Photoprotective and anti-skin-aging effects of eicosapentaenoic acid in human skin in vivo

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Abstract Skin aging can be attributed to photoaging (extrinsic) and chronological (intrinsic) aging. Photoaging and intrinsic aging are induced by damage to human skin attributable to repeated exposure to ultraviolet (UV) irradiation and to the passage of time, respectively. In our previous report, eicosapentaenoic acid (EPA) was found to inhibit UV-induced matrix metalloproteinase-1 (MMP-1) expression in human dermal fibroblasts. Therefore, we investigated the effects of EPA on UV-induced skin damage and intrinsic aging by applying EPA topically to young and aged human skin, respectively. By immunohistochemical analysis and Western blotting, we found that topical application of EPA reduced UV-induced epidermal thickening and inhibited collagen decrease induced by UV light. It was also found that EPA attenuated UV-induced MMP-1 and MMP-9 expression by inhibiting UV-induced c-Jun phosphorylation, which is closely related to UV-induced activator protein-1 activation, and by inhibiting JNK and p38 activation. EPA also inhibited UV-induced cyclooxygenase-2 (COX-2) expression without altering COX-1 expression. Moreover, it was found that EPA increased collagen and elastic fibers (tropoelastin and fibrillin-1) expression by increasing transformin growth factor-β expression in aged human skin. Together, these results demonstrate that topical EPA has potential as an anti-skin-aging agent.—Kim, H. H., S. Cho, S. Lee, K. H. Kim, K. H. Cho, H. C. Eun, and J. H. Chung. **Photoprotective and anti-skin-aging effects of eicosapentaenoic acid in human skin in vivo. *J. Lipid Res.* 2006. 47: 921–930.**

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Skin aging can be attributed to extrinsic aging and intrinsic (chronological) aging and is commonly related to increased wrinkling, sagging, and laxity (1). Extrinsic aging is generally referred to as photoaging and is caused by repeated exposure to ultraviolet (UV) light. Whereas naturally aged skin is smooth, pale, and finely wrinkled, photoaged skin is coarsely wrinkled and associated with dyspigmentation and telangiectasia (2). Alterations in collagen, the major structural component of the skin, have been considered to be a cause of skin aging and are observed in naturally aged and photoaged skin (3, 4). However, the mechanisms of collagen destruction in aged skin have not been fully clarified.

Collagen destruction is, in part, related to the induction of matrix metalloproteinase (MMP) secreted by epidermal keratinocytes and dermal fibroblasts. MMP levels are increased by various stimuli, such as UV light, oxidative stress, and cytokines. UV light rapidly activates activator protein-1 (AP-1) DNA binding and induces MMPs, including MMP-1 (collagenase), stromelysin (MMP-3), and gelatinase (MMP-9) (5). UV-induced MMP-1 expression induces the cleavage of fibrillar collagen (types I and III) at a single site. Once collagen is cleaved by MMP-1, it is further degraded by MMP-3 and MMP-9, which are also increased by UV light exposure (6). In human skin, UV-induced AP-1 transcriptional activity that is closely related to MMP expression is limited by c-Jun expression, because c-Fos is constitutively expressed (7). Moreover, whereas c-Fos expression levels in young (18–28 years old) and aged (>80 years old) skin are not different, c-Jun expression is increased more so in aged skin than in young skin (8). Decreased expression of procollagen is partly mediated by c-Jun expression, which is induced by UV and interferes with procollagen transcription (9).

Intrinsic skin aging is largely dependent on genetic factors and is associated with increased fragility and loss of elasticity (10). With increasing age, collagen levels are reduced and MMP secretions increased in sun-protected

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Abbreviations: AA, arachidonic acid; AP-1, activator protein-1; COX-2, cyclooxygenase-2; DHA, docosahexaenoic acid; ECM, extracellular matrix; EPA, eicosapentaenoic acid; MAPK, mitogen-activated protein kinase; MED, minimal erythma dose; MMP, matrix metalloproteinase; PG, prostaglandin; TGF-β, transforming growth factor-β; UV, ultraviolet.

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skin compared with young skin (4, 11). Collagen decrease attributable to natural skin aging may arise from its reduced synthesis and increased degradation, with a concomitant increase of MMP expression. Moreover, in aged sun-protected skin, both the number of fibroblasts and their capacity to synthesize procollagen are reduced compared with young skin (4).

Essential fatty acids are divided into two categories depending on their saturation state. Omega-3 (ω-3) or omega-6 (ω-6) fatty acids have an unsaturated carbon at the third or sixth carbon in the methyl terminus, respectively. The ω-3 fatty acids include α-linolenic acid (18:3), stearidonic acid (18:4), eicosatetraenoic acid (20:4), eicosapentaenoic acid (EPA; 20:5), docosapentaenoic acid (22:5), and docosahexaenoic acid (DHA; 22:6) (12). One of these ω-3 PUFAs, EPA, is abundant in fish oil and is used to treat various diseases, such as inflammatory diseases and cancers. EPA competes with arachidonic acid (AA) for incorporation into cell membrane phospholipids and for the substrate of cyclooxygenase-2 (COX-2) (13). Prostaglandins (PGs) are derived from membrane PUFAs and play important roles in inflammation, immune response, and wound healing (14). In our previous report, we demonstrated that EPA inhibits UV-induced MMP-1 expression in human dermal fibroblasts and that it is mediated by the inhibition of the MEK1/ERK/c-Fos and SEK1/JNK/c-Jun pathways (15).

In this study, we investigated whether topical application of EPA prevents UV-induced skin damage and attenuates features of intrinsic aging in human skin in vivo. It was found that topical application of EPA inhibited UV-induced decreases in collagen and that it attenuated UV-induced MMP-1 and MMP-9 expression by inhibiting UV-induced c-Jun phosphorylation. We also found that topical application of EPA inhibited not only JNK and p38 activation but also COX-2 expression, which is also induced by UV irradiation. Moreover, we found that topical application of EPA increases the expression of extracellular matrix (ECM) proteins, such as procollagen, tropoelastin, and fibrillin-1, by increasing transformin growth factor-β (TGF-β) expression in aged human skin in vivo.

MATERIALS AND METHODS

Materials

To detect procollagen, monoclonal anti-type I procollagen N-terminal extension peptide (SP1.D8) antibody was obtained from hybridoma culture media (Developmental Studies Hybridoma Bank, Iowa City, IA) and monoclonal anti-human PIC antibody was purchased from Takara (Shiga, Japan). Monoclonal anti-MMP-1 antibody and monoclonal TGF-β1, -β2, and -β3 antibodies were purchased from Oncogene (Boston, MA); monoclonal anti-COX-1 and polyclonal anti-COX-2 antibodies were from Cayman Chemical Co. (Ann Arbor, MI) and Oxford Biomedical Research (Oxford, MI), respectively. Polyclonal anti-p-ERK, anti-t-ERK, anti-p-JNK, anti-t-JNK, anti-p-p38, anti-t-p38, and anti-p-c-Jun antibodies were from Cell Signal Technology (Beverly, MA). Polyclonal anti-β-actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal anti-human tropoelastin antibody and monoclonal anti-human fibrillin-1 antibody were from Elastin Products (Owensville, MO) and Neomarkers (Fremont, CA), respectively.

