TPA (10 nmol per mouse per 200 µl acetone) was applied topically to the shaved skin of C3H/HeN mice. The TPA was either applied three times on alternate days (multiple treatments) and the mice were killed 6 h after the last treatment or the TPA was applied once and the mice were killed at 6, 12 or 24 h later (acute treatment). The mice were either provided the unsupplemented standard AIN76A diet or the AIN76A diet supplemented with 0.5% (wt/wt) GSPs. This concentration of GSPs was used as we had found that it significantly inhibited tumor promotion in the skin tumorigenesis protocol. The GSP-supplemented diet was provided at least 1 week before the topical application of TPA. Skin samples from the treated areas were collected at the time of killing for determining the effect of dietary GSPs on TPA-induced inflammatory responses. In some experiments, structurally different tumor promoters (mezerein, benzoylperoxide, anthralin) were used in the place of TPA.

Evaluation of tumor growth

The skin of the mice that had been subjected to the DMBA/TPA two-stage skin tumor protocol was examined once a week for the appearance of papillomas or tumors until the yield and size of the tumors had stabilized. Growths that were >1 mm in diameter and that persisted for at least 2 weeks were defined as tumors and recorded. The dimensions of all the tumors were recorded at the termination of the experiment and tumor volumes were calculated using the hemiellipsoid model formula: tumor volume $= 1/2(4\pi/3)(l/2)(w/2)h$, where l = length, w = width and h = height.

Pathological evaluation of skin tumors

At the termination of the skin tumor protocol, representative biopsies from all the skin tumors were collected, fixed in 10% formaldehyde and embedded in paraffin. Deparafinized sections (5 µm thick) were stained routinely with hematoxylin and eosin for pathological evaluation by three independent observers who were blinded to the source of the tissues. The specimens were classified as tumors or non-neoplastic lesions according to the following criteria: loss of keratinization or keratinized centers, the presence of horn pearls and atypical cells.

Immunohistochemical detection of COX-2 and PCNA

Five micrometer thick frozen sections were hydrated in phosphate-buffered saline (PBS) and then non-specific binding sites were blocked with 1% bovine serum albumin and 2% goat serum in PBS. The sections were incubated with anti-COX-2 or anti-PCNA antibodies for 2 h at room temperature, washed and then incubated with biotinylated secondary antibody for 45 min followed by horseradish peroxidase-conjugated streptavidin. After washing in PBS, sections were incubated with diaminobenzidine substrate and counterstained with hematoxylin. Representative pictures were taken using a Nikon Eclipse E400 inverted microscope and DXM1200 digital camera.

Enzyme immunoassay for PGE_2

Skin or tumor samples were homogenized in 100 mM phosphate buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid and 10 µM indomethacin using a polytron homogenizer (PT3100, Fisher Scientific, Atlanta, GA). The supernatants were collected and the concentration of PGE_2 was determined in supernatants using the Cayman PGE_2 Enzyme Immunoassay Kit (Ann Arbor, MI) following the manufacturer's protocol.

Preparation of tissue lysates and western blot analysis

Epidermis or tumor samples were washed with cold PBS and lysed with ice-cold lysis buffer supplemented with protease inhibitors, as detailed previously (6,9). Epidermis was separated from the whole skin as described earlier (14). The epidermis or tumor tissue samples were pooled from at least three mice in each group, and three sets of pooled samples from each treatment group were used to prepare lysates, thus $n = 10$. For immunoblotting, proteins (20–35 µg) were resolved on 10% Tris–glycine gels and transferred onto a nitrocellulose membrane. After blocking the non-specific binding sites, the membrane was incubated with the primary antibody at 4°C overnight. The membrane was then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody and the immunoreactive bands were visualized using the enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ). Membranes were then stripped and reprobed with anti-β-actin antibody to verify equal protein loading. The relative density (arbitrary) of each band after normalization for β-actin is shown under each immunoblot as a fold-change compared with non-TPA-treated control, which has been assigned an arbitrary unit 1 in each case.

Leukocyte infiltration and myeloperoxidase assay

Leukocyte infiltration and the levels of myeloperoxidase (MPO) were assessed in skin samples obtained from the mice subjected to the short-term *in vivo* analysis protocol. MPO was measured as a marker of tissue infiltration in skin homogenate samples following the procedure of Bradley *et al.* (15). Briefly, the skin samples were homogenized in 50 mM potassium phosphate buffer, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide followed by sonication of the homogenate at 4°C for three 10 s bursts with a heat system sonicator equipped with a microprobe. The resulting supernatants were used for MPO estimation. MPO activity in the supernatant (0.1 ml) was assayed by mixing with 50 mM phosphate buffer (2.9 ml), pH 6.0, containing 0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance resulting from decomposition of H_2O_2 in the presence of *o*-dianisidine was measured at 460 nm using a Beckman Coulter DU 530 spectrophotometer. The data are expressed as mean MPO U/mg protein.

Skin edema

Skin edema was assessed in the mice subjected to the short-term *in vivo* analysis protocol using the weight of 1 cm diameter skin punches and measurement of bi-fold skin thickness. The 1 cm diameter skin punch biopsies were collected 6 h after treatment to the TPA or other inducers and immediately weighed on an electronic balance. Bi-fold skin thickness was measured using spring-loaded micrometer.

