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Protective effect of pomegranate derived products on UVB-mediated damage in human reconstituted skin

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Abstract

Solar ultraviolet (UV) radiation, particularly its UVB (290-320 nm) component, is the primary cause of many adverse biological effects including photoaging and skin cancer. UVB radiation causes DNA damage, protein oxidation and induces matrix metalloproteinases (MMPs). Photochemoprevention via the use of botanical antioxidants in affording protection to human skin against UVB damage is receiving increasing attention. Pomegranate, from the tree *Punica granatum* contains anthocyanins and hydrolyzable tannins and possesses strong anti-oxidant and anti-tumor promoting properties. In this study, we determined the effect of pomegranate derived products POMx juice, POMx extract and pomegranate oil (POMo) against UVB-mediated damage using reconstituted human skin (EpiDerm™ FT-200). EpiDerm was treated with POMx juice (1-2 µl/0.1 ml/well), POMx extract (5-10 µg/0.1 ml/well), and POMo (1-2 µl/0.1 ml/well) for 1 h prior to UVB (60 mJ/cm²) irradiation and was harvested 12 h post-UVB to assess protein oxidation, markers of DNA damage and photoaging by western blot analysis and immunohistochemistry. Pretreatment of Epiderm with pomegranate derived products resulted in inhibition of UVB-induced (i) cyclobutane pyrimidine dimers, (ii) 8-dihydro-2'-deoxyguanosine, (iii) protein oxidation, and (iv) PCNA protein expression. We also found that pretreatment of Epiderm with pomegranate derived products resulted in inhibition of UVB-induced (i) collagenase (MMP-1), (ii) gelatinase (MMP-2, MMP-9), (iii) stromelysin (MMP-3), (iv) marilysin (MMP-7), (v) elastase (MMP-12), and (vi) tropoelastin. Gelatin zymography revealed that pomegranate derived products inhibited UVB-induced MMP-2 and MMP-9 activities. Pomegranate derived products also caused a decrease in UVB-induced protein expression of c-Fos and phosphorylation of c-Jun. Collectively, these results suggest that all three pomegranate derived products may be useful against UVB-induced damage to human skin.

Introduction

Ultraviolet radiation (UV) from the sun is one of the most prominent environmental factor that has serious adverse effects including erythema, edema, hyperplastic responses, immunosuppression, hyperpigmentation, premature aging of skin and majority of cutaneous malignancies (1-5). Solar UV radiation is divided into three categories: UVA or (UV 320-400nm), UVB (280-320nm) and UVC (100-280nm). Both UVB and UVA are the causative factors for sunlight-induced skin disorders (6-7). UVB radiation is the most damaging component of the solar radiation reaching the earth and acts mainly on the epidermal basal cell layer of the skin. UVB wavelength photon is absorbed by DNA, and

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induces DNA damage by formation of cyclobutane pyrimidine dimers (CPD) and 8-dihydro-2'-deoxyguanosine (8-OHdG) (6,8). UVB-mediated DNA damage causes mutation of oncogenes and tumor suppressor genes and is an obligatory step for progress towards skin carcinogenesis (6,8-10). UVB radiation is considered to be a complete carcinogen, and initiates a photooxidative reaction, which impairs the antioxidant status and increases the level of reactive oxygen species (ROS) accompanied by activation of signaling pathways (3,11,12). To counteract this, skin has efficient antioxidant defense mechanisms but when the generation of ROS overwhelms this defense capacity, it impairs the ability of the skin to protect itself from the damaging effects of ROS resulting in oxidative damage of DNA, proteins and other macromolecules in the skin (6,8,11).

In recent years, botanical antioxidants have attracted considerable attention, because of their potential to quench ROS and inhibit UV-induced signal transduction pathways (1,3). Thus, it is suggested that a regular intake of antioxidants or treatment of the skin with products containing antioxidant ingredients may be a useful strategy for the prevention of UV-mediated cutaneous damage (13-15). This has sparked the use of exogenous supplementation of antioxidants, notably of botanical origin in skin care products.

