

Oral Feeding of Pomegranate Fruit Extract Inhibits Early Biomarkers of UVB Radiation-induced Carcinogenesis in SKH-1 Hairless Mouse Epidermis

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ABSTRACT

Pomegranate from the plant *Punica granatum* L. possesses strong antioxidant and anti-inflammatory properties. Recently, we have demonstrated that treatment of normal human epidermal keratinocytes with pomegranate fruit extract (PFE) inhibited UVB-mediated activation of nuclear factor kappa B (NF- κ B) and mitogen activated protein kinases pathways. Here, we evaluated the effect of PFE on early biomarkers of photocarcinogenesis employing SKH-1 hairless mice. PFE was provided in drinking water (0.2%, wt/vol) to SKH-1 hairless mice for 14 days before a single UVB (180 mJ cm⁻²) irradiation. We found that oral feeding of PFE inhibited UVB-induced: (1) skin edema; (2) hyperplasia; (3) infiltration of leukocytes; (4) lipid peroxidation; (5) hydrogen peroxide generation; (6) ornithine decarboxylase (ODC) activity; and (7) ODC, cyclooxygenase-2 and proliferating cell nuclear antigen protein expression. Oral feeding of PFE enhanced repair of UVB-mediated formation of cyclobutane pyrimidine dimers (CPDs) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG). Importantly, PFE treatment further enhanced UVB-mediated increase in tumor suppressor p53 and cyclin kinase inhibitor p21. Furthermore, oral feeding of PFE inhibited UVB-mediated: (1) nuclear translocation of NF- κ B; (2) activation of IKK α ; and (3) phosphorylation and degradation of I κ B ζ . Taken together, we provide evidence that oral feeding of PFE to mice affords substantial protection from the adverse effects of UVB radiation *via* modulation in early biomarkers of photocarcinogenesis and provide suggestion for its photochemopreventive potential.

INTRODUCTION

Nonmelanoma skin cancer accounts for more than 1 million cases of human malignancies annually in the United States and the incidence continues to rise (1,2). Solar UV radiation, particularly its UVB (290–320 nm) component, is the foremost cause of nonmelanoma skin cancers in humans, particularly in Caucasian individuals (2,3). UVB radiation is a complete carcinogen and causes excessive generation of reactive oxygen species (ROS) thus resulting in an oxidative stress in the skin

(4,5). Studies have shown that UVB radiation produces a variety of adverse effects that includes DNA damage (6,7), mutations in key regulatory genes (7,8), inflammation (9,10), immunosuppression (11,12), photoaging and skin cancer (2,13). UVB is directly absorbed by cellular DNA leading to the formation of DNA lesions primarily cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6-4)-pyrimidone photoproducts (4,14). In contrast, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) is induced by ROS and has been proposed as a key biomarker of oxidative DNA damage relevant to carcinogenesis (15,16).

Mechanisms that contribute to UVB-induced mutagenesis and carcinogenesis comprise inactivation of tumor suppressor genes and/or activation of oncogenes (17,18). Cellular responses to DNA damage by UVB radiation are usually multifaceted and often regulated by more than one transcription factor, for instance by both p53 and nuclear factor kappa B (NF- κ B). Studies have shown that DNA damage induced by UVB radiation triggers a rapid increase in p53, enhancing p21 synthesis and shutting off cell replication and DNA synthesis, allowing extended time for either DNA repair or apoptosis induction in the cells carrying UVB-damaged DNA (19,20). In both cases, the risk of UVB-induced skin cancer is reduced. Studies have shown that UVB-mediated activation of NF- κ B plays an imperative role in inflammation, cell proliferation and skin carcinogenesis (21,22).

As skin cancer is a significant problem associated with mortality and morbidity intensive efforts are required to develop novel strategies for the prevention of UV responses. There has been substantial interest in the identification of botanical agents capable of affording protection to skin from the adverse effects of solar UVB radiation. These botanical antioxidants for human use should have the ability to ameliorate the adverse biological effects of UV radiation. Pomegranate (*Punica granatum* L.) fruit widely consumed fresh and in beverage as juice or wine has been extensively used in traditional medicine in various parts of the world. Pomegranate fruit extract (PFE) derived from the tree *P. granatum* L. contains several polyphenols (such as catechins, gallic and ellagic acids) and anthocyanidins (such as delphinidin, cyanidin and pelargonidin) (23), and its antioxidant activity is superior than that of red wine and green tea (24). Studies have shown that PFE possesses strong anti-inflammatory (23), antiproliferative (25,26) and antitumorigenic properties (23,25). We recently demonstrated that PFE treatment of

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normal human epidermal keratinocytes resulted in inhibition of UVB-mediated activation of NF- κ B and mitogen activated protein kinases (MAPK) pathways (27). The objective of this study was to investigate the effect of oral feeding of PFE on early biomarkers of photocarcinogenesis employing SKH-1 hairless mice.

MATERIALS AND METHODS

Materials. The mono- and polyclonal antibodies (proliferating cell nuclear antigen [PCNA], ornithine decarboxylase [ODC], p53 and IKK α) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). CPD antibody was procured from Kamiya Biomedical Company (Seattle, WA). The monoclonal and polyclonal antibodies for cyclooxygenase-2 (COX-2) and p21 were obtained from Cell Signaling Technology (Beverly, MA). The 8-oxodG antibody was purchased from Chemicon International, Inc. (Temecula, CA). I κ B α and I κ B β (Phospho) antibodies were obtained from New England Biolabs (Beverly). NF- κ B/p65 antibody was procured from Geneka Biotechnology (Montreal, Canada). Antimouse secondary horse radish peroxidase conjugate was obtained from GE Healthcare (Piscataway, NJ). The DC BioRad Protein assay kit was purchased from BioRad Laboratories (Hercules, CA). Novex precast Tris-Glycine gels were obtained from Invitrogen (Carlsbad, CA).

Preparation of pomegranate fruit extract. Fresh fruit of pomegranate was peeled and its edible portion (seed coat and juice) was squeezed in 70% acetone–30% distilled water (1:20, by w/v). The red extract was filtered through filter paper (Whatman No. 1). The filtrate was condensed, freeze-dried and stored at 4°C (23).

Animals and treatment. Female SKH-1 hairless mice (6 weeks old) obtained from Charles River Laboratories (Wilmington, MA) were used in this study. After their arrival in the animal facility, the animals were allowed to acclimatize for 1 week before the start of the experiments. The animals were fed Purina Chow diet and water *ad libitum*. The mice were maintained at standard conditions: temperature of 24 \pm 2°C, relative humidity of 50 \pm 10% and 12 h room light per 12 h dark cycle. For UVB irradiation, the mice were housed in specially designed cages where they were held in dividers separated by Plexiglas. Sixty-four female SKH-1 hairless mice, maintained as described, were divided into four groups of 24 animals each (except in control and PFE alone groups, where eight animals were used). The mice in the first group received drinking water and served as a control, and those in the second group received PFE (0.2%, wt/vol) dissolved in drinking water. The mice in the third group received a single UVB (180 mJ cm⁻²) exposure with a custom designed Research Irradiation Unit (Daavlin, Bryan, OH) that consists of a fixture mounted on fixed legs. Mounted within the exposure unit are four UVB lamps and the exposure system is controlled using Daavlin Flex Control Integrating Dosimeters. The UV lamps emit UVB (280–320 nm; ca 80% of total energy) and UVA (320–375 nm; ca 20% of total energy), with UVC emission being insignificant. In this system dose units can be entered in mJ cm⁻² for UVB; variations in energy output are automatically compensated for the delivery of the desired dose. Using this system, the mice were exposed to accurate dosimetry of UVB radiation. The mice in the fourth group received PFE (0.2%, wt/vol) for 14 days before a single UVB (180 mJ cm⁻²) irradiation and continued until the termination of the experiment. The mice were then sacrificed at 1, 8 and 24 h post UVB exposure and skin biopsies were harvested for biochemical analysis.