Statistical analysis

Statistical analysis of tumor data was performed at the termination of the experiment. Tumor incidence in the TPA alone and GSPs + TPA-treated groups was compared using the χ^2 test. Tumor multiplicity data were analyzed using the Wilcoxon rank-sum test. The results of PGE_2 and MPO are expressed as means ± SDs. The statistical significance of difference between the values of control and treatment groups was determined using the Student's *t*-test.

Results

Dietary GSPs inhibit TPA-induced skin tumor promotion in DMBA-initiated mouse skin

The GSP-supplemented diet reduced TPA-promoted skin tumor development in DMBA-initiated mouse skin in terms of tumor incidence (Figure 1A), tumor multiplicity (Figure 1B) and tumor size (Figure 1C) as compared with the control diet. The tumor incidence was 35% ($P < 0.05$) lower in the mice fed the diet supplemented with 0.5%

GSPs than in the mice fed the unsupplemented control diet at the termination of the experiment (27th week). Although supplementation of the diet with the lower dose (0.2%) of GSPs resulted in a 20% lower tumor incidence, this effect did not reach statistical significance. Importantly, the tumors that developed in the mice fed the GSP-supplemented diets exhibited an increased latency period with a 4-week delay in the mice administered 0.2% GSPs and a 10-week delay in the mice administered 0.5% GSPs in the diet under the experimental conditions used in these studies. A total of 23 tumors were recorded in the group of mice that did not receive GSPs, whereas 13 tumors (43% inhibition, $P < 0.01$) were recorded in the group of mice fed 0.2% GSPs and only seven tumors (70% inhibition, $P < 0.001$) were recorded in the group of mice fed 0.5% GSPs (Figure 1B and Table I). Additionally, the tumor size was significantly lower in the mice that were provided GSPs in the diet (32%, $P < 0.01$ in mice receiving 0.2% GSPs and 70%, $P < 0.001$ in mice receiving 0.5% GSPs) than in the mice receiving the control diet (Figure 1C and Table I). Overall, both the rate of appearance of the TPA-induced tumors and their development in the GSP-treated mice was significantly lower ($P < 0.05$, Fisher–Irwin exact test) than in the mice that were not fed GSPs. The mice in the control group that were treated with vehicle alone or treated with GSPs did not develop tumors within the 27-week study period. During the carcinogenesis protocol, the body weights and food and water intakes did not differ among the experimental groups (data not shown), suggesting that administration of dietary GSPs does not produce any apparent signs of toxicity in mice at least at the concentrations used and within the time frame of the current experiments.

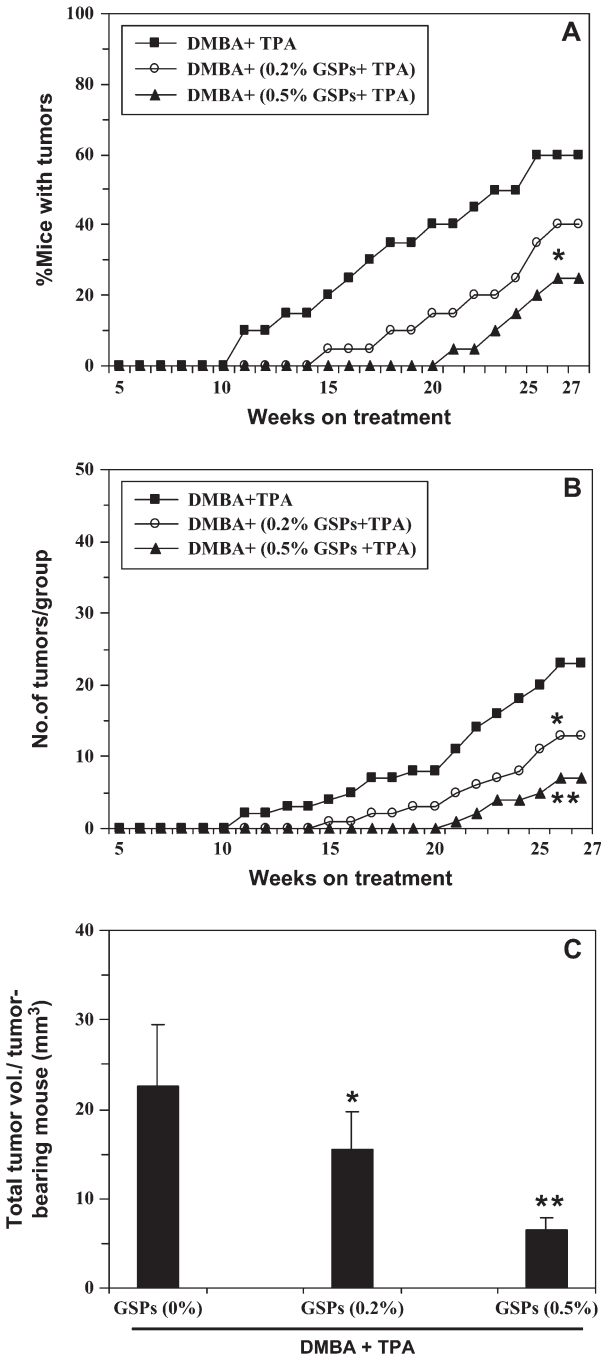

Fig. 1. Dietary GSPs inhibit TPA-induced skin tumor promotion in DMBA-initiated C3H/HeN mouse skin. The tumor data were recorded in terms of percent mice with tumors (**A**) and total number of tumors per group (**B**), and data were plotted as a function of the number of weeks on treatment. At the termination of the experiment at 27th week, tumor volume per tumor-bearing mouse was measured (**C**), and expressed as mean ± SD, $n = 20$. *Significant difference versus control (DMBA + TPA) group, $P < 0.05$. **Significant difference versus control group, $P < 0.001$.

