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# Astaxanthin attenuates the UVA-induced up-regulation of matrix-metalloproteinase-1 and skin fibroblast elastase in human dermal fibroblasts

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ABSTRACT

*Background:* Repetitive exposure of the skin to UVA radiation elicits sagging more frequently than wrinkling, which is mainly attributed to its biochemical mechanism to up-regulate the expression of matrix-metalloproteinase (MMP)-1 and skin fibroblast elastase (SFE)/neutral endopeptidase (NEP), respectively.
*Objective:* In this study, we examined the effects of a potent antioxidant, astaxanthin (AX), on the induction of MMP-1 and SFE by UVA treatment of cultured human dermal fibroblasts.
*Methods:* Those effects were assessed by real-time RT-PCR, Western blotting and enzymic activity assays.
*Results:* UVA radiation elicited a significant increase in the gene expression of MMP-1 as well as SFE/NEP (to a lesser extent) which was followed by distinct increases in their protein and enzymatic activity levels. The addition of AX at concentrations of 4–8 μM immediately after UVA exposure significantly attenuated the induction of MMP-1 and SFE/NEP expression elicited by UVA at the gene, protein and activity levels although both the UVA stimulation and the subsequent AX inhibition were greater for MMP-1 than for SFE/NEP. Analysis of the UVA-induced release of cytokines revealed that UVA significantly stimulated only the secretion of IL-6 among the cytokines tested and that AX significantly diminished only the IL-6 secretion.
*Conclusion:* These findings indicate that, based on different effective concentrations of AX, a major mode of action leading to the inhibition elicited by AX depends on inhibition of UVA effects of the reactive oxygen species-directed signaling cascade, but not on interruption of the IL-6-mediated signaling cascade. We hypothesize that AX would have a significant benefit on protecting against UVA-induced skin photo-aging such as sagging and wrinkles.


## 1. Introduction

Because of the deep penetration of UVA radiation into the dermis where fibroblasts (which produce matrix-proteins as well as their degrading enzymes) are located, compared with UVB radiation which penetrates only the epidermis, UVA-induced photo-damage of the skin is believed to be derived from effects on dermal fibroblasts. Repetitive UV exposure of the skin is well known to elicit photo-aging mainly consisting of wrinkling and sagging of the facial skin [1]. This photo-damage has been ascribed to the up-regulation of skin fibroblast elastase (SFE) and matrix-metalloproteinase (MMP)-1 (collagenase type I) in dermal fibroblasts [2–4], which leads to the degradation of major dermal components (elastin and collagen) which in turn results in wrinkling and sagging, respectively. SFE is a 94 kDa membrane-bound type metalloprotease with a neutral optimum pH [5–12]. Although there are several candidate enzymes for skin fibroblast elastase, such as 92, 72 kDa type IV collagenase, neutrophil elastase, cathepsin G and protease 3, none of those have properties which match its known characteristics. We recently reported that SFE activity, which is significantly up-regulated in the dermis of UV exposed skin, is a phosphoramidon-sensitive metalloproteinase [13,14] and is identical to neutral endopeptidase (NEP) [15,16]. Consistent with the in vivo chronic effects of UVA exposure [17], it has already been reported that direct UVA exposure of cultured human fibroblasts elicits an increased expression of MMP-1 at the gene and protein levels, which in turn leads to the enhancement of MMP-1 activity [18,19], although the UVA effect on the gene expression of NEP in cultured human fibroblasts has not yet been clarified. Those UVA effects are known to be mediated by the over-generation of reactive oxygen species (ROS) which leads to the imbalance or loss of cellular redox homeostasis, resulting in

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oxidative stress [20,21] although ROS are also part of normal regulatory circuits. Apart from permanent genetic changes involving protooncogenes and tumor suppressor genes, ROS (including singlet oxygen, hydroxyl radicals and hydrogen peroxides) have been identified as early events in the signaling sequence which eventually leads to the induction of MMP-1 after UVA exposure [20].

