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An *in vitro* Model for Fibroblast Photoaging Comparing Single and Repeated UVA Irradiations

Wongnapa Nakyai[1], Aurasorn Saraphanchotiwitthaya[1], Céline Viennet[2], Philippe Humbert*[2,3] and Jarupa Viyoch*[1]

[1]Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences and Center of Excellence for Innovation in Chemistry, Naresuan University, Phitsanulok, Thailand
[2]Engineering and Cutaneous Biology Laboratory, INSERM UMR 1098, University of Franche-Comte, Besancon, France
[3]Department of Dermatology, University Hospital, Besancon, France

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ABSTRACT

The current method for efficient evaluation of antiphotoaging compounds is an *in vitro* skin culture model using a single ultraviolet A (UVA) irradiation of fibroblasts. However, skin photoaging is caused by repeated exposure to UVA radiation. The objective of this study was to develop an appropriate model for *in vitro* skin photoaging by comparing the different effects of single (5 J cm^{-2}) and repeated exposures (5 J cm^{-2} × 3 times) of fibroblasts to UVA irradiation. Our results demonstrated that a single and repeated exposure to UVA irradiation had different effects on fibroblasts. In the single UVA-irradiated group, collagen lattice contraction and the protein levels of type I procollagen and matrix metalloproteinase-1 (MMP-1) increased, while the levels of fibronectin and alpha-smooth muscle actin (α-SMA) were unchanged, compared to levels in the non-UVA-irradiated group (control). In contrast, repeated UVA exposure significantly induced G0/G1 cell cycle arrest, reduced collagen lattice contraction and type I procollagen and fibronectin expression, and increased MMP-1 expression. There was no difference in α-SMA expression when comparing repeatedly irradiated and non-UVA-irradiated fibroblasts. Our findings clearly indicate that repeated UVA irradiation of cells induces malfunctions found in photoaged skin and is an appropriate *in vitro* skin model of photoaging.

INTRODUCTION

Cumulative ultraviolet A (UVA) exposure can cause skin photoaging from the biomolecular to the cellular level (1–3). Many studies have found that chronic UVA irradiation damages the skin structure by decreasing collagen synthesis (4,5) and stimulating the expression of matrix metalloproteinase enzymes (MMPs) (6–8). Moreover, collagen fibers and other extracellular matrices (ECMs) such as fibronectin, elastin and laminin are destroyed (9).

Fibronectin is a major cell surface glycoprotein of fibroblast cells that plays a role in cell–cell and cell-substratum adhesion, while maintaining cell morphology and motility (10). Fibronectin mediates cell adhesion primarily through heterodimeric integrin receptors and connects cells to collagen fibers (11,12). Previous studies have found that UV irradiation of mouse skin, human skin and *in vitro* fibroblast cultures stimulated fibronectin production (5,13–15). However, *in vitro* experiments using cultured human fibroblasts have shown that exposure to UVA irradiation does not change fibronectin expression (16), and other studies have found that UV suppresses the expression of fibronectin (17,18). These studies present conflicting results that require further clarification. Of further interest, we were unable to find any research reports on the expression of α-smooth muscle actin (α-SMA) in photoaged fibroblasts. α-SMA, a cytoskeletal protein, regulates the mechanical activities of fibroblasts and their production of collagen and other EMCs (19,20).

Although prior studies have clarified the processes of skin photoaging using *in vitro* models (6,7,21–24), almost all such studies used a single dose of UVA irradiation. However, skin photoaging is caused by repeated exposure to UV radiation. It remains unknown whether a single dose (5 J cm^{-2}) or a repeated dose (5 J cm^{-2} × 3 times) of UVA irradiation causes differing effects on the function of skin fibroblasts. In this study, we observed different effects on collagen organization and the expression of type I procollagen, MMP-1, fibronectin and α-SMA in fibroblasts exposed to single or repeated doses of UVA irradiation. The fibroblasts subjected to repeated UVA irradiation exhibited alterations in collagen organization and in type I collagen, MMP-1 and fibronectin expressions. These alterations are similar to the skin aging phenomena, which occurs in photoaged skin.

MATERIALS AND METHODS

Cell isolation and culture. Normal human dermal fibroblasts were isolated from skin tissues from five donors aged 47–62 years undergoing facial cosmetic surgery at Naresuan University Hospital. The study adhered to the protocol approved by the Naresuan University IRB, No. 242/57. Tissues were digested with dispase solution (5 mg ml^{-1}) at 4°C. After 16 h of incubation, the epidermal layer was removed, and the dermis was cut into small pieces (~2 mm^3). Dermal explants were allowed to adhere to 25-cm^2 culture flasks for 30 min at 37°C in 5% CO_2/95% air before addition of DMEM complete medium (Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin solution and 0.4% Amphotericin solution (all from Invitrogen, New York, USA)). After 2 weeks of culture, fibroblasts

*Corresponding authors' e-mails: philippe.humbert@univ-fcomte.fr (Philippe Humbert), jarupav@nu.ac.th or jarupaviyoch4@yahoo.com (Jarupa Viyoch)

migrated out of the explants and proliferated. They were routinely subcultured by trypsinization. To obtain sufficient cells for all experiments, fibroblasts were repassaged 4–9 times.

