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Original Article

Dietary compound ellagic acid alleviates skin wrinkle and inflammation induced by UV-B irradiation

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Abstract: Ellagic acid, a polyphenol compound present in berries and pomegranate, has received attention as an agent that may have potential bioactivities preventing chronic diseases. This study examined photoprotective effects of ellagic acid on collagen breakdown and inflammatory responses in UV (ultraviolet)-B irradiated human skin cells and hairless mice. Ellagic acid attenuated the UV-B-induced toxicity of HaCaT keratinocytes and human dermal fibroblasts. Non-toxic ellagic acid markedly prevented collagen degradation by blocking matrix metalloproteinase production in UV-B-exposed fibroblasts. Anti-wrinkle activity of ellagic acid was further investigated in hairless mice exposed to UV-B, in which it attenuated UV-B-triggered skin wrinkle formation and epidermal thickening. Topical application of 10 μmol/l ellagic acid diminished production of pro-inflammatory cytokines IL-1β and IL-6, and blocked infiltration of inflammatory macrophages in the integuments of SKH-1 hairless mice exposed to UV-B for 8 weeks. In addition, this compound mitigated inflammatory intracellular cell adhesion molecule-1 expression in UV-B-irradiated keratinocytes and photoaged mouse epidermis. These results demonstrate that ellagic acid prevented collagen destruction and inflammatory responses caused by UV-B. Therefore, dietary and pharmacological interventions with berries rich in ellagic acid may be promising treatment strategies interrupting skin wrinkle and inflammation associated with chronic UV exposure leading to photoageing.

Key words: ellagic acid – inflammation – photoageing – skin – wrinkle

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Introduction

Ultraviolet (UV) radiation is one of the causative factors of DNA damage and inflammatory responses, and induces various cutaneous lesions such as photoageing and photocarcinogenesis (1,2). The damage of the extracellular matrix (ECM) integrity in skin tissues is responsible for the blister formation and skin wrinkle, indicative of photoageing (3). The matrix metalloproteinases (MMP) production and subsequent ECM alteration were observed in pre-mature skin ageing and in aged skin (2,4). Collagenolytic MMP enzymes attack fibrillar collagen and elastin involved in the dermal strength and resiliency (2). Accordingly, the MMP inhibition is one of the strategies to prevent UV-triggered skin photodamage.

UV irradiation produces reactive oxygen species (ROS), which in turn regulates a variety of cellular functions including collagen fragmentation and MMP secretion (5). Thus, antioxidants scavenging and quenching ROS have been proposed to be photoprotective agents. Antioxidant *N*-acetyl cysteine inhibited UV-induced human skin ageing through mitogen-activated protein kinase signalling pathways (6). UV increased release of pro-inflammatory mediators from a variety of skin cells and infiltration and activation of immune cells into the skin, resulting in MMP activation (2). Inflammation activated various matrix-degrading MMP, which leads to abnormal matrix degradation and accumulation of non-functional matrix components (7).

In recent years, the application of botanical agents in skin care products has been increasing. Phenolic compounds are potential agents protecting the skin against adverse effects of UV radiation (5,8). The skin photoprotection appears to pertain to their antioxidant, anti-inflammatory and anti-carcinogenic properties (8). Administration of dietary grape seed proanthocyanidins inhibited UV-B-induced skin photocarcinogenesis and pigmentation in animal models (9,10). The green tea polyphenols prevented UV-induced oxidative genotoxic damage in keratinocytes and mouse skin (11,12). A recent human

study showed that topical green tea extract reduced UV-B-mediated epithelial damage at low concentrations without tachyphylaxis, suggesting green tea extract as suitable photochemopreventive agents (13). In addition, polyphenol-rich plant extracts were effective in dampening the oxidative cellular damage of UV radiation and preventing skin cancer cell proliferation (14).

Ellagic acid, a polyphenolic compound mostly found in berries and pomegranate, is shown to possess growth-inhibiting and apoptosis-promoting activities in cancer cells (14–16). However, inhibitory actions of ellagic acid in the photoageing were not well defined. Pomegranate fruit extract (PFE) full of phytochemicals exhibited anti-oxidative activity, which was thought to be resulting from the action of ellagic acid (17). It was also shown that PFE was an effective photochemopreventive agent ameliorating UV-A-mediated damages via modulating cellular pathways in normal human epidermal keratinocytes (18). Recently, it was revealed that PFE prevented UV-A- and UV-B-induced damage in human skin fibroblast cells (14).

Considering that PFE inhibited UV-induced cellular damage, it was hypothesized that ellagic acid (Fig. 1a) blocked UV light-induced wrinkle formation and skin inflammation. To test this hypothesis, this study elucidated the inhibitory activity of ellagic acid in the collagen degradation and inflammatory responses of skin cells and hairless mice induced by UV-B irradiation. We examined whether ellagic acid manipulated the production of collagenolytic MMP, expression of intracellular cell adhesion molecule-1 (ICAM-1) and secretion of inflammatory interleukin (IL)-1β and IL-6 in UV-B-exposed HaCaT keratinocytes, human dermal fibroblasts and dorsal skin of SKH-1 hairless mice.