Histopathologic examination of the tumors at the termination of the experiment revealed that of the 23 tumors in the group of mice that received the unsupplemented control diet, four were squamous cell carcinomas, 15 squamous cell papillomas and four keratoacanthomas. Of the 13 tumors in the group of mice that received the diet supplemented with 0.2% GSPs, one was squamous cell carcinoma, 10 were squamous cell papillomas and two keratoacanthomas; whereas, of the seven tumors in the group of mice that were provided 0.5% GSPs, there were six squamous cell papillomas and one keratoacanthoma. Thus, the majority of tumors were squamous cell papillomas and were of epidermal origin.

Dietary GSPs delayed the malignant progression of papillomas to carcinomas

Although 20% of the mice that were provided the control diet developed carcinoma, only 5% of the mice that were provided 0.2% GSPs developed carcinoma, and none of the mice which were provided 0.5% GSPs developed carcinoma during the entire treatment protocol. The total number of carcinomas at the termination of the experiment in the group of mice that received the control diet was four; therefore, 17% of the papillomas were converted into carcinomas in this group; whereas, only one carcinoma was recorded in the group of mice that were provided 0.2% GSPs and only 7.7% of the

Table I. Protective effect of dietary GSPs on TPA-induced skin tumor promotion in DMBA-initiated mouse skin; data were recorded at the end of chemical carcinogenesis protocol[a]

Parameters	Treatment groups		
	Control	0.2% GSPs + DMBA + TPA	0.5% GSPs + DMBA + TPA
Tumor-bearing mice per group, *n*	12	8	5
Total tumors per group, *n*	23	13 (43)[b]	7 (70)[c]
Total tumor volume per group (mm^3)	270	119 (55)[d]	33 (87)[c]
Total tumor volume per tumor-bearing mouse (mm^3)	22.5 ± 7.2	15.5 ± 4.2 (32)[b]	6.6 ± 1.2 (70)[c]
Mean tumor volume per tumor (mm^3)	11.7 ± 6	9.1 ± 4.1 (23)[b]	4.7 ± 2.0 (60)[c]

[a]Total number of tumors and tumor volume in different treatment groups were recorded at 27th week when tumor yield and growth had stabilized, $n = 20$. The indicated doses of GSPs were given in AIN76A control diet, and the values in parentheses indicate the percent inhibition.
[b]Significant versus control group, $P < 0.01$.
[c]Significant versus control group, $P < 0.001$.
[d]Significant versus control group, $P < 0.005$.