UVA irradiation. Fibroblasts were seeded at a density of 3×10^4 cells cm^{-2} and were incubated in DMEM complete medium for 24 h followed by 24 h in serum-free DMEM, after which the cells were washed twice with phosphate-buffered saline (PBS) and covered with a thin layer of PBS. UVA irradiation was applied either as (i) a single dose of 3, 5, 9 or 15 J cm^{-2}, or (ii) as three repeated doses of 5 J cm^{-2} (5 J cm^{-2} × 3 times) with each exposure separated by a 48-h interval. UVA irradiation was performed using an F-lamp UVASPOT 1000 with a 315–400 nm H-1 bandpass filter (Dr. Hönle, UV Technology, Germany). UVA radiation was uniformly exposed to samples at a distance of 30 cm, and the intensity of UVA was measured at the level of the culture plates by a UVA meter (Dr. Hönle, UV Technology, Germany). Cells were immersed in prewarmed PBS (37°C) during each irradiation period, and the temperature during irradiation (108 s for 3 J cm^{-2}, 180 s for 5 J cm^{-2}, 324 s for 9 J cm^{-2} and 540 s for 15 J cm^{-2} doses) was maintained at 37°C. After irradiation, the cells were placed in fresh serum-free medium. Cells and supernatants were collected 24 h after the first and third irradiation for analyzes. The collection time was based on our previous study indicating the maximal change in biomolecules secreted and/or produced by fibroblasts occurred 24 h after UVA exposure (25). Two control cell groups followed the same single or triple exposure protocols but without irradiation. The protocol for cell irradiation is shown in Scheme 1.

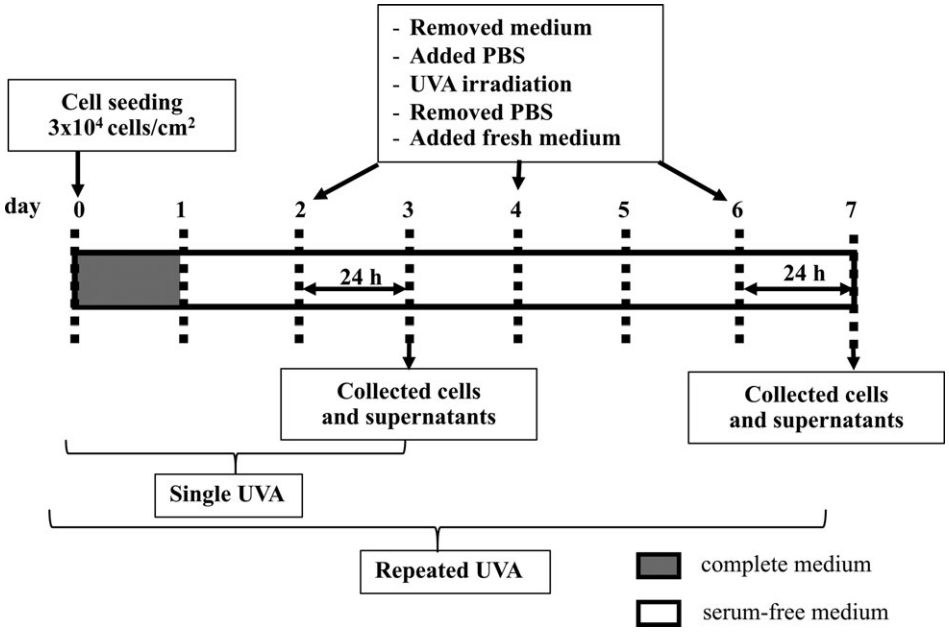
Cell viability. UVA irradiation was performed in 96-well plates (3×10^4 cells cm^{-2}). A total of 50 µl of XTT solution (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt, 0.2 mg ml^{-1}) with 200 µl of serum-free medium were added immediately or 24 h after the first or the third irradiation. Microtiter plates were incubated for 4 h at 37°C. Colorimetric changes were measured at 490 nm using a microplate reader (Ceres UV 900 HDI, Bio-Tek Instrument Inc, Vermont, USA). The absorbances of the controls (non-UVA irradiated) were taken as 100% viable cells.

Cell cycle analysis. Cells were trypsinized from 25-cm^2 flasks (3×10^4 cells cm^{-2}) 24 h after UVA irradiation, washed twice with PBS and then fixed in ice-cold 70% ethanol. They were washed twice with PBS, stained with 200 µl Muse cell cycle reagent (Muse cell cycle kit, EMD Millipore Co., MA, USA) and incubated at room temperature for 30 min in the dark. The cell cycle distribution was analyzed using the Muse Cell Analyzer (EMD Millipore Co., California, USA).

Enzyme-linked immunosorbent assay (ELISA). Cell culture supernatants were collected from 25-cm^2 flasks (3×10^4 cells cm^{-2}) 24 h after the last UVA irradiation. MMP-1 and type I procollagen concentrations were quantified using a Human MMP-1 ELISA Kit (Ray Biotech, Inc. Georgia, USA) and a Human Procollagen type I C-peptide (PIP) ELISA Kit (Takara Bio, Inc., Shiga, Japan), respectively. The experiment was performed in triplicate.

Western blot analysis. Cells were lysed from 25-cm^2 flasks (3×10^4 cells cm^{-2}) at 24 h after the first and the third irradiation and stored at −80°C. Lysates were electrophoresed on 10% SDS-PAGE gels and transferred onto a polyvinylidene fluoride membrane (PVDF). Membranes were blocked with 5% skimmed milk powder for 2 h at room temperature, and then incubated for 1 h with primary anti-MMP-1 monoclonal (1:1000 dilution, Millipore, Massachusetts, USA), anti-type I procollagen monoclonal (1:1000 dilution, M011, Takara Bio, Inc.), antifibronectin monoclonal (1:500 dilution, FN12-8, Takara Bio, Inc.) or β-actin monoclonal (A5441, Sigma-Aldrich, St. Louis, Missouri, USA) antibodies, followed by secondary antibodies (1:2000 dilution, goat anti-mouse-HRP, Millipore) for 1 h. Membranes were then incubated with the chemiluminescent HRP substrate Luminata Forte (Millipore), and bands were detected using the Bio-Rad ChemiDoc XRS+ system. Densitometric analysis was performed using the Bio-Rad Image Lab Software. The experiment was performed in triplicate.