Edema and hyperplasia. To assess the inhibitory effect of oral feeding of PFE on UVB-induced skin edema the mice were exposed to UVB (180 mJ cm⁻²) and 24 h post UVB exposure bifold-skin thickness was measured. At least four determinations were made at different dorsal skin sites per mouse in each group. For the hyperplasia study, skin was removed, fixed in 10% formalin and embedded in paraffin. Vertical sections (5 μ m) were cut, mounted on a glass slide, and stained with hematoxylin and eosin.

Immunohistochemical detection of CPDs and 8-oxodG. The skin biopsies were frozen in optimal cutting temperature (OCT) compound under liquid nitrogen immediately after removal and were stored at -80°C for further use. Briefly, 5 μ m thick frozen skin sections were thawed and fixed in cold acetone for 10 min and washed in PBS (pH:

7.4). The sections were then kept in 70 mmol L⁻¹ NaOH in 70% ethanol for 2 min to denature nuclear DNA followed by neutralization for 1 min in 100 mmol L⁻¹ Tris-HCl (pH 7.5) in 70% ethanol. Nonspecific binding sites were blocked by 30 min incubation at room temperature in PBS containing 10% goat serum. Sections were then incubated with mouse monoclonal antibodies to 8-oxodG and CPD. For negative control the sections were incubated without primary antibody. After overnight incubation at 4°C and three washes in PBS, the sections were incubated for 30 min with biotinylated rabbit antimouse link antibody and then for 30 min with streptavidin conjugated to horse radish peroxidase. After washing, sections were incubated with diaminobenzidine (DAB) plus peroxidase substrate and counterstained with methyl green.

Preparation of epidermal skin lysate and nuclear lysate. Epidermis from the whole skin was separated as described earlier (23) and was homogenized in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na₃VO₄, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF [pH 7.4]) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem, La Jolla, CA). The homogenate was then centrifuged at 14 000 g for 25 min at 4°C and the supernatant (total cell lysate) was collected, aliquoted and stored at -80°C. For the preparation of nuclear lysate, 0.2 g of the epidermis was homogenized into 1.0 mL of ice-cold phosphate-buffered saline (pH 7.6) and centrifuged at 12 000 g for 5 min at 4°C. The pellet was resuspended in 1 mL of cold buffer containing 10 mM HEPES (pH 7.9), 2 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA and 0.1 mM PMSF. After homogenization in a tight-fitting Dounce homogenizer, the homogenates were left on ice for 10 min then were centrifuged at 25 000 g for 10 min. The nuclear pellet was resuspended in 0.1 mL of the buffer containing 10 mM HEPES (pH 7.9), 300 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF and 10% glycerol with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III). The suspension was gently shaken for 20 min at 4°C. After centrifugation at 25 000 g for 10 min, the nuclear extracts (supernatants) were collected and quickly frozen at -80°C. The protein content in the lysates was measured by DC BioRad assay (BioRad Laboratories) as per the manufacturer's protocol.

Western blot analysis. For Western blot analysis, 25–50 μ g of the protein was resolved over 8–12% Tris-glycine gels and transferred to a nitrocellulose membrane. The blot containing the transferred protein was blocked in blocking buffer (5% nonfat dry milk, 1% Tween 20; in 20 mM TBS, pH 7.6) for 1 h at room temperature followed by incubation with appropriate monoclonal primary antibody in blocking buffer for 2 h to overnight at 4°C. This was followed by incubation with antimouse or antirabbit secondary antibody horse radish peroxidase for 1 h and then washed several times and detected by chemiluminescence and autoradiography using XAR-5 film obtained from Eastman Kodak Co.

Ornithine decarboxylase enzyme activity. The epidermis from the dissected skin was separated as described earlier (23) and homogenized at 4°C in a glass-to-glass homogenizer in 10 volumes of ODC buffer (50 mM Tris-HCl buffer [pH 7.5] containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM pyridoxal-5-phosphate, 1 mM 2-mercaptoethanol and 0.1% Tween 80). The homogenate was centrifuged at 100 000 g at 4°C and the supernatant was used for enzyme determination. ODC enzyme activity was determined in epidermal cytosolic fraction by measuring the release of ¹⁴CO₂ from the D,L-[¹⁴C] ornithine by the method described earlier (23). Briefly, 400 μ L of the supernatant was added to 0.95 mL of the assay mixture (35 mM sodium phosphate [pH 7.2], 0.2 mM pyridoxal phosphate, 4 mM dithiothreitol, 1 mM EDTA, 0.4 mM L-ornithine containing 0.5 μ Ci of DL-[¹⁴C] ornithine hydrochloride) in 15 mL corex centrifuge tube equipped with rubber stoppers and central well assemblies containing 0.2 mL ethanolamine and methoxyethanol in 2:1(v/v) ratio. After incubation at 37°C for 60 min, the reaction was terminated by the addition of 1.0 mL of 2 M citric acid, using a 21G needle per syringe. The incubation was continued for 1 h. Finally, the central well containing the ethanolamine: methoxyethanol mixture to which ¹⁴CO₂ has been trapped was transferred to a vial containing 10 mL of toluene-based scintillation fluid and 2 mL of ethanol. The radioactivity was measured in a Beckman LS 6000 SC liquid scintillation counter. Enzyme activity was expressed as picomoles CO₂ released per hour per mg protein.

Immunostaining of hydrogen peroxide. Immunohistochemical detection of hydrogen peroxide (H_2O_2) in normal as well as UV-irradiated skin was performed following the procedure as described earlier (28). Briefly, 6 μ m thick frozen skin sections were incubated with 0.1 M Tris-HCl buffer, pH 7.5, containing 1 mg mL⁻¹ glucose and 1 mg mL⁻¹ DAB for 5–6 h at 37°C. Sections were then washed in distilled water and counterstained with methyl green (2% for 60 min). The DAB-peroxidase reaction gave a brown reaction product and methyl green a blue nuclear counterstain.

Lipid peroxidation assay. After treatment, skin samples were harvested and washed with PBS, and microsomal fraction was prepared as described earlier (28). Briefly, cells were homogenized with a Polytron homogenizer in PBS buffer containing potassium chloride (1.19%, w/v) and centrifuged at 18 000 g for 15 min at 4°C to prepare microsomal fraction. The lipid peroxidation (LPO) assay was performed in microsomal fraction obtained from the different treatment groups. The generation of malondialdehyde (MDA) was employed as a marker of LPO and estimated by the method of Wright *et al.* (29). Briefly, microsomal fraction (2.0 mg protein) was incubated for 1 h at 37°C in the presence of ferric ions (1 mM) and ADP (5 mM) in Ca²⁺-free phosphate buffer (0.1 M; pH: 7.4) containing MgCl₂ (0.1 mM). The reaction was terminated by addition of 0.6 mL of 10% (w/v) trichloroacetic acid followed by 1.2 mL of 0.5% (w/v) 2-thiobarbituric acid. The mixture was heated for 20 min at 90°C in a water bath. After cooling, the MDA levels were measured in the clear supernatant by recording absorbance at 532 nm. The final concentration of MDA generated during the reaction was calculated using a molar extinction coefficient of 1.56×10^5 M cm⁻¹.