Polyphenolics, widely distributed in botanicals with their significant amounts in vegetables, fruits and beverages, form an integral part of diet and possess strong free radical scavenging and antioxidant properties (16,17). *Punica granatum* L fruit commonly known as pomegranate is widely consumed fresh and in beverage form, as juice or wine has been used in various parts of the world as a traditional medicine and is also gaining popularity in the USA. Pomegranate is a rich source of many phenolic compounds, which include flavanoids and hydrolyzable tannins (18). Extracts from different parts of pomegranate fruit such as juice (19), seed (20) and peel (21) have been reported to exhibit strong antioxidant activity. Studies have shown that pomegranate juice possesses antiproliferative (22), antiatherogenic (23), antiinflammatory and antitumorigenic (22,24,25) properties. These effects of pomegranate and its derived products are attributable to its free radical scavenging and antioxidant properties (26). Recently, we have shown that treatment of immortalized HaCaT cells with POMx extract resulted in inhibition of UVB-mediated oxidative stress and markers of photoaging (27). For relevance of this work to human skin here we utilized three dimensional full thickness reconstituted human skin equivalent (EpiDermTMF_{FT}-200) that sustains differentiation and exhibit morphological, metabolic and growth characteristics similar to that of human skin *in vivo*. In the present study, we determined the effect of pomegranate derived products such as POMx juice, POMx extract and POMo against UVB-mediated damage in EpiDermTMF_{FT}-200. Specifically, we determined the effect of these products on UVB-induced protein oxidation and markers of DNA damage and photoaging.

Material and Methods

Materials

EpiDermTMF_{FT}-200 and EFT culture media were purchased from MatTeck Corp. (Ashland, MA). HRP-labelled antibody for CPD was purchased from Kamiya Biomedical Company (Seattle, WA). Antibody for 8-OHdG was obtained from Millipore (Billerica, MA). Antibodies for MMP-1, MMP-2 and MMP-9 were procured from Lab Vision Corporation (Fremount, CA). Antibodies for MMP-7, MMP-11 and MMP-12 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antimouse or antirabbit secondary antibody horseradish peroxidase conjugate and ECL western blotting detection reagent were purchased from Amersham Life Science (Arlington Height, IL). Protein carbonyl immunoblot kit was obtained from Cell Biolabs Inc. (San Diego, CA). Protease inhibitor cocktail set III was obtained from Calbiochem (La Jolla, CA). Novex precast tris-glycine gels were purchased from Invitrogen (Carlsbad, CA). BCA protein assay kit was obtained

from Pierce Biotechnology Inc. (Rockford, IL). All other chemicals used were at least of analytical grade. POMx juice, POMx extract and pomegranate oil (POMo) were provided by POM Wonderful, Inc. (Los Angeles, CA).

Human reconstituted Skin (EpiDerm™ FT-200)

This model system consists of normal human epidermal keratinocytes (NHEK) derived from neonatal-foreskin and normal human dermal fibroblasts (NHDF) derived from neonatal skin of same donor. These were co-cultured to form a multilayered, highly differentiated model of the human skin with functional dermis and epidermis (28-29). EpiDerm™ FT-200 consists of organized basal, spinous, granular, and cornified epidermal layers analogous to those found *in vivo* (28-30). The dermal compartment is composed of a collagen matrix containing viable NHDF. Ultrastructurally, the EpiDerm™ FT-200 closely resembles human skin, thus providing a useful *in vitro* model for study. Histochemical analysis shows the presence of basement membrane along with signaling proteins. This model also serves for *in vitro* evaluation of skin phenomenon where fibroblast-keratinocyte interactions (paracrine signaling) are important and modulate cell response, which play important role in regulating tissue homeostasis (31,32). The EpiDerm™ FT-200 was supplied as single well tissue culture plate inserts with each insert containing functionally and metabolically active reconstituted skin with surface area 1.0 cm² shipped at 4°C on medium-supplemented, agarose gel. Upon receipt the EpiDerm was equilibrated at 37 °C, 5% CO₂, in EFT media supplied along with the kit for 24 h and maintained. Through out the experiment EpiDerm™ FT-200 was maintained in 6 well culture plates at air liquid interface with lower dermal side of tissue exposed to media and the upper epidermal stratum corneum exposed to air.

POM products

POM products were provided by POM Wonderful, Inc. (Los Angeles, CA). Pomegranate fruit was sliced and squeezed for the juice and the remaining material including squeezed arils, rind and other parts were processed to remove the seeds before undergoing a series of concentration steps to produce a polyphenol rich POMx. POMx is a 70 Brix commercial grade of pomegranate extract concentrate with a polyphenol content of 135 000 p.p.m. gallic acid equivalent and ellagitannins as its major constituent. POMx juice contains anthocyanins ellagitannins and hydrolyzable tannins. The source of POMo is pomegranate seed.