Immunofluorescence staining. Cells were cultured on glass coverslips in 6-well plates (3×10^4 cells cm^{-2}). At 24 h after the first and the third irradiation, cell-bearing coverslips were washed twice with PBS, fixed in ice-cold methanol for 15 min at room temperature, washed again with PBS and permeabilized with 0.2% Triton-X in PBS for 10 min followed by PBS washing. Cells were then blocked with 0.1% Triton-X, 5% BSA in PBS for 30 min and incubated with rabbit anti-α-SMA polyclonal (1:50 dilution, ab5694, Abcam plc., Cambridge, UK) or mouse antifibronectin monoclonal (1:100 dilution, ab6328, Abcam plc., Cambridge, UK) antibodies at 4°C overnight in a humidified chamber. Cells were washed twice with PBS, and then incubated in the dark at room temperature for 1 h with goat anti-mouse polyclonal (1:500 dilution, FITC, ab6785, Abcam) or goat anti-rabbit polyclonal (1:500 dilution, Cy5, ab6564, Abcam) antibodies. After washing twice with PBS, nuclei were stained with DAPI solution for 10 min at room temperature in the dark, rinsed twice with PBS, and finally the coverslips were coated with Fluoromount (Diagnostic BioSystems, California, USA). Cells were visualized under a fluorescence microscope (Axio Observer Z1, Carl Zeiss Microscopy Ltd., Cambridge, UK).



Scheme 1. Representative diagram of the study protocol.

Measurement of contraction generated by fibroblasts embedded in collagen lattices. Cells were pre-irradiated in 25-cm^2 flasks (3×10^4 cells cm^{-2}) with single or triple UVA exposures. Collagen gels were prepared as described previously (26) using rat tail type I collagen (Gibco, Invitrogen) dispersed in DMEM/10% FBS (pH adjusted to 7.4 with NaOH/$NaCO_3$) containing pre-irradiated fibroblasts (8×10^5 cells ml^{-1}). These mixed solutions were placed into 60 mm culture dishes and then incubated at 37°C. Collagen lattices were imaged with a document scanner every day for 1 week, and gel areas were calculated. Culture medium was changed every 2 days with fresh serum-free medium. The experiment was performed in triplicate.

Statistical analysis. For each condition, all quantitative data are expressed as the mean ± standard deviation (SD). Student's unpaired *t*-test was used to compare groups, and $P < 0.05$ was considered significant. For the collagen lattice experiment, data were analyzed using one-way ANOVA with post hoc Tukey HSD.

RESULTS

Viability and cell cycle analysis of fibroblasts singly or repeatedly exposed to UVA

First, to clarify the effects of UVA intensity on cell growth and function, cultured fibroblasts were singly exposed to UVA at doses of 3, 5, 9 and 15 J cm^{-2}. The viability of UVA-irradiated cells was determined 24 h after irradiation. A large and significant decrease in cell viability was found in fibroblasts irradiated with UVA at doses of 9 and 15 J cm^{-2}, while UVA radiation at doses of 3 and 5 J cm^{-2} had no influence on the viability of the cells (Fig. 1a). The cells irradiated with UVA at 3 and 5 J cm^{-2} were further analyzed for cell cycle effects. As shown in Table 1, UVA radiation at 3 and 5 J cm^{-2} did not alter the cycle distribution of the fibroblasts.

Next, to compare the effects of single and repeat irradiation on the viability of fibroblasts, cells were exposed to a single 5 J cm^{-2} dose of UVA or exposed three times to 5 J cm^{-2} UVA and were then collected immediately or 24 h after the last UVA irradiation. As described above, the selected 5 J cm^{-2} dose did not cause a loss of cell viability either immediately or 24 h after exposure (Fig. 1b). For cell cycle analysis, as compared to a single dose, three repeated doses (5 J cm^{-2} × 3 times) of UVA irradiation induced a dramatic increase in the percentage of cells in the G0/G1 phase (85.7 ± 0.7 for a single dose; 92.3 ± 0.8 for repeat doses) with a decrease in the S phase (6.8 ± 0.4 for a single dose; 3.1 ± 0.6 for repeat doses) and G2/M phase (7.5 ± 0.5 for a single dose; 4.6 ± 0.4 for repeat doses). The cell cycle distribution in the singly irradiated group was indistinguishable from the distribution in the control group (Table 2).

MMP-1, type I procollagen and fibronectin produced by fibroblasts singly or repeatedly exposed to UVA

Although a single 3 or 5 J cm^{-2} UVA dose did not alter the viability or cell cycle of fibroblasts, the effect of these single UVA doses on cell function was further analyzed. We first focused on the effect of UVA on MMP-1 secretion because an increase in MMP-1 is usually found in photoaged fibroblasts (7,27). Using ELISA to analyze MMP-1 levels in medium collected 24 h after irradiation, we determined that a 3 J cm^{-2} UVA dose did not significantly affect the MMP-1 level, while a large and significant increase in MMP-1 was observed in media from fibroblasts irradiated with 5 J cm^{-2} of UVA (Fig. 2a).

To compare the effect of single and repeat doses on the production of MMP-1, type I procollagen and fibronectin, these protein levels in singly (5 J cm^{-2}) and repeatedly (5 J cm^{-2} × 3 times) UVA-irradiated fibroblasts were determined using ELISA and Western blotting and/or immunofluorescence techniques. As described above for ELISA, a single UVA irradiation dramatically increased the amount of MMP-1 secreted during the postradiation period of 24 h (Fig. 2b). Repeated doses also increased the MMP-1 level relative to the level in the non-UVA-irradiated group, but the increase was lower than that found for a single dose. The levels of intracellular MMP-1 characterized by Western blotting were in accordance with the secreted MMP-1 levels measured by ELISA (Fig. 2c).

Table 1. Cell cycle distribution of fibroblasts after 24 h of single UVA irradiation at 3 and 5 J cm^{-2}. Mean ± SD, $n = 3$.