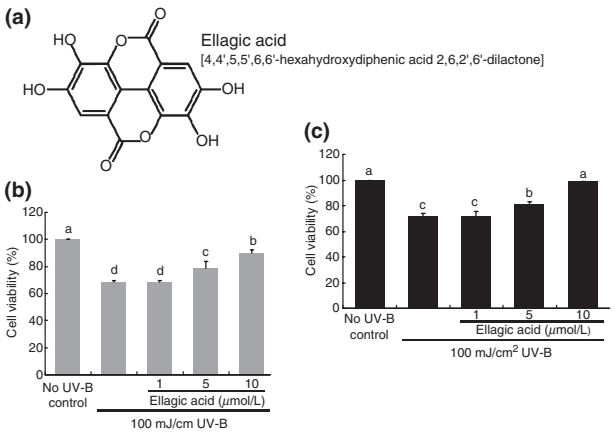

Figure 1. Chemical structure of ellagic acid (a) and cell viability of HaCaT keratinocytes (b) and dermal fibroblasts (c) treated with ellagic acid and challenged with UV-B irradiation. Confluent cells were left untreated or stimulated with UV-B prior to 24 h incubation with 1–10 μmol/l ellagic acid. Cell viability was measured using MTT assay and presented as mean ± SEM from three independent experiments with multiple estimations (b and c). Values not sharing a letter are different at $P < 0.05$.

Materials and methods

Materials

Human dermal fibroblasts were obtained from Clonetics (San Diego, CA, USA) and human keratinocyte HaCaT cell line was kindly provided by Professor Norbert E. Fusenig (German Cancer Research Center, Germany). Ellagic acid and Dulbecco's modified Eagle's media and culture reagents were purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA). Foetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were provided from Lonza (Walkersville, MD, USA). 3-(4,5-Dimetylthiazol-yl)-diphenyl tetrazolium bromide (MTT) was obtained from DUCHEFA Biochemie (Haarlem, Netherlands). Antibodies against human MMP-1, human MMP-8, human MMP-13, human type1 collagen, human ICAM-1 and mouse scavenger receptor A (SR-A) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit and donkey anti-goat IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Cyanine 3-conjugated donkey anti-goat IgG and goat anti-rabbit IgG were provided by Rockland (Gilbertville, PA, USA).

Cell culture and UV-B irradiation

HaCaT keratinocyte cell line and human dermal fibroblasts were cultured in Dulbecco's modified Eagle's media containing 10% FBS, 2 mmol/l glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C humidified atmosphere of 5% CO_2 in air. Keratinocytes were plated at 70–80% and fibroblasts were plated at 90–95% confluence in all experiments. The UV-B light source (λ_{max} = 312, no UV-A and UVC emission) was provided from Bio-Sun lamps (Vilber Lourmat, France). Keratinocytes and fibroblasts were pre-treated with 1–10 μmol/l ellagic acid, exposed to 100 mJ/cm^2 UV-B and incubated for 24 or 48 h. In our previous study (5), the irradiation energy of UV-B at ≥100 mJ/cm^2 significantly and dose-dependently reduced the viability of 48 h-cultured cells in a range of 50–3000 mJ/cm^2.

After the challenge of HaCaT keratinocyte and dermal fibroblasts for 24 h with UV-B and 1–10 μmol/l ellagic acid, the MTT assay was performed to quantitatively determine cellular viability (19). The cells were incubated in a fresh medium containing 1 mg/ml MTT for 3 h. After removal of unconverted MTT, the purple formazan product was dissolved in isopropanol. Absorbance of formazan dye was measured at λ = 570 nm.

Western blot analysis

Western blot analysis was conducted using whole cell lysates and culture media prepared from human dermal fibroblasts and keratinocytes (20). Whole cell extracts were prepared in a lysis buffer containing 1% β-mercaptoethanol, 1 mol/l β-glycerophosphate, 0.1 mol/l Na_3VO_4, 0.5 mol/l NaF and protease inhibitor cocktail. Cell extracts of equal amounts of total proteins or equal volumes of culture supernatants were electrophoresed on 6–10% SDS-PAGE and transferred onto a nitrocellulose membrane. Non-specific binding was blocked by soaking the membrane in a TBS-T buffer [50 mmol/l Tris–HCl (pH 7.5), 150 mmol/l NaCl and 0.1% Tween 20] containing 5% skim milk for 3 h. The membrane was incubated overnight at 4°C with monoclonal mouse anti-human MMP-1, polyclonal rabbit anti-human ICAM-1 and polyclonal goat antibodies (human MMP-8, human MMP-13 and human type1 collagen). The membrane was then incubated with a secondary antibody goat anti-rabbit IgG- or donkey anti-goat IgG-conjugated with horseradish peroxidase. The protein levels were determined by using Supersignal West Pico Chemiluminescence detection reagents (Pierce Biotechnology, Rockford, IL, USA) and Konica X-ray film (Konica, Tokyo, Japan). Incubation with polyclonal mouse anti-human β-actin antibody was performed for endogenous control.