Treatment and UVB irradiation of EpiDerm™ FT-200

EpiDerm™ FT-200 was topically treated with or without POMx juice (1-2µl), POMx extract (5-10 µg) or POMo (1-2µl) diluted in EFT media (100 µl/tissue/well) containing 0.1% DMSO for 1 h. These doses of pomegranate products were decided by doing dose-response studies in terms of its photoprotective effects against UVB-mediated DNA damage (data not shown). After that, the media containing pomegranate products were removed and the skin samples were gently washed with PBS 2-3 times via gentle pipetting of the apical tissue surface to remove any non-absorbed products. A control with 0.1% DMSO in EFT medium was maintained for all experiments. The culture media were then replaced with PBS and exposed to UVB (60mJ/cm²). After UVB exposure, the culture inserts containing the skin samples were placed in fresh media and were harvested 12 h post-UVB irradiation for western blotting and RT-PCR analyses, and media was collected for gelatin zymography.

Immunostaining for CPD

For detection of CPD, EpiDerm™ FT-200 was collected 12 h post UVB irradiation, was frozen in OCT medium and sectioned. 5µm thick sections were fixed in chilled acetone for 20 min. To denature nuclear DNA, sections were treated with 70mM NaOH in 70% ethanol and neutralized for 1 min in 100mM Tris-HCl in 70% ethanol. Endogenous peroxidase was

quenched by incubation in 0.3% hydrogen peroxide in methanol and washed with PBS. Nonspecific binding sites were blocked by incubating the sections with goat serum blocking solution for 1 h and incubated overnight at 4°C with anti-thymine dimer HRP-labeled antibody. After washing with PBS, the sections were incubated with DAB peroxidase substrate solution for 2 min at room temperature, washed with distilled water, followed by counterstaining with Mayers Hematoxylin solution. Sections were rinsed in tap water, dehydrated through graded alcohol, cleared in xylene and mounted in permanent mounting medium.

Immunostaining for 8-OHdG

For detection of 8-OHdG, EpiDerm™ FT-200 was collected 12 h post UVB were fixed in 10% neutralized formalin and embedded in paraffin. Sections 5µm in thickness were deparaffinized in xylol and rehydrated, through graded ethanol solutions to 70% and washed in PBS. For antigen retrieval sections were heated at 95°C for 30 min in EDTA buffer (pH 8.0) and then cooled for 20 min and washed in PBS. To denature nuclear DNA, sections were treated with 70mM NaOH and neutralized for 1 min in 100mM Tris-HCl (pH 7.5). Endogenous peroxidase was quenched by incubation in 0.3% hydrogen peroxide. Nonspecific binding sites were blocked by incubating the sections with goat serum blocking solution for 1 h and incubated with primary antibody against 8-OHdG overnight at 4°C followed by incubation with HRP-labeled secondary antibody for 1 h at room temperature. After washing with PBS, the sections were incubated with DAB peroxidase substrate solution (Dako) for 2 min at room temperature, rinsed with distilled water followed by counterstaining with Mayers Hematoxylin solution. Sections were rinsed in tap water, dehydrated through graded alcohol cleared in xylene and mounted in permanent mounting medium.

Immunostaining for PCNA and tropoelastin

EpiDerm™ FT-200 was collected 12 h after UVB irradiation, was fixed in 10% neutralized formalin and embedded in paraffin. 5µm sections were cut, deparaffinized in xylol and rehydrated, through graded ethanol to 70% and washed in PBS. For antigen retrieval, sections were heated at 95°C for 30 min in citrate buffer (pH 6.0) and then cooled for 20 min and washed in PBS. Endogenous peroxidase was quenched by incubation in 0.3% hydrogen peroxide, for 20 min and washed in washing buffer (PBS + Tween). Nonspecific binding sites were blocked by incubating the sections with goat serum blocking solution for 1 h. Sections were incubated with primary antibody against PCNA and tropoelastin overnight at 4°C followed by incubation with specific HRP-labeled secondary antibody for 1 h at room temp. After washing in wash buffer, the sections were incubated with DAB peroxidase substrate solution for 2 min at room temperature, rinsed with distilled water followed by counterstaining with Mayers Hematoxylin solution. Sections were rinsed in tap water, dehydrated through 70-100 % graded alcohol cleared in xylene and finally mounted in permanent mounting medium.