Statistical analysis. The results are expressed as the mean \pm SE. Statistical analysis of all the data between groups receiving UVB exposure alone and that with PFE treatment plus UVB exposure was performed by Student's *t*-test. The *P* value <0.05 was considered statistically significant.

RESULTS

Oral feeding of PFE inhibits UVB-induced cutaneous edema, hyperplasia and infiltration of leukocytes

Acute exposure of skin to UVB irradiation leads to cutaneous edema, hyperplasia, erythema, infiltration of leukocytes, dilation of dermal blood vessels and vascular hyperpermeability (21,30,31). In the present study, we assessed the effect of oral feeding of PFE on skin edema in UVB exposed SKH-1 hairless mouse. Bifold-skin thickness as an indicator of vascular permeability and edema was measured 24 h post UVB radiation. Exposure of mouse skin to UVB radiation resulted in a significant increase in bifold-skin thickness compared with control and PFE alone treated animals (Fig. 1a). Oral feeding of PFE resulted in a significant inhibition against UVB-induced bifold-skin thickness when compared with UVB alone group (Fig. 1a). We next evaluated the effect of oral feeding of PFE on UVB-mediated induction of epidermal hyperplasia and infiltration of leukocytes, 24 h post UVB radiation. Hematoxylin-eosin staining of mouse dorsal skin sections revealed that UVB irradiation to the mouse skin resulted in hyperplasia, and mixed cell infiltration in the dermis, which comprised mostly neutrophils with some mononuclear cells admixed when compared with control treated animals (Fig. 1b). However, these effects of UVB-induced infiltration of leukocytes and epidermal hyperplasia were inhibited by oral feeding of PFE (Fig. 1b).

Oral feeding of PFE inhibits UVB-induced lipid peroxidation and generation of H_2O_2 in SKH-1 hairless mice

One of the hallmarks of UVB-induced oxidative stress is the formation of oxidized macromolecules, including LPO (28,32).

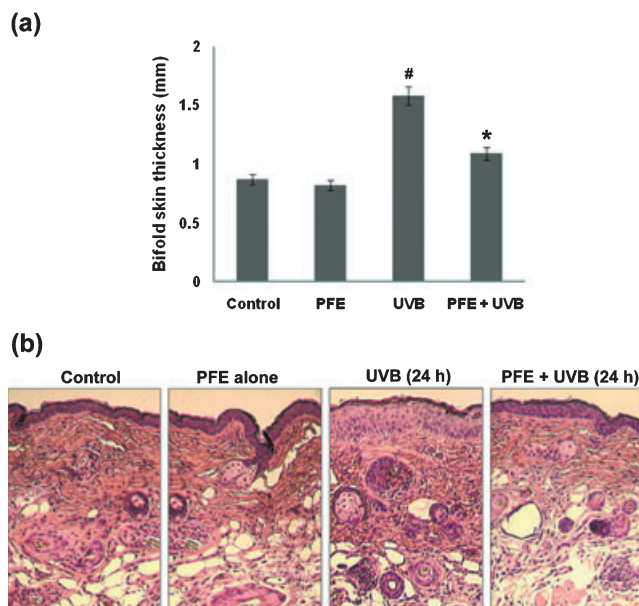


Figure 1. Inhibitory effect of oral feeding of pomegranate fruit extract (PFE) on UVB-induced cutaneous edema, hyperplasia and infiltration of leukocytes in SKH-1 hairless mice. The groups of mice were either unexposed (control), received PFE in drinking water (0.2%, wt/vol), exposed to UVB radiation (180 mJ cm⁻²), received PFE in drinking water (0.2%, wt/vol) for 14 days before a single UVB (180 mJ cm⁻²) irradiation and continued until the termination of the experiment. (a) Twenty-four hours after UVB irradiation, the skin edema was determined by measuring the bifold-skin thickness. At least four determinations were made at different dorsal skin sites per mouse in each group. The data represents the mean \pm SE of eight mice ([#]*P* < 0.001 vs control; ^{*}*P* < 0.001 vs UVB). (b) At 24 h post UVB irradiation, the animals were sacrificed and skin biopsies were processed for hyperplasia study by hematoxylin and eosin staining. Representative pictures are shown.

Therefore, we evaluated the effect of oral feeding of PFE on UVB-mediated increase in LPO, which is a well-accepted marker of photo-oxidative damage. UVB irradiation to mouse skin resulted in a significant induction of LPO in the epidermal microsomal fraction 24 h after UVB irradiation. Oral feeding of PFE significantly inhibited the UVB-mediated enhancement of LPO (Fig. 2a). We next assessed the effect of oral feeding of PFE on UVB-mediated generation of H_2O_2 in mouse skin. Immunohistochemical analysis data revealed that UVB irradiation of mouse skin resulted in increased production of H_2O_2 in the skin, 24 h post UVB irradiation compared with control and PFE alone groups (Fig. 2b). In skin samples obtained 24 h after UVB exposure the numbers of H_2O_2 positive cells were significantly higher when compared with control group (Fig. 2c). Oral feeding of PFE abrogated the numbers of H_2O_2 -producing cells compared with the UVB-irradiated animals (Fig. 2b,c).

Oral feeding of PFE inhibits UVB-induced epidermal ODC activity and protein expression

Ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis is upregulated in skin tumors compared with normal skin (33). Studies have shown that increase in ODC activity plays a critical role in skin tumor promotion in

