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Ferulic acid, a phenolic phytochemical, inhibits UVB-induced matrix metalloproteinases in mouse skin via posttranslational mechanisms

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Abstract

Matrix metalloproteinases MMP-2 and -9 are known to be overexpressed in ultraviolet B (UVB)-irradiated skin tissues and contribute to the acceleration of photoaging and the development of skin cancer. But the specific molecular mechanisms that can control or interfere with the expression and regulation of these MMP-2 and -9 activities in skin are not clearly understood. The aim of the present study was to analyze the suppressive effects of ferulic acid (FA), an abundant phenolic compound present in various dietary and medicinal plants, on UVB radiation-induced MMP-2 and -9 activities in mouse skin. For attenuation of chronic UVB irradiation damage to skin, inhibition of MMP-2 and -9 protein expression was detected using immunohistochemistry analysis. However, the *in situ* suppressive effects of FA did not interfere with the transcription or translation of MMP-2 and -9, suggesting that its action could be mediated via the proteasome pathway. Histological analyses showed that FA attenuates the degradation of collagen fibers, abnormal accumulation of elastic fibers and epidermal hyperplasia induced by UVB, demonstrating the functional and physiological relevance of FA effects in UVB-irradiated skin tissues. Together, our findings provide a novel and increased insight into the *in vivo* action of FA and suggest a possible clinical application in skin pathophysiological conditions associated with overexpression of MMP-2 and -9.

Keywords: Cell signaling; Matrix metalloproteinase; Inflammation; Invasion; Proteasome

1. Introduction

Matrix metalloproteinases (MMPs) were initially characterized as matrix-degrading proteases, which play an important role in remodeling the extracellular matrix in many physiological and pathological processes [1]. However, an increasing number of studies have revealed a wide variety of nonmatrix substrates for MMPs, such as adhesion molecules, receptors, chemokines, cytokines, growth factors, intercellular junction proteins and structural molecules. Proteolytic modification of these molecules by MMPs can confer diverse cellular and molecular activities under normal and disease conditions [2]. Research in recent years has demonstrated a strong association between the deregulated MMP expression and various pathophysiological conditions such as cancer, arthritis, cardiovascular diseases, neurodegenerative disorders and others [3]. The contribution of MMPs to disease processes is further supported by studies of relevant disease models in MMP-deficient animals [4]. Specific MMPs are, therefore, now being considered important therapeutic targets for a number of diseases.

Skin cancer, a very prevalent form of cancer in humans, typically occurs in photoaged skin [5,6]. Chronic exposure to UV radiation is the main environmental factor that causes photoaging and photocarcinogenesis [7]. Increased expression of MMPs and their activities are observed in photodamaged skin [8]. Several different types of cells in the skin such as fibroblasts, keratinocytes, macrophages, endothelial cells, mast cells and eosinophils are known to produce MMPs [9]. Ultraviolet B (UVB) radiation induces the expression of two important MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), which have been implicated in skin photoaging and photocarcinogenesis [10,11]. Recent evidence further suggests that premature aging can predispose skin to cancer development [6]. Basal cell carcinoma (BCC), the most common form of skin cancer, arises within the basal layer of the epidermis and typically occurs in areas of chronic sun exposure [12]. Overexpression of MMP-2 and -9 is observed in stromal cells surrounding malignant epithelial cells in BCC [11]. Squamous cell carcinoma (SCC) is a malignant tumor of keratinocytes of the spinous layer of the epidermis and frequently arises in UV-exposed skin [13]. The growth and aggressiveness of SCC are correlated with a high expression of MMP-2 and -9 [14]. In addition to nonmalignant melanoma, UV radiation has also been implicated in the progression

Abbreviations: UVB, ultraviolet B radiation; FA, ferulic acid; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; NF-κB, nuclear factor kappa B; Akt, protein kinase B; mTOR, mammalian target of rapamycin; 4E-BP1, eIF4E-binding protein 1; eIF4E, eukaryotic initiation factor 4E; p70S6K, ribosomal p70 S6 protein kinase; S6, ribosomal protein S6; Raptor, regulatory associated protein of mTOR; EGFR, epidermal growth factor receptor.

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of cutaneous melanoma [15]. Matrix metalloproteinases 2 and 9 have been shown to play a crucial role in cutaneous melanoma progression, invasion and metastasis [16]. These findings strongly suggest that strategies to inhibit the aberrant expression of gelatinases in the skin may help prevent premature skin aging and cancers.

Recently, increasing effort has been put into finding novel approaches to manage risk factors for skin cancers, including the damage from frequent exposure to solar UV radiation, in particular its UVB component. Phytocompounds from various fruits, vegetables and tea leaves are known to contain antioxidant and anti-inflammatory phytochemicals that may confer anticarcinogenic activities. The US Food and Drug Administration (FDA) recently approved a new drug application for the marketing of a tea leaf extract fraction, Veregen (sinecatechins), as a topical, botanical drug treatment remedy for skin condyloma [17]. With these developments in mind, in this study, we evaluated the potential clinical benefit of ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid, FA). Ferulic acid is a naturally occurring hydroxycinnamic acid derivative and is one of the major phenolic compounds present in cereal grains including rice, wheat, barley and oats, various citrus fruits, tomatoes, a range of vegetables and medicinal herbs [18,19]. Ferulic acid has been shown to inhibit the expression of cytotoxic and inflammation-associated enzymes, including inducible nitric oxide synthase, caspases and cyclooxygenase-2 [20]. Previous studies have reported that FA has diverse medicinal effects including anti-inflammatory, anticancer, antioxidant, neuroprotective and cardioprotective activities [19]. Although very limited evidence is available of its effects on the skin, two previous studies have reported beneficial effects of FA against skin tissue damage. *In vivo* experiments with FA showed a protective effect against UV radiation-induced erythema [21], and addition of FA stabilized a topical formulation of vitamin C and E, which doubled the protection against UV-induced erythema in skin [22]. Protective effects of FA against photoaging and photocarcinogenesis, however, have not been reported. The present study investigated the potential anticancer and antiaging effects of FA and the possible mechanistic basis of such action by targeting two important gelatinases, MMP-2 and -9, in UVB-irradiated mouse skin. Our findings suggest that FA strongly suppresses MMP-2 and -9 expression *in vivo* and that this suppression is mediated via specific posttranslational mechanisms. The potential applications of this result in skin care and disease are discussed.