Gelatin zymography

For zymography, culture media in which EpiDerm™ FT-200 was grown after UVB exposure was subjected to substrate gel electrophoresis for detection of gelatinolytic activity. Samples were concentrated using centricon YM-30 centrifugal filter unit which retains proteins greater than 30 KD. The samples with equal protein content were mixed with non reducing sample buffer and electrophoresed in precasted 10% SDS-polyacrylamide gels containing 1% gelatin and run in Novex tris glycine SDS running buffer. To eliminate SDS content, gels were washed twice with Novex zymogram renaturing buffer for 30 min at room temperature with gentle agitation. Afterward, the gels were incubated at 37°C overnight in Novex developing buffer (50 mM Tris-HCl, pH 8, 5 mM CaCl₂, 1 µM ZnCl₂,

and 0.02% NaN_3 , which allows gelatinolytic enzymes to act. Gels were stained for 3 h in 40% methanol and 10% glacial acetic acid containing 0.5% Coomassie Brilliant Blue and were destained in the same solution without dye. The gelatinolytic activity of MMPs was evident as a clear band against the blue background of stained gelatin.

Whole cell lysate preparation

EpiDerm™ FT-200 lysates for western blot analysis was prepared by homogenizing the EpiDerm™ FT-200 in lysis buffer (10mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 1 mM EGTA) containing 0.2 mM sodium vanadate, 2 mM PMSF, 0.5% NP-40, and 0.2 U/ml aprotinin with freshly added protease inhibitor cocktail at 4°C for 15 min. The lysates were centrifuged at 13,000 rpm for 25 min at 4°C to remove cell debris. Clear supernatant was collected, and protein estimation performed by BCA method.

SDS-polyacrylamide gel electrophoresis and western blot analysis

For western blot analysis, equal amount (30-40 μg) of protein was resolved electrophoretically over 12% Tris glycine gel, and transferred to a nitrocellulose membrane. The blot containing the transferred protein was blocked in blocking buffer (5% nonfat dry milk in 20mM Tris-buffered saline, pH 7.6 containing 1% Tween 20 - TBST) for 1 h at room temperature followed by incubation with appropriate primary antibody in blocking buffer for 2 h to overnight at 4°C. This was followed by incubation with specific secondary antibody horseradish peroxidase for 2 h at room temperature and then washed 3 times, 15 min each in TBST and detected by enhanced chemiluminescence and autoradiography using Blue Lite Autorad film obtained from ISC Bioexpress (Kaysville, UT).

Determination of protein oxidation

For determination of protein oxidation, Cell Biolabs' protein carbonyl immunoblot kit was used. Briefly gel proteins were transferred to the PVDF membrane. Following the electroblotting step, PVDF membrane was immersed in 100% methanol for 15 sec, and dried at room temperature for 5 min, then equilibrated in TBS containing 20% methanol for 5 min. Membrane was washed in 2N HCl for 5 min and incubated in dinitrophenylhydrazine (DNPH) solution for 5 min. Following derivitization with DNPH membrane was washed three times with 2N HCl, and then five times with 100% methanol, 5 min each. The blot containing the derivitized protein was blocked in blocking buffer and incubated with primary antibody against DNPH for 3 h at room temperature followed by incubation in HRP labeled secondary antibody for 2 h and detected by enhanced chemiluminescence and autoradiography.

Results

POMx juice, POMx extract and POMo inhibit UVB-mediated formation of CPD and 8-OHdG in human reconstituted skin

Photodamage to epidermal DNA is considered an important factor in the development of skin cancer (6,8). It is known that both CPDs and 8-OHdG are formed in epidermal DNA after UVB irradiation and are considered as important biomarkers of DNA damage. We, therefore, performed immunohistochemical staining using antibody specific for CPD and 8-OHdG to study the effect of the pomegranate derived products on UVB-mediated formation of CPD and 8-OHdG in human reconstituted skin, EpiDerm™ FT-200. Our result show stronger and intensive staining for CPD and 8-OHdG (Figure 1) in the nuclei of UVB (60mJ/cm²) irradiated EpiDerm™ FT-200, 12 h post-UVB (60mJ/cm²) exposure compared to those of non-irradiated control. However, topical treatment of EpiDerm™ FT-200 with POMx juice (2 μl), POMx extract (5 μg) or POMo (2 μl) prior to UVB irradiation resulted in

a significant reduction in both the number and intensity of CPD (Figure 1A) and 8-OHdG positive cells (Figure 1B). Treatment of EpiDerm™ FT-200 with POMx juice, POMx extract or POMo at the concentration used did not show any effect as compared to non-irradiated control in any of the parameter studied (data not shown).