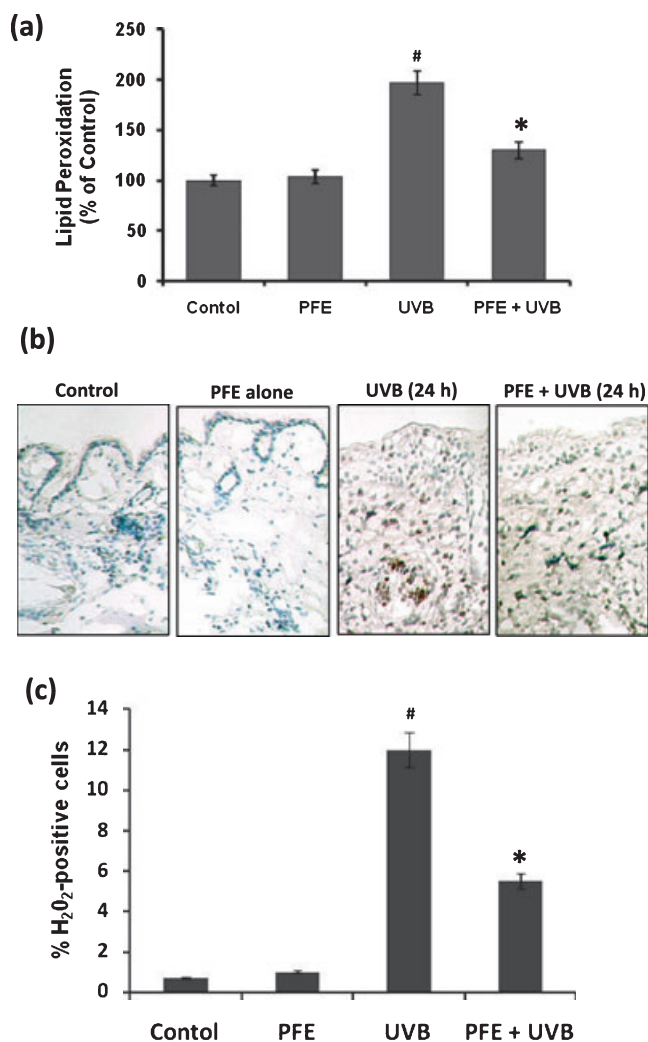


Figure 2. Inhibitory effect of oral feeding of pomegranate fruit extract on UVB-induced generation of hydrogen peroxide (H₂O₂) and lipid peroxidation in SKH-1 hairless mice. Animals in each group were treated as described in Fig. 1. (a) Twenty-four hours after UVB irradiation, the animals were sacrificed, and epidermal microsomal fraction was prepared. Generation of malondialdehyde was used as a marker of lipid peroxidation. The data represents the mean \pm SE of eight mice ([#] $P < 0.001$ vs control; $*P < 0.001$ vs UVB). (b) Twenty-four hours after UVB irradiation, the animals were sacrificed, and immunohistochemical detection of hydrogen peroxide (H₂O₂) was performed using the DAB-peroxidase reaction. Representative pictures are shown. (c) The numbers of H₂O₂ positive cells were counted in five different areas of the sections under a microscope. The numbers of H₂O₂ positive cells are represented as percent of H₂O₂ positive cells in the control group. The data represents the mean \pm SE of eight mice ([#] $P < 0.001$ vs control; $*P < 0.001$ vs UVB).

animal models (34). Therefore, we studied the effect of oral feeding of PFE on UVB-mediated induction of epidermal ODC activity in SKH-1 hairless mice. UVB irradiation to SKH-1 hairless mouse skin resulted in a significant increase in epidermal ODC activity, at 24 h post UVB treatment compared with control and PFE alone groups. Oral feeding of PFE significantly inhibited UVB-induced epidermal ODC activity when compared with UVB alone group (Fig. 3a). We next examined the effect of oral feeding of PFE on UVB-induced epidermal ODC protein expression. UVB exposure

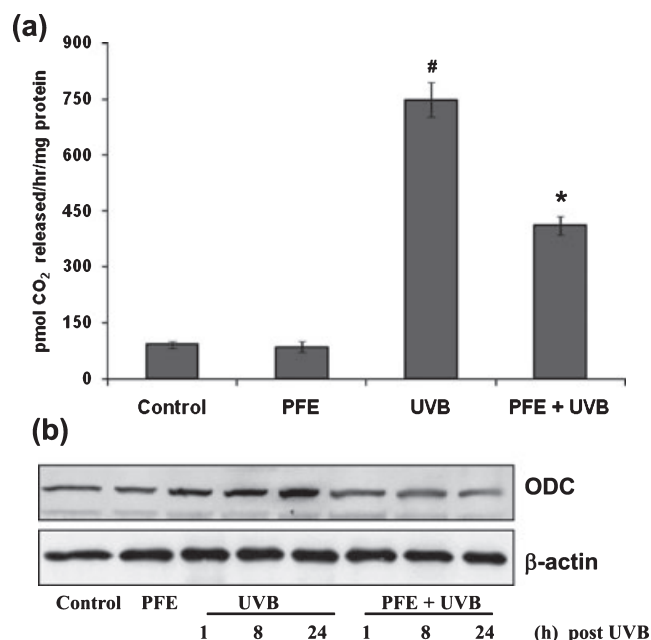


Figure 3. Inhibitory effect of oral feeding of pomegranate fruit extract on UVB-induced epidermal ornithine decarboxylase (ODC) activity and protein expression in SKH-1 hairless mice. Animals in each group were treated as described in Fig. 1. (a) At 24 h post UVB irradiation, the animals were sacrificed, epidermal cytosolic fraction was prepared and ODC activity was determined. The data represents the mean \pm SE of eight mice ([#] $P < 0.001$ vs control; $*P < 0.001$ vs UVB). (b) At different time points post UVB irradiation, animals were sacrificed. The epidermis was separated, epidermal protein lysates were prepared and Western blot analysis was performed to determine the protein expression of ODC. Equal loading was confirmed by stripping the Western blot and reprobing it for β -actin. The representative blots are shown from three independent experiments.

to SKH-1 hairless mice resulted in a marked increase in epidermal ODC protein expression in time-dependent manner (*i.e.* at 1, 8 and 24 h post UVB) compared with control group as determined by Western blot analysis (Fig. 3b). Oral feeding of PFE resulted in inhibition in ODC protein expression when compared with UVB alone groups at all time points (Fig. 3b).

Oral feeding of PFE inhibits UVB-induced epidermal COX-2 and proliferating cell nuclear antigen protein expression

Studies have demonstrated that the expression of proinflammatory enzyme COX-2 is induced by UVB exposure (28,35). Therefore, we determined the effect of oral feeding of PFE on UVB-induced epidermal COX-2 protein expression. Western blot analysis revealed that UVB exposure to SKH-1 hairless mice resulted in a marked increase in epidermal COX-2 protein expression in a time-dependent manner compared with control group (Fig. 4). Oral feeding of PFE resulted in inhibition COX-2 protein expression (Fig. 4). It is well known that exposure of skin to UVB radiation induces proliferative potential of epidermal cells (36). We, therefore next investigated the effect of oral feeding of PFE on UVB-induced expression of PCNA (marker of proliferation). We found that oral feeding of PFE resulted in inhibition of PCNA protein expression when compared with UVB alone group (Fig. 4).

Oral feeding of PFE inhibits UVB-induced formation of CPDs and 8-oxodG in SKH-1 hairless mice

It is well documented that both CPDs and 8-oxodG are formed after UVB irradiation and considered as important biomarkers of DNA damage (6,15). Employing immunohistochemical analysis, we assessed the effect of oral feeding of PFE on UVB-mediated DNA damage in mouse epidermis. UVB irradiation to SKH-1 hairless mouse skin resulted in

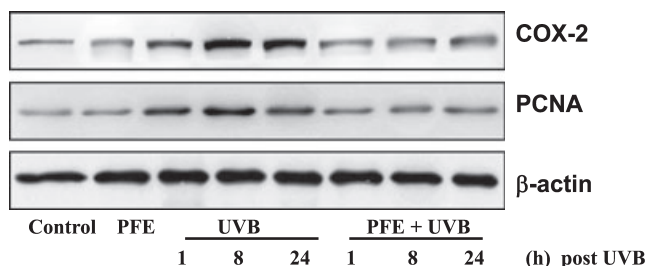


Figure 4. Inhibitory effect of pomegranate fruit extract on UVB-induced epidermal cyclooxygenase-2 (COX-2) and proliferating cell nuclear antigen (PCNA) protein expression in SKH-1 hairless mice. At different time points post UVB irradiation, animals were sacrificed. The epidermis was separated, epidermal protein lysates were prepared and Western blot analysis was performed to determine the protein expression of COX-2 and PCNA. Equal loading was confirmed by stripping the Western blot and reprobing it for β -actin. The representative blots are shown from three independent experiments.

increased formation of both CPDs (Fig. 5a) and 8-oxodG (Fig. 5c) when compared with their respective control groups. In skin samples obtained 24 h after UVB exposure the numbers of CPD positive cells (Fig. 5b) and 8-oxodG positive cells (Fig. 5d) were significantly higher when compared with their respective control groups. Oral feeding of PFE to SKH-1 hairless mice resulted in marked inhibition of UVB-induced formation of CPDs (Fig. 5a,b) and 8-oxodG (Fig. 5c,d) when compared with their respective UVB alone groups. These observations suggest that endogenous defense mechanism may play a role in the repair of UVB-induced DNA damage in mice that received PFE.