Analyses of real-time-polymerase chain reaction (PCR)

Following cell culture protocols, total RNA of human dermal fibroblasts was extracted using a commercially available Trizol reagent kit (Invitrogen, Carlsbad, CA, USA). The RNA (2 μg) was reversibly transcribed with 200 units of reverse transcriptase and 0.5 g/l oligo-$(dT)_{15}$ primer (Bioneer, Korea). The mRNA transcript levels of MMP-1 (forward primer: 5′-ACGGATACCCCAAGGACATCT-3′, reverse primer: 5′-CTCAGAAAGAGCAGCATCGATATG-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, forward primer: 5′- GAAGGTGAAGGTCGGAGTC-3′, reverse primer: 5′-GAAGATGGTGATGGGATTTC-3′) were quantified by real-time PCR (Corbett Research, Australia) using SYBR Green PCR kit (Quagen, Valencia, CA). The PCR and melting curve analyses were carried out as previously described (5). The housekeeping gene GAPDH was used for internal normalization. Data analysis of real-time RT-PCR results and calculation of the relative quantitation were carried out using the Delta CT analysis using the Rotor-gene software version 6.0 (Corbett Research, Australia).

Immunocytochemistry

After human dermal fibroblasts or HaCaT keratinocytes grown on 24-well glass slides were washed with PBS containing 0.2% Tween 20 (PBS-T), cells were fixed with 4% ice-cold formaldehyde for 30 min and treated for 2 min with 0.1% Triton-X100 and 0.1% citric acid in PBS. To block any non-specific binding, cells were incubated with 20% FBS for 1 h. After washing fixed fibroblasts with PBS-T, polyclonal goat anti-human collagen type1 was sufficiently added to cells and incubated overnight at 4°C. For the ICAM-1 expression, HaCaT cells were incubated with polyclonal rabbit anti-human ICAM-1. The cells were incubated with cyanine 3-conjugated anti-goat IgG (1:10000) for collagen type1 and cyanine 3-conjugated anti-rabbit IgG (1:10000) for ICAM-1 as a secondary antibody. Fluorescent images were obtained by a fluorescence microscopy with an AXIOIMAGER (Zeiss, Göttingen, Germany) fluorescent microscope.

Animals and UV-B radiation

Male SKH-1 hairless mice (4-week-old) obtained from Charles River Laboratory (Wilmington, MA, USA) were kept on a 12 h light/12 h dark cycle at 23 ± 1°C with 50 ± 10% relative humidity under specific pathogen-free conditions and fed a standard diet (CJ Feed, Korea) and water *ad libitum* at the animal facility of Hallym University. The animals were allowed to acclimatize for a week before beginning the experiments. Eighteen mice were divided into three groups. The first group of six mice was not irradiated with UV-B and used as ellagic acid–vehicle acetone controls. The other 12 mice were exposed to UV-B three times a week and the irradiation intensity was increased weekly by 1 MED (minimum erythema dose) to 3 MED, and then continued at 3 MED until the 8th week. Total irradiation intensity was 63 MED for 8 weeks, and about 7 min was required to reach 1 MED. The dorsal skin surface of these animals was irradiated with two TL2-W/01 RS UV-B lamps (Philips, Somerset, NJ, USA). Among UV-B exposed animals, one group of six mice was treated with 10 μmol/l ellagic acid dissolved in 100 μl acetone. Long-term topical application of ellagic acid was performed on the dorsal skin of UV-B-irradiated mice every day. All experiments were approved by the Committee on Animal Experimentation of Hallym University and performed in compliance with the University's Guidelines for the Care and Use of Laboratory Animals.

Skin characteristics

A photograph of the dorsal skin of each mouse was taken using an optical system in digital camera (Canon, Japan) and CCD camera (Visioscan VC98, CK Electronics, Germany), just before animals were killed. The skin characteristics were analysed using Skin-Visiometer SV 600 software. Arbitrary Units for the skin roughness/smoothness and scaliness were based on the principle of measuring the depth of furrows according to shadow size and brightness because of inflection under illumination.

Histology and microscopy

Mice were killed by cervical dislocation under anaesthesia at the termination of experimental protocols. For histological analyses, skin specimens from mouse central dorsum were obtained at the end of the experiments, fixed in 10% buffered formalin. The paraffin-embedded skin specimens were sectioned at 5 μm, deparaffinized and stained with Masson-trichrome for the visualization of collagen fibres and also processed with haematoxylin and eosin staining for the light microscopic evaluation. The stained tissue sections were examined using an optical microscope AXIOIMAGER (Zeiss, Germany) and five images (200×) were taken per section. Epidermal thickness was determined as the distance from the basal layer to the stratum granulosum/stratum corneum junction. The thickness was measured in each photograph at 10 random sites.

Enzyme-linked immunosorbent assay (ELISA)

The cytokine levels of IL-1β and IL-6 in the UV-B-irradiated SKH-1 hairless mouse skin tissue were determined by using ELISA. The skin tissue samples were homogenized in tissue protein reagent buffer containing 1% β-mercaproethanol, 1 mol/l β-glycerophosphate, 0.1 mol/l Na_3VO_4, 0.5 mol/l NaF with protease inhibitor cocktail and homogenates were centrifuged at 2000 *g* for 10 min. After the centrifugation, IL-1β and IL-6 in the tissue supernatants were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA). The protein concentrations of tissue supernatants were determined and the levels of IL-1β and IL-6 in the skin tissues were normalized and expressed as μg/g tissue protein.