2. Materials and methods

2.1. Mice

Female BALB/cAnN.Cg-Foxn1^{nu}/CrNarl nude mice were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. All mice used in our experiments were 7–8 weeks old. Mice were maintained in pathogen-free conditions and fed on standard laboratory chow and water in the animal facilities of the Institute of Biological Chemistry, Academia Sinica, Taiwan. Animal experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee, Academia Sinica, Taiwan.

2.2. Chemical reagents

Ferulic acid (Fig. 1A) was purchased from Kishida Co. (Tokyo, Japan). Rapamycin and MG-132 were purchased from Calbiochem (La Jolla, CA, USA). Solutions of the above compounds were freshly prepared in a mixture of 98% acetone and 2% dimethyl sulfoxide prior to use.

2.3. UVB irradiation

Mice were irradiated using a UV109 apparatus (Waldmann, Medizintechnik, Germany) equipped with a single UV compact fluorescent lamp (narrow band UVB, TL01), as we previously described [23]. One hundred percent energy emission was obtained at wavelength 312 nm with a radiation intensity of 1.06 mW/cm^2 and at a lamp-to-target distance of 10 cm as measured by a Variocontrol Digital Radiometer (Waldmann). A restricted area (3.14 cm^2) of mouse abdominal skin was exposed to a single or multiple doses of UVB radiation (4000 J/m^2) as required. In brief, female BALB/c mice abdomen were cleanly shaved and disinfected with 70% ethanol. No visual damage to epidermal skin tissue was observed. Mouse skin area was marked with an ink stamp containing a circular area 2 cm in diameter (3.14 cm^2) followed by exposure to UVB irradiation (4000 J/m^2). Different dosages of FA (0.01, 0.05, 0.25, 0.5 and 1 mg/3.14 cm^2 tissue site/mouse) were then applied within the circular area, and further functional assays were carried out. The dose of UVB radiation used in the present study was suberythemogenic to the experimental mice. Chronic UVB irradiation experiments were performed by exposing test mouse skin to a single dose of UVB every day for 5 days/week over a period of 4 weeks.

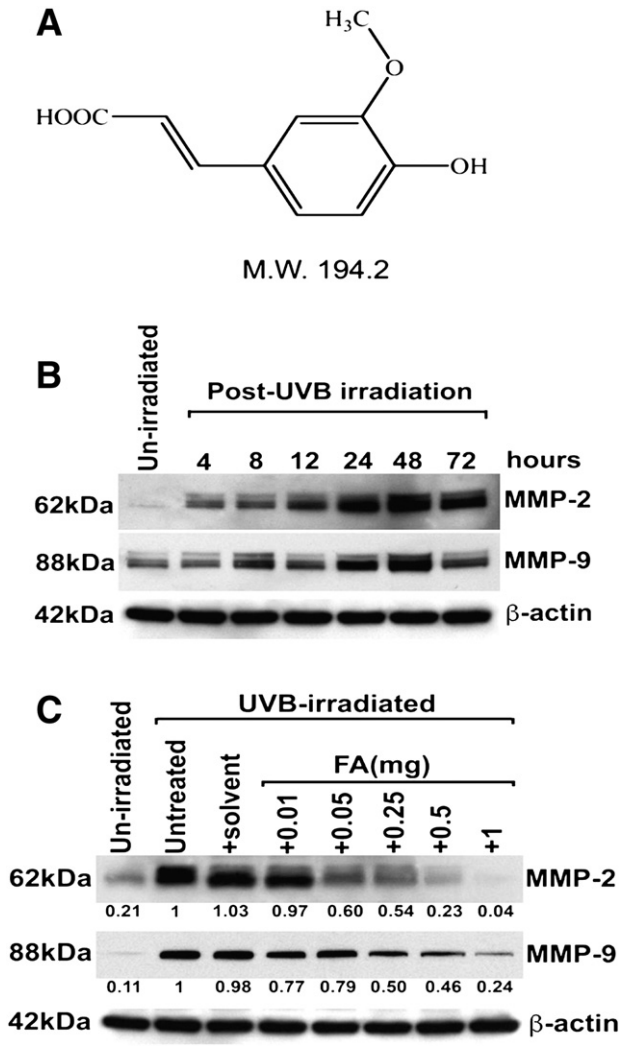


Fig. 1. Ferulic acid suppresses UVB-induced MMP-2 and -9 protein expression in mouse. (A) Chemical structure of FA. (B) Mouse skin was unirradiated or UVB irradiated, and skin samples were collected at different time points, processed and analyzed for MMP-2 and -9 levels by Western blot. (C) Ultraviolet-B-irradiated mouse skin was left untreated or immediately treated with solvent alone or with different concentrations of FA (0.01, 0.05, 0.25, 0.5, 1 mg/site/mouse). Data are representative of three independent experiments.

2.4. Reverse transcription–polymerase chain reaction analysis

Reverse transcription–polymerase chain reaction (RT-PCR) was used to analyze the expression of MMP-2 and -9 mRNAs in mouse skin tissue. Isolation of total RNA from frozen skin samples obtained after various treatments and subsequent RT-PCR reactions using AccessQuick RT-PCR system (Promega, Madison, WI, USA) were carried out as described previously [23]. The primers contained the following sequences: mouse *MMP-2* sense primer 5′-AGCAGCTGTACAGACACTGGTC-3′ and antisense primer 5′-ATCCTTGGTCAGGACAG AAGCC-3′; mouse *MMP-9* sense primer 5′-CTAGTGAGA-GACTCTACACGGAG-3′ and antisense primer 5′-GAGCCACGACCATACAGATACTG-3′; mouse *GAPDH* sense primer 5′-CATCACTGCCACCCAGAAGACTGTGGA-3′ and antisense primer 5′-TACTCCTTGGAGGCCATGTAGGCCATG-3′. Gel images were scanned, and

densitometry analysis of the captured image was performed using Gene Tools software (Syngene, Cambridge, UK).