POMx juice, POMx extract and POMo inhibit UVB-mediated increase in protein carbonyl group in human reconstituted skin

UV irradiation generates irreversible oxidation of the side chains of certain amino acids resulting in the formation of carbonyl groups on proteins (a marker of protein oxidation) (33). To study the effect of pomegranate derived products on UVB-mediated changes on generation of protein carbonyl groups, we performed immunoblot analysis after derivitization of protein carbonyl group on PVDF membrane with DNPH. Our results show that UVB (60mJ/cm²) irradiation resulted in an increase in the expression of protein with carbonyl groups whereas treatment of EpiDerm™ FT-200 topically with POMx juice (1-2μl), POMx extract (5-10 μg) or POMo (1-2μl) for 1 h prior to UVB (60mJ/cm²) resulted in a decreased expression of protein with carbonyl groups (Figure 2).

POMx juice, POMx extract and POMo inhibit UVB-mediated cell proliferation in human reconstituted skin

Proliferating cell nuclear antigen is an active nuclear protein involved in both DNA damage and repair (34,35). To study the effect of pomegranate derived products on UVB-induced changes on PCNA in human reconstituted skin, EpiDerm™ FT-200, we performed immunohistochemical staining and immunoblotting using antibody specific for PCNA. Our result show that UVB (60 mJ/cm²) irradiation to EpiDerm™ FT-200 resulted in an increase in protein expression of PCNA as compared to non-irradiated control. (Figure 3A,B). Pretreatment of EpiDerm™ FT-200 topically with POMx juice (1-2μl), POMx extract (5-10 μg) or POMo (1-2μl) for 1 h prior to UVB (60mJ/cm²) diminished PCNA protein expression of EpiDerm as compared to non irradiated control (Figure 3A,B).

POMx juice, POMx extract and POMo inhibit UVB-mediated increase in tropoelastin levels in human reconstituted skin

UVB irradiation stimulates the synthesis of elastin, one of the important components of extracellular matrix (ECM) in the skin of humans and experimental animals (36-38). To study the effect of pomegranate derived products on UVB-induced changes on elastin in human reconstituted skin, EpiDerm™ FT-200, we performed immunoblotting and immunohistochemical staining for tropoelastin, a monomer precursor of elastin. Our result showed that UVB (60mJ/cm²) caused an increase in the protein expression along with immunostaining of tropoelastin as compared to non-irradiated control (Figure 4A,B). Pretreatment of EpiDerm™ FT-200 topically with POMx juice (1-2μl), POMx extract (5-10 μl) or POMo (1-2μl) for 1 h prior to UVB (60mJ/cm²) decreased tropoelastin protein expression as compared to UVB (Figure 4A,B).

POMx juice, POMx extract and POMo inhibit UVB-mediated increase in the protein levels and activity of matrix metalloproteinases (MMPs) in human reconstituted skin

Exposure to UVB radiation is known to upregulate the synthesis of matrix degrading enzymes, MMPs. MMPs are a family of structurally related zinc-dependent endopeptidases, which play a role in degrading a wide variety of ECM components and play an important role in tumor invasion and photoaging (27,39). We therefore evaluated the effect of pomegranate derived products on UVB-induced MMPs activities and protein expression in human reconstituted human skin, EpiDerm™ FT-200. UVB (60 mJ/cm²) irradiation of EpiDerm™ FT-200 caused an increase in the protein expressions of MMPs-1, -2, -3, -7, -9,

-11 and -12 (Figure 5A). UVB also resulted in an increase in gelatinase activity of MMP-2 and MMP-9 in the surrounding media (Figure 5B). Our data show that pretreatment of EpiDerm™ FT-200 with POMx juice (1-2µl), POMx extract (5-10µg) or POMo (1-2µl) for 1 h prior to UVB (60mJ/cm²) exposure inhibited the UVB mediated increase of MMPs-1, -2, -3, -7, -9, -11 and -12 protein expressions (Figure 5A) and decreased MMP-2, and MMP-9 gelatinase activities (Figure 5B).