Topical application of EPA

EPA was purchased from Sigma (St. Louis, MO) and dissolved in ethanol-polyethylene glycol (70:30) containing 1% (w/v) tocopherol for topical application to human skin in vivo. To investigate the effects of topical EPA on UV-induced skin damage, young Korean adults (20–30 years old), all volunteers without current or prior skin disease, were enrolled. Young human volunteers (male, average age = 28 years; n = 7) were treated twice (24 h intervals; 24 h after the first treatment, the second treatment was performed) with vehicle or 2% EPA under occlusion. Ethanol-polyethylene glycol (70:30) containing 1% (w/v) tocopherol was used as vehicle. Twenty-four hours after the second treatment, buttock skin was irradiated with UV light [2 minimal erythma doses (MEDs)]. Forty-eight hours after irradiation, buttock skin was obtained by punch biopsy for immunohistochemical analysis and Western blot analysis of procollagen and MMP. To investigate the effects of EPA on UV-induced c-Jun phosphorylation, mitogen-activated protein kinase (MAPK) activation, and COX expression, skin biopsy was performed 6 h after UV irradiation. To investigate the effects of topical EPA on intrinsically aged skin, aged Korean adults (>75 years old), all volunteers without current or prior skin disease, were enrolled. Aged human volunteers (male, average age = 76.5 years; n = 4) were treated with vehicle or 2% EPA for 2 weeks (total of six times; treated on Monday, Wednesday, and Friday) under occlusion. Ethanol-polyethylene glycol (70:30) containing 1% (w/v) tocopherol was used as vehicle. Twenty-four hours after the last treatment, buttock skin was biopsied for immunohistochemical analysis and Western blot analysis. Skin protein was prepared by extracting whole skin biopsied to the upper dermis.

This study was conducted according to Declaration of Helsinki principles. All procedures received prior approval from the Institutional Review Board at Seoul National University Hospital, and all subjects provided written informed consent.

UV irradiation

A Waldmann UV-800 (Waldmann, Villingen-Schwenningen, Germany) phototherapy device and F75/85W/UV21 fluorescent lamp with emmission spectrum between 285 and 350 nm (peak at 310–315 nm) were used as the UV light source, as described previously (16). The strength of UV irradiation at the skin surface was measured using a Waldmann UV meter (model 585100). Buttock skin was irradiated with filtered UV light filtered by a Kodacel filter (TA401/407; Kodak, Rochester, NY), and the MED was determined at 24 h after irradiation. MED ranged between 70 and 90 mJ/cm^2 for the brown skin of Koreans.

Immunohistochemical analysis

Immunohistochemical analyses were performed as described previously (17). Human skin samples were fixed in 10% formalin for 24 h and embedded in paraffin. Serial sections (4 μm) were mounted onto silane-coated slides (Dako, Glostrup, Denmark). Sections were stained with hematoxylin and eosin and Masson-Trichrome. Acetone-fixed frozen sections were stained with the following primary antibodies in a humidified chamber at 4°C for 18 h: monoclonal anti-procollagen type I (SP1.D8) antibody, monoclonal anti-human PIC antibody, polyclonal rabbit anti-tropoelastin antibody, monoclonal anti-fibrillin-1 antibody, and anti-TGF-β1, -β2, and -β3 antibodies. Control staining was performed with normal rabbit and mouse immunoglobulin, which demonstrated no immunoreactivity (data not shown).

Average epidermal thickness was determined using an image-analysis program (BMI plus software; BumMi Universe Co., Ltd.). The ratio of tropoelastin and fibrillin (fiber area to dermis area) was measured from the dermoepidermal junction to the dermis (100 μm depth) at ×400.

Western blot

Western blot analysis of biopsied skin samples was performed as described previously (11). Briefly, punch-biopsied skin samples were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 5 mM PMSF, and 1 mM DTT) containing 1% (w/v) Triton X-100. Lysates were centrifuged at 12,000 *g* for 15 min, and supernatants were collected for Western blot and zymography. Protein concentration of samples was determined by Bradford assay. Equal amount of proteins was loaded onto Tris-glycine gels and then electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were subsequently blocked with 5% skim milk in TBS/T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.05% Tween-20) and incubated with the indicated antibodies. Blotting proteins were visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, England).

Zymography

To assess the gelatinolytic activity of MMP-9, soluble protein was extracted from punch-biopsied skin samples as described above. Equal amounts of protein were subjected to gelatin zymography using zymogram gels containing 10% gelatin, according to the manufacturer's protocol (Novex, San Diego, CA). After electrophoresis, gels were renatured by incubating in renaturing buffer [50 mM Tris-HCl, pH 7.4, and 2% (v/v) Triton X-100] for 30 min at room temperature. Gels were then incubated in a developing buffer [50 mM Tris-HCl, pH 8.0, 2.5 mM $CaCl_2$, and 0.02% (w/v) sodium azide] for 24 h at 37°C. Proteolytic bands were visualized by staining gels with 0.5% (w/v) Coomassie brilliant blue solution.

Statistical analysis

Statistical analyses were performed using Student's *t*-test. $P < 0.05$ was considered statistically significant. All analyses were performed using Statistical Analysis Software (SAS, Inc., Cary, NC). Results are presented as means ± SEM.

RESULTS

EPA inhibited UV-induced epidermal thickening in young human skin in vivo

Because UV irradiation has been shown to induce epidermal thickening in human skin, we investigated the effects of EPA on UV-induced epidermal thickening and epidermal growth. EPA was applied topically to young human buttock skin, and then skin was treated with UV light (2 MEDs). Forty-eight hours after irradiation, skin was biopsied. Serially sectioned samples were stained with hematoxylin and eosin, as described in Materials and Methods. UV light induced epidermal thickening (**Fig. 1**) by 214 ± 19.6% ($P < 0.05$ vs. the UV-untreated control group; n = 7), and topical EPA decreased the epidermal thickness induced by UV treatment by 72 ± 12.6% ($P < 0.05$ vs. the UV-only-treated group; n = 7). In EPA-only-treated skin, epidermal thickness increased slightly to 130 ± 9% ($P < 0.05$ vs. the UV-untreated control group; n = 7).

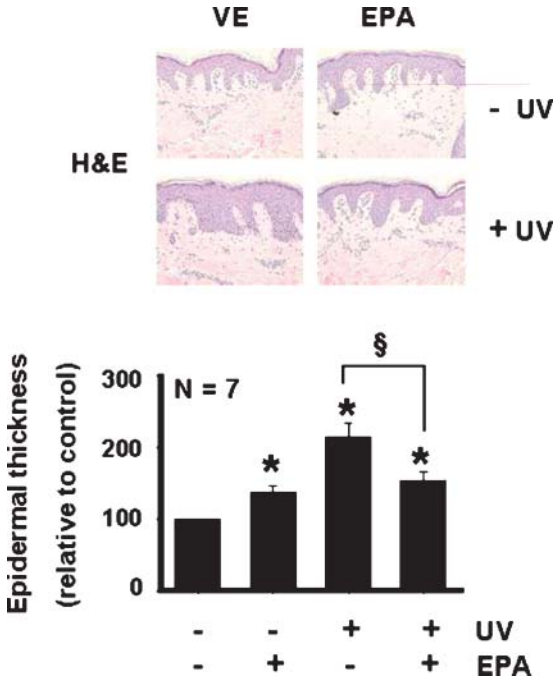

Fig. 1. Eicosapentaenoic acid (EPA) inhibits ultraviolet (UV) light-induced epidermal thickening in young human skin in vivo. Young human buttock skin was topically treated with 2% EPA or its vehicle (VE), as described in Materials and Methods, and then irradiated with UV light [2 minimal erythma dose (MEDs)]. Forty-eight hours after irradiation, skin was biopsied. Serial sections (4 μm) were mounted onto silane-coated slides and stained with hematoxylin and eosin (H&E). Results are shown as means ± SEM. * $P < 0.05$; § $P < 0.05$, versus UV-treated group.