AX is a carotenoid found mainly in plants and marine seafood such as salmon, lobster, shrimp or crab and is well known to have antioxidant [22,23], anti-inflammatory and immunomodulatory activities [24,25]. The major mode of action of AX is to scavenge ROS [26]. In the LPS-stimulated murine macrophage cell line RAW264.7, AX has been reported to have a potent capacity to block the nuclear translocation of the NF-κB p65 subunit and IκBα degradation through its inhibitory effect on NκB kinase (IKK) activity [27]. Due to its scavenger potential for singlet oxygen (which is generated by UVA exposure), AX has been implicated in the marked inhibitory effects on UVA-induced oxidative cell damage [28–30]. Thus, it is anticipated that the addition of AX to UVA-exposed fibroblasts in culture will prevent the UVA-stimulated up-regulation of MMP-1. Recently, a redox imbalance has been shown to be closely linked to a variety of altered cellular responses in which the intracellular redox condition profoundly affects intracellular signaling pathways [31] The redox state of each cell is reflected by the precise balance between the levels of oxidizing and reducing equivalents, such as ROS and endogenous antioxidants such as glutathione. ROS act as second messengers in intracellular signal transduction, and control the action of several signaling pathways, including mitogen-activated protein (MAP) kinases [31]. Thus, owing to its inhibitory effects on NF-κB transcription activity which occurs downstream of the generation of ROS elicited by some stimuli [32], it is of considerable interest to determine the mode of action of AX during intracellular signaling downstream of the ROS-directly triggered activation of some kinases such as IKK kinase. In this study, which focuses on the effects of AX on intracellular signaling induced after ROS generation which leads to the increased expression of metalloproteases, we used AX immediately after UVA exposure and examined the subsequent effects on the induction of MMP-1 and SFE/NEP expression in cultured human fibroblasts.

## 2. Materials and methods

### 2.1. Materials

The synthetic substrate for elastase, N-succinyl-tri-alanyl-p-nitroaniline (STANA) was purchased from the Peptide Institute Inc. (Osaka, Japan). Anti-human NEP (neutral endopeptidase), anti-human CD 10 mouse antibody, and SN5c were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse IgG; PP54 was purchased from Chemicon (Tokyo, Japan). Rabbit anti-human MMP-1 (collagenase type 1) rabbit antibody was purchased from Sigma (St. Louis, MO, USA). Horseradish peroxidase-conjugated goat polyclonal anti-mouse IgG was obtained from Transduction Laboratories (NJ, USA), DAB substrate was obtained from Funakoshi (Tokyo, Japan), and ELISA Kits (for IL-1α, IL-1β, IL-6, IL-8, GM-CSF, and ET-1) were obtained from Endogen (Thermo Fisher Scientific, Yokohama, Japan). Other chemicals including AX, of reagent grade, were purchased from Sigma.

### 2.2. Cell cultures

Human dermal fibroblasts derived from human foreskins (Cell Applications, Inc., San Diego, CA, USA), were cultivated in Eagle's medium (EMEM) with 10% fetal calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B at 37 °C in a 95% air, 5% $CO_2$ atmosphere.

### 2.3. Cell viability assay

Cell viability assay was performed using Cell Proliferation Kit II (XTT) (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacture's instruction.

### 2.4. Extraction of enzyme

Cultured fibroblasts at 80% confluence were washed once with phosphate-buffered saline (PBS), scraped into PBS, and centrifuged at 4 °C, 1000 rpm for 5 min. The cell pellets were lysed with 0.1 M Tris–HCl (pH 7.6) buffer containing 0.1% Triton-X 100 and 1 mM PMSF, followed by ultrasonication for 5 min on ice. Cleared supernatants after the removal of cell residues by centrifugation (2000 rpm, 10 min) were used as the fibroblast enzyme solution.

### 2.5. UVA irradiation

Human dermal fibroblasts were washed twice with PBS. The UVA source was a FL20S/BLB fluorescent lamp (Clinical Supply, Tokyo, Japan) that emitted an energy spectrum with high fluency in the UVA region (300–430 nm), with a peak at 352 nm. A 6-mm thick glass plate was used to block UVB emissions. The emitted dose was calculated using a UVA radiometer photodetector (Torex, Tokyo, Japan). The cells were washed with PBS, then resuspended in PBS, and subjected to UVA irradiation. The duration of UV irradiation delivered to cells was altered by sliding a plastic lid covered with aluminum foil onto a flat-bottomed plate. After irradiation, the cells were cultured in EMEM without FCS at 37 °C. Control samples were mock irradiated and maintained under the same culture conditions as those used for the UVA-irradiated specimens.

### 2.6. MMP-1 activity assay

For MMP-1 activity analyses, fibroblast culture media were concentrated approximately 10-fold with a Centricon at 4 °C and 80 μl of each concentrate was incubated for 15 min at 37 °C with trypsin solution (0.05 mg/ml) to activate MMP-1. After the addition of 10 μl trypsin inhibitor solution (0.25 mg/ml) to inactivate the trypsin, the concentrated media were subjected to the measurement of MMP-1 activity using a type I collagenase assay kit (Code No. AK37; Primary Cell Co., LTD) which measures the MMP-1-mediated cleavage of fluorescently labeled collagen type I, according to the manufacturer's instruction.

