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RESEARCH PAPER

Dietary Grape Seed Proanthocyanidins Inhibit UVB-Induced Cyclooxygenase-2 Expression and Other Inflammatory Mediators in UVB-Exposed Skin and Skin Tumors of SKH-1 Hairless Mice

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ABSTRACT

Purpose The purpose of this study was to determine the chemopreventive mechanism of dietary grape seed proanthocyanidins (GSPs) against ultraviolet (UV) radiation-induced skin tumor development in mice.

Methods Six-to-seven-week-old SKH-1 hairless mice were subjected to photocarcinogenesis protocol, and exposed to UVB radiation (180 mJ/cm^2) three times/week for 24 weeks. Mice were fed a standard AIN76A control diet with or without supplementation with grape seed proanthocyanidins (GSPs; 0.2% or 0.5%, *w/w*). At the termination of the experiment, mice were sacrificed, and skin and skin tumor samples were harvested and subjected to the analysis of biomarkers related to inflammation using immunostaining, western blot analysis, ELISA and real-time PCR.

Results Dietary GSPs inhibited UVB-induced infiltration of proinflammatory leukocytes and the levels of myeloperoxidase, cyclooxygenase-2 (COX-2), prostaglandin (PG) E_2, cyclin D1 and proliferating cell nuclear antigen (PCNA) in the skin and skin tumors compared to non-GSPs-treated UVB irradiated mouse skin and skin tumors. GSPs also significantly inhibited the levels of proinflammatory cytokines, tumor necrosis factor-α ($P < 0.01$), IL-1β ($P < 0.001$) and IL-6 ($P < 0.001$), in UVB-exposed skin and skin tumors.

Conclusion The results from this study clearly suggest that dietary GSPs inhibit photocarcinogenesis in mice through the inhibition of UVB-induced inflammation and mediators of inflammation in mouse skin.

KEY WORDS chemoprevention · COX-2 · grape seed proanthocyanidins · skin cancer · ultraviolet radiation

ABBREVIATIONS

COX-2	cyclooxygenase-2
IL	interleukin
MPO	myeloperoxidase
PCNA	proliferating cell nuclear antigen
PGE_2	prostaglandin E_2
TNF-α	tumor necrosis factor-α

INTRODUCTION

The incidence of cutaneous malignancy is equivalent to the incidence of malignancies in all other organs combined (1), and thus represents a major public health problem. The depletion of the ozone layer that allows more solar ultraviolet (UV) radiation to reach the surface of the Earth, a continuing increase in life expectancy, and changing dietary and lifestyle habits appear to be contributing factors for the increasing risk of skin cancer. Skin cancer 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 United States cost $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 dietary phytochemicals, is becoming increasingly popular as a means to protect against skin diseases, including skin cancers.

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Long-term exposure of the skin to solar UV radiation induces inflammatory responses, oxidative stress, immunosuppression, DNA damage and gene mutations, which altogether have been implicated in various skin diseases, including the risk of skin cancers (2–5). UV-induced inflammatory responses, which are characterized by increased blood flow and vascular permeability, result in the development of edema, erythema, hyperplastic responses, and increases in the levels of cyclooxygenase-2 (COX-2) and prostaglandin (PG) metabolites (5–8). UV-induced inflammation is considered an early and important event in tumor promotion or the growth of skin tumors. Chronic inflammation plays an important role in all three stages of tumor development, *i.e.*, initiation, promotion and progression (7). Therefore, the control of UVB-induced inflammatory responses is considered an important strategy to prevent skin cancer risk.

Proanthocyanidins are naturally occurring compounds that are widely found in fruits, vegetables, nuts, seeds, flowers, and barks of some plants. They are a class of phenolic compounds that take the form of oligomers or polymers of polyhydroxy flavan-3-ol units, such as (+) catechin and (−)-epicatechin (9). The seeds of the grape (*Vitis vinifera*) are a particularly rich source of proanthocyanidins. The grape seeds are available as byproducts of the industrial production of grape juice and wine and are rich with 60–70% polyphenols. The seeds contain a larger fraction of proanthocyanidins, which are primarily composed of dimers, trimers and oligomers of monomeric catechins or epicatechins (10–12). These grape seed proanthocyanidins (GSPs) have been shown to have antioxidant (13,14), anti-mutagenic, anti-inflammatory and anti-carcinogenic (15,16) properties. GSPs are cytotoxic to various cancer cell lines (17–19) with no apparent adverse biological effects on normal cells, *e.g.*, human epidermal keratinocytes (13,20), and long-term feeding of a GSPs-supplemented diet did not result in any apparent signs of toxicity in mice (16,17). We have shown previously that supplementation of the diet with GSPs inhibits the growth and development of UV radiation-induced skin tumors in SKH-1 hairless mice (16); however, a molecular mechanism underlying this inhibitory effect by dietary GSPs is not clearly understood.

As UV-induced inflammation and its mediators, such as the induction of COX-2, production of prostaglandins (PG) and proinflammatory cytokines, and enhancement in cellular proliferating potential, have been implicated in skin tumor development, we sought to determine whether the chemopreventive effects of dietary GSPs on photocarcinogenesis are mediated, at least in part, through the inhibition of UVB-induced inflammation and inflammatory mediators in mouse skin.