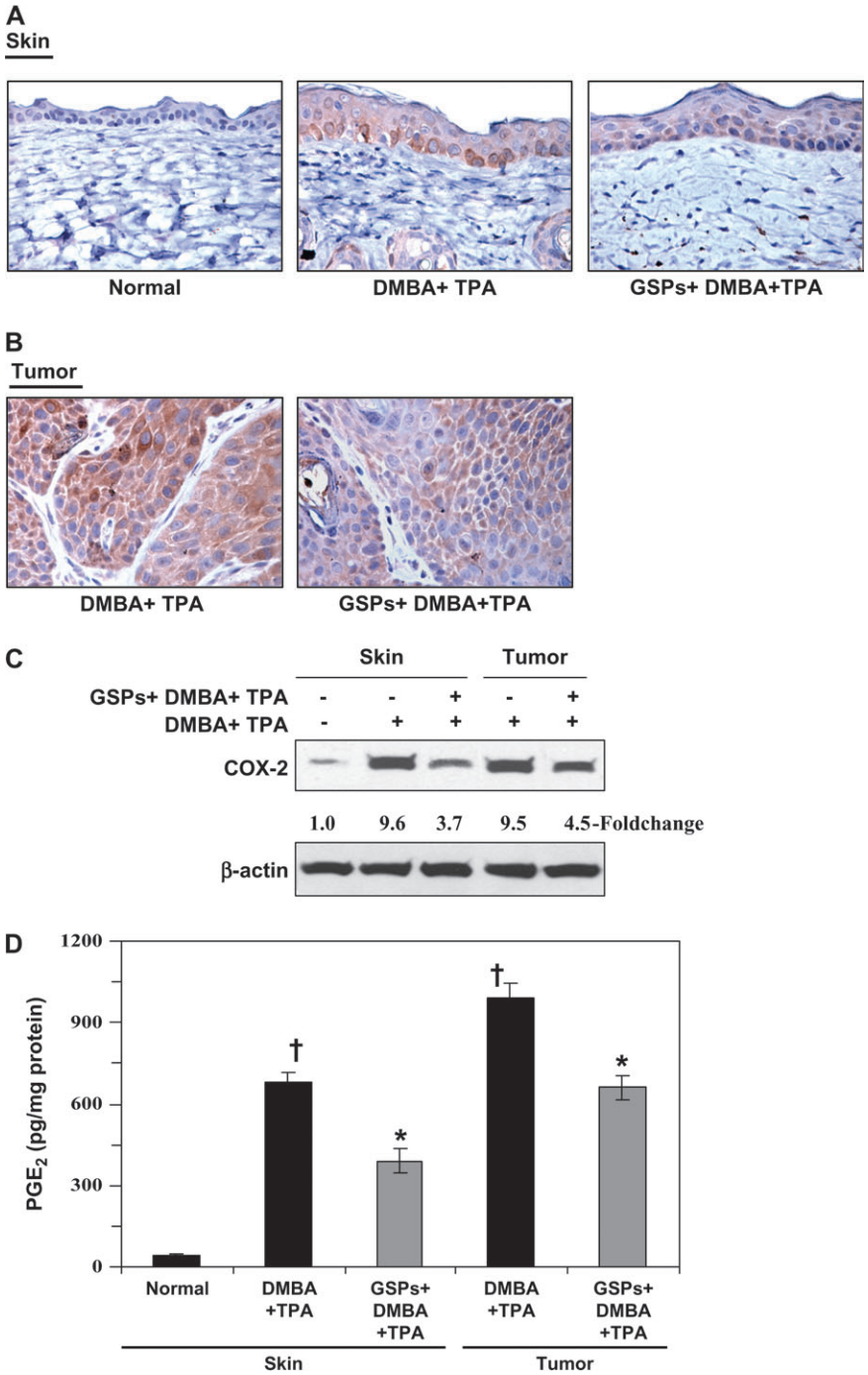

Fig. 2. Dietary GSPs (0.5%, wt/wt) inhibit the expressions of COX-2 and PGE_2 in DMBA-initiated and TPA-promoted skin and skin tumors. Tumor uninvolved skin and skin tumor samples were collected at the termination of the experiment and processed for the examination of COX-2 expression and PGE_2 production. Immunohistochemical detection and localization of COX-2 expression in skin (**A**) and tumors (**B**). (**C**) COX-2 expression in the epidermal skin and in skin tumors was determined using western blot analysis, as described in Materials and Methods. A representative blot is shown from three independent experiments with identical observations. The relative density (arbitrary) of each band after normalization for β-actin is shown under each immunoblot as a fold-change compared with non-TPA-treated control, which has been assigned an arbitrary unit 1 in each case. (**D**) The levels of PGE_2 were determined in the homogenates of epidermal skin or tumor lysates by an enzyme-linked immunosorbent assay. The concentration of PGE_2 was expressed in terms of pg/mg protein as mean ± SD, $n = 10$. *Significant difference versus DMBA + TPA-treated control groups, $P < 0.01$. †Significantly higher versus normal (control) skin, $P < 0.001$.

papillomas were converted into carcinomas in this group. Taken together with the absence of carcinomas in the group of mice that were provided 0.5% GSPs, these data suggest that the risk of malignant progression of papillomas into carcinomas in GSP-treated mice was significantly reduced compared with the control mice that were not given GSPs in the diet.

Effect of dietary GSPs on TPA-induced inflammation and its mediators

Chronic inflammation has been shown to promote tumor development (13,16). As supplementation of the AIN76A control diet with 0.5% GSPs significantly inhibited TPA-induced skin tumor promotion in mice in the chemical carcinogenesis protocol (Figure 1), we used the

skin and tumor samples from this group and control group for further mechanistic analysis.

Dietary GSPs inhibit the levels of COX-2 expression and PGE_2 production in mouse skin and skin tumors

A characteristic response of keratinocytes to tumor promoters is enhanced COX-2 expression and a subsequent increase in the production of PG metabolites in the skin (13,16); moreover, elevated expression of COX-2 and prostaglandin (PG) metabolites has been observed in squamous and basal cell carcinomas of the skin (16,17). Of the PG metabolites, PGE_2 appears to play a pivotal role in tumor promotion. Immunohistochemical analysis confirmed that, in mice that were fed the unsupplemented control diet, the expression of COX-2 was higher in the skin of DMBA/TPA-treated mice than in skin of the mice that were not treated with DMBA/TPA (Figure 2A). The expression of COX-2 in the skin of the DMBA/TPA-treated mice that were provided the GSP-supplemented diet was lower than that in the skin of the DMBA/TPA-treated mice that were fed the unsupplemented control diet (Figure 2A). Similarly, the levels of COX-2 expression in the skin tumors were lower in the DMBA/TPA-treated mice that were provided the GSP-supplemented diet than in the tumors in the mice that were provided the control diet (Figure 2B). These data were confirmed by western blot analysis, which showed higher expression levels of COX-2 protein in DMBA/TPA-treated mouse skin and skin tumors and GSPs inhibition of this DMBA/TPA-induced elevation in the expression levels of COX-2 in the mouse skin and skin tumors (Figure 2C). As shown in Figure 2D, we also found that the levels of PGE_2 in the skin and tumors of the DMBA/TPA-treated mice were significantly higher ($P < 0.001$) than non-DMBA/TPA-treated mouse skin samples. The administration of GSPs significantly inhibited ($P < 0.01$) the DMBA/TPA-induced elevation in the levels of PGE_2 in both skin and skin tumors.