	% of cell cycle phases		
Sample	G0/G1 (%)	S (%)	G2/M (%)
Control	85.2 ± 1.5	6.6 ± 0.4	8.2 ± 1.1
Single 3J/cm^2 UVA dose	84.5 ± 1.3	6.7 ± 0.1	8.8 ± 1.4
Single 5J/cm^2 UVA doses	84.9 ± 1.1	6.8 ± 0.1	8.3 ± 1.3

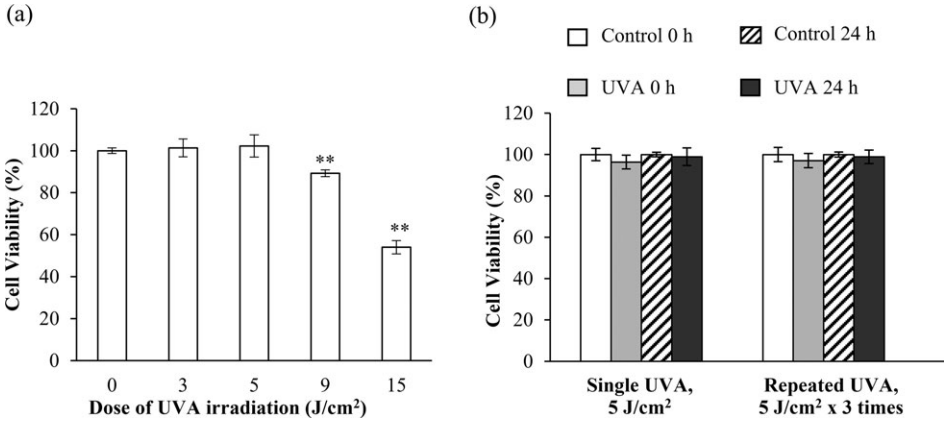


Figure 1. Effect of UVA irradiation on cell viability. (a) Cells were irradiated with the indicated single doses of UVA radiation, and then their viability was determined by the XTT assay at 24 h. (b) Cell viability of fibroblasts after a single (5 J cm^{-2}) or repeated doses of UVA (5 J cm^{-2} × 3 times) irradiation at 0 and 24 h determined by the XTT assay. The data represent the mean ± SD, $n = 3$, $^{**}P < 0.01$.

By Western blotting and ELISA, a single dose of UVA irradiation was found to increase intracellular and secreted forms of type I procollagen during the postradiation period of 24 h. Conversely, a repeated dose decreased the level of type I procollagen accumulated in fibroblasts and secreted into the extracellular medium (Fig. 3a,b).

A single dose of UVA irradiation did not significantly change the intracellular level of fibronectin analyzed by Western blotting (Fig. 4a). This result correlated with the immunofluorescence staining of fibronectin on the surface of fibroblasts, which was similar in the singly irradiated and control groups (Fig. 4b). Repeated UVA irradiation induced a decrease in fibronectin production, which was observed both by Western blotting and immunofluorescence staining (Fig. 4a,b).

Table 2. Cell cycle distribution of fibroblasts after 24 h of single or repeated low dose UVA irradiation. Mean ± SD, $n = 3$. $^{**}P < 0.01$ compared to controls (Student's *t*-test)

	% of cell cycle phases		
Sample	G0/G1 (%)	S (%)	G2/M (%)
Control	84.7 ± 0.6	6.8 ± 0.1	8.5 ± 0.6
Single UVA dose (5 J cm^{-2})	85.7 ± 0.7	6.8 ± 0.4	7.5 ± 0.5
Repeated UVA doses (5 J cm^{-2} × 3 times)	92.3 ± 0.8**	3.1 ± 0.6**	4.6 ± 0.4**

α-SMA expression in fibroblasts singly or repeatedly exposed to UVA

Because we detected a difference in extracellular matrix production between cells irradiated singly (5 J cm^{-2}) or repeatedly (5 J cm^{-2} × 3 times) with UVA, we further tested how these UV doses affected the expression of cytoskeletal α-SMA. UVA irradiation at the selected dose and number of exposures did not alter the expression of α-SMA in fibroblasts examined by immunostaining (Fig. 5). However, fibroblast cell morphology was changed after a single or repeated UVA irradiation. A single UVA (5 J cm^{-2}) exposure induced an enlarged cell shape, while repeated doses (5 J cm^{-2} × 3 times) of UVA irradiation resulted in a collapsed appearance with a small area of cytoplasm (Fig. 5).

The contraction of collagen lattices incorporating fibroblasts singly or repeatedly exposed to UVA

Fibroblasts pre-exposed to UVA were embedded in collagen gels, and their ability to contract the matrix was measured. Fibroblasts irradiated with a single dose (5 J cm^{-2}) of UVA consistently demonstrated greater contractility, while in contrast, repeated UVA (5 J cm^{-2} × 3 times) exposures were deleterious (Fig. 6a,b). The morphologies of UVA-irradiated cells cultured in the collagen lattices for 7 days were also observed. Singly irradiated cells had an enlarged and flattened cell shape, while