MATERIALS AND METHODS

Animals, Antibodies and Reagents

The six-to-seven-week-old female SKH-1 hairless mice used in this study were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed five per cage and acclimatized for at least one week before the start of the experiment. All mice were housed under the following conditions: 12 h dark/12 h light cycle, 24±2°C temperature and 50±10% relative humidity. The mice were fed a standard AIN76A diet (Harlan Teklad, Madison, WI) with or without GSPs (0.2% or 0.5%, *w/w*) 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.

The antibodies specific to COX-2 and a kit for 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 corresponding secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals used in this study were of analytical grade.

Dietary Administration of GSPs

The GSPs were obtained from the Kikkoman Corporation (Tokyo, Japan), and the chemical composition has been described earlier (16). Experimental diets containing GSPs (0.2 or 0.5%, *w/w*) were commercially prepared in pellet form in the AIN76A powdered control diet by TestDiet (Richmond, IN) for our research using the GSPs that we provided for this purpose. The GSPs were given in the diet of the mice at least two weeks before UVB-irradiation and continued until the termination of the experiment.

UVB Irradiation and Photocarcinogenesis Protocol

Female SKH-1 hairless mice of 6–7 weeks of age were used in this study. Mice were divided randomly into three separate treatment groups with 20 mice in each group for photocarcinogenesis experiments. All groups were exposed to UVB radiation. Mice in group 1 were fed non-GSPs-supplemented standard AIN76A diet, mice in group 2 received the standard AIN76A diet supplemented with GSPs (0.2%, *w/w*), and mice in group 3 received the standard AIN76A diet supplemented with 0.5% (*w/w*) GSPs. Mice were UVB-irradiated as described earlier (16). Briefly, the dorsal skin was exposed to UV radiation from a band of four FS24T1 UVB lamps (Daavlin, UVA/UVB Research Irradiation Unit, Bryan, OH) equipped with an electronic controller to regulate UV dosage. The UVB lamps primarily emit UVB (290–320 nm, >80% of

total energy) and UVA (320–375 nm, <20% of total energy) radiation with peak emission at 314 nm as monitored (16). Under the standard photocarcinogenesis protocol, mice were UVB irradiated (180 mJ/cm^2) three times a week for a total of 24 weeks. Mice in group 4 ($n=10$) received control AIN 76A diet and were not exposed to UVB radiation and served as a negative control to assess spontaneous tumor induction. At the termination of the experiment at the 24th week after UVB irradiation, tumor samples and tumor-uninvolved skin samples were collected for the analysis of various biomarkers of inflammation.

Histological Evaluation of Infiltrating Leukocytes and Myeloperoxidase (MPO) Activity

Skin samples were fixed in 10% buffered formalin and processed for routine H&E staining for detecting infiltrating cells microscopically. The levels of MPO also were assessed in skin samples obtained from the mice subjected to the photocarcinogenesis protocol. MPO was assessed as a marker of tissue infiltration in skin homogenate samples following the procedure of Bradley *et al.* (21) and as used previously by us (22). 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. The data are expressed as mean MPO units/mg protein.

Immunohistochemical Detection of COX-2 and PCNA

Five-μm 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.

PGE_2 Immunoassay for Quantitation of Prostaglandin E_2

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

Assay for Pro-inflammatory Cytokines by ELISA

Epidermal or tumor homogenates from each treatment group were prepared for the analysis of cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, using cytokine-specific ELISA Kit (BioSource International, Camarillo, CA) following the manufacturer's protocol.

Preparation of Skin or Tumor Tissue Lysates and Western Blot Analysis

Epidermis or tumor samples were washed with cold PBS and lysed with ice-cold lysis buffer supplemented with cocktail of protease inhibitors (*e.g.*, aprotinin and leupeptin), as detailed previously (8,14). Epidermis was separated from the whole skin as described earlier (23). The epidermis or tumor tissue samples were pooled from at least three mice in each group, and 3 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 protein expression was detected and visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). To ensure equal protein loading, the membranes were stripped and reprobed with anti-β actin antibodies. The relative density (arbitrary) of each band after normalization for β-actin is shown under each immunoblot as a fold-change compared with non-UVB-exposed control, which has been assigned an arbitrary unit 1 in each case.

RNA Extraction and Quantitative Real-Time PCR

The total RNA was extracted from the mouse epidermis using TRIzol reagent (Invitrogen, CA) following the protocol recommended by the manufacturer. The mRNA expression of PCNA and cyclin D1 in skin samples was determined using real-time PCR, as detailed previously (24). Briefly, for the mRNA quantification, complementary DNA (cDNA) was synthesized using 3 μg RNA through a reverse transcription reaction (iScript^{TM} cDNA Synthesis Kit, BIO-RAD, CA). Using SYBR Green/Fluorescein PCR Master Mix (SuperArray Bioscience Corporation, MD), cDNA was amplified using real-time PCR with a

BioRad MyiQ thermocycler and SYBR green detection system (BioRad, CA). Samples were run in triplicate to ensure amplification integrity. Manufacturer-supplied (SuperArray, Bioscience Corporation, MD) primer pairs were used to measure the mRNA expression of PCNA and cyclin D1. The standard PCR conditions were 95°C for 15 min, then 40 cycles at 95°C, 30 sec; 55°C, 30 sec; and 72°C, 30 sec, as recommended by the primer's manufacturer. The expression levels of genes were normalized to the expression level of the β-actin mRNA in each sample. The threshold for positivity of real-time PCR was determined based on negative controls. For mRNA analysis, the calculations for determining the relative level of gene expression were made using the cycle threshold (C_t) method. The mean C_t values from duplicate measurements were used to calculate the expression of the target gene with normalization to a housekeeping gene used as internal control (β-actin), and using the $2^{-\Delta Ct}$ formula.

