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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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 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 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 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 morbidity and mortality associated with this disease.

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 E2 (PGE2) 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 GSP product contains ~89% proanthocyanidins, with dimers...
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 3-5 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 pmol per mouse) in 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.

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. Growth 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 hemispherical model formula: tumor volume = \(\frac{4}{3}\pi l^2w\), where \(l = \text{length}, w = \text{width} \) and \(h = \text{height}\).

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. Deparaffinized 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.
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.

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 protocola

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Treatment groups</th>
<th>Data recorded at the end of carcinogenesis protocol</th>
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</thead>
<tbody>
<tr>
<td>Tumor-bearing mice per group, n</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Total tumors per group, n</td>
<td>23</td>
<td>13 (43)b</td>
</tr>
<tr>
<td>Total tumor volume per group (mm³)</td>
<td>270</td>
<td>119 (55)b</td>
</tr>
<tr>
<td>Total tumor volume per tumor-bearing mouse (mm³)</td>
<td>22.5 ± 7.2</td>
<td>15.5 ± 4.2 (32)b</td>
</tr>
<tr>
<td>Mean tumor volume per tumor (mm³)</td>
<td>11.7 ± 6</td>
<td>9.1 ± 4.1 (23)b</td>
</tr>
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</table>

aSignificant versus control group, $P < 0.01$.

bSignificant versus control group, $P < 0.001$.

cSignificant versus control group, $P < 0.005$.
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 PGE₂ 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, PGE₂ 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 PGE₂ 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 PGE₂ 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, $n = 9–10$. 

<table>
<thead>
<tr>
<th></th>
<th>Skin</th>
<th>Tumor</th>
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<tbody>
<tr>
<td>GSPs+ DMBA/TPA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DMBA/TPA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PCNA</td>
<td>1.0</td>
<td>3.9</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>5.4</td>
<td>2.4</td>
</tr>
<tr>
<td>β-actin</td>
<td>1.0</td>
<td>5.2</td>
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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...
An influx of leukocytes into the inflamed skin. The provision of skin samples after treatment with TPA (Figure 4B), suggesting an increase in MPO activity in various treatment groups. We found an increase in MPO activity in dermal homogenate samples from the skin treated with TPA. To inhibit TPA-induced infiltration of leukocytes in the treated sites, we determined the levels of MPO in skin homogenate samples from the skin treated 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 treated 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 supports that the GSPs act, at least in part, to inhibit the TPA-induced inflammatory responses in the skin.

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

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dose of tumor promoter</th>
<th>Skin punch weight (mg)</th>
<th>% Inhibition</th>
<th>Bi-fold skin thickness (mm)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>0.80 ± 0.08</td>
<td>20.9 ± 1.0</td>
<td></td>
<td>0.80 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>GSPs</td>
<td>0.80 ± 0.09</td>
<td>20.8 ± 1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute TPA (1×) TPA</td>
<td>1.10 ± 0.10</td>
<td>30.9 ± 1.3</td>
<td>46</td>
<td>1.10 ± 0.10</td>
<td>26.2 ± 1.3</td>
</tr>
<tr>
<td>GSPs + TPA</td>
<td>0.95 ± 0.10</td>
<td>31.5 ± 1.3</td>
<td></td>
<td>1.31 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Multiple TPA (3×)</td>
<td>1.10 ± 0.08</td>
<td>28.9 ± 2.0</td>
<td>56</td>
<td>1.10 ± 0.08</td>
<td>24.4 ± 1.3</td>
</tr>
<tr>
<td>Acute mezerein (1×)</td>
<td>0.94 ± 0.09</td>
<td>23.2 ± 1.2</td>
<td>48</td>
<td>0.92 ± 0.10</td>
<td>22.0 ± 1.2</td>
</tr>
<tr>
<td>Mezerein + GSPs</td>
<td>0.86 ± 0.10</td>
<td>21.3 ± 1.5</td>
<td></td>
<td>0.90 ± 0.08</td>
<td>21.5 ± 1.5</td>
</tr>
<tr>
<td>Acute BPO (1×)</td>
<td>0.84 ± 0.10</td>
<td>21.3 ± 1.5</td>
<td></td>
<td>0.90 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>GSPs + BPO</td>
<td>0.84 ± 0.10</td>
<td>21.3 ± 1.5</td>
<td></td>
<td>0.90 ± 0.08</td>
<td></td>
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<tr>
<td>Acute anthralin (1×)</td>
<td>0.84 ± 0.10</td>
<td>21.3 ± 1.5</td>
<td></td>
<td>0.90 ± 0.08</td>
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<tr>
<td>Anthralin + GSPs</td>
<td>0.84 ± 0.10</td>
<td>21.3 ± 1.5</td>
<td></td>
<td>0.90 ± 0.08</td>
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*Significant versus tumor promoter treatment alone, P < 0.01.

**Inhibitions 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 PGE2 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

**Dietary GSPs inhibit TPA-induced COX-2 expression and PGE2 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 PGE2 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.
Veterans Administration Merit Review Award to S.K.K.

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References


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