EPA inhibited UV-induced decrease of procollagen expression in young human skin in vivo

Photoaging is caused by repeated UV irradiation, and reductions in collagen expression have been considered to be a cause of wrinkles in photoaged skin. We investigated the effect of EPA on decreased procollagen expression induced by UV light by immunohistochemical analysis and Western blotting in young human skin in vivo.

Immunohistochemistry revealed that UV decreased procollagen expression in fibroblasts in the upper dermis and in the dermoepidermal junction. Immunohistochemical results show that EPA increased procollagen staining in UV-untreated skin and that it inhibited the decrease of procollagen staining in UV-treated skin (**Fig. 2A**). We also found by Western blot analysis that UV light reduced the level of procollagen expression to 18 ± 4.5% ($P < 0.05$ vs. the UV-untreated control group; n = 7) of UV-untreated control levels (Fig. 2B). EPA restored the level of procollagen that was decreased by UV light to 46 ± 12% ($P < 0.05$ vs. the UV-only-treated group; n = 7) of UV-untreated control levels, as observed by immunohistochemistry. These results indicate that EPA counteracted the downregulating effects of UV light on procollagen and inhibited the decrease of procollagen. Consistent with the procollagen immunohistochemical profile, collagen fiber staining was increased substantially in EPA-pretreated, UV-irradiated

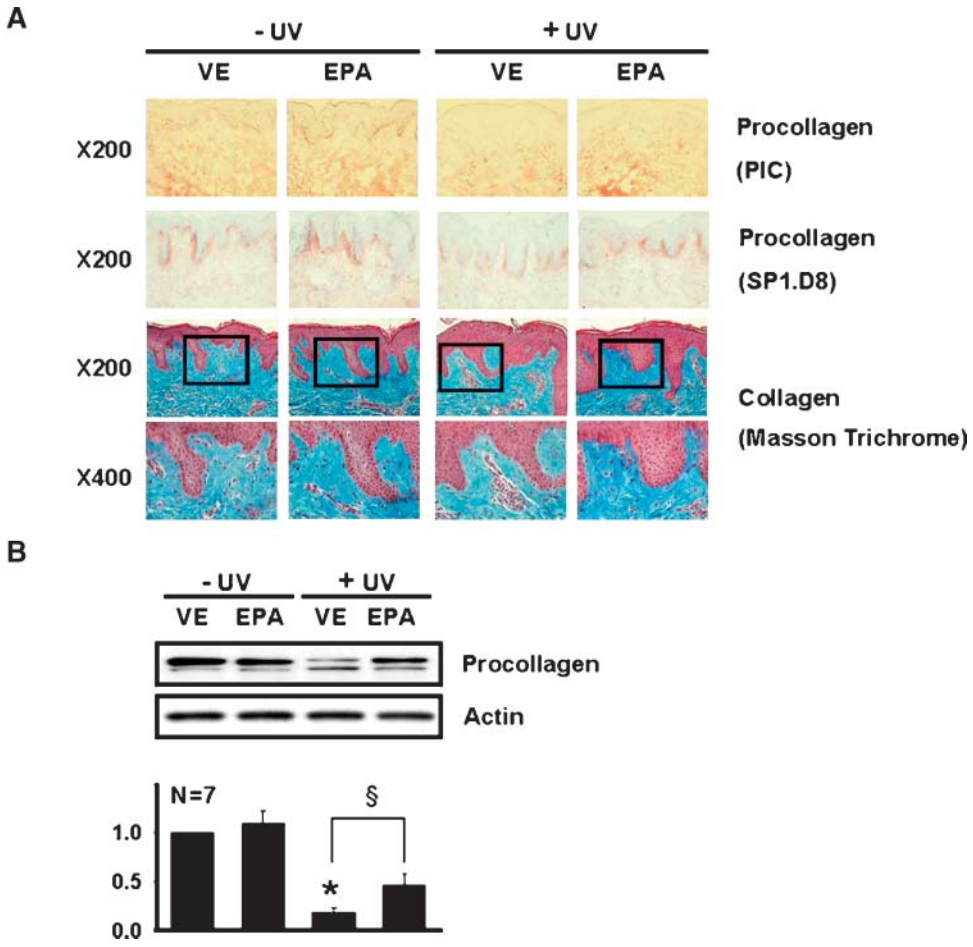

Fig. 2. EPA prevents UV-induced procollagen decreases in young human skin in vivo. Young human buttock skin (n = 7) was topically treated with 2% EPA or its vehicle (VE), as described in Materials and Methods, and then irradiated with UV light (2 MEDs). Forty-eight hours after irradiation, skin was biopsied. A: Serial sections (4 μm) were mounted onto silane-coated slides and stained with two antibodies against type I procollagen (PIC and SP1.D8) and with Masson-Trichrome to detect collagen fibers, as described in Materials and Methods. Areas in boxes are shown as 2-fold enlargements. B: Proteins were extracted from biopsied skin samples as described in Materials and Methods. The expression of procollagen was determined by Western blotting. Results are shown as means ± SEM. * $P < 0.05$; $^{\S}P < 0.05$, versus UV-treated group.

skin compared with vehicle-pretreated, UV-irradiated skin, as revealed by Masson-Trichrome stain (Fig. 2A). These results demonstrate that topical application of EPA inhibited UV-induced decreases of procollagen expression in human skin in vivo.

EPA inhibited UV-induced MMP-1 and MMP-9 expression in young human skin in vivo

We investigated the effects of EPA on UV-induced MMP-1 and MMP-9 expression by Western blotting and zymography in young human skin in vivo. UV light increased the levels of MMP-1 ($P < 0.05$ vs. the UV-untreated control group; n = 5) (**Fig. 3A**) and MMP-9 ($P < 0.05$ vs. the UV-untreated control group; n = 6) (Fig. 3B). However, EPA significantly inhibited this UV-induced MMP-1 expression by 55 ± 13% ($P < 0.05$ vs. the UV-only-treated group; n = 5) and MMP-9 expression by 75 ± 7% ($P < 0.05$ vs. the UV-only-treated group; n = 6). These results show that topical EPA inhibits UV-induced MMP-1 and MMP-9 expression in human skin.

EPA inhibited UV-induced c-Jun phosphorylation in young human skin in vivo

AP-1 is closely related to MMPs such as MMP-1 and MMP-9 that induce the decrease of collagen. For the formation of AP-1 complex, Jun proteins form homodimers or heterodimers with Fos protein (18). The transcriptional activity of AP-1 is also dependent on the degree of phosphorylation of c-Jun and expression of c-Fos as well as their abundance. However, because the level of c-Fos is not altered in photodamaged human skin (8), UV-induced c-Jun expression results in decreased collagen (9). Therefore, we investigated the effect of EPA on UV-induced c-Jun phosphorylation, which stabilizes c-Jun protein and then sustains its expression, in young human skin in vivo. UV was found to increase the level of phosphorylated c-Jun ($P < 0.05$ vs. the UV-untreated control group; n = 6) (**Fig. 4**), and EPA inhibited UV-induced c-Jun phosphorylation by 79 ± 11% ($P < 0.05$ vs. the UV-only-treated group; n = 6). These results show that EPA inhibits UV-induced MMP-1 and MMP-9 expression and that this