Statistical Analysis

The results of cytokine levels and PGE_2 are expressed as means ± SD. 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 UVB-Induced Skin Tumor Development

We have shown previously that dietary GSPs inhibit UVB-induced skin tumor development in SKH-1 hairless mice (16). The data presented in Fig. 1a indicated that dietary administration of GSPs at the level of 0.2% and 0.5% (*w*/*w*) supplemented with AIN76A control diet significantly lowered the tumor multiplicity by 47% and 65% ($P<0.05$ & $P<0.001$) respectively, and growth or size of the tumor by 49% and 66% ($P<0.001$), respectively.

GSPs Inhibit UVB-Induced Infiltration of Inflammatory Leukocytes in the Skin

As UVB-induced infiltration of leukocytes is the major source of inflammatory reactions, and chronic inflammation has been shown to promote tumor development (7,25), we examined the effect of GSPs on UVB-induced infiltration of leukocytes in mouse skin. Also, as inhibition of UVB-induced skin tumor development in mice by 0.5% GSPs was highly significant (Fig. 1a), we used the skin and tumor samples from this group and control group for further mechanistic analysis. The chronic exposure of skin to UVB radiation for 24 weeks induces infiltration of leukocytes at UVB-irradiated sites of the mouse skin compared with control non-UVB-exposed mouse skin (Fig. 1b). However, the administration of GSPs inhibited UVB-induced infiltration of leukocytes at the UVB-irradiated skin sites, particularly in the dermis. To further confirm whether GSPs inhibited UVB-induced infiltration of leukocytes, the levels of MPO activity in cytosolic fractions of the skin samples of different treatment groups was determined. MPO is commonly employed as a marker of infiltrating leukocytes, which includes activated monocytes/macrophages and neutrophils. A significant increase in MPO activity in the skin samples of mice exposed to UVB radiation was observed compared with the skin samples of non-UVB-exposed mouse skin; however, the levels of MPO were significantly reduced (64%, $p<0.001$) in the skin samples of GSPs-fed mice than non-GSPs-fed UVB-irradiated mice (Fig. 1c). The reduction in MPO activity in GSPs-fed group of mice after UVB exposure indicates the inhibition of influx of leukocytes to the inflamed skin.

Further, it was evident from the H&E staining (Fig. 1b) of the skin samples that the thickness of the epidermis is greater in the UVB-irradiated skin than in the non-UVB-irradiated control mouse skin, which suggests a hyperplastic response in the skin; however, the dietary GSPs inhibited UVB-induced hyperplastic response in the skin. To compare the hyperplastic responses among the treatment groups, we measured the epidermal thickness at 5 equidistant places along the entire length of the skin section from the dermo-epidermal junction to the top of stratum corneum, and all 5 values were averaged and reported as the mean epidermal thickness in micrometer. 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 data summarized in Fig. 1d, there was a significant increase in mean epidermal thickness (68 ± 12 μm) and mean vertical thickness of epidermal cell layers (7 ± 2) compared to the non-UVB-irradiated control mouse skin (20.0 ± 5.0 μm thick and 2.0 ± 0.5 cell layers). In mice given the GSPs-supplemented diet, there was a significant reduction (>60%, $P<0.01$) in UVB-induced increase in epidermal thickness (35 ± 7 μm) and vertical thickness of epidermal cell layers (4 ± 2, $P<0.05$), as shown in Fig. 1d.

GSPs Inhibit UVB-Induced Enhancement of COX-2 Expression and PGE_2 Production

Elevated expression of COX-2 and PG metabolites have been observed in squamous- and basal-cell carcinomas of the skin (7,26). Additionally, UVB-induced COX-2 expression and subsequent increase in the production of PG metabolites in the skin is a characteristic response of keratinocytes to tumor promoters (7,25). Immunohistochemical detection analysis