Oral feeding of PFE enhances UVB-mediated increase in p53 and p21 protein expression in SKH-1 hairless mice

Studies have shown that the expression levels of p53 as well as its downstream target p21 were increased in response to UVB radiation (20). Therefore, we evaluated the effect of oral feeding of PFE on UVB-mediated increase in the expression of p53 and p21 proteins. The Western blot data demonstrated that UVB irradiation resulted in an increase in p53 and p21 proteins in time-dependent manner compared with their respective control groups (Fig. 6). Oral feeding of PFE resulted in a further enhancement in UVB exposure-mediated increase in these proteins in SKH-1 hairless mouse epidermis (Fig. 6).

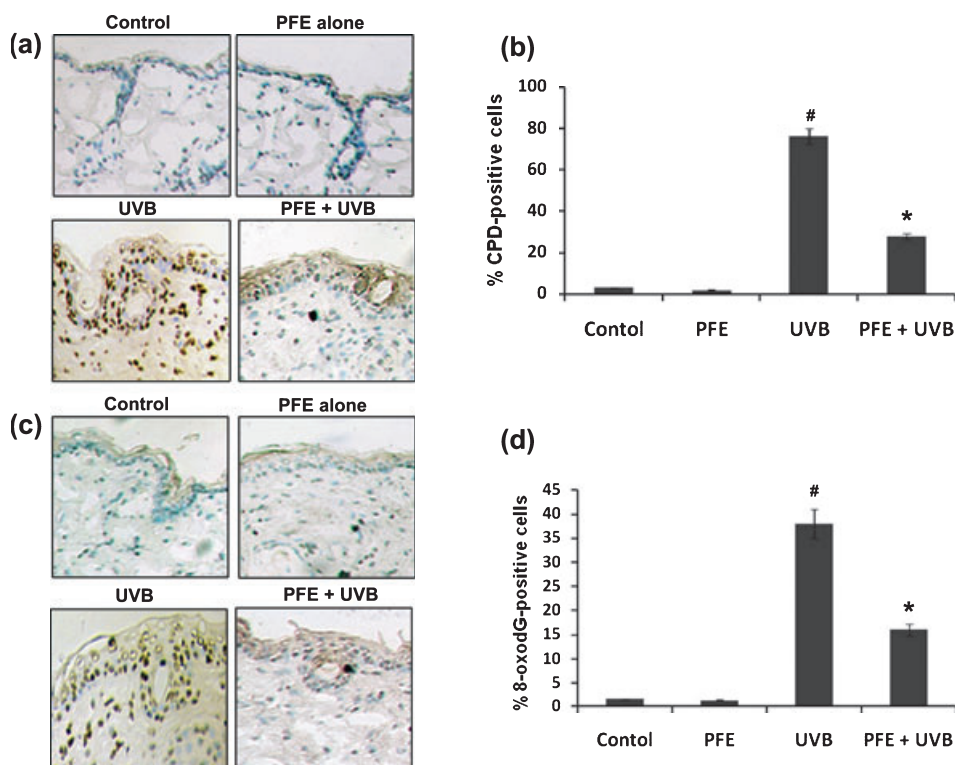


Figure 5. Inhibitory effect of pomegranate fruit extract on UVB-induced formation of cyclobutane pyrimidine dimers and 8-oxo-7,8-dihydro-2'-deoxyguanosine in SKH-1 hairless mice. Twenty-four hours post UVB irradiation, the animals were sacrificed, skin biopsies were taken and frozen in OCT. Skin sections, 6 μ m thick, were cut and processed for immunostaining. Immunohistochemical staining for cyclobutane pyrimidine dimers (CPDs) (a), and 8-oxodG (c) was performed using appropriate antibodies. Representative pictures are shown. The number of CPD positive cells (b) and 8-oxodG positive cells (d) after immunostaining were counted in five different areas of the sections under a microscope. The numbers of CPD and 8-oxodG positive cells are represented as percent of CPD and 8-oxodG positive cells, respectively. The data represent the mean \pm SE of eight mice ([#] $P < 0.001$ vs control; ^{*} $P < 0.001$ vs UVB).

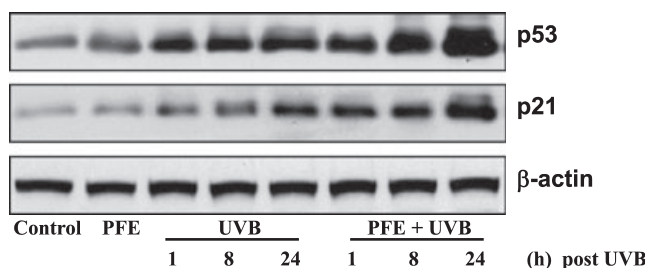


Figure 6. Oral feeding of pomegranate fruit extract enhances UVB-mediated increase in p53 and p21 protein expression in SKH-1 hairless mice. At different time points post UVB irradiation, animals were sacrificed. The epidermis was separated, epidermal protein lysates were prepared and Western blot analysis was performed to determine the protein expression of p21 and p53. Equal loading was confirmed by stripping the Western blot and reprobing it for β -actin. The representative blots are shown from three independent experiments.

Oral feeding of PFE inhibits UVB-induced activation of IKK α and NF κ B, and phosphorylation and degradation of I κ B α in SKH-1 hairless mice

Nuclear factor kappa B is present in the cytosol as a heterodimer usually consisting of its p50 and p65 subunits bound to its inhibitory proteins I κ B. One of the critical events in NF- κ B activation is its dissociation from inhibitory protein I κ B (4). We determined whether oral feeding of PFE to SKH-1 hairless mice inhibits UVB-mediated activation of NF- κ B. UVB irradiation resulted in phosphorylation and degradation of I κ B α protein in time-dependent manner in the epidermal cytosol. Our data clearly demonstrated that oral feeding of PFE markedly inhibited UVB-mediated phosphorylation and degradation of I κ B α (Fig. 7). Because IKK α activity is

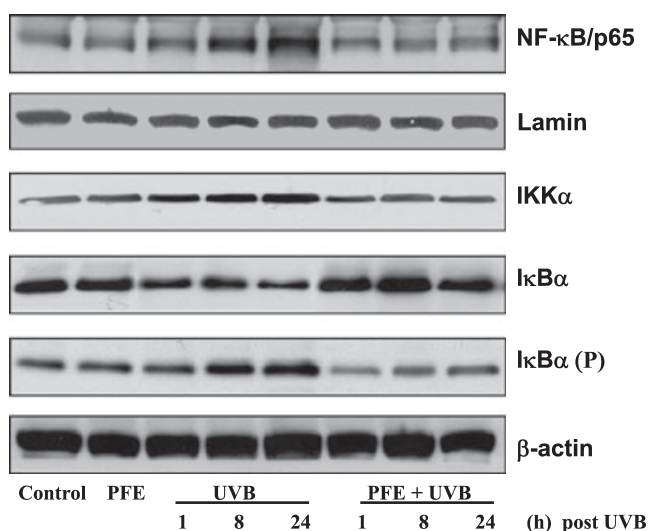


Figure 7. Inhibitory effect of oral feeding of pomegranate fruit extract on UVB-induced activation of IKK α and nuclear factor kappa B, and phosphorylation and degradation of I κ B α in SKH-1 hairless mice. At different time points post UVB irradiation, animals were sacrificed. The epidermis was separated and epidermal cytosolic and nuclear protein lysates were prepared and Western blot analysis was performed to determine the protein expression. Equal loading was confirmed by stripping the Western blot and reprobing it for β -actin or lamin. The representative blots are shown from three independent experiments.

necessary for I κ B α protein phosphorylation/degradation, we also measured IKK α protein level. UVB radiation resulted in the activation of IKK α protein that in turn phosphorylate and degrade I κ B α protein. Oral feeding of PFE inhibited UVB-induced activation of IKK α (Fig. 7). We then investigated whether oral feeding of PFE inhibits UVB-induced activation and nuclear translocation of NF- κ B/p65 in skin of SKH-1 hairless mice. Employing Western blot analysis, we found that UVB exposure resulted in the activation and nuclear translocation of NF- κ B/p65, which was markedly inhibited by oral feeding of PFE (Fig. 7). Lamin was used as a loading control for NF κ B.