2.5. Western blot analysis

Skin samples from experimental mice, subjected to various treatments, were collected at indicated time points. Total protein was isolated from each skin sample as we previously described [23]. Where required, ubiquitinated proteins were column-enriched as per the manufacturer's (Calbiochem, CA, USA) instructions. Proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by transfer to PVDF membrane (Novex, San Diego, CA, USA). The membranes were incubated overnight at 4°C with mouse specific antibodies against MMP-2, MMP-9 (Abcam, Cambridge, UK), phospho-Akt (Ser-473), phospho-mTOR (Ser-2448 and -2481), phospho-eIF4E (Ser-209), phospho-4E-BP1 (Ser-65), phospho-p70S6K (Thr-389), phospho-S6 (Ser-235/236), Raptor (Cell Signaling Technology, Danvers, MA, USA) or ubiquitin (Pierce, Rockford, IL, USA). Equal protein loading was assessed using mouse β-actin antibodies (Abcam) as internal controls. The blots were incubated with appropriate horseradish-peroxidase-conjugated secondary antibodies and developed with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Images were scanned, and densitometry analysis was performed as described earlier.

2.6. Histological analysis

Frozen skin tissues collected from experimental mice were cut into 6-μm sections and fixed in Tissue-Tek OCT compound (polyvinyl alcohol 10.24%, polyethylene glycol 4.26% and nonreactive ingredient 85.50%) obtained from Sakura Finetek (Torrance, CA, USA). These skin tissue sections were used to carry out various analyses. Immunohistochemistry was performed using MMP-2 monoclonal and MMP-9 polyclonal antibodies (Abcam) at 1:500 dilution. Skin tissue sections were incubated in 3% H_2O_2 for 30 min followed by incubation with primary antibody for another 30 min. Sections were washed in phosphate-buffered saline (PBS) and incubated for 10 min with biotin-conjugated secondary antibody. After washing in PBS, sections were incubated in 3,3′ diaminobenzidine substrate (Chemicon, Temecula, CA, USA) for 20 min followed by counterstaining with hematoxylin. For detection of collagen and elastic fibers, skin tissue sections were stained with Van Gieson's solution for 5 min and Verhoeff's hematoxylin for 30 min (Sigma-Aldrich, St. Louis, MO, USA), respectively. Sections were washed with tap water and dehydrated before mounting the cover slips. Skin epidermal hyperplasia was observed using hematoxylin and eosin staining. Tissue sections were incubated with Mayer's hematoxylin (Muto Pure chemicals, Tokyo, Japan) for 5 min followed by washing and dehydration. Sections were then stained with eosin for 5 min, washed and dehydrated. All the above skin sections were visualized and photographed using a Eclipse E800 microscope (Nikon, Tokyo, Japan).

3. Results

3.1. FA suppresses UVB-induced expression of MMP-2 and -9 proteins in mouse skin

In our previous studies, UVB irradiation of mouse skin with a single dose of 4000 J/m^2 produced significant molecular changes [23]. In order to determine whether exposure to a similar single dose of UVB radiation could induce expression of MMP-2 and -9 proteins, we studied the kinetics of their expression in the skin of mice. Significant time-dependent induction of MMP-2 and -9 protein expression was observed in UVB-irradiated skin as compared with unirradiated control (Fig. 1B). A high level of induction was observed from 24 h through 48 h, with a slight reduction in MMP-2 and -9 expression from 72 h. We thus chose the 24-h time point to carry out the subsequent experiments in this study.

We next looked at the possible effects of topically applied FA on UVB-induced MMP-2 and -9 expression after UVB radiation. Ultraviolet-B-irradiated mice were left untreated or immediately treated with solvent alone or FA at different doses (0.01, 0.05, 0.25, 0.5 and 1 mg/site/mouse). Unirradiated, untreated mice again served as a negative control. Ferulic acid application produced a dose-dependent inhibition of the UVB-induced expression of both MMP-2 and -9 proteins (Fig. 1C). The most significant inhibition was observed at the dose of 1 mg/site/mouse, and therefore this dosage was used for subsequent experiments. A gelatin zymography assay confirmed that FA significantly inhibited MMP-2 and -9 expression, approximately 37% and 83%, respectively, as compared to the levels of test skin from UVB-irradiated and solvent-treated mice (data sets not shown).

3.2. FA does not interfere with UVB-induced transcription of MMP-2 and -9 genes

Several previous studies have reported the transcriptional induction of MMP-2 and -9 genes in UVB-irradiated skin. To confirm whether UVB radiation would indeed induce the transcription of MMP-2 and -9 in the skin of our experimental mice, we analyzed the kinetics of their mRNA expression. Induction of MMP-2 and -9 mRNA expression compared to the levels in unirradiated control mice was detected as early as 4 h after UVB irradiation. Maximum induction was observed at 12 h through 24 h post-UVB irradiation (Fig. 2A). To determine whether the inhibitory effect of FA on UVB-induced MMP-2 and -9 protein expression was due to an interference with mRNA transcription, mice were treated with solvent alone or FA, and the experiment was repeated. Fig. 2B shows that neither solvent nor FA application had any effect on UVB-induced expression of MMP-2 and -9 mRNAs at 24 h.

3.3. FA inhibits UVB-induced activation of specific key components of the translational machinery

Our data indicated that the suppressive effects of FA on UVB-induced MMP-2 and -9 protein expression did not occur at the

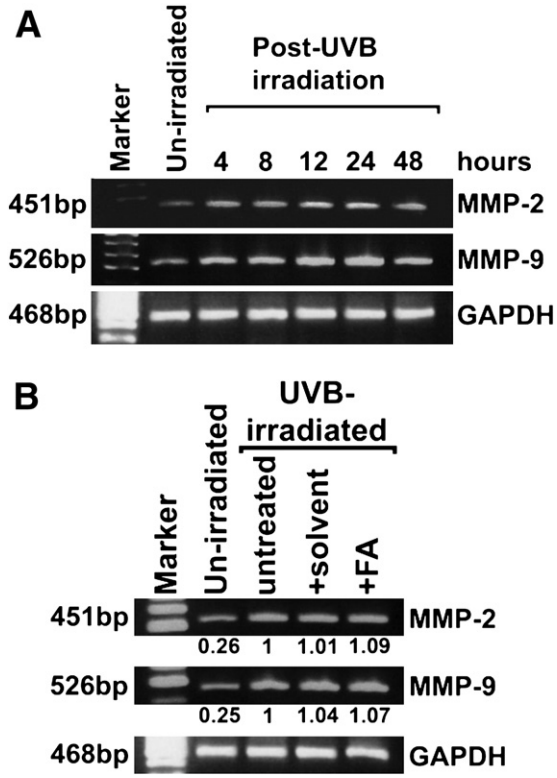


Fig. 2. Ferulic acid does not suppress UVB-induced MMP-2 and -9 mRNA expression. Mouse skin was left unirradiated or UVB irradiated, and total RNA was extracted from tissues and analyzed by RT-PCR. (A) Samples were collected at different time points as indicated after UVB irradiation and analyzed for MMP-2 and -9 mRNA expression. (B) Ultraviolet-B-irradiated mouse skin was untreated or immediately treated with solvent alone or with FA at a concentration of 1 mg/site/mouse. Samples were collected at 24 h postirradiation and analyzed for mRNA expression. Unirradiated and UVB-irradiated but untreated mice served as negative and positive controls, respectively. The signal intensities of each test gene mRNA were quantified, and the changes relative to the respective positive control value (designated as 1) are shown under each gel image. Representative data from three independent experiments are shown.