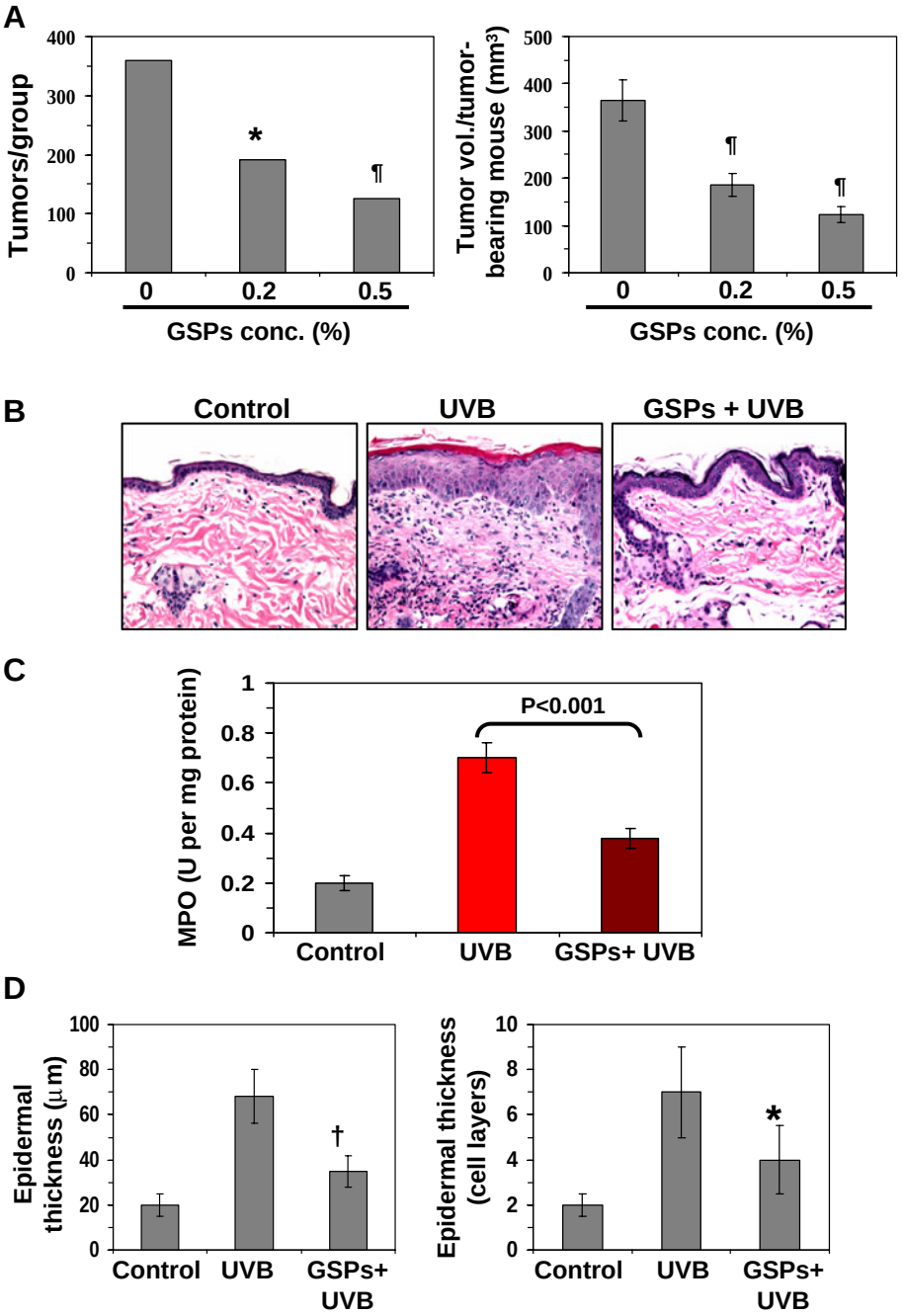

Fig. 1 Dietary GSPs inhibit UVB radiation-induced skin tumor development, cutaneous infiltration and hyperplastic responses in mouse skin. All data were recorded and summarized at the termination of the experiment at 24th week of UVB irradiation. **a** Dietary GSPs inhibit UVB-induced skin tumor development in terms of total number of tumors per group and total tumor volume per tumor-bearing mouse. Tumor data were summarized in terms of total number of tumors/group, and tumor volume is represented as mean (mm^3) ± SD (16), as shown ($n = 20$). Significant inhibition *versus* UVB alone at the termination of the experiment, $^{*}P < 0.05$, $^{¶}P < 0.001$. **b** GSPs (0.5%, *w/w*) inhibit UVB-induced cellular infiltration, as evident by the number of blue-colored nuclei. Representative micrographs of H&E staining are shown from experiments conducted using skin samples and had identical patterns ($n = 10$). **c** MPO was determined as a marker of UVB-induced cutaneous infiltration. The levels of UVB-induced MPO were lower in GSPs (0.5%, *w/w*)-treated mouse skin than non-GSPs-treated UVB-irradiated mouse skin. Data are reported as the mean unit/mg protein ± SD ($n = 10$). Significant difference *versus* UVB alone; $P < 0.001$. **d** Dietary GSPs inhibit UVB-induced hyperplastic response in terms of epidermal thickness (μm) and epidermal thickness in terms of cell layers. Significant difference *versus* UVB alone; $^{*}P < 0.05$, $^{†}P < 0.01$.

indicated that chronic exposure of the skin to UVB radiation resulted in enhanced expression of COX-2 as compared to the non-UVB-exposed mouse skin (Fig. 2a). Administration of GSPs in diet reduced the expression of COX-2 in UVB-exposed skin as evidenced by the intensity pattern of immunostaining of COX-2. These observations were further confirmed by western blot analysis that showed higher expression levels of COX-2 protein in UVB-exposed mouse skin than non-UVB-exposed control skin, and the GSPs inhibited UVB-induced expression of COX-2 protein compared with non-GSPs-treated UVB-exposed skin, as shown in Fig. 2b. The levels of PGE_2 were also determined in these samples. As shown in Fig. 2c, the levels of PGE_2 in the skin of the UVB-exposed mouse skin were significantly higher ($P < 0.001$) than non-UVB-exposed mouse skin samples. The administration of dietary GSPs significantly inhibited ($P < 0.001$) UVB-induced elevation in the levels of PGE_2 in the mouse skin.