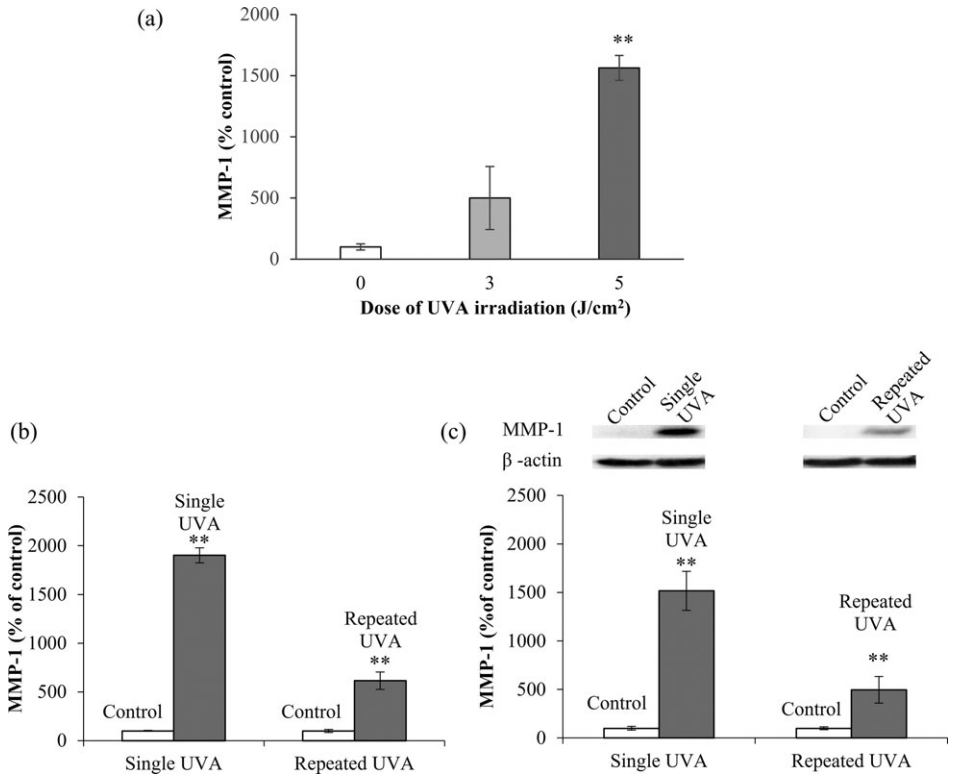


Figure 2. Effect of UVA on MMP-1 levels in human skin fibroblasts. (a) Cells were irradiated with a single 3 or 5 J cm^{-2} dose of UVA, culture media were collected 24 h after UVA exposure, and the MMP-1 protein levels were assessed by ELISA. (b and c) Levels of MMP-1 in media and whole cells 24 h after a single or repeated UVA irradiation. (b) MMP-1 levels in the culture medium were analyzed by ELISA. (c) MMP-1 levels were determined by Western blotting of whole cell lysates. Western blot analyzes were normalized using *β*-actin as a loading control. Data are expressed as the mean ± SD, $n = 3$, $^{**}P < 0.01$.

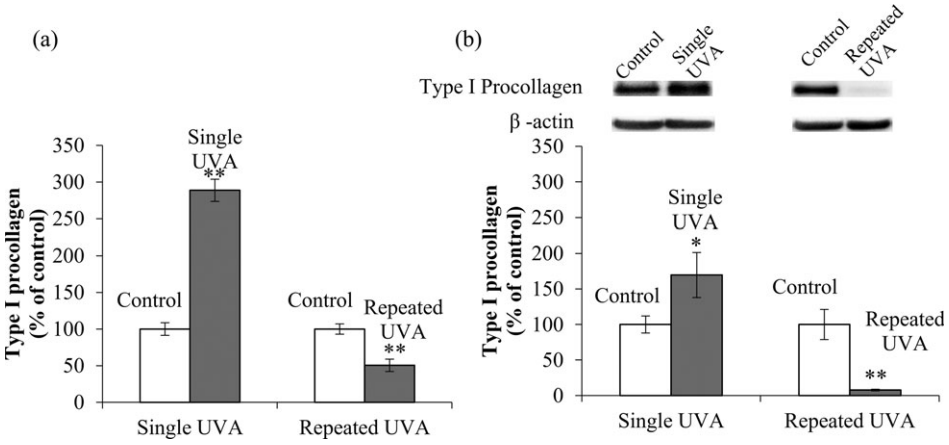


Figure 3. Levels of Type I procollagen in media and whole cells 24 h after a single or repeated UVA irradiation. (a) Type I procollagen levels in the culture medium were analyzed by ELISA. (b) Type I procollagen levels were determined by Western blotting of whole cell lysates. Western blot analyzes were normalized using β-actin as a loading control. Data are expressed as the mean $\pm$ SD, $n = 3$, $^*P < 0.05$ and $^{**}P < 0.01$.

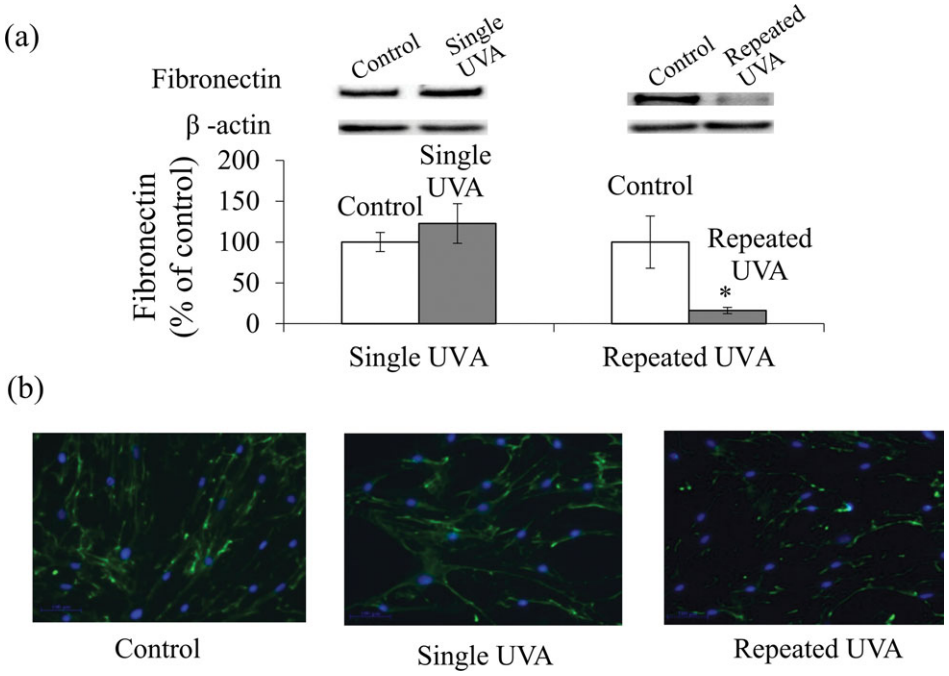


Figure 4. Fibronectin expression in fibroblasts 24 h after a single or repeated UVA irradiation. (a) Fibronectin was assessed by Western blotting and normalized to β-actin as a loading control. Data are expressed as the mean $\pm$ SD, $n = 3$, $^*P < 0.05$. (b) Fibronectin was assessed by immunofluorescence. The secondary antibody used for fibronectin was labeled by FITC (green), and nuclei were counterstained with DAPI (blue).

repeatedly irradiated cells had an elongated appearance with a small area of cytoplasm (Fig. 6c).