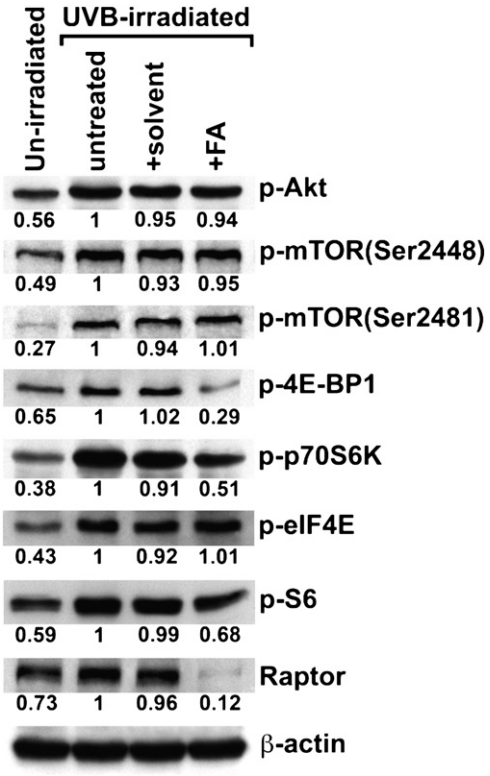


Fig. 3. Ferulic acid inhibits UVB-induced activation of some key components of translational machinery. Mouse skin was unirradiated, UVB irradiated or UVB irradiated and immediately treated with solvent or FA at a concentration of 1 mg/site/mouse. Total protein extracts were prepared from skin samples harvested at 2 h posttreatment and subjected to Western blot analysis. Phosphorylated Akt, mTOR, 4E-BP1, p70S6K, eIF4E, S6 and Raptor were probed with respective mouse specific antibodies. Unirradiated and UVB-irradiated but untreated mice served as negative and positive controls, respectively. Mouse β-actin served as a control for protein input. The changes in the signal intensity of each test protein were estimated as described in Fig. 1. Data are representative of two independent experiments. p, phospho.

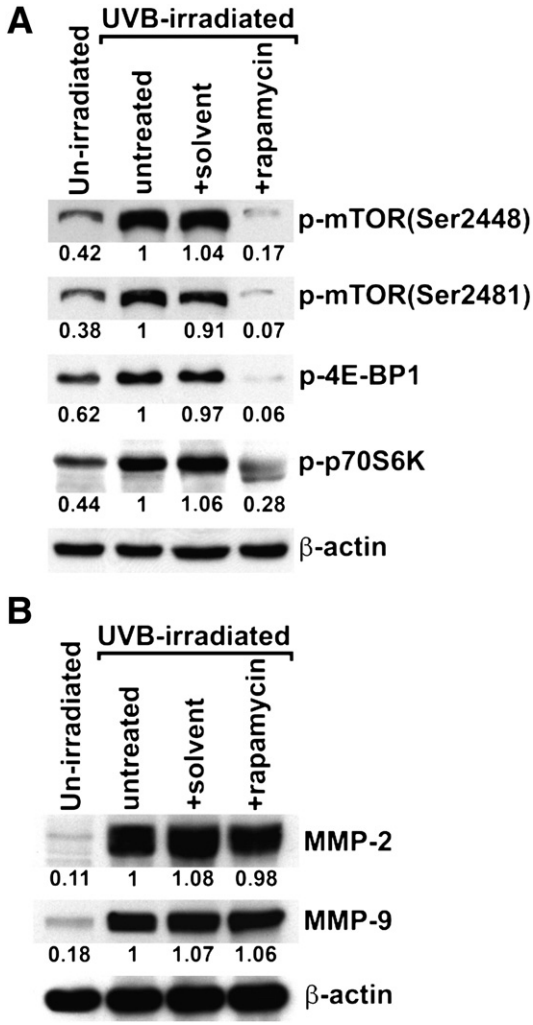


Fig. 4. Rapamycin inhibits UVB-induced activation of translational factors but has no effect on MMP-2 and -9 protein expression. Mouse skin was unirradiated, UVB irradiated or UVB irradiated and immediately treated with solvent or rapamycin at a concentration of 0.5 mg/site/mouse. Total protein extracts were prepared from skin samples harvested at required time points and subjected to Western blot analysis. (A) Samples collected at 2 h posttreatment were used to detect the phosphorylation of mTOR, 4E-BP1 and p70S6K by respective antibodies. (B) Samples collected at 24 h posttreatment were used to detect MMP-2 and -9 protein expression. Unirradiated and UVB-irradiated but untreated mice served as negative and positive controls, respectively. Mouse β-actin served as a control for protein input. The changes in the signal intensity of each test protein were estimated as described in Fig. 1. Data are representative of two independent experiments. p, phospho.

transcriptional level. Since expression of MMP-2 and -9 has been shown to be regulated at the translational level in certain cancer systems [24,25], we sought to investigate whether FA could interfere with translational machinery and thereby inhibit UVB-induced MMP-2 and -9 expression. In these experiments, UVB irradiation of mouse skin increased the phosphorylation of several key components of protein translation apparatus including Akt, mTOR, 4E-BP1, p70S6K, eIF4E and S6 and augmented the expression of Raptor protein at 2 h postirradiation (Fig. 3), levels that then gradually decreased to the control level over the following 2–3 h (data not shown). The effects of FA on UVB-induced phosphorylation and expression of these components of the translational machinery were then determined. Ferulic acid significantly inhibited UVB-induced phosphorylation of 4E-BP1 and reduced the expression of Raptor protein (Fig. 3). Ferulic acid also showed moderate inhibition of p70S6K and S6 phosphorylation.