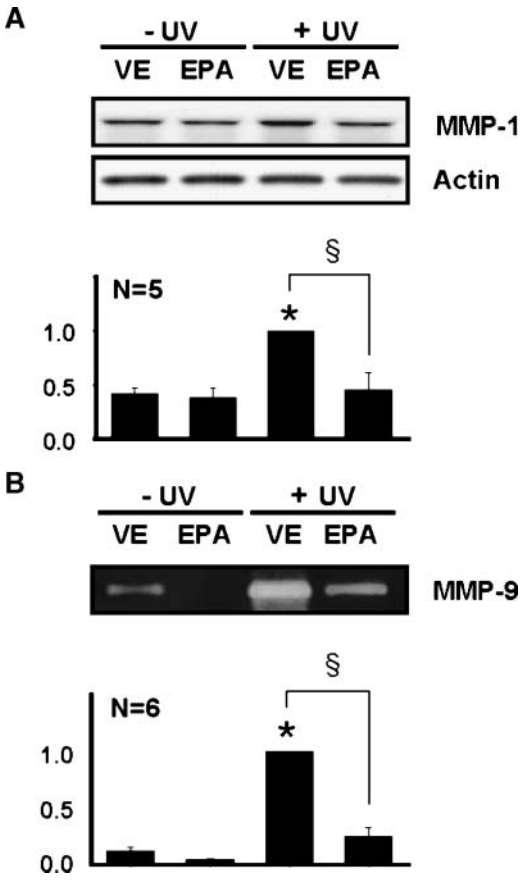

Fig. 3. EPA inhibits UV-induced matrix metalloproteinase-1 (MMP-1) and MMP-9 expression in young human skin in vivo. Young human buttock skin was topically treated with 2% EPA or its vehicle (VE), as described in Materials and Methods, and then irradiated with UV light (2 MEDs). Forty-eight hours after irradiation, skin was biopsied. Proteins were extracted from biopsied skin samples as described in Materials and Methods. MMP-1 (A, n = 5) and MMP-9 (B, n = 6) expression was determined by Western blotting and zymography. Results are shown as means ± SEM. * $P < 0.05$; $^{\S}P < 0.05$, versus UV-treated group.

inhibition may be mediated by a reduction in the level of phosphorylated c-Jun, which is known to be closely associated with UV-induced AP-1 activation in human skin.

EPA inhibited UV-induced JNK and p38 activation but not ERK activation in young human skin in vivo

From the results described above, we found that EPA inhibits UV-induced MMP-1 and MMP-9 expression in human skin in vivo, which may be mediated by abolishing c-Jun phosphorylation. Thus, we investigated the effects of EPA on UV-induced MAPK in young human skin in vivo. UV irradiation induced the activation of three MAPKs, ERK, JNK, and p38 ($P < 0.05$ vs. the UV-untreated control group; n = 6) (**Fig. 5**). Although EPA did not inhibit UV-induced ERK activation (Fig. 5A), EPA inhibited UV-induced JNK (Fig. 5B) and p38 (Fig. 5C) activation by 54 ± 9% ($P < 0.05$ vs. the UV-only-treated group; n = 6) and 68 ± 12% ($P < 0.05$ vs. the UV-only-treated group; n = 6), respectively. Because JNK and p38 are closely related to c-Jun phosphorylation and expression, these findings suggest that the inhibition of UV-induced c-Jun phosphorylation by EPA is mediated by inhibition of the activation of JNK and p38.

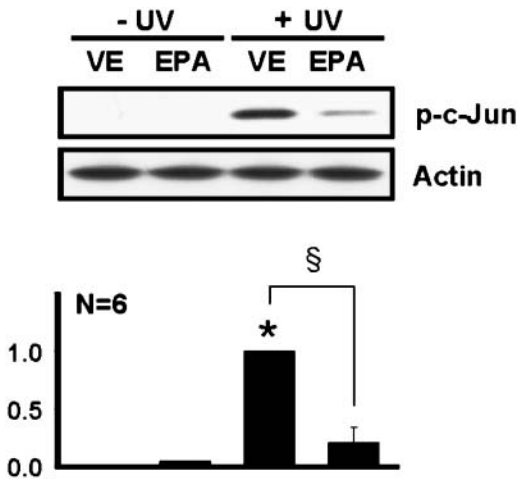

Fig. 4. EPA inhibits UV-induced c-Jun phosphorylation in young human skin in vivo. Young human buttock skin (n = 6) was topically treated with 2% EPA or its vehicle (VE), as described in Materials and Methods, and then irradiated with UV light (2 MEDs). Six hours after irradiation, skin was biopsied. Proteins were extracted from biopsied skin samples as described in Materials and Methods. Phosphorylated (p-) c-Jun was detected by Western blotting. Results are shown as means ± SEM. * $P < 0.05$; $^{\S}P < 0.05$, versus UV-treated group.

EPA inhibited UV-induced COX-2 expression in young human skin in vivo

UV induces COX-2 expression that produces PG from AA in human skin. PG is known to play an important role in MMP expression (19–21) and is also a well-known inhibitor of collagen synthesis (22). Therefore, we investigated the effect of EPA on UV-induced COX-2 expression in young human skin in vivo. UV irradiation dramatically induced COX-2 expression ($P < 0.05$ vs. the UV-untreated control group; n = 4) (**Fig. 6A**). EPA significantly inhibited UV-induced COX-2 expression by 76 ± 4% ($P < 0.05$ vs. the UV-treated group; n = 6) in young human skin in vivo (Fig. 6A). However, EPA did not affect COX-1, which is constitutively expressed in human skin (Fig. 6B). This result indicated that EPA inhibited UV-induced COX-2 expression.

EPA increased the expression of ECM in aged human skin in vivo

Histological changes in sun-protected aged human skin (intrinsic aged skin) include reduced collagen levels and reduced expression of tropoelastin and fibrillin-1 (components of elastic fibers) (23). We investigated the effect of EPA on collagen and elastic fiber levels in aged human skin in vivo. Compared with vehicle-treated skin, EPA-treated skin demonstrated increased procollagen staining in fibroblasts throughout the dermis as well as denser extracellular staining in the dermoepidermal junction, as shown by PIC and SP1.D8 staining, respectively (**Fig. 7A**).