Dietary GSPs inhibit DMBA/TPA-induced increases in the levels of PCNA and cyclin D1 in the skin and skin tumors

The proliferation potential of epidermal cells (i.e. the hyperplastic response) is another marker of the TPA-induced inflammatory reaction in the skin. Immunohistochemical analysis revealed that DMBA/TPA application enhances the proliferation potential of epidermal keratinocytes as indicated by the PCNA staining pattern in the epidermis and that GSPs inhibited this DMBA/TPA-induced expression of PCNA in both skin (Figure 3A) and skin tumor (Figure 3B) samples. These data were further confirmed by western blot analysis, as shown in Figure 3C. Similarly, western blot analysis revealed that dietary GSPs inhibited DMBA/TPA-induced increase in the expression levels of cyclin D1 in both skin and skin tumor samples (Figure 3C).

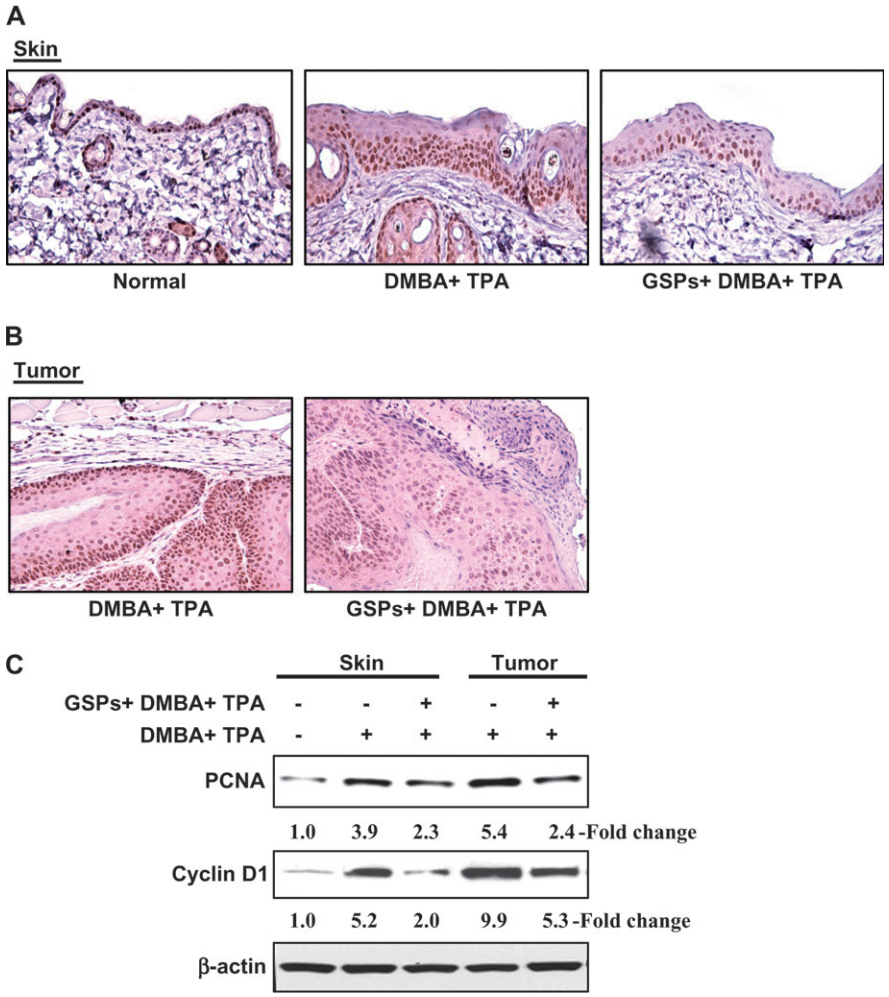

Fig. 3. Dietary GSPs inhibit DMBA-initiated and TPA-promoted markers of inflammation (PCNA and cyclin D1) in the mouse skin and skin tumors. Immunohistochemical detection of PCNA expression in skin (**A**) and tumor (**B**) samples. Data were compared between GSP-fed and non-GSP-fed control groups of mice, $n = 6$. (**C**) PCNA and cyclin D1 expressions were determined using western blot analysis, as described under Materials and Methods. A representative blot is shown from three independent experiments with identical observations, $n = 9$–10.

Short-term in vivo studies

To further verify whether antitumor promotion effect of GSPs is associated with the inhibition of TPA-induced inflammatory responses, short-term experiments were performed.