3.4. Inhibition of 4E-BP1 and p70S6K phosphorylation does not result in suppression of UVB-induced MMP-2 and -9 protein expression

In order to determine whether the inhibition of 4E-BP1 and p70S6K phosphorylation would result in suppression of UVB-induced MMP-2 and -9 protein expression, we conducted an experiment with rapamycin, a known inhibitor of mTOR, which is an upstream activator of 4E-BP1 and p70S6K. In UVB-irradiated skin treated with 0.5 mg/site/mouse rapamycin for 2 or 24 h, there was significant inhibition of the phosphorylation of UVB-induced mTOR, 4E-BP1 and p70S6K (Fig. 4A). However, UVB-induced MMP-2 and -9 protein expression was clearly not inhibited by rapamycin (Fig. 4B), suggesting that their expression does not require an increased level of 4E-BP1 and p70S6K phosphorylation.

3.5. FA causes degradation of UVB-induced MMP-2 and -9 proteins through the proteasome pathway

The results obtained so far suggest the possibility that FA may act through a posttranslational mechanism. A balance between the synthesis and degradation of a protein regulates the protein level and related activity of specific protein molecules. Therefore, we examined the possible involvement of the proteasome, a known enzyme complex that degrades ubiquitinated proteins, in FA

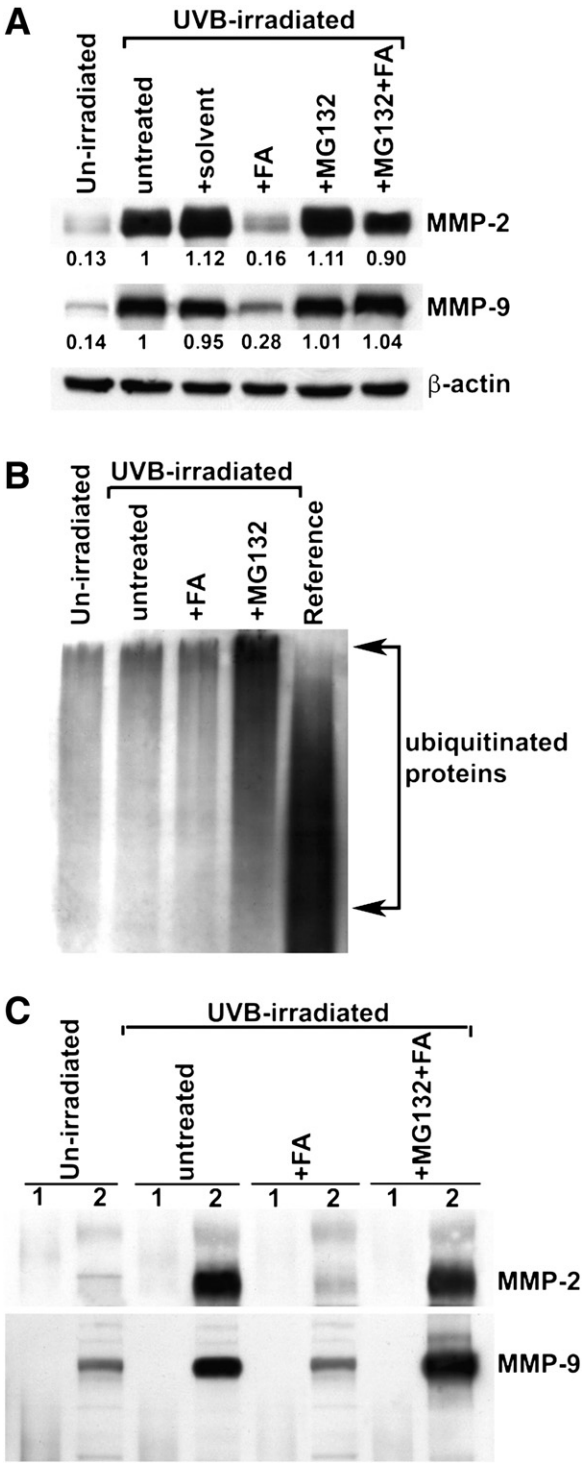


Fig. 5. Ferulic acid causes the degradation of UVB-induced MMP-2 and -9 proteins through the proteasome pathway. Mouse skin was unirradiated or UVB irradiated. Ultraviolet-B-irradiated mouse skin was untreated or immediately treated with solvent alone, FA (1 mg/site/mouse) or MG-132 (1 mg/site/mouse) alone or MG-132 (1 mg/site/mouse) plus FA (1 mg/site/mouse). Skin samples were collected at 24 h postirradiation, processed and analyzed by Western blotting. (A) The MMP-2 and -9 protein levels were analyzed using specific antibodies. Unirradiated and UVB-irradiated but untreated mice served as negative and positive controls, respectively. Mouse β-actin served as a control for protein input. The changes in the signal intensity of each test protein were estimated as described in Fig. 1. (B) Enrichment and presence of ubiquitinated proteins were confirmed using antiubiquitin antibody. Reference, commercial positive control. (C) Proteins present in the eluted samples from polyubiquitin-affinity beads (lane 1) and in the supernatants (lane 2) were detected using MMP-2 and -9 specific antibodies. Data are representative of two independent experiments.