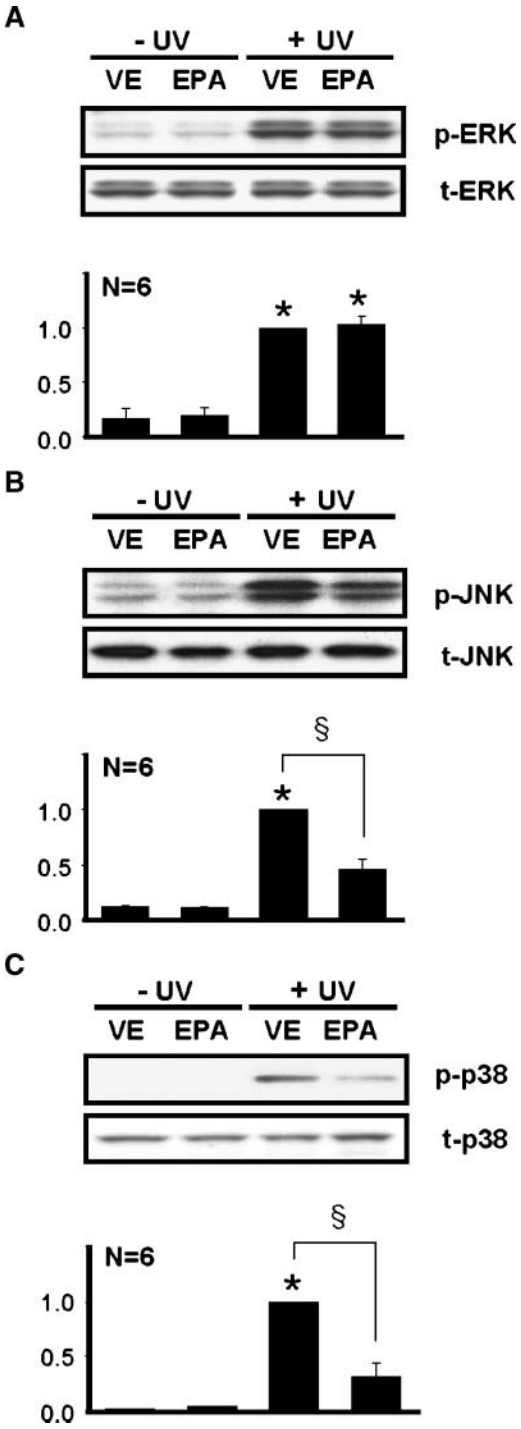

Fig. 5. EPA inhibits UV-induced JNK and p38 activation but not ERK activation in young human skin in vivo. Young human buttock skin (n = 6) was topically treated with 2% EPA or its vehicle (VE), as described in Materials and Methods, and then irradiated with UV light (2 MEDs). Six hours after irradiation, skin was biopsied. Proteins were extracted from biopsied skin samples as described in Materials and Methods. Activation of ERK (A), JNK (B), and p38 (C) was determined by Western blotting using phosphospecific (p-) antibodies. Total forms (t-) of ERK, JNK, and p38 were used as loading controls. Results are shown as means ± SEM. * $P < 0.05$; $^{\S}P < 0.05$, versus UV-treated group.

Consistent with the findings of procollagen immunohistochemistry, collagen staining with Masson-Trichrome also revealed increased collagen fibers throughout the dermis in EPA-treated aged human skin (Fig. 7A). Western blot analysis also showed that EPA increased the level of procollagen by 218 ± 39% ($P < 0.05$ vs. the vehicle-treated control group; n = 4) (Fig. 7B).

In addition to increased collagen expression, EPA increased tropoelastin and fibrillin-1 levels (**Fig. 8**). Compared with vehicle treatment, EPA treatment substantially increased the intricate network of tropoelastin-immunoreactive elastic fibers in the dermoepidermal junction by 145 ± 22% ($P < 0.05$ vs. the vehicle-treated control group; n = 4) and fibrillin-1-immunoreactive fibers by 696 ± 138% ($P < 0.05$ vs. the vehicle-treated control group; n = 4) (Fig. 8A, C). Western blots also revealed that EPA

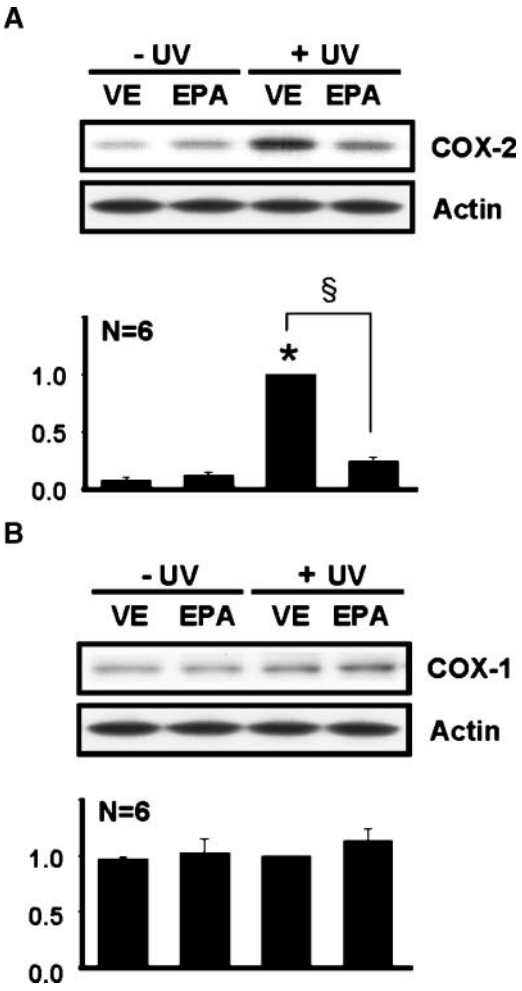

Fig. 6. EPA inhibits UV-induced cyclooxygenase-2 (COX-2) expression without altering COX-1 expression in young human skin in vivo. Young human buttock skin (n = 6) was topically treated with 2% EPA or its vehicle (VE), as described in Materials and Methods, and then irradiated with UV light (2 MEDs). Six hours after irradiation, skin was biopsied. Proteins were extracted from biopsied skin samples as described in Materials and Methods. Expression of COX-1 (A) and COX-2 (B) was determined by Western blotting. Results are shown as means ± SEM. * $P < 0.05$; $^{\S}P < 0.05$, versus UV-treated group.

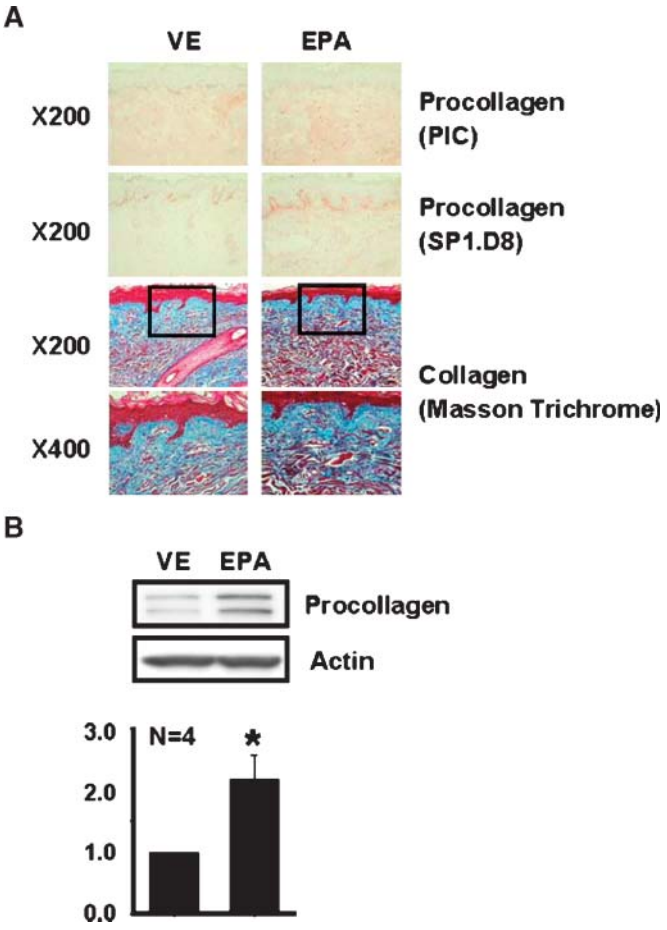

Fig. 7. EPA increases the level of procollagen expression in aged human skin in vivo. Aged human buttock skin (n = 4) was topically treated with 2% EPA or its vehicle (VE), as described in Materials and Methods. A: Serial sections (4 μm) were mounted onto silane-coated slides and stained with two antibodies against type I procollagen (PIC and SP1.D8) and with Masson-Trichrome to detect collagen fibers, as described in Materials and Methods. Areas in boxes are shown as 2-fold enlargements. B: Proteins were extracted from biopsied skin samples as described in Materials and Methods. The expression of procollagen was determined by Western blotting. Results are shown as means ± SEM. * $P < 0.05$.

increased the level of tropoelastin and fibrillin-1 by 420 ± 53% ($P < 0.05$ vs. the vehicle-treated control group; n = 4) and 131 ± 18% ($P < 0.05$ vs. the vehicle-treated control group; n = 4), respectively (Fig. 8B, D). These results show that topical application of EPA to aged human skin increased procollagen, tropoelastin, and fibrillin-1 expression in the ECM and thus demonstrate that EPA has preventive and therapeutic effects on intrinsic skin aging.