### 2.7. Measurement of elastase activity

Elastase activity using the synthetic substrate STANA was measured as previously described by Nakagawa et al. [8]. In brief, 100 μl of enzyme solution was dispensed into 96-well plates, which were pre-incubated for 15 min at 37 °C. After addition of 2 μl 62.5 mM STANA, the plates were further incubated for 1 h at 37 °C. The release of p-nitroaniline was measured by absorbance at 405 nm and enzymatic activity is expressed as unit per mg protein, one unit representing the activity that releases 1 nmol of nitroaniline per hour.

### 2.8. Western blotting

For NEP determination, cell lysates were separated on 10% SDS-PAGE gels, and were blotted to PVDF membranes (Bio-Rad, Hercules, CA, USA). After blocking with 3% bovine serum

**Table 1**
Primer sequences used in this study.

| Primer | Sequence | Size | $T_m$ |
|---|---|---|---|
| GAPDH | Forward: 5′-GAAGGTGAAGGTCGGAGTCAACG-3′<br>Reverse: 5′-AGTCCTTCCACGATAACCAAAGTTG-3′ | 384 bp | 53 °C |
| Neutral endopeptidase (NEP) | Forward: 5′-GTCCTGCTCCTCACCATCATAGC-3′<br>Reverse: 5′-CGATCTTCAGGTTTAGCCGTAGC-3′ | 731 bp | 53 °C |
| Collagenase-I (MMP-1) | Forward: 5′-GCTGGGAGCAAACACATCTGAGGT-3′<br>Reverse: 5′-TGAGCCGCAACACGATGTAAGTTG-3′ | 311 bp | 53 °C |

albumin-containing Tris/HCl (pH 7.5, 100 mM NaCl), the membranes were treated with mouse monoclonal anti-human NEP antibody at room temperature for 1 h then with anti-mouse IgG conjugated to horseradish peroxidase, and finally with Amersham ECL reagents and were exposed to X-ray film for specified times to detect bands. For MMP-1 determination, fibroblast media were concentrated in Centricon™ centrifugal filter devices (Mr cutoff 30,000) for 35 min at 5000 × *g* at 4 °C. Volume equivalents were assayed for MMP-1 by SDS-PAGE and immunoblotting with the anti-MMP-1 antibody. In each case, immunoblots were incubated with the horseradish peroxidase-conjugated anti-rabbit antibody, then treated with Amersham ECL reagents and exposed to X-ray film for specified times to detect bands.

### 2.9. Real-time RT-PCR analysis

After UVA exposure with or without subsequent AX treatment, the expression of MMP-1 and NEP in cultured human dermal fibroblasts was examined using real-time RT-PCR and was normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Total RNAs from treated cells were isolated using an RNeasy Mini Kit (Qiagen, CA, USA). cDNAs were then synthesized using a Rever Tra Ace qPCR RT Kit (Toyobo, Tokyo, Japan) by reverse transcription of 1 μg total RNA using oligo dT and Moloney murine leukemia virus reverse transcriptase. The sets of oligonucleotide primers used are shown in Table 1. Real-time RT-PCR with SYBR Green was performed using Power SYBR Green PCR (Applied Biosystem Japan, Tokyo, Japan) in a DNA engine Opticon Real-Time PCR detection system (MJ Japan Ltd., Tokyo, Japan). Briefly, each 25 μl reaction volume contained cDNA templates, primer pairs and 2× Master mix. Amplification in the DNA engine Opticon after initial denaturation at 94 °C for 15 min was performed for 40 cycles at 94 °C for 15 s, 53 °C for 30 s and 74 °C for 1 min.

### 2.10. Statistics

Data for statistical analysis are presented as means ± SD of at least three separate experiments. Comparisons between groups were analyzed by Student's *t*-test. *P* values of 0.05 or less are considered statistically significant.

## 3. Results

### 3.1. Effects of UVA exposure on cell viability

When cultured human fibroblasts were exposed to UVA radiation at doses of 5 or 10 J/cm$^2$, there was no significant change in cell viability by 24 h post-irradiation compared with non-radiation (Fig. 1).

### 3.2. Effects of UVA exposure on gene expression of NEP and MMP-1

When cultured human fibroblasts were exposed to UVA radiation at doses of 5 or 10 J/cm$^2$, levels of mRNAs encoding NEP were significantly enhanced at 12 h post-irradiation at both UVA doses and at 24 h with the UVA dose of 5 J/cm$^2$ compared with the mock irradiation control (Fig. 2A). Similarly, the MMP-1 mRNA levels were significantly increased at 6, 9, 12 and 24 h post-irradiation at a UVA dose of 10 J/cm$^2$ (Fig. 2B).