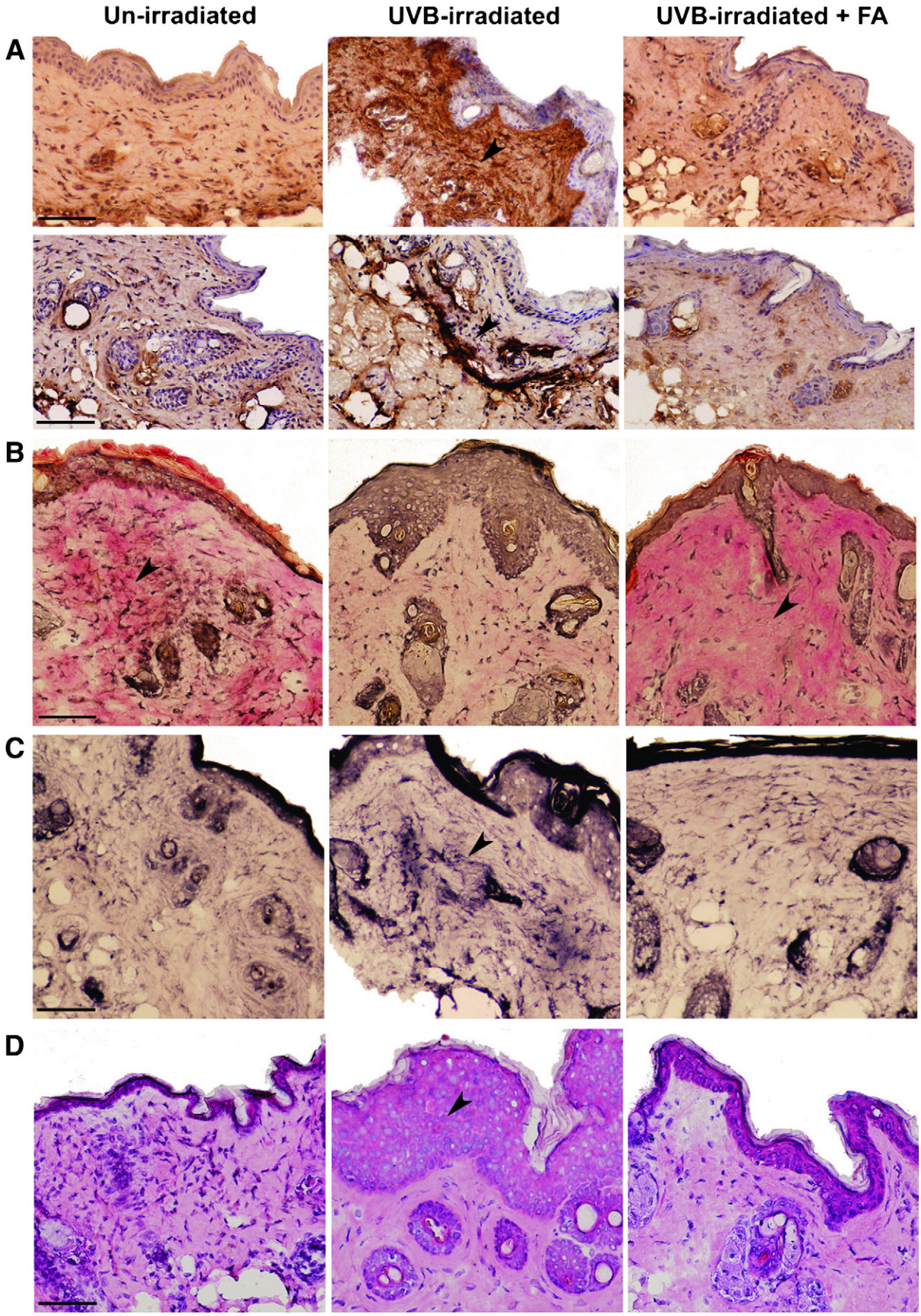
suppression of UVB-induced MMP-2 and -9 proteins by blocking its function using a known proteasome inhibitor, MG-132. Ultraviolet-B-irradiated mouse skin was treated with solvent, FA or MG-132 (1 mg/site/mouse) alone, or FA and MG-132 together, and protein levels of MMP-2 and -9 were analyzed. The inhibitory effect of FA on UVB-induced MMP-2 and -9 protein expression was prevented by MG-132 (Fig. 5A). This result strongly suggests that FA causes the degradation of MMP-2 and -9 proteins through the proteasome pathway. To determine whether ubiquitination of MMP-2 and -9 is responsible for their degradation, we isolated ubiquitin-conjugated proteins from the above-mentioned treatment samples and analyzed their levels by Western blot using antiubiquitin as a probe. We observed an accumulation of ubiquitinated proteins in eluate from the MG-132-treated skin sample (Fig. 5B, right lanes), indicating an inhibition of proteasome function. However, we did not detect any ubiquitinated MMP-2 and -9 proteins in ubiquitin-enriched eluates from samples treated with MG-132 plus FA (Fig. 5C, lane 1). In contrast, we observed the presence of significant amounts of unconjugated MMP-2 and -9 proteins in the supernatant fractions (Fig. 5C, lane 2), a similar trend to that observed in Fig. 5A. This result hence suggests that FA effects may not occur via the ubiquitination of MMP-2 and -9 proteins.

3.6. FA suppresses MMP-2 and -9 proteins in chronically UVB-irradiated mouse skin

It is evident from the aforementioned results that FA suppress the levels of MMP-2 and -9 proteins in mouse skin exposed to a single dose of UVB radiation. However, it is known that the deleterious effects of UVB radiation on skin are cumulative over time. Therefore, we sought to determine whether FA could prevent the effect of increased expression of MMP-2 and -9 in chronically UVB-irradiated skin tissues. For this purpose, mouse abdominal skin was irradiated as described in Materials and Methods. In the treatment group, mouse skin was topically treated with FA immediately after each UVB exposure. To minimize animal usage, treatment with solvent alone was excluded since solvent did not show any significant effect on UVB-induced MMP-2 and -9 expression. Skin samples were processed and analyzed by immunohistochemistry with specific antibodies. A significant increase in expression of MMP-2 and -9 was observed in UVB-irradiated skin as compared with unirradiated control skin (Fig. 6A). Matrix metalloproteinase 2 was predominantly present in the dermis, whereas MMP-9 was present at the junction of the dermis and epidermis (Fig. 6A). The skin treated with FA showed a significant reduction in chronic UVB radiation-induced levels of MMP-2 and -9, indicating its possible long-term beneficial effects against the deleterious effects of UVB radiation.

3.7. FA attenuates chronic UVB radiation-induced histopathological changes in mouse skin

Ultraviolet-B-induced MMP-2 and -9 are known to degrade the collagen and damage the basement membrane in skin. To explore the functional relevance of suppressive effects of FA on UVB-induced MMP-2 and -9, we examined its effect on collagen fiber degradation in chronically UVB-irradiated mouse skin. Fig. 6B shows that UVB irradiation significantly decreased the collagen fibers in the dermis compared to the unirradiated control mice, and FA treatment clearly reduced the UVB-induced degradation of collagen fibers. In addition to collagen fiber degradation, chronic UVB irradiation increased the accumulation of elastic fibers in the dermis as compared with unirradiated control. Topical treatment with FA significantly decreased the UVB-induced elastic fiber deposition to a level similar to that observed in unirradiated skin (Fig. 6C). We also observed a characteristic histological change, a hyperplastic response, with


Un-irradiated
UVB-irradiated
UVB-irradiated + FA
A
B
C
D

thickening of the epidermis containing more than 10 cell layers, in mice exposed to chronic UVB radiation. Treatment with FA completely prevented the hyperplastic response induced by UVB radiation (Fig. 6D). Furthermore, the dermis of the chronic UVB-irradiated skin exhibited enlarged sebaceous glands, dermal cyst proliferation and an increased number of vacuoles as compared with control mice skin, and FA treatment effectively inhibited these changes. Therefore, FA can protect both the epidermis and the dermis of skin exposed to chronic UVB radiation.