Immunohistochemical staining

For immunohistochemical analyses, paraffin-embedded integument sections (5-μm thick) were employed. The sections were placed on glass slides, deparaffinated and hydrated with xylene and graded alcohol. The tissue sections were then subjected to incubation with 0.5% H_2O_2 in methanol for the removal of endogenous peroxidase. The non-specific antibody binding site was blocked by using 3% BSA in PBS-T for 1 h, followed by 1 h incubation with goat anti-mouse SR-A (1:100) or goat anti-mouse ICAM-1 (1:100). The tissue sections were incubated for 1 h with peroxidase-conjugated anti-goat IgG (1:200). The integuments were developed with 3,3′-diaminobenzidine as a substrate for 1 min and counterstained with haematoxylin.

Data analysis

The results are presented as mean ± SEM for each treatment group. Statistical analyses were conducted using Statistical Analysis Systems. Significance was determined by one-way ANOVA followed by Duncan range test for multiple comparisons and were considered significant at $P < 0.05$.

Results

Inhibitory effects of ellagic acid on viability of UV-B-exposed skin cells

To examine cell viability in UV-B-irradiated HaCaT keratinocytes for 24 h (Fig. 1b) and human dermal fibroblasts for 48 h (Fig. 1c), the MTT analysis was conducted. The UV-B irradiation at 100 mJ/cm^2 reduced cell viability up to 30–40%. When ellagic acid in concentrations between 1 and 10 μmol/l was added to UV-B-exposed keratinocytes and fibroblasts, the viability was dose-dependently enhanced and attained the full inhibition with 10 μmol/l ellagic acid in UV-B-induced skin cells.

Blockade of MMP secretion and collagen degradation in UV-B-exposed and ellagic acid-treated dermal fibroblasts

Figure 2a shows the inhibitory effects of ellagic acid on the MMP production in UV-B-induced human dermal fibroblasts. Western blot data revealed that 48 h-UV-B irradiation augmented the production of collagenolytic enzymes of MMP-1, MMP-8 and MMP-13. Adding ≥5 μmol/l ellagic acid to UV-B-exposed fibroblasts abolished the secretion of all of the enzymes. There was a low basal mRNA expression of MMP-1 in quiescent fibroblasts (Fig. 2a). The expression of MMP-1 mRNA was elevated in 24 h-UV-B-stimulated dermal fibroblasts, as confirmed by quantitative real-time PCR (Fig. 2b). The MMP-1 transcript expression UV-B was sharply induced by ≈fivefold, compared with that of no-UV-B control cells. The UV-B-induced mRNA expression level of MMP-1 was diminished in the cells treated with 10 μmol/l ellagic acid. Indeed, this was consistent with a substantial attenuation of MMP-1 secretion caused by ellagic acid.

This study attempted to examine whether ellagic acid attenuated the collagen breakdown of dermal fibroblasts induced by UV-B radiation. As expected, the UV-B irradiation dampened the cellular levels of type 1 collagen, as evidenced by Western blots (Fig. 2c). The MMP production appeared to be responsible for the collagen levels down-regulated by UV-B irradiation. By contrast, the collagen levels were dose-dependently enhanced by the treatment with 1–10 μmol/l ellagic acid. Immunocytochemical data with a type 1 collagen antibody were consistent with Western blot data (Fig. 2d). A heavy cytoplasmic staining was observed in UV-B-untreated control fibroblasts, whereas the staining was almost vanished in UV-B-exposed cells. The cytoplasmic staining of cells treated with ≥5 μmol/l ellagic acid was comparable with, if distinguishable from, that of UV-B-untreated cells (Fig. 2d). In