DISCUSSION

Many studies have reported that UV radiation alters the expressions of several biomarkers related to the skin aging phenomenon. The biomarkers produced by dermal fibroblasts include the MMP family (e.g. MMP-1, 2, 3 and 9), which breakdown collagen fibrils (1,7,27), ECMs (e.g. collagen I, III, IV, elastin, proteoglycans and laminins), which maintain fibroblast shape and function (28–31) and cytokines and growth factors, which influence cytoskeletal functions (32–34). In this study, we evaluated the different effects of single and repeat UVA irradiation on dermal fibroblasts and focused on cell viability and cell cycle, MMP-1, type I procollagen, fibronectin and α-SMA production, and the contractility of fibroblasts.

We first tested UVA dose of 3, 5, 9 and 15 J cm^{-2} for single irradiation. We found a large and significant decrease in viability of fibroblasts irradiated with 9 or 15 J cm^{-2} UVA, while there was no alteration of viability or cell cycle in fibroblasts irradiated with 3 or 5 J cm^{-2} UVA. Similarly, other studies have found no changes in cycling and/or viability of cells at low doses ($\leq$5 J cm^{-2}) of UVA exposure (35–37) and death of cells at high doses ($\geq$15 J cm^{-2}) of UVA exposure (38–40). However, the biological functions and morphology of fibroblasts were altered

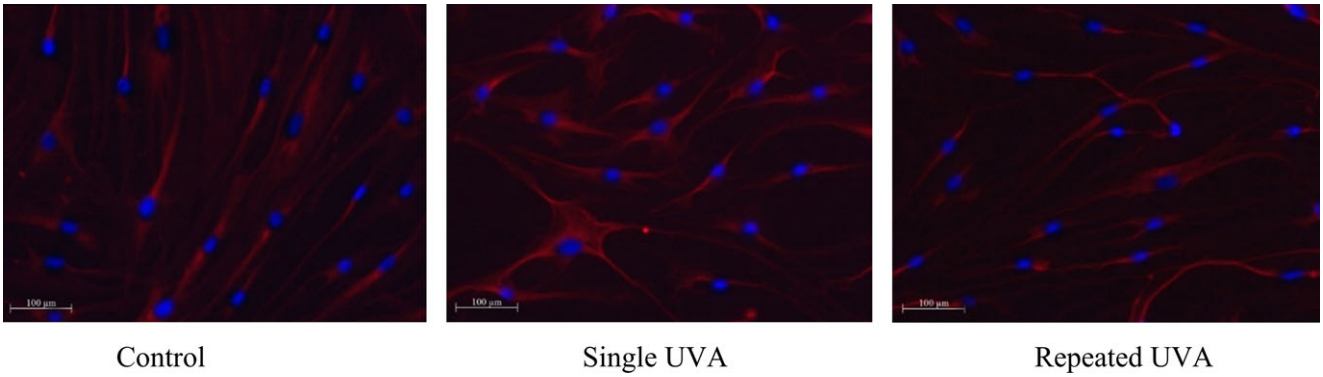


Figure 5. Immunofluorescence for α-SMA (Cy5, red) expressed 24 h after a single or repeated doses of UVA irradiation in fibroblasts cultured on coverslips. Nuclei were counterstained with DAPI (blue).