GSPs Inhibit UVB-Induced Increases in the Levels of Proliferating Markers in the Skin

UVB-induced inflammatory responses lead to cellular proliferation in skin cells; therefore, we then determined the proliferation potential of epidermal cells as another marker of the UVB-induced inflammatory reaction in the skin. For this purpose, the levels of PCNA and cyclin D1 were determined. Using immunohistochemical detection analysis (Fig. 3a), we found that chronic exposure of the skin to UVB enhanced the expression of PCNA in epidermal cells as evidenced by the intenser dark brown staining of PCNA-positive cells than non-UVB-exposed control skin, and the dietary GSPs inhibited UVB-induced expression of PCNA in epidermal cells. These data were further confirmed by the analysis of mRNA expression by real-time PCR and protein expression levels by western blot analysis, as shown in Fig. 3b and c. Real-time PCR and western blot analyses revealed that dietary GSPs inhibited UVB-induced increases in the expression of levels of PCNA in the epidermal samples. Similarly, we determined the levels of cyclin D1, another marker of cellular proliferation. As shown in Fig. 3b and c (right panels), the mRNA and protein levels of cyclin D1 were higher in UVB-exposed skin than non-UVB-exposed control mouse skin, and dietary GSPs inhibited the expression levels of cyclin D1 in UVB-exposed mouse skin compared to non-GSPs-treated UVB-exposed skin.

GSPs Inhibit the Levels of UVB-Induced Increases of Proinflammatory Cytokines in Mouse Skin

Exposure of the skin to UVB radiation resulted in the induction of inflammatory responses, including the induction of proinflammatory cytokines, and these responses are further enhanced by UVB-induced infiltrating leukocytes at the UVB-irradiated site of the skin. Therefore, we examined and compared the effects of GSPs on the UVB-induced proinflammatory cytokines in the mouse skin. Skin samples were analyzed for the levels of TNF-α, IL-1β and IL-6 using cytokine-specific ELISA kits following the manufacturer's protocol. As shown in Table I, chronic exposure of the skin to UVB radiation resulted in significantly higher accumulation of TNF-α ($P<0.001$), IL-1β ($P<0.001$) and IL-6 ($P<0.001$) in the skin of mice as compared to non-UVB-exposed control mice. However, the levels of these proinflammatory cytokines were significantly inhibited (TNF-α, 47%; IL-1β, 59%; IL-6, 58%) by dietary GSPs in the UVB-irradiated skin of mice compared to non-GSPs-treated UVB exposed mice.

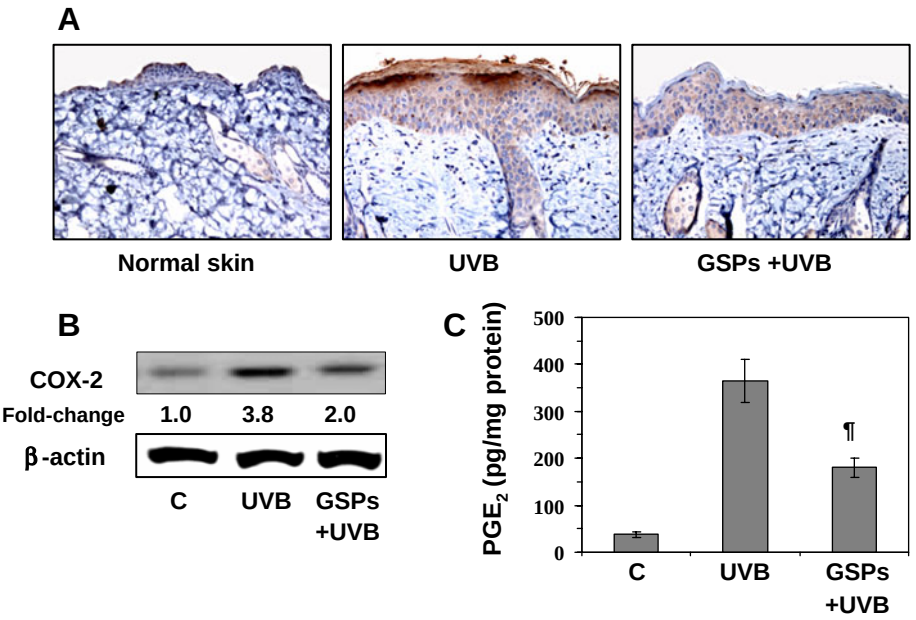

Fig. 2 GSPs inhibit the expression of COX-2 and PGE_2 in UVB-irradiated mouse skin. a Frozen skin sections (5 μm thick) were subjected to immunoperoxidase staining to detect COX-2 expression that is dark brown. b The levels of COX-2 were determined in epidermal lysate samples using western blot analysis. Equal loading of protein samples was confirmed using β-actin. Experiments were conducted and repeated separately using samples obtained from ten animals in each group with almost identical observations. A representative blot is shown. The relative density (arbitrary) of each band after normalization for β-actin is shown under immunoblot as a fold-change compared with non-UVB-exposed control, which has been assigned an arbitrary unit 1. c Epidermal PGE_2 was determined as a marker of inflammation using PGE_2 immunoassay kit as described in Materials and Methods, and the concentration of PGE_2 is expressed in terms of pg per mg protein as a mean ± SD, n = 10. Significant difference versus UVB alone, $^{¶}P<0.001$.