POMx juice, POMx extract and POMo inhibit UVB-induced phosphorylation of c-jun and expression of c-Fos in human reconstituted skin

Activator protein-1 (AP-1) is closely related to matrix degrading enzymes that induce breakdown of collagen. Jun proteins form homodimers or heterodimers with fos proteins to form AP-1 complexes. The transcriptional activity of AP-1 is dependent on the degree of phosphorylation of c-jun and expression of c-fos (1,3). We therefore investigated the effect of pomegranate derived products on UVB-induced phosphorylation of c-jun protein and expression of c-fos protein. Our results show that UVB (60mJ/cm²) irradiation of EpiDerm™ FT-200 increased the level of phosphorylated c-jun and c-fos proteins. Topical treatment of EpiDerm™ FT-200 with POMx juice (1-2µl), POMx extract (5-10 µg) or POMo (1-2µl) for 1 h prior to UVB (60mJ/cm²) inhibited UVB-mediated phosphorylation of c-jun protein and expression of c-fos protein (Figure 6).

Discussion

Exposure of skin to solar UV radiation, particularly its UVB component, is believed to be the major cause of a variety of cutaneous disorders including photoaging and skin cancers (15,40). Studies have demonstrated that UV radiation can act as a potent inducer of ROS, which are responsible for the photooxidative damage to nucleic acids, lipids and proteins. As ROS are implicated in skin damage by UVB, scavenging of these reactive species could prevent the oxidative reactions and subsequently protect skin from the damaging effects of UVB. Therefore, the use of antioxidants to reduce the harmful effect of UV by scavenging ROS is a novel approach to prevent the damage caused by UV radiation. In recent years, considerable attention is being focused on the use of naturally occurring botanicals for their potential preventive effect against UV radiation mediated damages referred to as “photochemopreventive effects” (41). One such natural product is pomegranate, which is widely consumed fresh and in beverage forms and has been used extensively in ancient cultures for various medicinal properties (42). Pomegranate is a rich source of anthocyanins and hydrolyzable tannins and possesses potent antioxidant and anti-inflammatory properties (18,43). Studies have shown that pomegranate and other naturally occurring antioxidant-rich botanicals are effective in reducing the harmful effect of UVB-mediated skin damage (6,24).

Our results show that UVB caused damage to DNA and proteins as evident from increased formation of CPD, 8-OHdG and protein carbonyl groups (Figures 1 and 2). UVB-radiation can cause DNA damage, directly by absorption of high energy photons by DNA and indirectly through ROS. Absorption of UVB energy results in DNA photoproducts such as CPD whereas ROS result in 8-OHdG formation. These photoproducts can be repaired by the nucleotide excision repair system or the base excision repair system (44). Pomegranate derived products POMx juice, POMx extract and POMo protect the EpiDerm™ FT-200 from UVB-mediated DNA damage by its strong antioxidant activity (Figure 1). It may also cause an increase in DNA repair mechanisms and therefore play a significant role in ameliorating or preventing UVB-induced DNA damage. Studies show that antioxidant such as vitamin D and EGCG inhibit DNA damage by increasing DNA repair mechanisms (45, 46).

UV irradiation, which is part of the skin damage process, causes irreversible damage to proteins by ROS generation (47). Our data (Figure 2) confirms other studies which show that UV irradiation causes oxidation of certain amino acid resulting in formation of carbonyl groups on proteins with the accumulation of oxidatively modified protein (33). The toxic effects of ROS are counteracted by antioxidants and antioxidant enzymes. Other antioxidant rich botanicals have also been reported to protect skin cells against UV-induced oxidative damage to proteins (48). Therefore topical and/or systemic application of antioxidants could support physiological mechanisms to maintain or restore protein integrity thereby maintaining a healthy skin barrier.