EPA increased the expression of TGF-β1, -β2, and -β3 in aged human skin in vivo

Because TGF-β is an important cytokine that induces the synthesis of ECM, we investigated the effect of EPA on the expression of TGF-β1, -β2, and -β3 in aged human skin in vivo (**Fig. 9**). In vehicle-treated skin, TGF-β1 was weakly expressed in the epidermis and dermis, whereas TGF-β2 and -β3 were stained mainly in the lower epidermis. It was found that topical application of EPA increased not only TGF-β1, which is primarily stained in dermis, but also TGF-β2 and -β3, which are stained mainly in epidermis. These results indicate that topical application of EPA to aged human skin increases ECM expression by stimulating TGF-β signaling.

DISCUSSION

In our previous report, we demonstrated that EPA inhibits UV-induced MMP-1 expression in human dermal fibroblasts (15) and that treating human dermal fibroblasts with EPA inhibited UV-induced AP-1 activation, which has an important role in MMP-1 expression. Furthermore, we found that suppression of UV-induced AP-1 activation by EPA was mediated by inhibition of the MEK1/ERK/c-Fos and SEK1/JNK/c-Jun pathways. Therefore, in this study, we investigated the effect of EPA on the expression of MMPs and ECMs in young and aged human skin in vivo.

Skin aging can be attributed to extrinsic aging (photoaging) and intrinsic (chronological) aging. Photoaging concerns premature skin aging caused by repeated sun exposure (24–26). Recently, it was suggested that excessive matrix degradation by UV-induced MMPs secreted by various cells (e.g., keratinocytes, fibroblasts, and inflammatory cells) contributes substantially to the connective tissue damage that occurs during photoaging (5, 11, 26). The following mechanism of photoaging has been proposed. Initially, AP-1 is activated by UV light, and AP-1-driven MMPs such as MMP-1 and MMP-9 are induced (3, 5, 11). These MMPs then degrade collagen, which results in a collagen deficiency in photodamaged skin and eventually causes skin wrinkling (27). In human skin, UV-induced AP-1 transcriptional activity is determined by c-Jun expression, because c-Fos is expressed continuously (7). Whereas c-Fos expression in young and aged skin is unaltered, c-Jun expression is higher in aged skin than in young skin (8). Some recent reports have suggested that the major regulatory mechanism of procollagen expression involves transcriptional control (28–31). Decreased procollagen expression is partly mediated by c-Jun, which is induced by UV light and interferes with procollagen transcription (9). Moreover, all-*trans* retinoic acid, the best known anti-skin-aging agent, has been shown to interfere with the responses of skin to acute UV irradiation, which induces collagen degradation (32). All-*trans* retinoic acid does not inhibit UV-induced c-Jun mRNA but rather blocks c-Jun phosphorylation and accumulation (7). In this study, we found that topical EPA not only inhibited the UV-induced decrease of procollagen expression but also attenuated UV-induced MMP-1 and MMP-9 expression. Moreover, we also found that topical EPA inhibited UV-induced c-Jun phosphorylation and activation of JNK and p38, but not ERK, in young human skin in vivo. If EPA act as a sunscreen, its photoprotective effects on various target molecules should be similar. Therefore, we think that the photoprotective effects of EPA in human skin do not result from the sunscreen effect.

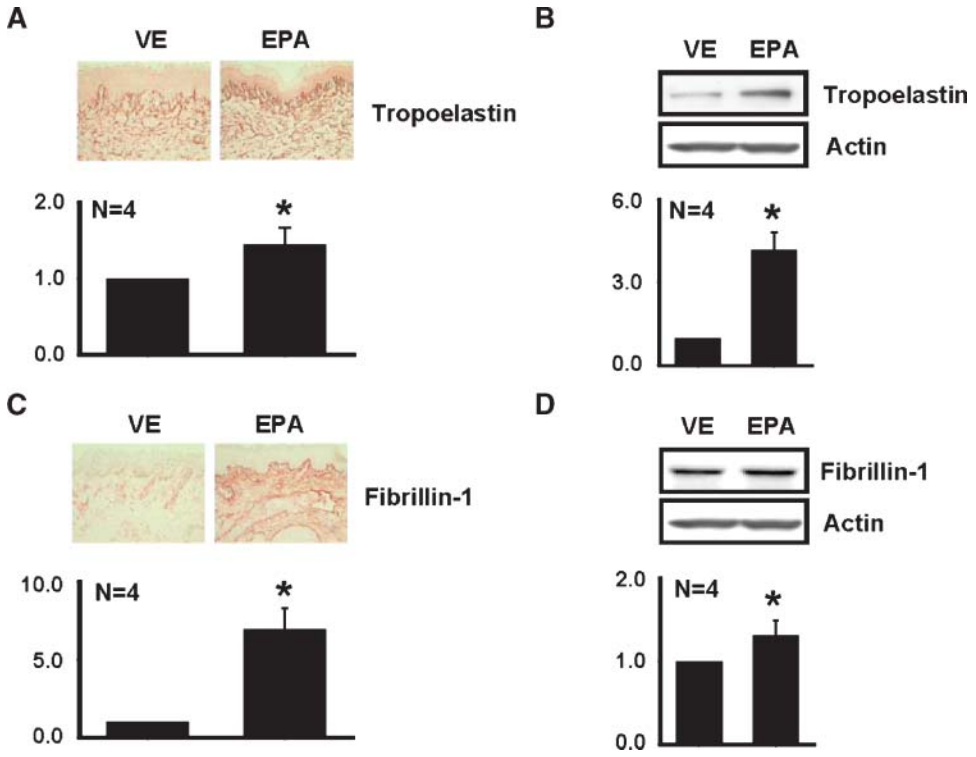

Fig. 8. EPA increases the level of tropoelastin and fibrillin-1 expression in aged human skin in vivo. Aged human buttock skin (n = 4) was topically treated with 2% EPA or its vehicle (VE), as described in Materials and Methods. A, C: Serial sections (4 μm) were mounted onto silane-coated slides and stained with tropoelastin (A) and fibrillin-1 (C) antibodies, as described in Materials and Methods. B, D: Proteins were extracted from biopsied skin samples as described in Materials and Methods. The expression of tropoelastin (B) and fibrillin-1 (D) was determined by Western blotting. Results are shown as means ± SEM. * $P < 0.05$.

PGs are known to play an important role in UV-induced skin responses, including erythma (33). Moreover, PGE_2 induces various MMPs, such as collagenase and stromelysin, in macrophages and fibroblasts (19–21, 26, 34). COX is a key enzyme that mediates PG production by converting AA to PGH_2, a main precursor of all prostanoids. In human skin, at least two isoforms of COX exist, the constitutive form (COX-1) and the inducible form (COX-2) (34). COX-2 expression is increased by various stimuli, such as UV, growth factors, and cytokines, and is closely implicated in inflammation, photocarcinogenesis, and aging (35). On the other hand, COX-1 is expressed constantly and is important in maintaining homeostatic functions. In this study, we found that EPA inhibits UV-induced COX-2 expression without altering COX-1 expression.