4. Discussion

Large volumes of evidence have demonstrated the beneficial effects of plant natural product factors present in whole grain products, fruits and vegetables in reducing the risk of various chronic diseases including cancers, cardiovascular diseases, diabetes and others. Along these lines, the US FDA has recently approved a new drug application for Veregen, a tea leaf extract containing phytocompound mixtures of sinecatechins, for topical treatment of perianal and genital condyloma [17]. In our present investigation, we contemplated a similar approach for potential skin care remedies. Here we present the protective effects of FA, a common dietary component, against the deleterious effects of UVB radiation of the skin. Consistent with previous findings of Inomata and his coworkers [10], we observed a time-dependent increase in MMP-2 and -9 protein expression in mouse skin exposed to a single dose of UVB radiation. Topical treatment of mouse skin with FA immediately after UV exposure drastically inhibited the UVB-induced increase in MMP-2 and -9 protein expression. Synthetic inhibitors of MMP-2 and -9 have been shown elsewhere to prevent basement membrane damage and premature aging of skin exposed to UV radiation [26]. In addition, studies on MMP-2 and -9 knockout mice have revealed in these animals a decreased incidence of skin carcinogenesis, tumor growth and metastasis [27,28]. These above findings together led us to hypothesize that FA may confer protection against the damaging effects of the MMP-2 and -9 overexpression in UVB-exposed skin.

Matrix metalloproteinase expression is known to be regulated at various levels including transcription and posttranscription [29]. Ultraviolet B radiation has been shown to induce activation of activator protein-1 and nuclear factor kappa B (NF-κB) transcription factors and thereby the transcription of downstream targets such as MMPs [30,31]. Consistent with these findings, we observed an increased expression of MMP-2 and -9 mRNAs in UVB-irradiated mouse skin, indicating the transcriptional up-regulation of these genes by UVB radiation. We then examined whether the inhibitory effects of FA on MMP-2 and -9 protein levels are due to its interference with UVB-induced activation of their mRNA transcription. We found that FA treatment did not show any effect on UVB-induced mRNA levels of MMP-2 and -9, suggesting that FA may inhibit MMP-2 and -9 proteins through posttranscriptional regulatory mechanisms such as protein translation and/or degradation.

Subsequently, the possible interference of FA with the translational machinery and its association with the suppression of MMP-2 and -9 expression were investigated. Translation of mRNAs is controlled primarily at the level of initiation, which is usually rate limiting. The initiation step involves the phosphorylation of two key initiation factors, the eIF4F complex and p70S6K [32]. Ultraviolet B radiation has been reported to induce the phosphorylation of specific translation factors and regulate their upstream signal transduction pathways [33,34]. In the present study, we show that exposure to UVB radiation can increase the phosphorylation of Akt and mTOR kinases and of the downstream effectors, such as 4E-BP1, eIF4E, p70S6K and S6, in mouse skin, and this is in agreement with previous findings. Next, we examined the effect of FA on UVB-induced phosphorylation of several critical components of the protein translational machinery mentioned above. Our data revealed a drastic inhibition of UVB-induced phosphorylation of 4E-BP1 and a moderate level of inhibition of p70S6K and S6 by FA. Interestingly, FA significantly reduced the expression of Raptor protein, which is required for mTOR-mediated phosphorylation of 4E-BP1 and p70S6K [34]. This result suggests that reduced expression of Raptor in response to FA may lead to the suppression of UVB-induced phosphorylation of 4E-BP1 and p70S6K. To follow up this idea, we used rapamycin to verify the role of phosphorylated 4E-BP1 and p70S6K in UVB-induced MMP-2 and -9 protein expression in mouse skin. Rapamycin is a specific inhibitor of mTOR, which suppresses the phosphorylation of 4E-BP1 and p70S6K and blocks the translation initiation of specific mRNAs [32]. In agreement with previous findings [33–35], we show here that rapamycin significantly inhibited the UVB-induced phosphorylation of mTOR and its downstream effectors 4E-BP1 and p70S6K. Surprisingly, however, rapamycin did not have any effect on UVB-induced expression of MMP-2 and -9 proteins in mouse skin. These results suggest that increased phosphorylation of specific translational factors in response to UVB radiation does not contribute to the increased expression of MMP-2 and -9 proteins *in vivo*. Together, our results show that the inhibition of UVB-induced phosphorylation of 4E-BP1 and p70S6K by FA may not play a role in its suppressive effects on MMP-2 and -9 proteins.

The fact that FA did not have any detectable effect on UVB-induced mRNA transcription or the protein synthesis of MMP-2 and -9 led us to speculate the possible involvement of posttranslational mechanisms. The proteasome pathway responsible for the degradation of a variety of cellular proteins is sensitive to proteasome inhibitors or ATP depletion. The peptide aldehyde MG-132 belongs to a group of proteasome inhibitors that can readily enter test cells and selectively inhibit the chymotrypsin-like activity of the proteasome [36]. It can effectively inhibit NF-κB activation by preventing NF-κB degradation and regulates ERK1/2 and JNK1 pathways. It blocks degradation of short-lived proteins, which in turn induces HSP and ER chaperone expression, leading to thermotolerance [37,38]. Our results (Fig. 5) demonstrate that, in the presence of MG-132, FA failed to inhibit the levels of UVB-induced MMP-2 and -9 proteins in mouse skin. This result thus suggests that FA may cause the degradation of UVB-induced MMP-2 and -9 proteins through mediation via the proteasome pathway. Posttranslational regulation of MMP levels by the modulation of proteasome activity has been previously reported in mouse mammary epithelial cells [39]. Ubiquitination is believed to be the trigger for the proteasome-mediated degradation of most proteins in eukaryotes [40]. In our present study, if the ubiquitin-dependent proteasome pathway is involved in the degradation of MMP-2 and -9 proteins by FA, the blockage of proteasome activity should then result in an accumulation of ubiquitinated MMPs. However, experimentally, we did not detect any ubiquitin-conjugated MMP-2 and -9 proteins. This result hence may indicate that