### 3.3. Effects of AX on the UVA-induced increase of gene expression of NEP and MMP-1

When AX was added at a concentration of 1, 4 or 8 μM immediately after UVA irradiation at 10 J/cm$^2$, the increased gene expression of NEP at 12 h post-irradiation was significantly reduced to the non-irradiated control level only at 4 μM AX (Fig. 3A). In contrast, the addition of AX at a concentration of 8 μM immediately after the mock irradiation did not affect the gene expression of NEP at 12 h post-mock irradiation. The UVA-induced increase in gene expression of MMP-1 at 6 h post-irradiation was significantly reduced to the non-irradiated control level at 4 and at 8 μM AX (Fig. 3B). In contrast, the addition of AX at a concentration of 8 μM immediately after the mock irradiation did not affect the gene expression of MMP-1 at 6 h post-mock irradiation.

### 3.4. Effects of AX on protein expression of NEP and MMP-1

When cultured human fibroblasts were exposed to UVA at a dose of 10 J/cm$^2$, levels of NEP protein in the cell lysates were slightly but not significantly increased at 72 h post-irradiation (Fig. 4A). When AX was added at a concentration of 1, 4 or 8 μM immediately after the UVA exposure, the slightly increased protein level of NEP at 72 h post-irradiation was slightly but not significantly decreased to the non-irradiated control level at all concentrations of AX (Fig. 4A). In contrast, the addition of AX at a concentration of 8 μM immediately after the mock irradiation did not affect the protein expression of NEP at 72 h post-mock irradiation. When cultured human fibroblasts were exposed to UVA at a dose of 10 J/cm$^2$, levels of MMP-1

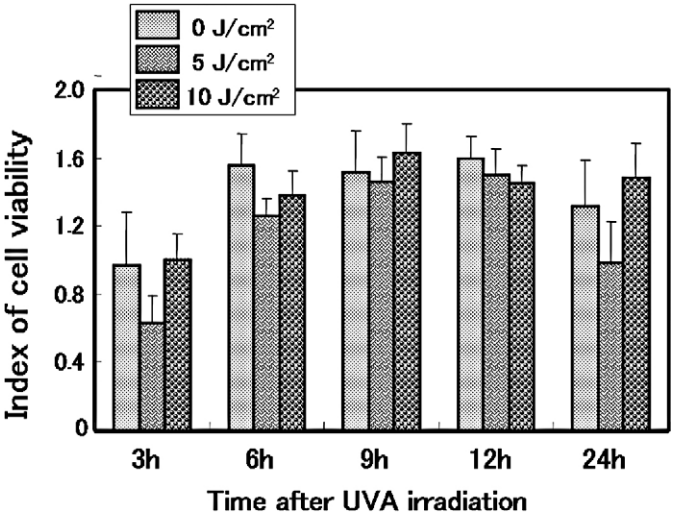


**Fig. 1.** Effects of UVA exposure on cell viability.

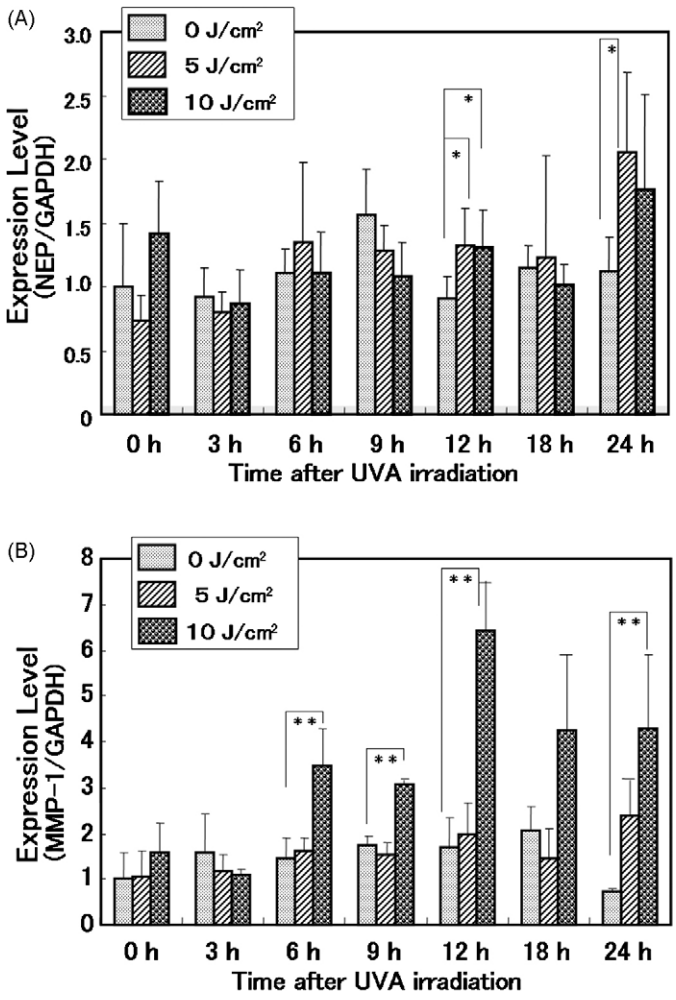


**Fig. 2.** (A) Effects of UVA radiation on levels of NEP mRNAs in cultured human dermal fibroblasts as revealed by real-time RT-PCR analysis. $^{*}p < 0.05$. (B) Effects of UVA radiation on levels of MMP-1 mRNAs in cultured human dermal fibroblasts as revealed by real-time RT-PCR analysis. $^{**}p < 0.01$.