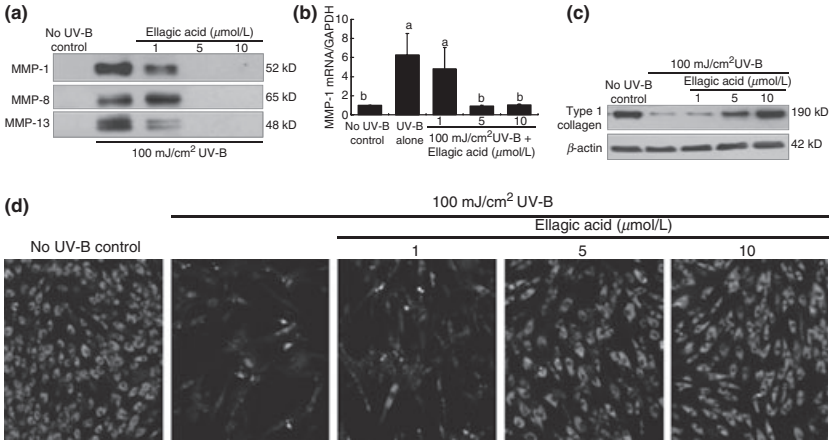

Figure 2. Inhibition of MMP production (a, b) and reduction of type 1 collagen level (c, d) by ellagic acid in human dermal fibroblasts challenged with UV-B irradiation. Confluent cells were left untreated or stimulated with UV-B prior to 48 h incubation with 1–10 μmol/l ellagic acid. Conditioned culture media were subjected to 10% SDS-PAGE, followed by Western blot analysis with respective primary antibody of MMP-1, MMP-8 and MMP-13 (a). Real-time-PCR data (b) showed transcriptional levels of MMP-1 mRNA in ellagic acid-treated and UV-B-exposed human dermal fibroblasts. The GAPDH gene was used as an internal control for the co-amplification with MMP-1. The bar graphs (mean ± SEM, $n = 3$) represent quantitative mRNA transcript levels of MMP-1 relative to GAPDH. Values not sharing a letter refer to significant difference at $P < 0.05$. For the measurement of cellular level of type 1 collagen, cell lysates were run for Western blot analysis with respective primary antibody against type 1 collagen or β-actin protein (c). Bands are representative of three independent experiments. In the immunocytochemical experiments (d), cells were fixed and then incubated with goat anti-human type 1 collagen. Antibody localization was detected with cyanine 3-conjugated donkey anti-goat IgG using a fluorescence microscopy. Magnification: 200-fold.

addition, submicromolar ellagic acid elevated collagen levels in a dose-dependent manner; it should be noted that ellagic acid inhibited MMP secretion up-regulated by UV-B irradiation completely (Fig. 2a).

Inhibition of UV-B-exposed skin wrinkle formation and collagen digestion by ellagic acid

Skin Wrinkle formation was examined in SKH-1 hairless mice exposed to chronic UV-B radiation for 8 weeks. Integumentary images and cutaneous characteristics for wrinkle were photographed and analysed by digital and CCD optical camera (Fig. 3). Long-term UV-B irradiation created wrinkle in the mouse skin (Fig. 3a), and rendered the skin rough and scaly (Fig. 3b), indicative of skin photoageing. When 10 μmol/l ellagic acid was topically applied to the dorsal skin of hairless mice, the wrinkle formation was noticeably attenuated and the dorsal skin attributes were improved.

Histological staining with masson-trichrome for dermal collagen fibres showed that 8-week UV-B radiation reduced the cellular collagen levels in the intra-dermis (Fig. 4a), indicating elevated digestion of dermal connective tissues. In contrast, there was a strong staining of collagen fibres in 10 μmol/l ellagic acid-treated hairless mice exposed to UV-B, compared with that in UV-B-alone mice (Fig. 4a). These staining data proved the cell culture data that ellagic acid up-regulated the cellular levels of type 1 collagen in UV-B-exposed dermal fibroblasts (Fig. 2).

Skin epidermal thickening was measured in UV-B-irradiated hairless mice by haematoxylin and eosin staining (Fig. 4b). A fourfold increase in the epidermal thickness of the dorsal skin of chronic UV-B-exposed hairless mice was observed. The 8-week topical application of ellagic acid substantially reduced the epidermal thickness of the dorsal skin (Fig. 4b). Accordingly, the topical treatment with ellagic acid diminished rough and thick wrinkles formed induced by the exposure to long-term UV-B irradiation.

UV-B-induced production of pro-inflammatory cytokines in hairless mouse skin

In this study, the production of the pro-inflammatory cytokines of IL-1β and IL-6 in skin tissues of hairless mice was examined using ELISA. The chronic UV-B exposure increased levels of IL-1β and IL-6 in the dorsal skin of hairless mice (Fig. 5a). By contrast, the topical treatment with 10 μmol/l ellagic acid significantly attenuated production of IL-1β and IL-6 proteins. Accordingly, the inhibition of ICAM-1 expression of keratinocytes by ellagic acid was most likely because of its inhibition of production of IL-1β and IL-6 pertaining to skin inflammation.

This study further investigated inflammatory macrophages crowded in the dermis during the UV-B irradiation. The UV-B irradiation increased the number of macrophages in mouse skin integuments (Fig. 5b), as evidenced by immunohistochemical staining using an SR-A antibody.

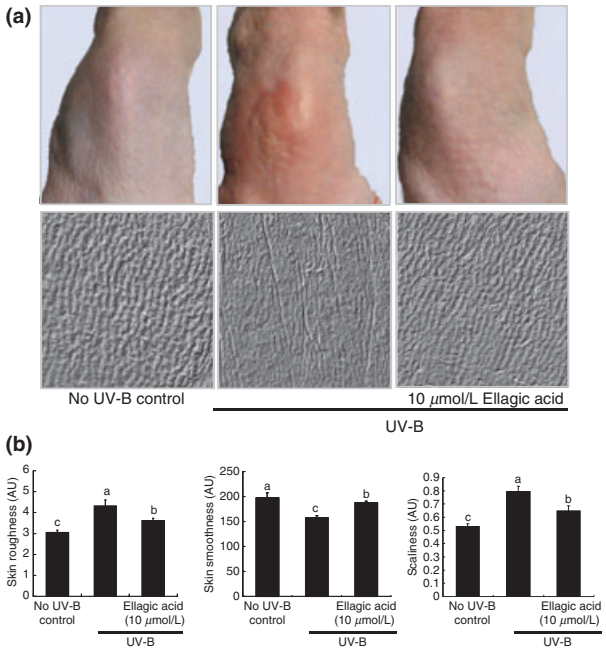

Figure 3. Wrinkle formation (a) and skin characteristics (b) after long-term topical treatment with ellagic acid on the dorsal skin of SKH-1 hairless mice exposed to UV-B radiation. The dorsal skin of hairless mice was topically treated with 10 μmol/l ellagic acid. Skin specimens were obtained from dorsal skin of mice that were killed at the end of the 8-week experiments. Skin wrinkle images were photographed by using a CCD optical camera and skin surface analysis was performed. Arbitrary units (AU) for the skin roughness/smoothness and scaliness were based on the principle of measuring the depth of furrows according to shadow size and brightness because of inflection under illumination.