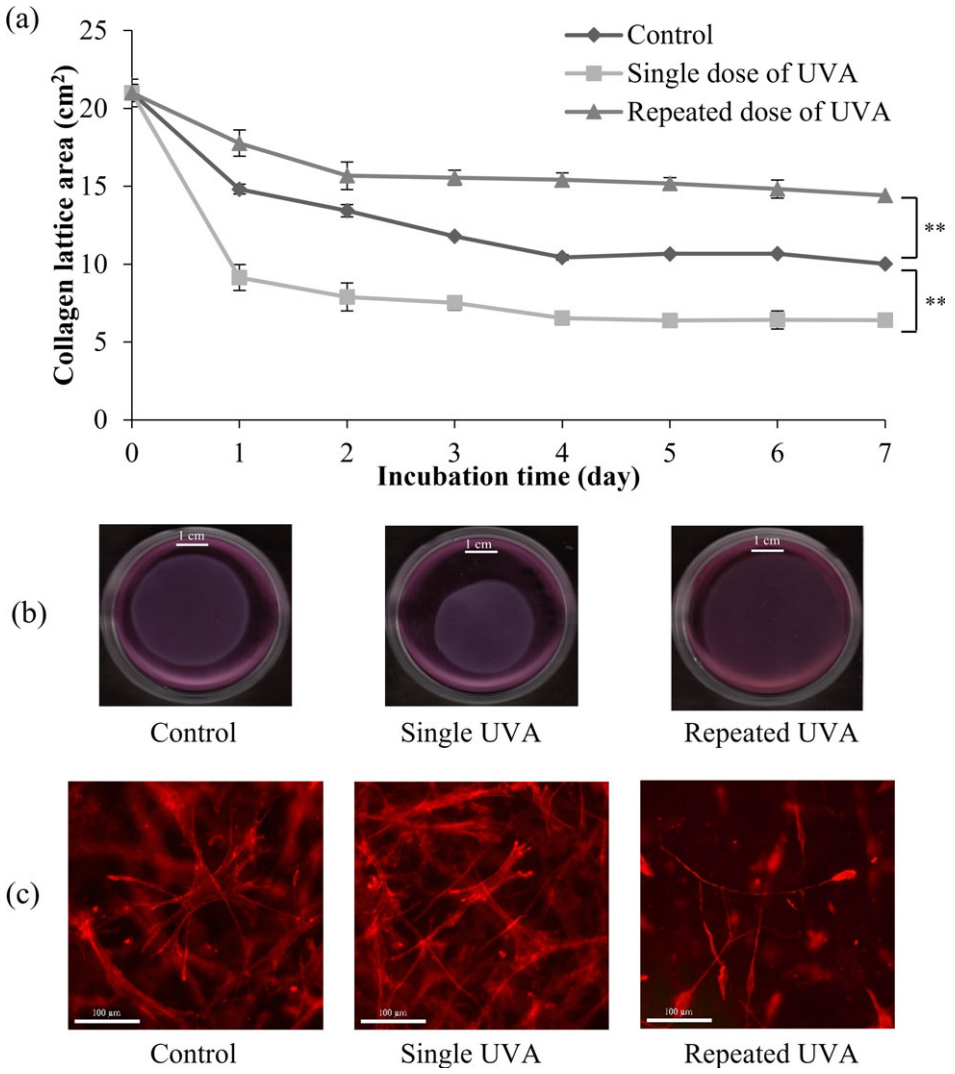


Figure 6. Contraction of collagen gels harboring fibroblasts pre-exposed to single or repeated UVA doses. (a) The area of each lattice was measured over seven days of culture. Each point represents the mean ± SD, $n = 3$, $^{**}P < 0.01$. (b) Photographs of collagen lattices at day 7. (c) Cell morphologies at day 7 in collagen lattices.

after a single or repeated 5 J cm^{-2} UVA exposure, and such alterations differed based on the pattern (single or repeat) of irradiation. In the present study, the viability and cycling of cells irradiated with a single UVA (5 J cm^{-2}) dose were compared with those of cells irradiated with repeated UVA (5 J cm^{-2} × 3 times) doses. We found that the viability of singly or repeatedly

UVA-exposed cells did not differ from the viability of control cells (non-UVA-irradiated group). However, repeated UVA irradiation induced cell cycle arrest in the G0/G1 phase, while single UVA irradiation did not alter the cell cycle distribution. Moreover, we found that repeated exposure resulted in cells with a collapsed appearance and a small area of cytoplasm. This cell shape coincides with the shape of fibroblasts found in severely photodamaged skin where the dermal fibroblasts lose their ability to contract and synthesize collagen (30,41). These results suggest that repeated doses of exposure induce irreversible damage with an alteration of cell function that might reflect cellular photoaging (1,3).

The photobiological effects of radiation on the synthesis of extracellular matrix proteins and their degradation are well characterized (2–5). Single ionizing radiation (IR, infrared radiation) can stimulate type I procollagen in human skin 24 h after exposure (42). Photodamaged dermis expresses elevated levels of MMPs and reduced production of type I collagen (43). Intriguingly, we found that a single exposure to UVA (5 J cm^{-2}) stimulates type I procollagen and MMP-1 expression. These results imply that this single UVA dose increases the capacity of fibroblasts to express MMP-1 and consequently disorganizes collagen fibrils but also increases the capacity of fibroblasts to synthesize new collagen to compensate for this effect. Repeated doses of UVA irradiation (5 J cm^{-2} × 3 times) reduced the level of type I procollagen production and increased MMP-1 expression. We hypothesize that the cumulative exposure to UVA at this dose alters the regulation of collagen homeostasis by fibroblasts. The multiply irradiated cells could not control the balance between collagen synthesis and degradation, consequently resulting in the degradation and/or disorganization of the ECMs. Therefore, the frequency of UV exposure is important for the collagen metabolic response.

Fibronectin is one of the major proteins involved in cell–cell or cell-ECM adhesion and in the contraction capacity of fibroblasts (44–46). Cell adhesion is not only dependent on the quantity but also on the quality of fibronectin. Aged fibroblasts produce defective fibronectin with an abnormal structure and function (47,48). Reports on fibronectin expression in skin photoaging are contradictory. Some studies demonstrated an increase in fibronectin expression in photoaging (5,13–15) or chronoaging models (49,50), while other studies reported that the suppression of fibronectin expression is responsible for skin photoaging (17,18). However, one study reported that UVA irradiation did not alter fibronectin production by fibroblasts (16). The present study expands on previous work by showing that a single UVA exposure did not change fibronectin expression, while repeated doses of UVA decreased fibronectin expression. The lower fibronectin expression in the repeatedly UVA-irradiated fibroblasts correlates well with the fibroblast dysfunctions observed in this work, including the reduction in contractility and irreversible damage to cell morphology.