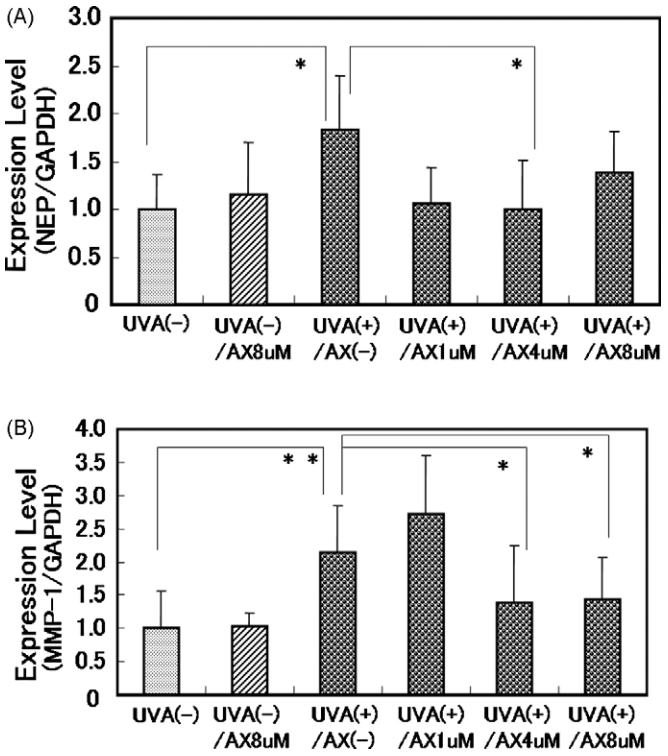


**Fig. 3.** (A) Effects of AX on NEP gene expression in UVA-exposed human fibroblasts; Levels of NEP mRNAs were assessed by real-time RT-PCR at 12 h post-irradiation. $^{*}p < 0.05$. (B) Effects of AX on MMP-1 gene expression in UVA-exposed human fibroblasts; Levels of MMP-1 mRNAs were assessed by real-time RT-PCR at 6 h post-irradiation. $^{*}p < 0.05$, $^{**}p < 0.01$.

protein expression in the conditioned medium significantly increased at 72 h post-irradiation (Fig. 4B). When AX was added at a concentration of 1, 4 or 8 μM immediately after the UVA exposure, the significantly increased expression of MMP-1 protein at 72 h post-irradiation was significantly decreased to the non-irradiated control level at 4 and at 8 μM AX (Fig. 4B). In contrast, the addition of AX at a concentration of 8 μM immediately after the mock irradiation did not affect the protein expression of MMP-1 at 72 h post-mock irradiation.

### 3.5. Effects of AX on enzymatic activity of NEP and MMP-1

While UVA at a dose of 10 J/cm$^2$ induced a significant increase in elastase activity, the addition of AX immediately after the UVA exposure slightly but not significantly diminished that increase in elastase activity (Fig. 5A). UVA irradiation at a dose of 10 J/cm$^2$ induced a significant increase of MMP-1 activity, while the addition of AX immediately after UVA exposure significantly abolished that increase in MMP-1 activity (Fig. 5B).

### 3.6. Secretion of cytokines by UVA-exposed fibroblasts and the effects of AX

When cultured human fibroblasts were exposed to UVA at a dose of 10 J/cm$^2$ and cytokines secreted into the conditioned medium were measured, only IL-6 was significantly increased at 48 h post-irradiation (Table 2). IL-1α, IL-1β, GM-CSF, TNFα and ET-1 were not affected,. The addition of AX immediately after UVA exposure significantly abolished the release of IL-6 only at 8 μM AX (Table 2). In contrast, the addition of AX at a concentration of 8 μM immediately after the mock irradiation did not affect the secretion levels of all cytokines tested except for IL-1β, the level of which was slightly decreased at 48 h post-mock irradiation.