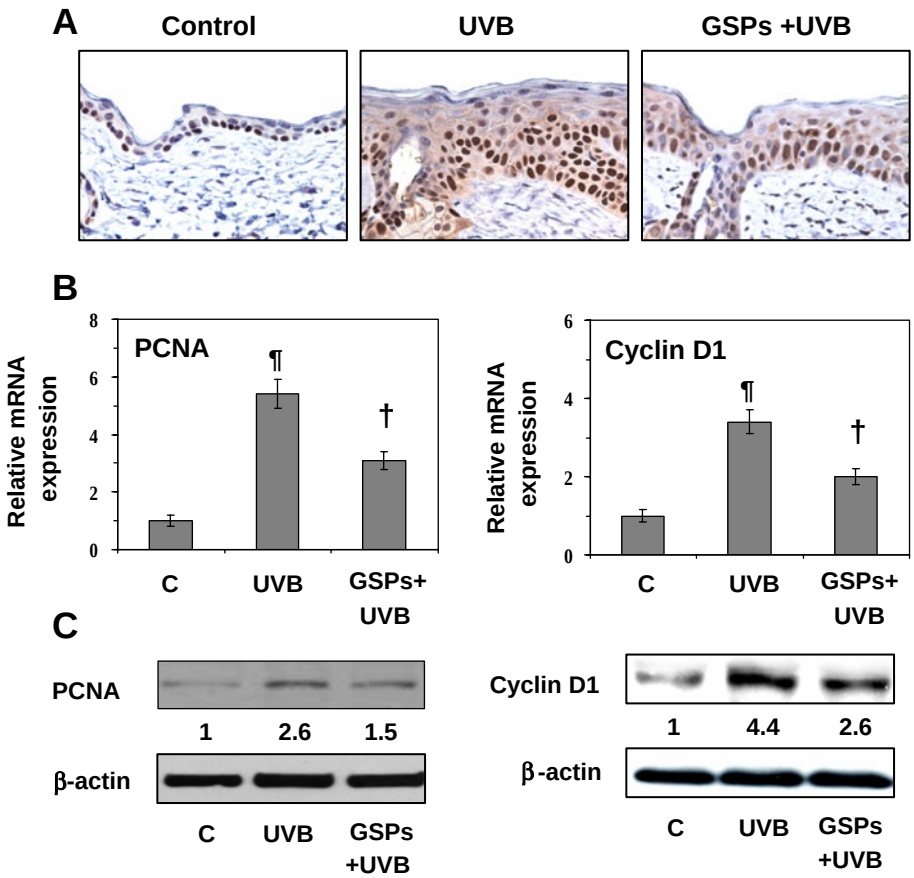

Fig. 3 Dietary GSPs inhibit the proliferation potential of epidermal cells in UVB-exposed mouse skin. Skin samples were collected for the analysis of biomarkers of proliferation at the termination of the photocarcinogenesis experiment at 24th week. **a** Frozen skin sections (5 μm thick) were subjected to immunoperoxidase staining to detect PCNA expression that is dark brown. **b**, The epidermal mRNA expression of PCNA and cyclin D1 was determined using real-time PCR. The mRNA expression of PCNA and cyclin D1 is presented after normalization to β-actin using the C_t method. $n = 10$. Significant inhibition *versus* UVB alone, $^{\dagger}P < 0.01$. Significant increases *versus* non-UVB-exposed control skin, $^{\P}P < 0.001$. **c**, The protein expression of epidermal PCNA and cyclin D1 was analyzed using western blotting. Representative examples of blots are shown from 3 sets of experiments. The samples in each set were prepared by pooling the epidermis from 3 different mice that showed identical results, $n = 10$. Data under each blot indicates the fold-change in band intensity compared to control.

Dietary GSPs Inhibit the UVB-Induced Enhancement of Expression of COX-2, PGE_2, Cellular Proliferation Potential and Proinflammatory cytokines in Skin Tumors

After examining the effect of dietary GSPs on the status of UVB-induced inflammation and their mediators in the skin of mice, we further checked the effect of GSPs on the status of these inflammatory mediators in the UVB-induced skin tumors. The UVB-induced skin tumors were subjected to the analysis of COX-2, PCNA, PGE_2 and proinflammatory cytokines expression using immunostaining, western blot analysis, ELISA and immunoassay for quantitation of PGE_2. Immunostaining analyses clearly indicated that GSPs inhibited the levels of COX-2 and PCNA in terms of intense staining pattern and the number of COX-2- and PCNA-positive cells in the skin tumors compared to non-GSPs-treated skin tumors (Fig. 4a). This observation was further confirmed by checking the status of COX-2 and PCNA proteins in the tumor lysate samples using western blot analysis. The intensities of the bands in western blot analysis indicated that the protein levels of COX-2 and PCNA were relatively lower in the tumors of GSPs-treated mice compared to the skin tumors of non-GSPs-treated mice (Fig. 4b). The levels of cyclin D1, another marker of cellular proliferation, were also inhibited by the administration of GSPs in the skin tumors compared to the tumors of non-GSPs-treated mice (Fig. 4b). PGE_2 immunoassay analysis in skin tumor samples indicated that dietary GSPs significantly reduced the levels of PGE_2 (54%, $P < 0.01$) in skin tumors compared to non-GSPs-treated skin tumors (Fig. 4c).