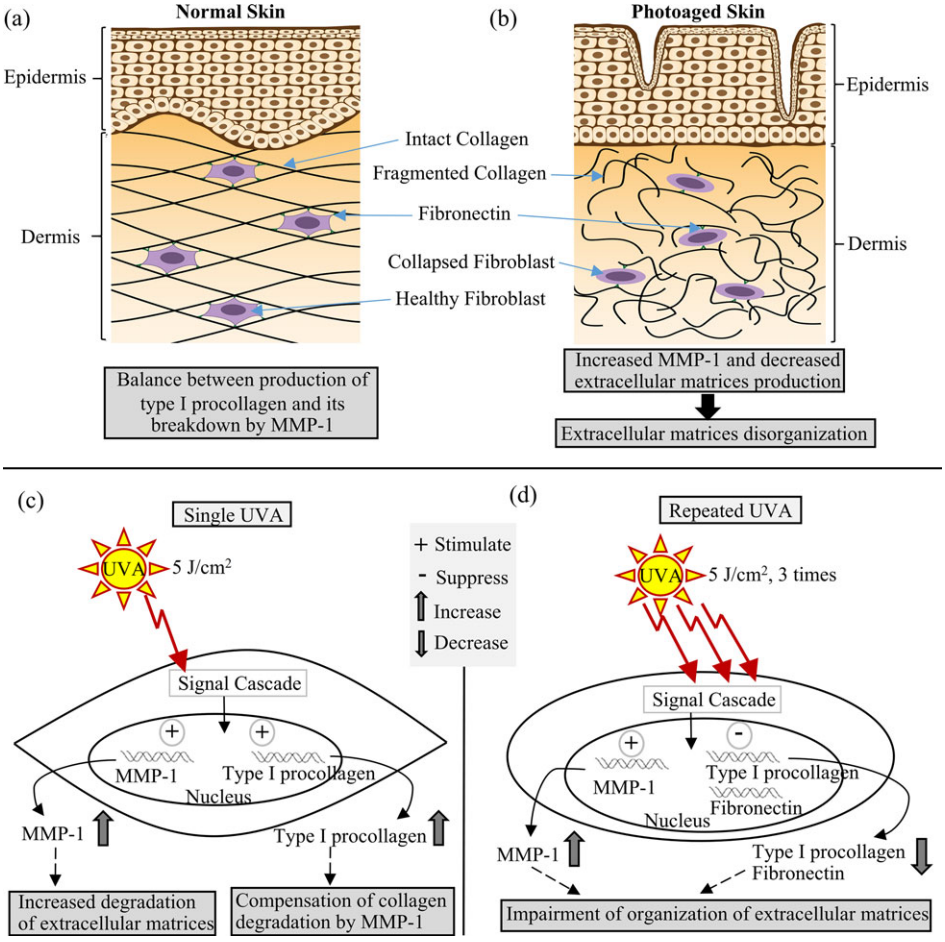


Figure 7. Illustration of (a) normal skin (b) photoaged skin, (c) the effects of single UVA exposure and (d) repeated UVA exposure to fibroblast.

In the present study, we also used the free-floating collagen lattice as a model to study the capacity of UVA-irradiated fibroblasts to reorganize the collagen network. When fibroblasts are placed within a three-dimensional collagen matrix, they move and form matrix adhesions, and their morphology and cytoskeletal organization change. To interact with collagen fibers, fibroblasts express cell surface adhesion receptors which promote linkage between extracellular adhesion molecules, such as fibronectin, and intracellular cytoskeletal proteins, such as α-SMA. A smaller reduction in the diameter/volume of free-floating collagen lattices implies a reduction in the ability of fibroblasts to interact and organize collagen fibers (51). In general, when cells cultured in monolayers are seeded in collagen lattices, the proliferation of fibroblasts is delayed (52,53). This results from the occurrence of cell migration and cell–collagen interactions mentioned above. This explains the low contractive capacity of the non-UVA-irradiated cells (control) found at the beginning of our collagen culture experiment. The single UVA-exposed cells were induced to upregulate the expression of type I procollagen and to have an enlarged and flattened cell shape that is favorable for cell–collagen fibril contacts (29,30). This explains the high organization of the collagen lattice (as detected by the large reduction in collagen diameter) by the singly UVA-irradiated fibroblasts on the first day of our collagen culture experiment. In contrast, repeated doses of UVA irradiation caused a collapsed cell shape with a small cytoplasm. Moreover, these fibroblasts expressed less type I procollagen and fibronectin, thus losing their potential to interact with collagen fibrils. The collapsed shape was still apparent 7 days after irradiation. This accounts for the reduced organizational capacity (as detected by the small reduction in collagen diameter) of the repeatedly UVA-irradiated fibroblasts. α-SMA is a stress fiber involved in fibroblast contractile activity and collagen reorganization (19,20). No prior study has examined α-SMA expression in UVA-irradiated fibroblasts. The single dose and repeated doses of UVA used in this study did not affect α-SMA expression. The cells enlarged in response to a single UVA exposure, but it is possible that cell morphology may sometimes not relate to the expression levels of cytoskeletal proteins. A prior study found that enlarged cells do not contain higher amounts of cytoskeletal components, such as actin (54).

It is likely that repeated doses of UVA irradiation irreversibly damaged the ability of cells to reorganize collagen, as the repeatedly UVA-irradiated cells exhibited no recovery in this activity during the 7-day culture period in our collagen lattice study. Moreover, as mentioned above, the collapsed shape of the repeatedly UVA-irradiated fibroblasts was still apparent 7 days after irradiation. Results from our previous study (25) and results from others (28,55) have demonstrated that 24 h after a single UV irradiation, the levels of MMP-1 and procollagen have markedly changed but after 72 h have returned to normal (before UV irradiation). Moreover, on day 7 of the collagen lattice study, the morphology of the singly UVA-irradiated cells was no different than that of the non-UVA-irradiated cells. These data indicate the reversible effect of the single dose of UVA irradiation.

It is well known that photoaged skin results from repeated UVA exposure which causes the overproduction of MMP-1 by fibroblasts which in turn causes collagen degradation which produces an accumulation of fragmented collagen fibers (Fig. 7b) (1,17,56). Decreased production of ECMs from the malfunction of fibroblasts found in skin dermal tissue also occurs from UVA exposure (3,30). In our study, the cultured fibroblasts were repeatedly exposed to UVA at the designed intensity and times (5 J cm^{-2} × 3 times). We observed impaired functioning of the cultured fibroblasts from an increase in the expression of MMP-1 and a decrease in the expression of ECMs, including type I procollagen and fibronectin (Fig. 7d). These changes correlate with phenomena which occurred in *in vivo* photoaged skin. However, a single UVA exposure at the designed dose (5 J cm^{-2}) induced the fibroblasts to synthesize a greater amount of collagen. This response was to compensate for the collagen degradation caused by secreted MMP-1 (Fig. 7c). Using a lower dose of single UVA exposure, such as 3 J cm^{-2}, had no effect on cell function, but higher doses (9 and 15 J cm^{-2}) caused cell death. Therefore, for an *in vitro* study on photoaging, UVA intensity and number of times the cells are exposed must be considered when attempting to identify cell functioning equivalent to that of the cells in *in vivo* photoaged skin. In our experimental design, we exposed the cultured fibroblasts repeatedly to UVA (5 J cm^{-2}) as an alternative model for evaluating antiphotoaging and photo-protective compounds. In addition, fibronectin has proven to be an appropriate marker for photoaged skin.

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