## 4. Discussion

In this study, we addressed the question as to whether the addition of an antioxidant immediately following exposure of human skin fibroblasts to UVA would modulate or interfere with the UVA initiated signal transduction cascades which lead to connective tissue degradation, a hallmark in skin photo-aging. For this purpose, we used AX immediately after the UVA irradiation of cultured human dermal fibroblasts to examine effects on the expression and activity of MMP-1 and SFE/NEP, the most important matrix-metalloproteinases associated with the degradation of the extracellular matrix which leads to photo-aging. In this study, we demonstrate that the exposure of cultured human dermal fibroblasts to UVA at a dose of 10 J/cm$^2$ significantly stimulates the gene expression of MMP-1 at 6–24 h post-irradiation without cytotoxicity. The increase in MMP-1 mRNA expression is followed by a significantly elevated level of MMP-1 protein and activity at 72 h post-irradiation. Those results are consistent with our previous in vivo studies on the UVA induction of MMP-1 [17] in which skin sagging is frequently elicited by repetitive exposure of hairless mouse skin to UVA, accompanied by enhanced MMP-1 activity in the dermis and is also in agreement with other in vitro UVA effects on MMP-1 in cultured human fibroblasts [33]. On the other hand, we show for the first time in

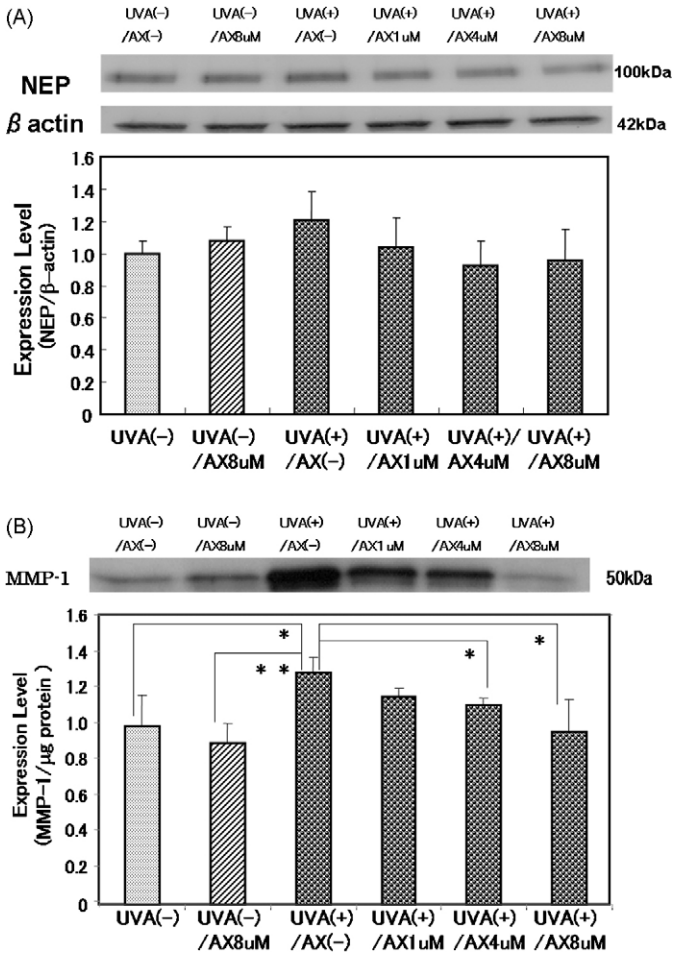


**Fig. 4.** (A) Effects of AX on NEP protein levels in UVA-exposed human fibroblasts assessed by Western blotting analysis at 72 h post-irradiation. (B) Effects of AX on MMP-1 protein levels in UVA-exposed human fibroblasts assessed by Western blotting analysis at 72 h post-irradiation. $^{*}p < 0.05$; $^{**}p < 0.01$.