Analysis of the levels of proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, in tumor samples was also performed by ELISA. As shown in Table II, dietary GSPs inhibited the levels of TNF-α (45% inhibition, $P < 0.001$), IL-6 (43% inhibition, $P < 0.01$) and IL-1β (39% inhibition, $P < 0.01$) in the skin tumors compared to non-GSPs-treated skin tumors.

DISCUSSION

Studies have shown that naturally occurring polyphenols, specifically those present in fruits and vegetables, beverages like green tea, and several herbs and plants with diverse pharmacological activities, are a promising class of phyto-

Table I Dietary GSPs (0.5%, *w/w*) Inhibit UVB-Induced Expression of Pro-inflammatory Cytokines in Mouse Skin

	TNF-α	IL-1β	IL-6
Control	7.0± 1.5	10.0± 2	5.0± 1.6
UVB alone	39.2± 3[a]	34.2± 3[a]	29.0±6.0[a]
GSPs + UVB	24.2 ± 4(47)[b]	20.0 ±4 (59)[c]	15.1 ± 4.0 (58)[c]

Mice were sacrificed at the termination of the photocarcinogenesis experiment at 24th week, and skin samples were collected and subjected to the analysis of cytokines using ELISA following the manufacturer's instructions.

Data on pro-inflammatory cytokines are presented as pg/mg protein. Values are means ± SD, $n=10$.

Data in parentheses indicate the percent inhibition by dietary GSPs.

Significant increase in UVB irradiated group *vs* non-UVB-exposed control group, [a] $p<0.001$.

Significant decrease in GSPs + UVB group *vs* UVB alone, [b] $p<0.01$; [c] $p<0.001$.

chemicals which inhibit tumor growth and development (16,27–30). GSPs differ from extensively studied polyphenols from green tea 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 (15,16), whereas green tea polyphenols are mainly composed of monomers, such as catechins, epicatechins and their gallate esters, including (−)-epigallocatechin-3-gallate (31). However, the chemopreventive action of dietary GSPs in inhibiting photocarcinogenesis appears identical to the activity of green tea polyphenols (15,23).

Long-term exposure of the skin to solar UV radiation is considered a major etiologic factor for the development of melanoma and non-melanoma skin cancers. In addition to several other adverse biologic effects of UV radiation, UV-induced chronic and sustained inflammation has been implicated in skin tumor development. In our continuing

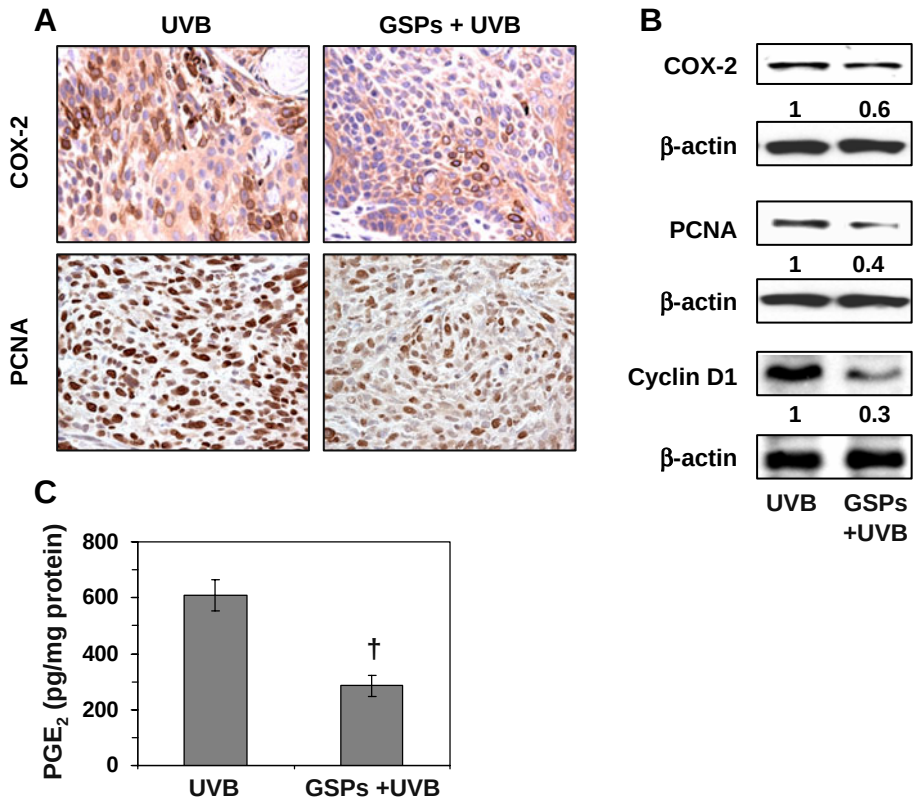

Fig. 4 Dietary GSPs inhibit inflammation in UVB-induced skin tumors. At the termination of the photocarcinogenesis experiment at 24th week (Ref. 16, Fig. 1a), mice were sacrificed, and skin tumors were harvested for the analysis of biomarkers of inflammation. a Paraffin-embedded tumor sections (5 μm thick) were subjected to immunoperoxidase staining to detect the expression level of COX-2-positive and PCNA-positive cells that is dark brown following the procedure as described in Materials and Methods. b Tumor lysates were used to analyze the protein levels of COX-2, PCNA and cyclin D1 using western blot analysis. The representative blots are shown from three independent experiments, and in each experiment, the tumor samples were pooled from at least three mice for preparation of lysates (n = 10), and equivalent protein loading was checked by probing stripped blots for β-actin as shown. c The levels of PGE_2 were determined using PGE_2 immunoassay kit, and the concentration of PGE_2 is expressed in terms of pg/mg protein as a mean ± SD, n = 10. Significant inhibition versus UVB alone, $^{\dagger}P<0.01$.