Conversely, the topical application of 10 μmol/l ellagic acid to UV-B-inflamed mouse skin substantially retarded the cutaneous gathering of macrophages.

Inhibition of ICAM-1 expression during UV-B-irradiation

Human keratinocytes during the course of inflammatory dermatoses are known to strongly express the surface adhesion molecule ICAM-1, which plays an important role in the generation of the epidermal inflammatory infiltrate by mediating leucocyte–keratinocyte interactions (21). This study examined whether UV-B irradiation elevated the ICAM-1 expression of HaCaT keratinocytes and whether this expression was inhibited by ellagic acid. There was a distinct increase in ICAM-1 expression in UV-B-exposed cells (Fig. 6a), as proved by Western blot analysis. When ellagic acid was added in concentrations between 1 and 10 μmol/l, the ICAM-1 induction was found to be dose-dependently inhibited. In addition, immunocytochemical staining revealed that ellagic acid inhibited the UV-B-elevated ICAM-1 expression in a similar manner (Fig. 6b).

This study attempted to further investigate inflammatory ICAM-1 expression in the epidermis during the UV-B irradiation (Fig. 6c). The UV-B irradiation enhanced the expression of ICAM-1 in mouse skin integuments, which was diminished by a topical treatment with 10 μmol/l ellagic acid.

Discussion

Polyphenolic ellagic acid mostly found in berries has been shown to possess growth-inhibiting and apoptosis-promoting activities in cancer cell lines (14–16). Ellagic acid prevented proteolytic degradation of existing dermal elastic fibres and efficiently enhanced elastogenesis in aged skin (22). However, the inhibitory actions of ellagic acid in the photoageing are not well defined. Pomegranate juice has

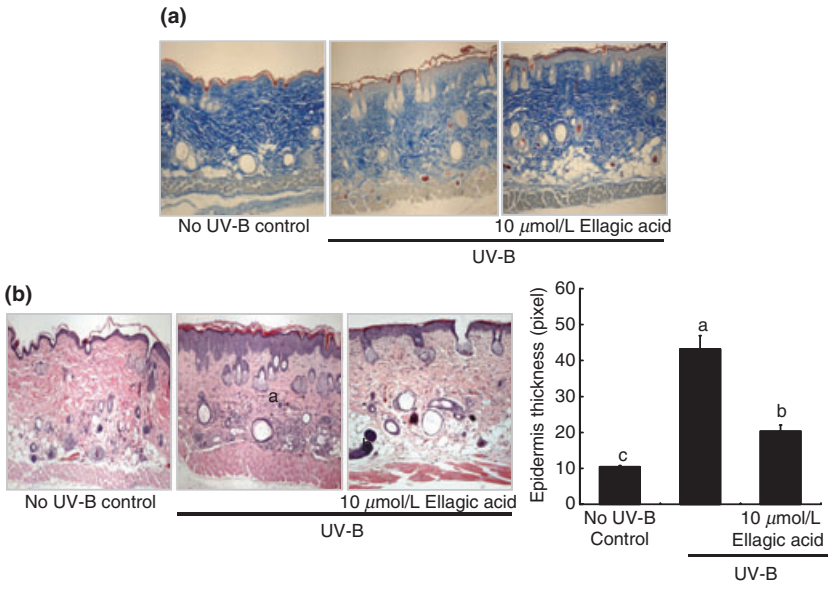

Figure 4. Effects of topical administration of ellagic acid on UV-B-induced wrinkle formation (a) and epidermal thickness (b) on the back of hairless mice skin at the end of week 8. After the UV-B irradiation for 8 weeks, histological sections of mouse dorsal skins were stained with Masson-trichrome (a). Each photograph is representative of at least six animals. Paraffin sections of biopsies were photographed after haematoxylin and eosin staining (b). The distance from the basal layer to the stratum granulosum/stratum corneum junction was measured for dermal thickness of dorsal skins. The respective values are mean ± SEM from six animals and the values not sharing a letter are different at $P < 0.05$. Magnification: 200-fold.