Fig. 6. Ferulic acid suppresses expression of MMP-2 and -9 and prevents histopathological changes in chronically UVB-irradiated mouse skin. Mouse skin was unirradiated, UVB irradiated or UVB irradiated and immediately treated with FA (1 mg/site/mouse) for 4 weeks (n=4 mice per group). Skin biopsies were collected 24 h after the last UVB exposure and processed. (A) Detection of MMP-2 and -9 by immunohistochemistry with specific antibodies. Positive staining was seen as brown for MMP-2 (top panel) in the dermis (arrow) and dark brown for MMP-9 (bottom panel) at the junction of epidermis and dermis (arrow). (B) Detection of collagen fibers with Van Gieson's staining. (C) Detection of elastic fibers with Verhoeff's staining. Positive staining was seen as pinkish red for collagen fibers (arrow) and black for elastic fibers (arrow) in the dermis. (D) Detection of hyperplastic response with hematoxylin and eosin staining. Increased numbers of cell layers in epidermis (arrow) and vacuoles in dermis were seen. Photographs of representative skin sections are shown at original magnification ×200. Bar, 100 μm. Data are representative of two independent experiments.

degradation of MMP-2 and -9 proteins might have resulted from mediation via an ubiquitin-independent proteasome pathway. Recent findings have indeed demonstrated the degradation of several proteins by either the 26S proteasome or the 20S proteasome through ubiquitin-independent mechanisms [41]. Further research is required to evaluate the possible involvement of ubiquitin-independent proteasome pathway in degradation of the UVB-induced MMP-2 and -9 proteins by FA.

Since the various deleterious effects of UVB radiation, photoaging and photocarcinogenesis are known to mainly result from the accumulated damage caused by repeated exposure, we investigated the potential beneficial effects of FA in chronic UVB exposure. In chronically UVB-irradiated skin, we observed increased accumulation of MMP-2 throughout the dermis and of MMP-9 in the basement membrane and around the sebaceous glands, results that are in agreement with previous findings [42,43]. Our results also clearly demonstrated the longer-term inhibitory effects of FA on UVB-induced MMP-2 and -9 expression. As gelatinases, MMP-2 and -9 are known to degrade type IV collagen of the basement membrane in skin [10]. In this study, we showed that the collagen fiber content of the dermis was drastically reduced after chronic UVB irradiation, whereas FA treatment effectively attenuated this degradative effect. Using zymography assay for enzymatic activity, we further observed that FA can significantly inhibit the specific activity of MMP-2 and -9 in skin of FA-treated mice as compared to activities in skin from UVB-irradiated and solvent-treated mice. These results therefore suggest that FA may attenuate the degradation of collagen fibers through suppression of the effects of UVB-induced MMP-2 and -9 overexpression. In addition to collagen degradation, abnormal accumulation of elastic fibers and epidermal hyperplasia are also considered to be key histopathological changes associated with the photoaging effect of UV radiation [44]. Similarly, we observed an accumulation of elastic fibers in the dermis and an increase in epidermal thickness in the chronically UVB-irradiated skin. Interestingly, FA treatment led to a significant suppression of UVB-induced accumulation of elastic fibers and epidermal hyperplasia. Although the direct role of UVB-induced MMPs in elastic fiber accumulation is still not clearly understood, it has been previously suggested that increased number of elastic fibers synthesized in response to UV radiation may replace the degraded collagen fibers in the dermis [45]. It is therefore possible that the suppression of collagen fiber degradation by FA may prevent the accumulation of elastic fibers. Recent studies have reported that UV radiation-induced keratinocyte proliferation and epidermal hyperplasia are dependent on activation of epidermal growth factor receptor (EGFR) [46]. Furthermore, EGFR activation induced by chronic UV radiation has been implicated in the development of skin cancer [47]. Matrix metalloproteinases are known to play a critical role in the activation of EGFR through the processing of a transmembrane growth factor precursor [48]. Ferulic acid has a high antioxidant capacity due to its resonance-stabilized phenoxy radical structure. The physicochemical property of FA of n-octanol to water partition coefficients has been reported as 0.375 and 0.489 at pH 3 and pH 10, respectively. A number of stability studies on FA estimated that the *t*90 value for this phytochemical is approximately 459 days at 25°C. Ferulic acid was also found to be unstable under a relative humidity of more than 76%, probably because of the hygroscopic nature of this compound. Particle size distribution of FA powder was in the range of 10–190 μm with a mean particle size of 61 μm. Flowability study of FA indicates that granulation may be necessary for the processing of this drug to solid physical forms [49]. The biological properties of FA *in vivo* may depend on its bioavailability, notably in a complex matrix such as cereals. Ferulic acid was found to improve chemical stability of vitamins (C and E) and to enhance the photoprotection against solar simulated irradiation of skin, from fourfold to approximately eightfold as measured by both erythema and sunburn cell formation [22]. For future potential application of FA as therapeutics, either to be applied topically or to be taken orally, the above physical, chemical and biological properties of FA will need to be considered.

Based on this evidence, we hypothesize that suppression of MMP-2 and -9 levels by FA may interfere with the activation of EGFR, and this in turn may result in the reduction of epidermal hyperplasia in UVB-irradiated skin. Future investigation is required to evaluate this hypothesis.

In summary, our study demonstrates for the first time that topical application of FA, a common dietary polyphenol, can effectively suppress the expression of UVB-induced MMP-2 and -9 proteins in mouse skin, and this effect is apparently mediated via posttranslational mechanisms involving proteasome-associated degradation. The inhibition of MMP-2 and -9 by FA can also lead to attenuation of the chronic effects of UVB radiation, including the degradation of collagen fibers, accumulation of elastic fibers and epidermal hyperplasia. Therefore, FA may protect the structural and molecular integrity of both the epidermal and the dermal tissues of the skin against the detrimental effects of UVB radiation. This new insight into the action of FA warrants future exploration of its possible therapeutic or preventive benefits for photoaging and photocarcinogenesis of the skin.

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