Dietary GSPs inhibit TPA-induced epidermal hyperplastic response

It was evident from the hematoxylin and eosin staining (Figure 4A) of the skin samples that the thickness of the epidermis is greater in the TPA-treated than in the non-TPA-treated mouse skin, which suggests a hyperplastic response to this tumor promoter; furthermore, the dietary GSPs inhibited this TPA-induced hyperplastic response in the skin. We therefore measured the epidermal thickness at five equidistant points along the entire length of the section from the dermo-epidermal junction to the top of stratum corneum, and all five values were averaged and reported as the mean epidermal thickness in micrometers. Similarly, the numbers of cell layers were counted from the dermo-epidermal junction to the bottom of the stratum corneum to determine the mean vertical thickness of cell layers in the epidermis. As shown in Figure 4A, 6 h after multiple TPA treatments, there was a significant increase in mean epidermal thickness (70 ± 10 μm) and mean vertical thickness of epidermal cell layers (6 ± 2) compared with the acetone-treated normal mouse skin (20.5 ± 5.0 μm thick and 2 ± 1 cell layers). In mice provided the GSP-supplemented diet, there was a significant reduction (>50%, $P < 0.01$) in this TPA-induced increase in epidermal thickness (39 ± 5 μm) and vertical thickness of epidermal cell layers (4 ± 1). Similar effects of GSPs were also observed when mice were treated once with TPA and the effects were determined 12 and 24 h later (Figure 4A). Dietary GSPs alone, however, did not induce an epidermal hyperplastic response in mouse skin (Figure 4A, upper panels).

Dietary GSPs inhibit structurally different skin tumor promoter-induced inflammatory responses

Development of edema is considered as a marker of inflammation. As determined by the weight of a 1 cm diameter punch of the skin, treatment of the skin with TPA resulted in a significantly higher skin punch weight (50% more, $P < 0.01$). The provision of the GSP-supplemented diet significantly reduced (46%, $P < 0.01$) the TPA-induced increase in punch weight, as shown in Table II. Similarly, provision of the GSP-supplemented diet reduced the increases in punch weight induced by multiple TPA applications. On analysis of the effects of dietary GSPs on the increase in punch weight induced by topical application of other structurally different skin tumor promoters, mezerein, benzoylperoxide and anthralin, we found that the GSPs also inhibited the induction of edema in terms of skin punch weight caused by these tumor promoters (Table II). To further verify the inhibitory effect of GSPs on tumor promoter-induced edema, we

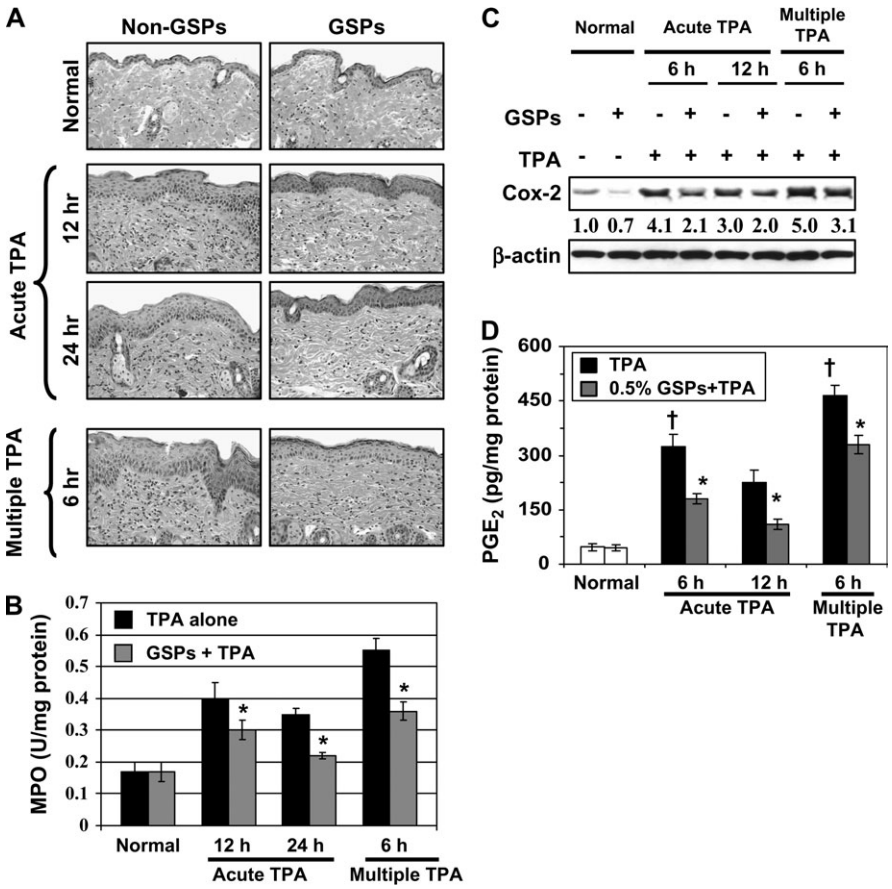

Fig. 4. Dietary GSPs inhibit TPA-induced inflammatory responses in mouse skin. TPA (10 nmol) was applied to the shaved dorsal skin of C3H/HeN mice and TPA was applied once or three times on alternate days. To determine the protective effect of GSPs, mice were fed AIN76A diet supplemented with 0.5% GSPs (wt/wt), as described in the Materials and Methods. The mice were killed at the indicated time points after application of the TPA and the dorsal, treated skin samples were collected. (**A**) Skin samples were processed for standard hematoxylin and eosin staining to examine the cellular infiltration and epidermal hyperplastic responses. Representative photomicrographs from each treatment group are shown, $n = 6$. (**B**) MPO was determined as a marker of tissue infiltration in skin homogenates. Data are reported as the mean ± SD ($n = 10$). (**C**) Samples were processed for the analysis of COX-2 expression using western blot analysis, as described under the Materials and Methods. A representative blot is shown from three independent experiments with identical observations. (**D**) The levels of PGE_2 were determined in the epidermal homogenate samples using enzyme immunoassay. The concentration of PGE_2 is expressed in terms of pg/mg protein as mean ± SD ($n = 10$). †Significant increase versus control skin, $P < 0.001$. *Significant inhibition versus TPA treatment alone, $P < 0.01$.