Table II Dietary GSPs (0.5%, *w/w*) Inhibit the Levels of Pro-inflammatory Cytokines in Mouse Skin Tumors

	UVB	GSPs + UVB	% Inhibition
TNF-α	29 ± 5	16 ± 4	45[b]
IL-1β	33 ± 5	20 ± 4	39[a]
IL-6	28 ± 4	16 ± 4	43[a]

Mice were sacrificed at the termination of the photocarcinogenesis experiment at the 24th week, and tumor samples were collected and subjected to the analysis of cytokines using ELISA following the manufacturer's instructions.

Data on pro-inflammatory cytokines are presented as pg/mg protein. Values are means ± SD, $n = 10$.

Significant decrease in GSPs + UVB group *vs* UVB alone, [a] $p < 0.01$; [b] $p < 0.001$.

efforts to discover newer and more effective dietary phytochemicals for the prevention of skin cancer, we have found that dietary GSPs have the ability to inhibit photocarcinogenesis in mice (16). To determine the chemopreventive mechanism of skin cancer by GSPs, we assessed the effect of GSPs on UVB-radiation-induced inflammation and inflammatory mediators as effective targets of chemoprevention. The finding of the present study is that the inhibition of UVB-induced skin tumor development by dietary GSPs is associated with the anti-inflammatory effects of the GSPs.

One of the most important enzymes in the process of inflammation and tumor development in UV-carcinogenesis is inducible COX-2. COX-2 is a rate-limiting enzyme for the generation of PG metabolites from arachidonic acid (32). COX-2 overexpression has been linked to the pathophysiology of inflammation and cancer (33) due to enhanced synthesis of PG metabolites, which have been shown to be a potential contributing factor in the development of nonmelanoma skin cancers (34,35). A number of studies have demonstrated overexpression of COX-2 in chronically UVB-irradiated skin, as well as in UVB-induced premalignant lesions and squamous-cell carcinomas (8,36,37). A role for COX-2 in photocarcinogenesis is also supported by several studies that demonstrate that inhibition of COX-2 activity by specific inhibitors can partially block carcinogenesis induced by long-term UVB exposures (38,39). In this study, we found that dietary administration of GSPs significantly inhibited UVB-induced inflammatory responses in terms of inhibition of COX-2 expression, PGE_2 production, and reduction in the levels of biomarkers of cellular proliferation, such as epidermal PCNA and cyclin D1. The inhibition of proliferating potential of epidermal keratinocytes by GSPs, which is indicated by the reduced expression of PCNA and cyclin D1 in UVB-exposed skin and skin tumors, may also be a contributing factor for the inhibitory effects of GSPs on the development of skin tumors in UV-irradiated mice.

The role of GSPs in ameliorating the UVB-induced inflammatory responses is further suggested by the finding of higher levels of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, in the non-GSPs-treated UVB-exposed skin of mice than the GSPs-treated UVB-exposed mouse skin. As elevated levels of proinflammatory cytokines have been implicated in skin cancer risk (7,40,41), the higher levels of pro-inflammatory cytokines would be expected to contribute to the tumor promotion process, and thus the development of tumors would be expected to occur earlier and progress more rapidly. This trend was observed in those mice which were not given GSPs in the diet (16). Our data indicated that administration of GSPs significantly inhibited UVB-induced expression of proinflammatory cytokines in mouse skin as well as skin tumors, and that may have contributed in inhibition of tumor development.

UVB-radiation-induced infiltration and accumulation of activated macrophages and neutrophils in 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 (21,42). The levels of MPO are commonly used as a quantitative marker of inflammatory infiltrates. Normal skin exhibits low background levels of MPO, whereas skin that is inflamed by an infection, wound, or exposure to UV radiation exhibits higher levels of MPO (21,24,42). Our analysis of the effects of GSPs showed that dietary GSPs inhibited UVB-induced infiltration of leukocytes in the mouse skin as well as reduced the levels of MPO, suggesting lower levels of inflammation in UVB-irradiated mouse skin by dietary GSPs.

Collectively, the data from this study suggest that anti-photocarcinogenic activity of GSPs is associated with the inhibition of UVB-induced inflammation and inhibition of inflammatory mediators in mouse skin. The outcome of this study, in conjunction with prior publications from our laboratory and others, suggests that GSPs may prove to be useful chemopreventive agent against UVB-radiation-induced inflammation-associated skin diseases, including melanoma and non-melanoma skin cancers in humans, and, therefore, more detailed studies with particular emphasis on molecular mechanisms could lead to new strategies for the prevention of inflammation-associated skin diseases in humans.

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