DISCUSSION

The UVB component of solar UV radiation is believed to be the major cause of the variety of cutaneous disorders including skin cancers (4,37,38). Botanical antioxidants consumed by the human population have gained considerable attention as photoprotective and photochemopreventive agents against skin cancers (2,4,37). Previously, we have shown that treatment of normal human epidermal keratinocytes with PFE inhibited UVB-mediated activation of NF- κ B and MAPK pathways (27). This study was designed to assess the photochemopreventive effect of PFE after single UVB irradiations to the skin of SKH-1 hairless mice, a well-accepted model of photodamage studies. Our data clearly demonstrate that oral feeding of PFE affords significant inhibition against UVB-induced skin edema and hyperplasia.

UVB radiation to mammalian skin is known to alter cellular function *via* oxidation of macromolecules, DNA damage, generation of ROS and alterations in signaling pathways (2,4,5). The incidence of LPO in the biological membrane is a free radical-mediated event that is regulated by the availability of substrates in the form of polyunsaturated fatty acids, oxidants which promote peroxidation (39,40). LPO is highly detrimental to cell membrane structure and function, and its elevated level has been linked to damaging effects such as loss of fluidity, inactivation of membrane enzymes and increases in cell membrane permeability which may ultimately lead to disruption of cell membrane potential (41,42). As we observed, UVB-induced leukocyte infiltration in the skin and inflammatory leukocytes are the major source of H₂O₂ production that plays an important role in inflammatory skin diseases and skin cancer. Our results clearly demonstrate that oral feeding of PFE would result in reduction of the risk factors associated with UVB radiation by inhibiting UVB-mediated LPO and production of H₂O₂.

Ornithine decarboxylase, a rate-limiting enzyme that catalyzes the biosynthesis of polyamines, plays an important role in the regulation of cell transformation and development of cancer (43,44). Aberrations in ODC regulation and subsequent polyamine accumulation are closely associated with neoplastic transformation (45). In addition, high levels of ODC gene products are consistently detected in virtually all animal tumors and in certain tissues predisposed to tumorigenesis (43,45). Agents that have the ability to block induction of ODC can prevent tumor formation. Therefore, ODC inhibition was shown to be a promising tool for screening inhibitors of skin tumorigenesis. In the present study, oral feeding of PFE resulted in a significant inhibition of UVB-mediated

induction of epidermal ODC activity and protein expression. It is logical to consider that oral feeding of PFE inhibited the action of the tumor promoter and/or the enzymatic pathway(s) that regulates the ODC induction.

Cyclooxygenase-2 has been implicated in UVB-induced skin inflammation and photocarcinogenesis, and its overexpression has been shown to enhance cell proliferation, induce angiogenesis, regulate antiapoptotic cellular defenses and augment immunological response through production of PGE₂ (46,47). A considerable body of evidence suggests that inhibition of COX-2 expression or activity is important for not only alleviating inflammation, but also for prevention of cancer. Our results demonstrate the inhibitory effect of oral feeding of PFE against UVB-mediated induction of epidermal COX-2 protein expression. These results indicate that, in addition to ODC, PFE also targets COX-2 expression in exerting its photoprotective effects.

Proliferating cell nuclear antigen is an auxiliary protein of DNA polymerase- δ and its high levels of expression correlate cell proliferation, suggesting that PCNA is an excellent marker of cellular proliferation, which also serves as an effective predictive indicator of initiated cancer cells (48). PCNA has been shown to play a role in the regulation of p21 activity by modulating the rate at which p21 is degraded (49). Studies have shown that phosphorylation of specific residues within the PCNA-binding motif can modulate the p21-PCNA interactions (50). Therefore, PCNA is regarded as an important target for p21 as well as a reliable biomarker for cell proliferation. We found that UVB irradiation enhanced epidermal PCNA protein expression compared with control and PFE alone fed groups. Oral feeding of PFE to mice inhibited UVB-induced PCNA protein expression. This suggests that inhibiting cell proliferation could be one of the mechanisms by which PFE protects epidermal damaged cells from entering the cell cycle, thus affording these cells additional time for DNA damage repair to avoid replication error that otherwise often results in carcinogenic mutation.

The p53 tumor suppressor gene plays a decisive role in protecting cells from DNA damage as a consequence of UVB exposure (51). In the keratinocytes, UV-DNA damage induces an elevation of p53 protein followed by the induction of its direct transcriptional target p21, a cyclin-dependent kinase inhibitor (20). It has been shown that p53 can play direct and indirect roles in UVB-induced, transcription-coupled DNA repair (52). DNA damage elicited by UVB triggers p53 accumulation and transcriptional activation, leading to cell cycle arrest allowing more time for the repair of damaged DNA or elimination of damaged cells by apoptosis (53). Studies have shown that early responses in the skin of SKH-1 mice after an acute UVB exposure include increased number of cells expressing both p21 and p53 (54). Our data demonstrated that UVB irradiation resulted in an increase in p53 and p21 proteins, and oral feeding of PFE resulted in a further enhancement in UVB exposure-mediated increase in these proteins in SKH-1 hairless mouse epidermis. UVB is a potent carcinogen known to damage DNA directly or through the generation of ROS. Both CPDs and 8-oxodG are formed in epidermal DNA after UVB irradiation and are considered as important biomarkers of DNA damage. Our data clearly demonstrated that oral feeding of PFE to SKH-1 hairless mice resulted in marked reduction in the number of CPDs and

8-oxodG positive cells and these may be due to enhanced DNA repair. Our data also suggest that the repair of 8-oxodG at the surface is better than at the base which is in agreement to the earlier published work on human skin (55).

Activated NF- κ B is a crucial factor for the immunoinflammatory responses and is also implicated in tumorigenesis (56). Therefore, NF- κ B has emerged as one of the most promising molecular targets in the prevention of cancer. NF- κ B is sequestered in the cytoplasm as a heterotrimer consisting of p50, p65 and I κ B α subunits. Upon phosphorylation and subsequent proteolytic degradation of I κ B α , NF- κ B activates and translocates to the nucleus where it binds to DNA and activates the target genes by binding to the DNA regulatory element (57). In the present study, we have demonstrated that oral feeding of PFE inhibited UVB-induced NF- κ B and IKK α activation, and phosphorylation and degradation of I κ B α protein. Because PFE inhibited I κ B α phosphorylation and degradation, this study suggests that the effect of PFE on NF- κ B/p65 is through inhibition of phosphorylation and subsequent proteolytic degradation of I κ B α .