Table II. Inhibitory effect of dietary GSPs on structurally different skin tumor promoter-induced skin edema in mice[a]

Treatments	Dose of tumor promoter	Skin punch weight (mg)	% Inhibition	Bi-fold skin thickness (mm)	% Inhibition
Acetone		20.9 ± 1.0[b]		0.80 ± 0.08	
GSPs		20.8 ± 1.0		0.80 ± 0.09	
Acute TPA (1×)					
TPA	5 μg	30.9 ± 1.3		1.10 ± 0.10	
GSPs + TPA		26.2 ± 1.3[c]	46	0.95 ± 0.10[c]	50
Multiple TPA (3×)					
TPA	5 μg	40.2 ± 1.4		1.31 ± 0.09	
GSPs + TPA		31.5 ± 1.3[c]	44	1.10 ± 0.08[c]	41
Acute mezerein (1×)					
Mezerein	5 μg	28.9 ± 2.0		1.10 ± 0.08	
GSPs + mezerein		24.4 ± 1.5[d]	56	0.94 ± 0.09[d]	54
Acute BPO (1×)					
BPO	20 mg	23.2 ± 1.2		0.92 ± 0.10	
GSPs + BPO		22.0 ± 1.2[c]	48	0.86 ± 0.10[c]	50
Acute anthralin (1×)					
Anthralin	50 μg	23.1 ± 1.5		0.90 ± 0.08	
GSPs + anthralin		21.5 ± 1.5[d]	68	0.84 ± 0.10[d]	60

[a]Tumor promoter-induced skin edema was determined by weighing the 1.0 cm diameter punch biopsies of treated skin as detailed in Materials and Methods. Bi-fold skin thickness of treated areas of the skin was measured 6 h after tumor promoter applications using spring-loaded micrometer. Mice were topically treated with various tumor promoters in 0.2 ml acetone per mouse with indicated doses of tumor promoters. In case of benzoylperoxide (BPO), the punch weight and bi-fold skin thickness was measured at 24 h after the treatment. The effect of GSPs on tumor promoter-induced edema was determined on acute topical application of tumor promoters. In case of TPA only, the effect of GSPs was also evaluated after multiple application of TPA.
[b]Mean ± SD of three individual values from each mouse, $n = 5$.
[c]Significant versus tumor promoter treatment alone, $P < 0.01$.
[d]Significant versus tumor promoter treatment alone, $P < 0.001$.

measured the bi-fold skin thickness before tumor promoter application and 6 h after acute treatment of various tumor promoters. As shown in Table II, the bi-fold skin thickness was increased significantly after acute (43%, $P < 0.01$) or multiple TPA treatment (73%, $P < 0.005$). Provision of the GSP-supplemented diet significantly inhibited TPA-induced edema in terms of bi-fold thickness of the skin after acute (50%, $P < 0.01$) and multiple treatment (41%, $P < 0.01$) with TPA. Dietary GSPs also significantly inhibited the development of edema in terms of bi-fold skin thickness caused by other structurally different skin tumor promoters, as shown in Table II. Dietary GSPs alone did not affect the thickness of the skin, suggesting that GSPs alone do not induce inflammation in the mouse skin.

Infiltrating leukocytes are considered to be a major source of inflammatory reactions and oxidative stress (13,16,18). Routine hematoxylin and eosin staining of skin samples revealed that the skin treatment with TPA induces infiltration of inflammatory leukocytes (activated monocytes/macrophages and neutrophils) that peaks around 12–24 h post-TPA application (Figure 4A). It was observed that provision of the GSP-supplemented diet markedly reduced the number of TPA-induced infiltrating leukocytes in the treated skin sites at 12 and 24 h post-TPA treatment. This inhibitory effect of GSPs on TPA-induced leukocyte infiltration was also evident after multiple treatment of skin with TPA. To confirm that dietary GSPs inhibit TPA-induced infiltration of leukocytes in the treated sites, we determined the levels of MPO in skin homogenate samples from the various treatment groups. We found an increase in MPO activity in skin samples after treatment with TPA (Figure 4B), suggesting an influx of leukocytes into the inflamed skin. The provision of the GSP-supplemented diet significantly inhibited ($P < 0.01$) TPA-induced MPO activity both after acute and multiple treatments of the skin with TPA and at all the time points studied. This GSP-induced reduction in MPO activity further suggests that the GSPs act, at least in part, to inhibit the TPA-induced inflammatory responses in the skin.

Dietary GSPs inhibit TPA-induced COX-2 expression and PGE_2 production in mouse skin

As tumor promoter-induced COX-2 expression and a subsequent increase in the production of PG metabolites in the skin are considered as characteristic responses to inflammation, we further determined whether dietary GSPs inhibit TPA-induced COX-2 expression and thereby inhibit PGE_2 production in mouse skin. Western blot analysis revealed that treatment of the mouse skin with TPA, either as a single or multiple applications, resulted in higher levels of COX-2 expression as compared with non-TPA-treated normal mouse skin (Figure 4C). Provision of the GSP (0.5%, wt/wt)-supplemented diet resulted in inhibition of this TPA-induced increase in COX-2 expression at all time points studied after a single or multiple applications of TPA. Similarly, dietary GSPs significantly inhibited ($P < 0.01$) TPA-induced increases in the levels of PGE_2 after both single and multiple TPA treatments (Figure 4D).