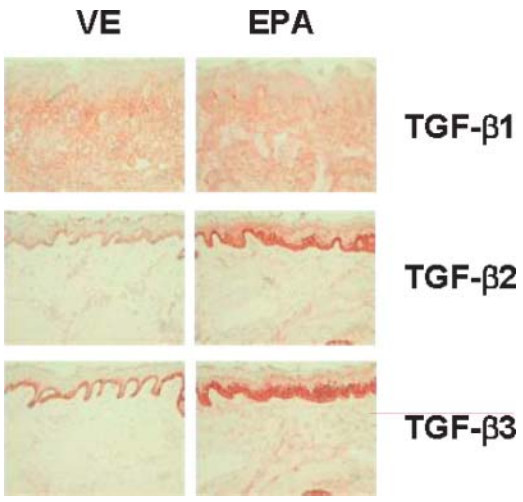

Fig. 9. EPA increases the levels of transformin growth factor-β1 (TGF-β1), -β2, and -β3 expression in aged human skin in vivo. Aged human buttock skin (n = 4) was topically treated with 2% EPA or its vehicle (VE), as described in Materials and Methods. Serial sections (4 μm) were mounted onto silane-coated slides and stained with TGF-β1, -β2, and -β3 antibodies, as described in Materials and Methods.

It was reported that the differential effects of ω-3 and ω-6 PUFAs might be related to the formation of PGE (13, 36). Whereas ω-6 PUFAs such as AA are converted to 2-series PGE (PGE_2) by COX-2, ω-3 PUFAs such as EPA and DHA are converted to 3-series PGE (PGE_3) by COX-2. It was also reported that ω-3 PUFAs compete with AA at the level of incorporation into cell membrane phospholipids, leading to the inhibition of PGE_2 production from AA and the increase of PGE_3 synthesis from EPA and DHA (13, 36). PGE_2 is more mitogenic and proinflammatory than PGE_3. It has been reported that fish oils, rich in ω-3 PUFAs, possess antiinflammatory properties in UV-exposed skin (37).

Skin aging in the sun-protected skin of elderly individuals results in a loss of collagen and an increase in MMP-1 expression (4, 26). This process is known as intrinsic

(chronological) skin aging, and collagen deficiencies during this process may arise from its reduced synthesis and increased degradation by concomitantly increased MMP expression. Also, intrinsically aged skin has decreased ECM and reduced elastin and fibrillin-1 expression. TGF-β is a multifunctional cytokine that plays an important role in the synthesis of extracellular connective tissue. TGF-β is also known to stimulate the proliferation of fibroblasts and to induce the synthesis and secretion of ECMs, such as collagen, tropoelastin, and fibrillin-1 (17, 38, 39). In addition, TGF-β downregulates the expression of proteolytic enzymes, such as collagenase and gelatinase, which degrade ECM (40, 41). We found here that topical EPA increased the expression of collagen, tropoelastin, and fibrillin-1. In addition, we also found that EPA increased the expression of TGF-β1, -β2, and -β3. These results demonstrate that topical EPA induces the expression of collagen, fibrillin-1, and tropoelastin by increasing the expression of TGF-β1, -β2, and -β3.

In conclusion, this study demonstrates that topical application of EPA inhibits UV-induced collagen decrease and attenuates MMP-1 and MMP-9 expression, which are induced by UV light, and that these effects of EPA are mediated by inhibiting UV-induced JNK and p38 activation and COX-2 expression. These results indicate that topical application of EPA prevents the photoaging process in human skin in vivo. Moreover, this study also shows that topical application of EPA to aged human skin induces ECM expression by increasing TGF-β expression. Therefore, we believe that topical application of EPA helps prevent and treat skin aging.

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REFERENCES

1. Jenkins, G. 2002. Molecular mechanisms of skin ageing. *Mech. Ageing Dev.* **123:** 801–810.
2. Gilchrest, B. A. 1989. Skin aging and photoaging: an overview. *J. Am. Acad. Dermatol.* **21:** 610–613.
3. Fisher, G. J., Z. Q. Wang, S. C. Datta, J. Varani, S. Kang, and J. J. Voorhees. 1997. Pathophysiology of premature skin aging induced by ultraviolet light. *N. Engl. J. Med.* **337:** 1419–1428.
4. Varani, J., R. L. Warner, M. Gharaee-Kermani, S. H. Phan, S. Kang, J. H. Chung, Z. Q. Wang, S. C. Datta, G. J. Fisher, and J. J. Voorhees. 2000. Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin. *J. Invest. Dermatol.* **114:** 480–486.
5. Fisher, G. J., S. C. Datta, H. S. Talwar, Z. Q. Wang, J. Varani, S. Kang, and J. J. Voorhees. 1996. Molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature.* **379:** 335–339.
6. Sternlicht, M. D., and Z. Werb. 2001. How matrix metalloproteinases regulate cell behavior. *Annu. Rev. Cell Dev. Biol.* **17:** 463–516.
7. Fisher, G. J., H. S. Talwar, J. Lin, P. Lin, F. McPhillips, Z. Wang, X. Li, Y. Wan, S. Kang, and J. J. Voorhees. 1998. Retinoic acid inhibits induction of c-Jun protein by ultraviolet radiation that occurs subsequent to activation of mitogen-activated protein kinase pathways in human skin in vivo. *J. Clin. Invest.* **101:** 1432–1440.
8. Chung, J. H., S. Kang, J. Varani, J. Lin, G. J. Fisher, and J. J. Voorhees. 2000. Decreased extracellular-signal-regulated kinase and increased stress-activated MAP kinase activities in aged human skin in vivo. *J. Invest. Dermatol.* **115:** 177–182.
9. Fisher, G. J., S. Datta, Z. Wang, X. Y. Li, T. Quan, J. H. Chung, S. Kang, and J. J. Voorhees. 2000. c-Jun-dependent inhibition of cutaneous procollagen transcription following ultraviolet irradiation is reversed by all-trans retinoic acid. *J. Clin. Invest.* **106:** 663–670.
10. Gilchrest, B. A. 1982. Age-associated changes in the skin. *J. Am. Geriatr. Soc.* **30:** 139–143.
11. Chung, J. H., J. Y. Seo, H. R. Choi, M. K. Lee, C. S. Youn, G. Rhie, K. H. Cho, K. H. Kim, K. C. Park, and H. C. Eun. 2001. Modulation of skin collagen metabolism in aged and photoaged human skin in vivo. *J. Invest. Dermatol.* **117:** 1218–1224.
12. Mirnikjoo, B., S. E. Brown, H. F. Kim, L. B. Marangell, J. D. Sweatt, and E. J. Weeber. 2001. Protein kinase inhibition by omega-3 fatty acids. *J. Biol. Chem.* **276:** 10888–10896.
13. Bagga, D., L. Wang, R. Farias-Eisner, J. A. Glaspy, and S. T. Reddy. 2003. Differential effects of prostaglandin derived from omega-6 and omega-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. *Proc. Natl. Acad. Sci. USA.* **100:** 1751–1756.
14. Herschman, H. R., W. Xie, and S. Reddy. 1995. Inflammation, reproduction, cancer and all that.... The regulation and role of the inducible prostaglandin synthase. *Bioessays.* **17:** 1031–1037.
15. Kim, H. H., C. M. Shin, C. H. Park, K. H. Kim, K. H. Cho, H. C. Eun, and J. H. Chung. 2005. Eicosapentaenoic acid inhibits UV-induced MMP-1 expression in human dermal fibroblasts. *J. Lipid Res.* **46:** 1712–1720.
16. Seo, J. Y., S. H. Lee, C. S. Youn, H. R. Choi, G. E. Rhie, K. H. Cho, K. H. Kim, K. C. Park, H. C. Eun, and J. H. Chung. 2001. Ultraviolet radiation increases tropoelastin mRNA expression in the epidermis of human skin in vivo. *J. Invest. Dermatol.* **116:** 915–919.
17. Son, E. D., J. Y. Lee, S. Lee, M. S. Kim, B. G. Lee, I. S. Chang, and J. H. Chung. 2005. Topical application of 17beta-estradiol increases extracellular matrix protein synthesis by stimulating TGF-beta signaling in aged human skin in vivo. *J. Invest. Dermatol.* **124:** 1149–1161.
18. Huang, C., P. C. Schmid, W. Y. Ma, H. H. Schmid, and Z. Dong. 1997. Phosphatidylinositol-3 kinase is necessary for 12-O-tetradecanoylphorbol-13-acetate-induced cell transformation and activated protein 1 activation. *J. Biol. Chem.* **272:** 4187–4194.
19. Wahl, L. M., and L. L. Lampel. 1987. Regulation of human peripheral blood monocyte collagenase by prostaglandins and antiinflammatory drugs. *Cell. Immunol.* **105:** 411–422.
20. Pentland, A. P., S. D. Shapiro, and H. G. Welgus. 1995. Agonist-induced expression of tissue inhibitor of metalloproteinases and metalloproteinases by human macrophages is regulated by endogenous prostaglandin E2 synthesis. *J. Invest. Dermatol.* **104:** 52–57.
21. Mauviel, A., C. Halcin, P. Vasiloudes, W. C. Parks, M. Kurkinen, and J. Uitto. 1994. Uncoordinate regulation of collagenase, stromelysin, and tissue inhibitor of metalloproteinases genes by prostaglandin E2: selective enhancement of collagenase gene expression in human dermal fibroblasts in culture. *J. Cell. Biochem.* **54:** 465–472.
22. Clark, J. G., K. M. Kostal, and B. A. Marino. 1982. Modulation of collagen production following bleomycin-induced pulmonary fibrosis in hamsters. Presence of a factor in lung that increases fibroblast prostaglandin E2 and cAMP and suppresses fibroblast proliferation and collagen production. *J. Biol. Chem.* **257:** 8098–8105.
23. Braverman, I. M., and E. Fonferko. 1982. Studies in cutaneous aging. I. The elastic fiber network. *J. Invest. Dermatol.* **78:** 434–443.
24. Uitto, J. 1986. Connective tissue biochemistry of the aging dermis. Age-related alterations in collagen and elastin. *Dermatol. Clin.* **4:** 433–446.
25. Gilchrest, B. A. 1996. A review of skin ageing and its medical therapy. *Br. J. Dermatol.* **135:** 867–875.
26. Chung, J. H., J. Y. Seo, M. K. Lee, H. C. Eun, J. H. Lee, S. Kang, G. J. Fisher, and J. J. Voorhees. 2002. Ultraviolet modulation of human macrophage metalloelastase in human skin in vivo. *J. Invest. Dermatol.* **119:** 507–512.
27. Gilchrest, B. A., and M. Yaar. 1992. Ageing and photoageing of the skin: observations at the cellular and molecular level. *Br. J. Dermatol.* **127 (suppl. 41):** 25–30.
28. Jimenez, S. A., J. Varga, A. Olsen, L. Li, A. Diaz, J. Herhal, and J. Koch. 1994. Functional analysis of human alpha 1(I) procollagen gene promoter. Differential activity in collagen-producing and