Our data suggest that oral feeding of PFE to mice affords substantial protection from the adverse effects of UVB radiation *via* modulation in early biomarkers of photocarcinogenesis and provide suggestion for its photochemopreventive potential.

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REFERENCES

1. Afaq, F., V. M. Adhami, N. Ahmad and H. Mukhtar (2002) Botanical antioxidants for chemoprevention of photocarcinogenesis. *Front. Biosci.* **7**, d784–792.
2. Bowden, G. T. (2004) Prevention of non-melanoma skin cancer by targeting ultraviolet-B-light signalling. *Nat. Rev. Cancer* **4**, 23–35.
3. Jemal, A., R. Siegel, J. Xu and E. Ward (2010) Cancer statistics, 2010. *CA Cancer J. Clin.* **60**, 277–300.
4. Afaq, F., V. M. Adhami and H. Mukhtar (2005) Photochemoprevention of ultraviolet B signaling and photocarcinogenesis. *Mutat. Res.* **571**, 153–173.
5. Bickers, D. R. and M. Athar (2006) Oxidative stress in the pathogenesis of skin disease. *J. Invest. Dermatol.* **126**, 2565–2575.
6. Afaq, F., D. N. Syed, A. Malik, N. Hadi, S. Sarfaraz, M. H. Kweon, N. Khan, M. A. Zaid and H. Mukhtar (2007) Delphinidin, an anthocyanidin in pigmented fruits and vegetables, protects human HaCaT keratinocytes and mouse skin against UVB-mediated oxidative stress and apoptosis. *J. Invest. Dermatol.* **127**, 222–232.
7. de Grujil, F. R. and H. Rebel (2008) Early events in UV carcinogenesis—DNA damage, target cells and mutant p53 foci. *Photochem. Photobiol.* **84**, 382–387.
8. Melnikova, V. O., A. Pacifico, S. Chimenti, K. Peris and H. N. Ananthaswamy (2005) Fate of UVB-induced p53 mutations in SKH-hr1 mouse skin after discontinuation of irradiation: Relationship to skin cancer development. *Oncogene* **24**, 7055–7063.
9. Halliday, G. M. and J. G. Lyons (2008) Inflammatory doses of UV may not be necessary for skin carcinogenesis. *Photochem. Photobiol.* **84**, 272–283.
10. Nichols, J. A. and S. K. Katiyar (2010) Skin photoprotection by natural polyphenols: Anti-inflammatory, antioxidant and DNA repair mechanisms. *Arch. Dermatol. Res.* **302**, 71–83.
11. Schwarz, T. (2008) 25 years of UV-induced immunosuppression mediated by T cells—From disregarded T suppressor cells to highly respected regulatory T cells. *Photochem. Photobiol.* **84**, 10–18.