Discussion

In our continuing efforts to develop newer and more effective dietary botanicals for the prevention of skin cancer, we first assessed the efficacy of dietary GSPs using a two-stage skin chemical carcinogenesis protocol. In this study, we used the inbred C3H/HeN strain of mice as these mice can be appropriately used to study the effects of the tumor promoters, and the modification of these effects by GSPs, on inflammatory responses. The central finding of the present study is that dietary GSPs afford significant protection against TPA-induced skin tumor development in DMBA-initiated mouse skin, and the antitumor promotion effects are associated with the anti-inflammatory effects of the GSPs. Further, as the development of papillomas was delayed and growth slowed with GSPs treatment, GSPs also delayed the malignant conversion of papillomas to carcinomas.

A wide range of studies have shown that naturally occurring polyphenols, specifically those present in fruits and vegetables, common beverages, like green tea, and several herbs and plants with diverse pharmacological activities, are a promising classes of agents with the potential to act to inhibit tumor promotion (19–23). Green tea is a widely consumed beverage worldwide and the composition of GSPs differs from green tea polyphenols in their unique combination of proanthocyanidins, which are polyphenols but with a higher molecular weight than the green tea polyphenols. GSPs are a mixture of dimers, trimers, tetramers and oligomers of monomeric catechin and epicatechin (7); whereas green tea polyphenols are mainly

composed of monomers, such as catechins, epicatechins and their gallate esters, including (−)-epigallocatechin-3-gallate (24). However, the activity of dietary GSPs in inhibiting tumor promotion in the skin chemical carcinogenesis model used in these studies appears identical to the activity of green tea polyphenols in inhibiting tumor promotion in the skin (20). With the notion that targeting the tumor promotion stage could be a better strategy for the prevention of cancer, regular consumption of vegetables and fruits that are good source of polyphenols has been associated with a reduced risk for several malignancies (25–29).

It has been recognized that inhibition of tumor promotion is most probably a better strategy for cancer chemoprevention than inhibition of the tumor initiation stage because initiation is a short irreversible event, whereas the tumor promotion stage is reversible during the early stages (13). It is well established that tumor promoters, such as TPA, induce inflammation and the mediators of inflammation are considered to be potent regulators of tumor promotion in skin cancers (13). We have shown earlier that topical application of TPA on the mouse skin enhances the expression of COX-2 (20), which is one of the most important enzymes responsible for the development of inflammation and tumors. COX-2 is a rate-limiting enzyme for generation of PG metabolites from arachidonic acid (30). COX-2 overexpression has been linked to the pathophysiology of inflammation and cancer due to enhanced synthesis of PG metabolites (31), which have been shown to be potential contributing factor in chemical carcinogenesis. In this study, we found that dietary GSPs inhibit the elevation in the expression of COX-2 and greater production of PGE_2 induced by chronic TPA exposure of DMBA-initiated mouse skin in both the skin and skin tumors, which may have contributed to the inhibition of the skin tumor development in the GSP-treated mice. The decreased proliferating potential of epidermal keratinocytes by dietary GSPs, which is indicated by the reduced expression of the PCNA and cyclin D1 proteins in the DMBA/TPA-induced skin and skin tumors, may also be contributing factors for the inhibitory effects of GSPs on the development of skin tumors.

The infiltration and accumulation of activated macrophages and neutrophils after TPA treatment of the mouse skin is a characteristic feature of skin inflammation, and the quantification of infiltrating leukocytes in skin is used routinely as a measure for the intensity of inflammation (15,32). The levels of MPO also are commonly used as a quantitative marker of inflammatory infiltrates since normal skin exhibits low background levels of MPO, whereas skin that is inflamed by an infection, wounding or by the application of phorbol ester and other tumor promoters enhances the levels of MPO (15,32). Our analysis of the effects of GSPs on the immediate responses induced by a single application of TPA in short-term *in vivo* experiments showed that dietary GSPs inhibited the infiltration of leukocytes in the mouse skin as well as reducing the levels of MPO activity at all the time points studied. Additionally, dietary GSPs reduced TPA-induced skin punch weight and bi-fold skin thickness, which were analyzed as markers of edema. TPA-induced edema is considered to be a marker of tissue inflammation. Notably, we confirmed that GSPs have the ability to inhibit various structurally different skin tumor promoter-induced inflammatory responses in the mouse skin.

Collectively, the data from the present study suggest that dietary GSPs have anti-skin tumor promotion activity and that the antitumor promotion activity of GSPs is associated with the inhibition of tumor promoter-induced inflammation in mouse skin. The results of the present study, in conjunction with prior publications from our laboratory and others, suggest that GSPs may prove to be useful chemopreventive agent against some forms of human cancers induced by environmental agents, and therefore, more detailed studies with particular emphasis on molecular mechanisms could lead to new strategies for cancer chemoprevention in humans.

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