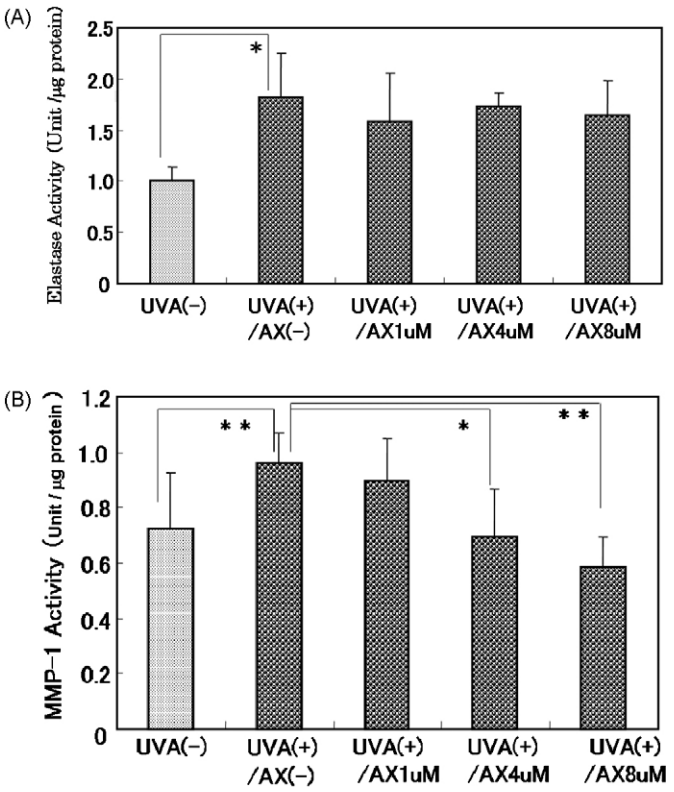


**Fig. 5.** (A) Effects of AX on SFE enzymatic activity in UVA-exposed human fibroblasts assessed by the enzymatic method described in Section 2 at 72 h post-irradiation. $^{*}p < 0.05$. (B) Effects of AX on MMP-1 activity in UVA-exposed human fibroblasts assessed by ELISA at 48 h post-irradiation. $^{*}p < 0.05$, $^{**}p < 0.01$.

this study that the exposure of cultured human dermal fibroblasts to UVA at a dose of 10 J/cm$^2$ significantly stimulates the gene expression of NEP at 12 h post-irradiation at UVA doses of 5 or 10 J/cm$^2$ as well as at 24 h with 5 J/cm$^2$ without cytotoxicity. The increase in NEP mRNA expression is followed by a slightly elevated level of NEP protein and SFE activity at 72 h post-irradiation. Those observed patterns for the UVA induction of MMP-1 and SFE/NEP are slightly different from our previous in vivo study [17] on the activities of MMP-1 and SFE in the dermis of repeatedly UVA-exposed hairless mice. While there was an induction of a marked sagging of the skin in those mice rather than wrinkling, both collagenase I and elastase activities were only slightly up-regulated (no statistically significant difference) in the UVA-exposed mice compared with the unexposed control mice after 14 weeks of daily UVA radiation. This is probably due to differences between in vivo and in vitro experiments with different numbers of UVA exposures. However, the more highly stimulated level of MMP-1 expression compared with SFE/NEP following a single UVA exposure in this study may reflect features in an early phase of photo-damage and suggests a preferential role of MMP-1 in UVA-photo-aging in which skin sagging is more frequently induced compared with wrinkling.

Since ROS have been identified as early events in the signaling sequence which eventually leads to the induction of MMP-1 after UVA exposure [20] and since AX is a well-known scavenger for singlet oxygen which interferes with the downstream signaling cascade highly accentuated in a redox imbalanced fashion [27], we examined the effects of AX on the UVA induction of MMP-1 and SFE/NEP in human dermal fibroblasts. We found that when added immediately after UVA exposure, AX significantly abrogates the UVA induction of MMP-1 and also has a weak inhibitory effect on

**Table 2**
Secretion levels of cytokines (pg/ml) in the medium of cultured skin fibroblast 72 h after UVA irradiation with 10 J/cm$^2$.

| Cytokine | UVA (−) | UVA (−) AX (8 μM) | UVA (+) AX (−) | UVA (+) AX (VM) | UVA (+) AX (4 μM) | UVA (+) AX (8 μM) |
|---|---|---|---|---|---|---|
| IL-a | 1.29 ± 0.48 | 1.77 ± 0.52 | 1.95 ± 0.45 | 1.53 ± 0.37 | 2.49 ± 0.63 | 1.17 ± 0.45 |
| IL-p | 5.71 ± 4.20 | 0.98 ± 0.74 | 2.63 ± 0.77 | 2.00 ± 0.30 | 1.78 ± 0.26 | 1.83 ± 0.61 |
| TNF-a | 58.22 ± 5.53 | 61.08 ± 2.63 | 54.21 ± 7.52 | 58.22 ± 2.63 | 59.56 ± 0.88 | 34.73 ± 10.07 |
| IL-6 | 10.19 ± 0.55 | 10.60 ± 1.27 | 14.78 ± 1.21** | 10.98 ± 2.10 | 11.63 ± 2.50 | 10.24 ± 2.00* |
| IL-8 | 5.92 ± 1.25 | 7.81 ± 2.30 | 6.07 ± 1.89 | 5.05 ± 2.95 | 6.50 ± 5.97 | 3.03 ± 1.74 |
| GM-CSF | 6.54 ± 2.98 | 7.42 ± 1.19 | 5.27 ± 1.62 | 2.81 ± 1.35 | 9.88 ± 3.28 | 7.82 ± 4.12 |
| ET-1 | 0.74 ± 0.06 | 0.89 ± 0.17 | 0.77 ± 0.06 | 1.45 ± 1.13 | 0.74 ± 0.17 | 0.89 ± 0.28 |

* $p < 0.05$ versus UVA (+)/AX (−).
** $p < 0.01$ versus UVA (−).

the UVA induction of SFE/NEP at the gene, protein and activity levels. Thus, the inhibitory effect of AX on the UVA induction occurs to a larger degree for MMP-1 than for SFE/NEP. This is probably due to the weaker stimulatory effect of UVA on SFE/NEP expression compared with MMP-1 expression.