12. Halliday, G. M. and S. Rana (2008) Waveband and dose dependency of sunlight-induced immunomodulation and cellular changes. *Photochem. Photobiol.* **84**, 35–46.
13. Afaq, F. and H. Mukhtar (2006) Botanical antioxidants in the prevention of photocarcinogenesis and photoaging. *Exp. Dermatol.* **15**, 678–684.
14. Melnikova, V. O. and H. N. Ananthaswamy (2005) Cellular and molecular events leading to the development of skin cancer. *Mutat. Res.* **571**, 91–106.
15. Arad, S., E. Zattra, J. Hebert, E. H. Epstein Jr, D. A. Goukassian and B. A. Gilchrist (2008) Topical thymidine dinucleotide treatment reduces development of ultraviolet-induced basal cell carcinoma in Ptch-1 +/- mice. *Am. J. Pathol.* **172**, 1248–1255.
16. Cooke, M. S., S. Loft, R. Olinski, M. D. Evans, K. Bialkowski, J. R. Wagner, P. C. Dedon, P. Møller, M. M. Greenberg and J. Cadet (2010) Recommendations for standardized description of and nomenclature concerning oxidatively damaged nucleobases in DNA. *Chem. Res. Toxicol.* **23**, 705–707.
17. Ming, M., W. Han, J. Maddox, K. Soltani, C. R. Shea, D. M. Freeman and Y. Y. He (2010) UVB-induced ERK/AKT-dependent PTEN suppression promotes survival of epidermal keratinocytes. *Oncogene* **29**, 492–502.
18. Lotti, L. V., S. Rotolo, F. Francescangeli, L. Frati, M. R. Torrisi and C. Marchese (2007) AKT and MAPK signaling in KGF-treated and UVB-exposed human epidermal cells. *J. Cell. Physiol.* **212**, 633–642.
19. van Schanke, A., G. M. van Venrooij, M. J. Jongsma, H. A. Banus, L. H. Mullenders, H. J. van Kranen and F. R. de Gruijl (2006) Induction of nevi and skin tumors in Ink4a/Arf Xpa knockout mice by neonatal, intermittent, or chronic UVB exposures. *Cancer Res.* **66**, 2608–2615.
20. Abd Elmageed, Z. Y., R. L. Gaur, M. Williams, M. E. Abdraboh, P. N. Rao, M. H. Raj, F. M. Ismail and A. Ouhtit (2009) Characterization of coordinated immediate responses by p16INK4A and p53 pathways in UVB-irradiated human skin cells. *J. Invest. Dermatol.* **129**, 175–183.
21. Afaq, F., N. Ahmad and H. Mukhtar (2003) Suppression of UVB-induced phosphorylation of mitogen-activated protein kinases and nuclear factor kappa B by green tea polyphenol in SKH-1 hairless mice. *Oncogene* **22**, 9254–9264.
22. Afaq, F., V. M. Adhami, N. Ahmad and H. Mukhtar (2003) Inhibition of ultraviolet B-mediated activation of nuclear factor kappaB in normal human epidermal keratinocytes by green tea Constituent (-)-epigallocatechin-3-gallate. *Oncogene* **22**, 1035–1044.
23. Afaq, F., M. Saleem, C. G. Krueger, J. D. Reed and H. Mukhtar (2005) Anthocyanin- and hydrolyzable tannin-rich pomegranate fruit extract modulates MAPK and NF-kappaB pathways and inhibits skin tumorigenesis in CD-1 mice. *Int. J. Cancer* **113**, 423–433.
24. Gil, M. I., F. A. Tomas-Barberan, B. Hess-Pierce, D. M. Holcroft and A. A. Kedar (2000) Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J. Agric. Food Chem.* **10**, 4581–4589.
25. Khan, N., N. Hadi, F. Afaq, D. N. Syed, M. H. Kweon and H. Mukhtar (2007) Pomegranate fruit extract inhibits prosurvival pathways in human A549 lung carcinoma cells and tumor growth in athymic nude mice. *Carcinogenesis* **28**, 163–173.
26. Malik, A., F. Afaq, S. Sarfaraz, V. M. Adhami, D. N. Syed and H. Mukhtar (2005) Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer. *Proc. Natl Acad. Sci. USA* **102**, 14813–14818.
27. Afaq, F., A. Malik, D. Syed, D. Maes, M. S. Matsui and H. Mukhtar (2005) Pomegranate fruit extract modulates UV-B-mediated phosphorylation of mitogen-activated protein kinases and activation of nuclear factor kappa B in normal human epidermal keratinocytes. *Photochem. Photobiol.* **81**, 38–45.
28. Afaq, F., V. M. Adhami and N. Ahmad (2003) Prevention of short-term ultraviolet B radiation-mediated damages by resveratrol in SKH-1 hairless mice. *Toxicol. Appl. Pharmacol.* **186**, 28–37.
29. Wright, J. R., H. D. Colby and P. R. Miles (1981) Cytosolic factors which affect microsomal lipid peroxidation in lung and liver. *Arch. Biochem. Biophys.* **206**, 296–304.
30. Yano, K., K. Kajijiya, M. Ishiwata, Y. K. Hong, T. Miyakawa and M. Detmar (2004) Ultraviolet B-induced skin angiogenesis is associated with a switch in the balance of vascular endothelial growth factor and thrombospondin-1 expression. *J. Invest. Dermatol.* **122**, 201–208.
31. Pearce, A. D., S. A. Gaskell and R. Marks (1987) Epidermal changes in human skin following irradiation with either UVB or UVA. *J. Invest. Dermatol.* **88**, 83–87.
32. Halliday, G. M. (2005) Inflammation, gene mutation and photo-immunosuppression in response to UVR-induced oxidative damage contributes to photocarcinogenesis. *Mutat. Res.* **571**, 107–20.
33. Gilmour, S. K. (2007) Polyamines and nonmelanoma skin cancer. *Toxicol. Appl. Pharmacol.* **224**, 249–256.
34. Feith, D. J., D. K. Bol, J. M. Carboni, M. J. Lynch, S. Sass-Kuhn, P. L. Shoop and L. M. Shantz (2005) Induction of ornithine decarboxylase activity is a necessary step for mitogen-activated protein kinase kinase-induced skin tumorigenesis. *Cancer Res.* **65**, 572–578.
35. Sharma, S. D. and S. K. Katiyar (2010) 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. *Pharm. Res.* **27**, 1092–1102.
36. Gu, M., S. Dhanalakshmi, R. P. Singh and R. Agarwal (2005) Dietary feeding of silibinin prevents early biomarkers of UVB radiation-induced carcinogenesis in SKH-1 hairless mouse epidermis. *Cancer Epidemiol. Biomarkers Prev.* **14**, 1344–1349.
37. Adhami, V. M., D. N. Syed, N. Khan and F. Afaq (2008) Phytochemicals for prevention of solar ultraviolet radiation-induced damages. *Photochem. Photobiol.* **84**, 489–500.
38. Erb, P., J. Ji, E. Kump, A. Mielgo and M. Wernli (2008) Apoptosis and pathogenesis of melanoma and nonmelanoma skin cancer. *Adv. Exp. Med. Biol.* **624**, 283–295.
39. Katiyar, S. K. and S. M. Meeran (2007) Obesity increases the risk of UV radiation-induced oxidative stress and activation of MAPK and NF-kappaB signaling. *Free Radic. Biol. Med.* **42**, 299–310.
40. Schneider, L. A., W. Bloch, K. Kopp, A. Hainzl, P. Rettberg, M. Wlaschek, G. Horneck and K. Scharfetter-Kochanek (2006) 8-Isoprostane is a dose-related biomarker for photo-oxidative ultraviolet (UV) B damage in vivo: A pilot study with personal UV dosimetry. *Br. J. Dermatol.* **154**, 1147–1154.
41. Bresgen, N., H. Jaksch, H. Lacher, I. Ohlenschläger, K. Uchida and P. M. Eckl (2010) Iron-mediated oxidative stress plays an essential role in ferritin-induced cell death. *Free Radic. Biol. Med.* **48**, 1347–1357.
42. Cuzzocrea, S., D. P. Riley, A. P. Caputi and D. Salvemini (2001) Antioxidant therapy: A new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. *Pharmacol. Rev.* **53**, 135–159.
43. Auvinen, M., A. Paasinen, L. C. Andersson and E. Hölttä (1992) Ornithine decarboxylase activity is critical for cell transformation. *Nature* **360**, 355–358.
44. Thomas, T. and T. J. Thomas (2003) Polyamine metabolism and cancer. *J. Cell Mol. Med.* **7**, 113–126.
45. Auvinen, M., A. Laine, A. Paasinen-Sohns, A. Kangas, L. Kangas, O. Saksela, L. C. Andersson and E. Hölttä (1997) Human ornithine decarboxylase-overproducing NIH3T3 cells induce rapidly growing, highly vascularized tumors in nude mice. *Cancer Res.* **57**, 3016–3025.
46. Athar, M., K. P. An, K. D. Morel, A. L. Kim, M. Aszterbaum, J. Longley, E. H. Epstein Jr and D. R. Bickers (2001) Ultraviolet B (UVB)-induced COX-2 expression in murine skin: An immunohistochemical study. *Biochem. Biophys. Res. Commun.* **280**, 1042–1047.
47. Herschman, H. R. (1994) Regulation of prostaglandin synthase-1 and prostaglandin synthase-2. *Cancer Metastasis Rev.* **13**, 241–256.
48. Lee, K. M., K. W. Lee, S. Byun, S. K. Jung, S. K. Seo, Y. S. Heo, A. M. Bode, H. J. Lee and Z. Dong (2010) 5-deoxykaempferol plays a potential therapeutic role by targeting multiple signaling pathways in skin cancer. *Cancer Prev. Res.* **3**, 454–465.
49. Cayrol, C. and B. Ducommun (1998) Interaction with cyclin-dependent kinases and PCNA modulates proteasome-dependent degradation of p21. *Oncogene* **17**, 2437–2444.
50. Scott, M. T., N. Morrice and K. L. Ball (2000) Reversible phosphorylation at the C-terminal regulatory domain of p21(Waf1/Cip1) modulates proliferating cell nuclear antigen binding. *J. Biol. Chem.* **275**, 11529–11537.

51. Smith, M. L. and A. J. Fornace Jr (1997) p53-mediated protective responses to UV irradiation. *Proc. Natl Acad. Sci. USA* **94**, 12255–12257.
52. McKay, B. C., M. A. Francis and A. J. Rainbow (1997) Wild type *p53* is required for heat shock and ultraviolet light enhanced repair of a UV-damaged reporter gene. *Carcinogenesis* **18**, 245–249.
53. Berg, R. J., H. J. van Kranen, H. G. Rebel, A. de Vries, W. A. van Vloten, C. F. Van Kreijl, J. C. van der Leun and F. R. de Gruijl (1996) Early p53 alterations in mouse skin carcinogenesis by UVB radiation: Immunohistochemical detection of mutant p53 protein in clusters of preneoplastic epidermal cells. *Proc. Natl Acad. Sci. USA* **93**, 274–278.
54. Kuerbitz, S. J., B. S. Plunkett, W. V. Walsh and M. B. Kastan (1992) Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl Acad. Sci. USA* **89**, 7491–7495.
55. Javeri, A., X. X. Huang, F. Bernerd, R. S. Mason and G. M. Halliday (2008) Human 8-oxoguanine-DNA glycosylase 1 protein and gene are expressed more abundantly in the superficial than basal layer of human epidermis. *DNA Repair (Amst.)* **7**, 1542–1550.
56. Chiang, Y. M., C. P. Lo, Y. P. Chen, S. Y. Wang, N. S. Yang, Y. H. Kuo and L. F. Shyur (2005) Ethyl caffeate suppresses NF-kappaB activation and its downstream inflammatory mediators, iNOS, COX-2, and PGE2 in vitro or in mouse skin. *Br. J. Pharmacol.* **146**, 352–363.
57. Karin, M. (2008) The IkappaB kinase—A bridge between inflammation and cancer. *Cell Res.* **18**, 334–342.