PCNA is an active nuclear protein involved in DNA replication, recombination and repair. UV-induced increase in cell proliferation is an early event associated with UV-mediated carcinogenesis that helps the exposed cells to proceed further into cell cycle (49), which could be prevented by arresting the cells at G₁ or S phase of the cell cycle (50). Our data from immunoblot and immunohistochemical analyses revealed that UVB radiation causes a significant upregulation in PCNA, a marker of cellular proliferation. However, UVB-induced PCNA expression were decreased by pomegranate derived products pretreatment (Figure 3). These results suggest that inhibition of cell proliferation by these pomegranate derived products could be one of the mechanisms by which these agents protects damaged cells from entering the cell cycle, thereby providing damaged cells additional time for repair and in case if the damage is severe preserving their entry into apoptotic pathway (51).

Solar UV damaged human skin is characterized by connective tissue damage that includes massive accumulation of abnormal elastic fibers. UVB irradiation is known to stimulate synthesis of elastin the major protein component of elastic fibers (52). Studies show that in human skin, tropoelastin the precursor monomer of elastin, are produced *in vivo* by both the epidermal keratinocytes and dermal fibroblast (53) and an interaction between epidermal keratinocytes and dermal fibroblast play a role in post-translational modification of elastin (54). Our result from immunoblot and immunohistochemical analyses show that UVB causes a significant increased in tropoelastin level whereas pretreatment of EpiDermTM FT-200 with POMx juice, POMx extract or POMo inhibited this UVB-mediated increase in tropoelastin levels (Figure 4). The reasons for UV induction in tropoelastin expression remain to be investigated further. It is possible however, that various cytokines and growth factors produced by inflammatory cells in photodamaged skin may play some role in the stimulation of cells to produce more tropoelastin (52).

Solar radiation causes cutaneous photodamage characterized by alterations in the quantity and structure of the extracellular matrix (55). Synthesis of ECM proteins and their degradation by MMPs are part of the dermal remodeling resulting from chronic exposure of skin to UV radiation. ROS generated upon UV exposure play a major role in dermal connective tissue transformations including degradation of skin collagen (56). MMP-mediated ECM damage has been shown to be a major contributor of photoaged human skin. Although UV-induced expression of MMP gene occurs predominantly in the epidermis, MMP proteins and their enzymatic activity are abundant in both the dermis and the epidermis (57,58). Our results show that UVB caused an increased in MMP-1, MMP-2, MMP-3, MMP-7 MMP-9, MMP-11 and MMP-12 protein expression along with an increase in the activity of secreted gelatinases in human reconstituted skin, whereas pretreatment with POMx juice, POMx extract or POMo abrogated this effect (Figures 5A and 5B) probably by inhibition of ROS generation due to their antioxidant activity. Therefore, inhibition of the induction of MMPs can alleviate UV-induced tumor invasion and photoaging.

Studies show that UV radiation-induced generation of ROS contributes significantly to signaling events that leads to gene expression (59). The *c-jun* gene which encodes the

nuclear phosphoprotein c-jun, in association with c-fos, binds to the activator protein-1 (AP-1) sites of DNA and acts as a regulatory factor for gene transcription (60). It has been reported that activation of AP-1 participates in the UVB-driven breakdown of ECM in human skin by inducing the expression of a series of MMPs responsible for ECM degradation (61). Our results show that UVB irradiation of EpiDerm™ FT-200 increased the level of phosphorylated c-jun along with c-fos protein, whereas pretreatment of EpiDerm™ FT-200 with POMx juice, POMx extract or POMo inhibited UVB-induced phosphorylation of c-jun and c-fos protein expression (Figure 6). These results explain that inhibition of c-jun phosphorylation along with c-fos, which is known to be closely associated with AP-1 activation, may contribute to the prevention of UVB-induced AP-1, which regulates MMPs expression in human skin.

In conclusion, this study demonstrates the photochemopreventive effect of pomegranate derived products. Our data suggest that pretreatment of EpiDerm™ FT-200 with POMx juice, POMx extract or POMo inhibited UVB-mediated DNA and protein damage, increase in PCNA and tropoelastin levels along with degradation of ECM proteins. Pomegranate derived products also attenuated UVB-induced phosphorylation of c-jun and increase in c-fos protein. These results suggest that all three pomegranate derived products may be useful against UVB-mediated damages to human skin. These results provide a basis for more in-depth studies to assess the effectiveness of pomegranate fruit and its derived products in the prevention of UVB-mediated damage and photoaging in humans.