-nonproducing cells and response to transforming growth factor beta 1. *J. Biol. Chem.* **269:** 12684–12691.
29. Rossert, J. A., and L. A. Garrett. 1995. Regulation of type I collagen synthesis. *Kidney Int. Suppl.* **49:** 34–38.
30. Inagaki, Y., S. Truter, S. Tanaka, M. Di Liberto, and F. Ramirez. 1995. Overlapping pathways mediate the opposing actions of tumor necrosis factor-alpha and transforming growth factor-beta on alpha 2(I) collagen gene transcription. *J. Biol. Chem.* **270:** 3353–3358.
31. Philips, N., R. I. Bashey, and S. A. Jimenez. 1995. Increased alpha 1(I) procollagen gene expression in tight skin (TSK) mice myocardial fibroblasts is due to a reduced interaction of a negative regulatory sequence with AP-1 transcription factor. *J. Biol. Chem.* **270:** 9313–9321.
32. Fisher, G. J., and J. J. Voorhees. 1998. Molecular mechanisms of photoaging and its prevention by retinoic acid: ultraviolet irradiation induces MAP kinase signal transduction cascades that induce Ap-1-regulated matrix metalloproteinases that degrade human skin in vivo. *J. Investig. Dermatol. Symp. Proc.* **3:** 61–68.
33. Carsberg, C. J., J. Ohanian, and P. S. Friedmann. 1995. Ultraviolet radiation stimulates a biphasic pattern of 1,2-diacylglycerol formation in cultured human melanocytes and keratinocytes by activation of phospholipases C and D. *Biochem. J.* **305:** 471–477.
34. Seo, J. Y., E. K. Kim, S. H. Lee, K. C. Park, K. H. Kim, H. C. Eun, and J. H. Chung. 2003. Enhanced expression of cyclooxygenase-2 by UV in aged human skin in vivo. *Mech. Ageing Dev.* **124:** 903–910.
35. Smith, W. L., E. A. Meade, and D. L. DeWitt. 1994. Pharmacology of prostaglandin endoperoxide synthase isozymes-1 and -2. *Ann. N. Y. Acad. Sci.* **714:** 136–142.
36. Liu, G., D. M. Bibus, A. M. Bode, W. Y. Ma, R. T. Holman, and Z. Dong. 2001. Omega 3 but not omega 6 fatty acids inhibit AP-1 activity and cell transformation in JB6 cells. *Proc. Natl. Acad. Sci. USA.* **98:** 7510–7515.
37. Pupe, A., R. Moison, P. De Haes, G. B. van Henegouwen, L. Rhodes, H. Degreef, and M. Garmyn. 2002. Eicosapentaenoic acid, a n-3 polyunsaturated fatty acid differentially modulates TNF-alpha, IL-1alpha, IL-6 and PGE2 expression in UVB-irradiated human keratinocytes. *J. Invest. Dermatol.* **118:** 692–698.
38. Massague, J. 1998. TGF-beta signal transduction. *Annu. Rev. Biochem.* **67:** 753–791.
39. Piek, E., C. H. Heldin, and P. Ten Dijke. 1999. Specificity, diversity, and regulation in TGF-beta superfamily signaling. *FASEB J.* **13:** 2105–2124.
40. Hall, M. C., D. A. Young, J. G. Waters, A. D. Rowan, A. Chantry, D. R. Edwards, and I. M. Clark. 2003. The comparative role of activator protein 1 and Smad factors in the regulation of Timp-1 and MMP-1 gene expression by transforming growth factor-beta 1. *J. Biol. Chem.* **278:** 10304–10313.
41. Eickelberg, O., E. Kohler, F. Reichenberger, S. Bertschin, T. Woodtli, P. Erne, A. P. Perruchoud, and M. Roth. 1999. Extracellular matrix deposition by primary human lung fibroblasts in response to TGF-beta1 and TGF-beta3. *Am. J. Physiol.* **276:** L814–L824.