As for the biological mechanism(s) underlying UVA-induced photo-aging, several hypotheses have been proposed for the elevated levels of MMP-1 expression in dermal fibroblasts as follows: (1) ROS generated by UVA radiation could trigger membrane lipids to induce a chain radical reaction, resulting in activation of the stress-related signaling pathway which leads to the stimulation of MMP-1 expression [34]. (2) The ROS could directly induce the release of primary inflammatory cytokines such as IL-1α and IL-1β which would act as autocrine cytokines that cause fibroblasts to produce and secrete several secondary cytokines (such as IL-6) which in turn could stimulates fibroblasts to up-regulate MMP-1 expression [19]. (3) Macrophage migration inhibitory factor (MIF), which is stimulated by IL-1α in an autocrine fashion, could trigger MMP-1 expression via signaling pathways including PKC, MAPK and AP-1 [35,36]. On the other hand, signaling mechanisms involved in the UVA induction of SFE/NEP (although to a lesser extent than MMP-1) remain to be elucidated due to the lack of enzymatic and genetic information about SFE. Since SFE/NEP also belongs to a family of matrix-metalloproteases, the most probable interpretation for the biochemical mechanisms involved in the observed inhibition by AX for both matrix-metalloproteases may be that the UVA-inducible ROS-directed lipid peroxidation chain reaction is interrupted by AX via its scavenger effect. However, at least pre-treatment with AX in UVA-exposed dermal fibroblasts is required to efficiently deplete UVA-generated ROS with short half-lives. It is easily predicted that post-treatment with AX immediately after UVA exposure is not adequate to efficiently deplete the UVA-generated ROS. Alternately, because of the well-known stimulatory effect of IL-6 on MMP-1 expression [19] and on SFE/NEP expression (unpublished data), it is also possible that the UVA-induced secretion of IL-6 is suppressed to some extent by AX, which would result in the down-regulation of MMP-1 and SFE/NEP expression via the IL-6-directed downstream signaling cascade. Since the observed inhibitory effect on IL-6 secretion was induced only at an AX concentration greater than 8 μM, in contrast to the down-regulation of MMP-1 or SFE/NEP expression which was seen even at an AX concentration of 4 μM, the interpretation concerning the inhibition of IL-6 secretion seems unlikely. In connection with the interruptive effect of AX on the intracellular signaling cascade mainly including protein kinases leading to MMP-1 expression, we have recently found that AX at concentrations ranging from 1 to 8 μM can markedly inhibit the phosphorylation of ERK or cAMP response element-binding protein (CREB) as well as microphthalmia associated transcription factor (MITF), a downstream target of ERK, in the stem cell factor-stimulated signaling cascade in non-UV exposed human melanoma cell lines (submitted). Since those phosphorylations are mediated by the kinase activities of MEK for ERK or protein kinase A for CREB and MAPK for MITF [37], the inhibitory effect of AX on those phosphorylations may be accounted for by a possible suppressive effect on those kinase activities. A similar selective inhibition of the activation of MAP kinases by antioxidants has been reported in the LPS induction of tissue factor [38]. Recent available evidence indicates that serine/threonine and tyrosine phosphatases can be regulated by altering the oxidation state of active-site functional groups, such as an active-site Fe ion in serine/threonine phosphatases or a cysteine residue in tyrosine phosphatases [39–41]. Thus, the inhibited phosphorylation by AX may be mediated through an increased dephosphorylation by MAP kinase phosphatases of cysteine residues at enzymatic active sites which are highly responsive to redox imbalance [41] although the precise mechanism(s) underlying the interruption of phosphorylation remains to be clarified.

The major finding of this study is that post-treatment with AX, which has a potent capacity to scavenge singlet oxygen, results in the complete abrogation of UVA-enhanced mRNA levels of MMP-1, accompanied by the specific down-regulation of increased protein expression and activity of MMP-1. Further, AX has a weak inhibitory effect on UVA-enhanced levels of NEP mRNA in concert with the specific down-regulation of increased protein expression and activity of SFE/NEP. This is of particular relevance for understanding UVA-associated photo-aging in which an AX-sensitive imbalance or loss of intracellular redox homeostasis is closely involved in the up-regulation of metalloprotease activity via MAP kinase signaling. Our results showing that AX can interfere with UVA-induced MMP-1 and SFE/NEP expression at concentrations as low as 4 μM suggest that topical or oral administration of AX might prevent UVA-associated photo-aging such as skin sagging or wrinkling.

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