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Figure 1. Effect of POMx juice, POMx extract and POMo on UVB-mediated formation of CPD in three dimensional human reconstituted skin (EpiDerm™ FT-200)

EpiDerm™ FT-200 was topically treated with or without POMx juice (2μl), POMx extract (10 μg) or POMo (2μl) diluted in EFT media (100 μl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm²). Twelve h post-UVB, skin were harvested and immunostaining was performed to detect UVB-induced DNA damage in the form of (A) CPD and (B) 8-OHdG positive cells as described in “Materials and Methods”. Representative sections of immunostaining are shown from three independent experiments with similar results.

Figure 2. Effect of POMx juice, POMx extract and POMo on UVB-mediated protein oxidation in three dimensional human reconstituted skin (EpiDerm™ FT-200)

EpiDerm™ FT-200 was topically treated with or without POMx juice (1-2μl), POMx extract (5-10 μg) or POMo (1-2μl) diluted in EFT media (100 μl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm²). Twelve h post UVB, EpiDerm™ FT-200 was harvested and cell lysates prepared and western blot analysis performed after derivitization of transferred protein PVDF membrane with DNPH. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The values above the figures represent relative density of the bands normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results.

Figure 3. Effect of POMx juice, POMx extract and POMo on UVB-mediated increase in protein expression of PCNA in three dimensional human reconstituted skin (EpiDerm™ FT-200)

(A) EpiDerm™ FT-200 was topically treated with or without POMx juice (1-2μl), POMx extract (5-10 μg) or POMo (1-2μl) diluted in EFT media (100 μl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm²). [A] Twelve h post-UVB, EpiDerm was harvested and cell lysates prepared for western blot analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The values above the figures represent relative density of the bands normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results. (B) EpiDerm™ FT-200 was topically treated with or without POMx juice (2μl), POMx extract (10 μg) or POMo (2μl) diluted in EFT media (100 μl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm²). Twelve h post-UVB, EpiDerm was harvested and immunostaining was performed to detect UVB-induced PCNA positive cells as described in “Materials and Methods”. Representative sections of immunostaining are shown from five independent experiments with similar results.

Figure 4. Effect of POMx juice, POMx extract and POMo on UVB-mediated increase in tropoelastin in three dimensional human reconstituted skin (EpiDerm™ FT-200)

(A) EpiDerm™ FT-200 was topically treated with or without POMx juice (1-2µl), POMx extract (5-10 µg) or POMo (1-2µl) diluted in EFT media (100 µl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm²). [A] Twelve h post-UVB, EpiDerm was harvested and cell lysates prepared for western blot analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The values above the figures represent relative density of the bands normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results. (B) EpiDerm™ FT-200 was topically treated with or without POMx juice (2µl), POMx extract (10 µg) or POMo (2µl) diluted in EFT media (100 µl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm²). Twelve h post-UVB, EpiDerm was harvested and immunostaining was performed to detect UVB-induced PCNA positive cells as described in "Materials and Methods". Representative sections of immunostaining are shown from five independent experiments with similar results.

Figure 5. Effect of POMx juice, POMx extract and POMo on UVB-mediated increase in MMPs protein and gelatinase activity in human reconstituted skin (EpiDermTMFT-200)

(A) EpiDermTM FT-200 was topically treated with or without POMx juice (1-2μl), POMx extract (5-10 μg) or POMo (1-2μl) diluted in EFT media (100 μl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDermTM FT-200 washed with PBS and exposed to UVB (60mJ/cm²). Twelve h post-UVB, EpiDerm was harvested and cell lysates prepared for western blot analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The values above the figures represent relative density of the bands normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results. (B) EpiDermTM FT-200 was topically treated as described above. Twelve h post-UVB, culture media was collected and gelatin zymography performed as described in "Materials and Methods". The gels shown here are representative of three independent experiments with similar results.

Figure 6. Effect of POMx juice, POMx extract and POMo on UVB-mediated phosphorylation of c-jun and expression of c-fos protein in human reconstituted skin (EpiDerm™ FT-200)
 EpiDerm™ FT-200 was topically treated with or without POMx juice (1-2μl), POMx extract (5-10 μg) or POMo (1-2μl) diluted in EFT media (100 μl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm²). Twelve h post-UVB, EpiDerm was harvested and cell lysates prepared for western blot analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The values above the figures represent relative density of the bands normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results.