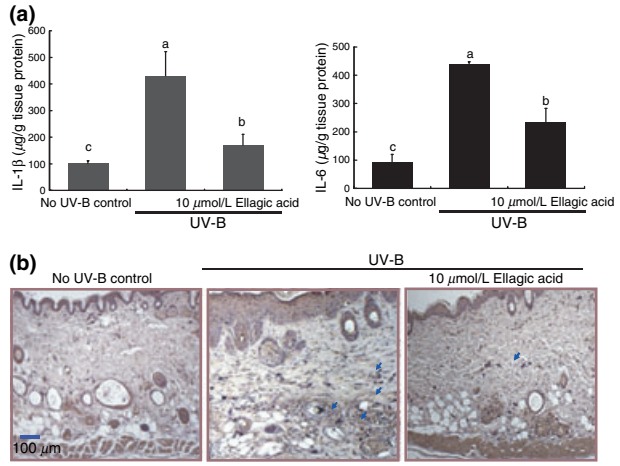

Figure 5. Effects of topical ellagic acid on secretion of IL-1β and IL-6 (a) and on infiltrate of inflammatory macrophages (b) in UV-B-exposed dorsal skin tissue of hairless mice. The production of IL-1β and IL-6 in dorsal skin tissues of hairless mice was measured. Respective data represent mean ± SEM from three animal skin tissues of each animal group (a). Values not sharing a letter are different at $P < 0.05$. After the UV-B irradiation for 8 weeks, histological sections of mouse dorsal skins were immunohistochemically stained using a goat anti-mouse SR-A and counterstained with haematoxylin (b). The arrows showing brown spots indicate macrophages infiltrated in mouse skin integuments inflamed by UV-B irradiation.

the highest concentration of ellagitannins among commonly consumed juices and contains the unique polyphenol ellagitannin, punicalagin. Ellagitannins are not absorbed intact into the blood stream but are hydrolysed to ellagic acid. PFE exhibited anti-oxidant properties, which was thought to be as a result of the action of ellagic acid (17). It was shown that PFE were effective in ameliorating UV-mediated damages in human skin fibroblasts and epidermal keratinocytes (14,18).

UV radiation is known to directly or indirectly damage DNA and to disturb ECM preservation with increased MMP activity (1). Histological and ultrastructural studies have revealed that the photodamaged skin is associated with increased epidermal thickness and alterations of connective tissue organization (23). The MMP activity was increased in the skin even with a brief UV irradiation, which led to collagen destruction and photoageing (5). This study showed that UV radiation augmented collagen degradation in dermal fibroblasts most likely via activated collagenolytic MMP. The MMP production and subsequent ECM alterations were observed in pre-mature skin ageing and in aged skin (2,4). Collagen plays an important role in the elasticity of skin connective tissues with diversified structures (24). Collagenolytic MMP enzymes attack fibrillar collagen and elastin responsible for the dermal strength and resiliency (2). It is deemed that the MMP inhibition is one of strategies to prevent UV-triggered photodamage. Oral administration of green tea polyphenols resulted in an inhibition of UV-B-induced expression of ECM degrading MMP in hairless mouse skin (12). This study found that ellagic acid inhibited the UV irradiation-induced MMP production in dermal fibroblasts and

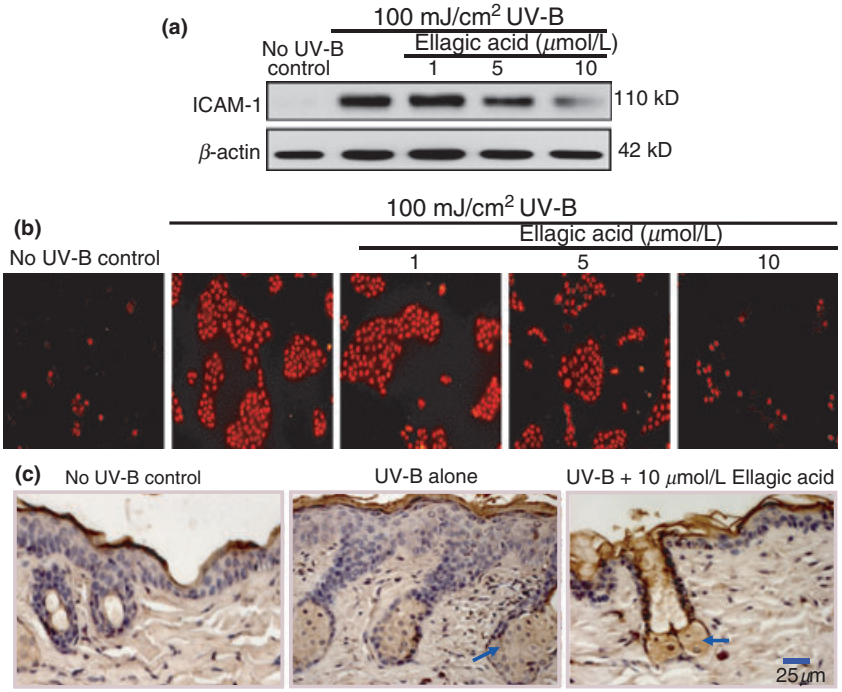

Figure 6. Inhibition of inflammatory ICAM-1 expression by ellagic acid in UV-B-exposed keratinocytes and dorsal skin tissue of hairless mice. Cell lysates were subject to Western blot analysis with a primary antibody against ICAM-1 (a). β-Actin protein was used as an internal control. Immunocytochemical experiments were performed using rabbit anti-human ICAM-1 (b). Antibody localization was detected with cyanine 3-conjugated goat anti-rabbit IgG using a fluorescence microscopy. Magnification: 200-fold. The expression of ICAM-1 in dorsal skin tissues of hairless mice topically treated with ellagic acid was measured using an ICAM-1 antibody (c). After the UV-B irradiation for 8 weeks, histological sections of mouse dorsal skins were stained using a goat anti-mouse ICAM-1 and counterstained with haematoxylin.

the subsequent loss of collagen fibres in the dermal layer of hairless mice.

UV-B-stimulated collagen fragmentation and MMP production were linked to induction of ROS (5). In addition, the ROS-triggered MMP induction was observed in photoaged skin accompanying connective tissue breakdown (4). Topically applied anti-oxidant *N*-acetyl cysteine and green tea extract prevented UV-induced signalling leading to photoageing in human skin (6,13). Accordingly, one approach to ameliorate UV photoageing is the use of anti-oxidants scavenging and quenching ROS (25). Botanical anti-oxidants may be most useful agents preventing UV-mediated photodamage (26). The green tea polyphenol epigallocatechin gallate prevented collagen degradation and genotoxic damage of UV radiation (5,8). Several studies have shown that botanical compounds with an anti-oxidative activity are potential agents reducing the risk of skin diseases (25,27,28). This study showed that ROS-dampening ellagic acid inhibited cellular collagen breakdown of dermal fibroblasts and enhanced the collagen fibres levels in the mouse dermis dropped by UV-B irradiation.

UV irradiation enhanced production of pro-inflammatory mediators from various skin cells and promoted activation and infiltration of immune cells such as neutrophils and other phagocytic cells into the skin (2). Elastases and cathepsin G released from neutrophils infiltrated into the skin cause further inflammation. UV-induced oxidative stress promoted the infiltration of CD11b+ macrophages in the skin (27). It is deemed that the inflammatory mechanisms may accentuate direct damageing effects on molecules and cells which cause photoageing. Thus, inhibition of inflammatory responses to photo-skin interactions is crucial to comprehensively protect the skin against adverse solar effects. Prevention of inflammation using anti-inflammatory compounds, including cytokine generation inhibitors appears to be one strategy assuageing photoageing (8,29). Anti-oxidant silymarin inhibited the infiltration of CD11b+ macrophages in the skin promoted by UV-induced oxidative stress (27). This study showed that prolonged UV-B irradiation provoked pro-inflammatory cytokines of IL-1β and IL-6 in the dermis, which was subsided by topical ellagic acid. It was also shown that red orange extracts efficiently counteracted UV-B-induced inflammation and apoptosis pertaining to cellular oxidative stress in human keratinocytes (26).

Wrinkle formation occurs because of accumulated skin damages such as matrix destruction and skin inflammation. Skin inflammation activated various MMP, leading to abnormal matrix degradation and accumulation of non-functional matrix components in the dermal and epidermal compartments (2,7). UV radiation-exposed fibroblasts underwent inflammatory responses through increased production of various inflammatory mediators (30). However, the inhibition of UV-induced inflammation leading to MMP activation is not clearly defined. In this study, ellagic acid diminished the release of pro-inflammatory cytokines in hairless mouse skin chronically exposed to UV-B. Together, the inhibition of cytokine release by ellagic acid was most likely responsible for reduction of collagenolytic MMP production and subsequent mitigation of collagen fibre degradation.

Alterations in cutaneous and systemic immunity occur as a result of the UV-induced inflammation (30,31). These changes lead to the generation of suppressor T cells, the induction of antigen-specific immunosuppression and the lowering of cell-mediated immunity. Cell adhesion molecules play a key role in the induction of the immune response and in the pathogenesis of inflammation. UV exposure induces inflammation and damage, including alteration of adhesion molecule expression and loss of antigen-presenting cell function within the skin (31).This study revealed that ellagic acid diminished the increased ICAM-1 expression of HaCaT keratinocytes caused by UV-B radiation. It has been previously shown that the expression of ICAM-1 on keratinocytes was involved in the skin pathogenesis of actinic prurigo, an inflammatory skin disease with an abnormal immune response (32). Accordingly, ellagic acid appeared to be effective in relieving the clinical symptoms of photodermatitis such as actinic prurigo in which UV light is implicated. Furthermore, ellagic acid attenuated the cutaneous gathering of inflammatory macrophages elevated in the UV-B-inflamed skin integuments. The topical epigallocatechin gallate application on mouse skin prior to the exposure to UV-B inhibited UV-B-induced infiltration of CD11b+ cells (33). Such effect was associated with induction of UV-B-induced suppression of contact hypersensitivity responses. Thus, the present *in vivo* observations suggest that topical ellagic acid may prevent solar UV-B light-induced skin disorders associated with immune suppression.

In conclusion, the present results demonstrate the photoprotective effects of anti-oxidant ellagic acid on skin wrinkle formation resulting from collagen breakdown through increasing MMP production. The mechanism by which ellagic acid alleviated UV-B-initiated photoageing should be further clarified in terms of cellular signalling components such as mitogen-activated protein kinase and nuclear transcriptional factors. Topical ellagic acid alleviated UV-B-induced dermal roughening and thickening leading to skin wrinkle. In addition, ellagic acid mitigated cutaneous accumulation of inflammatory cytokines and adhesion molecule ICAM-1, and inflammatory infiltrates of macrophages in hairless mice skin. Therefore, topical or dietary interventions with berries and pomegranate rich in ellagic acid and ellagitannins are promising strategies in curtailing skin wrinkling and cutaneous inflammation associated with chronic UV exposure leading to photoageing.

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