Involvement of Reactive Oxygen Species in Abnormal Tropoelastin Deposition Induced by UVA-Photosensitizers

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Chronically, sun-exposed human skin is characterized by dermal connective tissue damage with the accumulation of abnormal elastic fibers. However, little is known about the relationship between accumulation of abnormal elastic fibers and photodamaged skin. In the present study, we investigated the involvement of reactive oxygen species (ROS) in photoaged skin including abnormal accumulation of tropoelastin (TE) induced by ultraviolet A (UVA)-irradiation using an *in vitro* model of elastic fiber formation. Our data showed that the morphological appearances of TE deposition was observed following the addition of recombinant TE immediately after treatment with UVA-irradiation by immunofluorescence staining and semi-quantitative assay. Our data also revealed that treatment with hypoxanthine-xanthine oxidase, which generates superoxide radicals, stimulated TE deposition. Furthermore, we confirmed that abnormal TE deposition induced by UVA-irradiation was inhibited by treatment with Cu/Zn superoxide dismutase, a superoxide radical scavenger. Therefore, the data obtained suggest that superoxide radical is a candidate for inducing morphological changes and increasing TE deposition induced by UVA-irradiation in human skin fibroblast cells. The present study would be helpful for developing a prophylactic agent for photoaged skin.

Key words —— photoaged, reactive oxygen species, skin fibroblast cells, tropoelastin, ultraviolet A-irradiation

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INTRODUCTION

Elastic fibers are found in almost all tissues of the human body including the blood vessels, ligaments, lungs, and skin, and they play an important role in maintaining organ flexibility. Elastin is an insoluble extracellular matrix protein and the core protein of elastic fibers that imparts elasticity to tissues such as skin, lungs, ligaments, and arterial walls.[1,2] Elastin is secreted from cells as a soluble protein of approximately 70 kDa, referred to as tropoelastin (TE). TE is cross-linked in the extracellular space by one or more members of the lysyl oxidase gene family to form an elastin polymer, which is the functional form of the mature protein.[3] Aggregated tropoelastin are then deposited onto performed microfibrillar templates including fibrillin-1, which act as a molecular scaffold.[4] Finally, the oxidative deamination of peptidyl lysine residues in tropoelastin is catalyzed by lysyl oxidase (LOX), which can spontaneously condense with neighboring amino groups or other peptidyl aldehydes to form covalent cross-links such as desmosine or isodesmosine. Cross-linked forms tropoelastin contributes to the elastic properties of tissue.

Photoaged skin is clinically characterized by deep wrinkling and laxity and histologically displays prominent alterations in extracellular matrices in the dermis and a diminished number of fibrils with reduced electron density and cross-striations.[5–7] Chronically, sun-exposed human skin is characterized by dermal connective tissues damage including an abnormal accumulation of TE;[8] however, the detailed mechanisms are still unclear. The skin is continuously in contact with oxygen and is increasingly exposed to UV irradiation. Therefore, an interest in speculating the

role of reactive oxygen species (ROS) that causes photo-oxidative damage of the skin has recently increased.[9–12] Oxygen radicals including hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide radicals (O_2^-), and hydroxyl radical ($OH^\cdot$) emerge from the reduction of two unpaired electrons containing ground state O_2. They are very potent oxidants that variably damage resident cells and extracellular matrices.[13]

Longer wavelength ultraviolet A (UVA)-irradiation is less affected by environmental variables than UVB and easily penetrates cloud cover and glass windows because the short wavelength cutoff of glass is about 320 nm. Skin photon penetration is positively correlated with wavelength. Up to 50% of UVA is able to reach the depth of monocytes and the dermal compartment, whereas only 14% of UVB reaches the lower epidermis. It has been estimated that the upper dermis is 100 fold higher in the UVA region than in the UVB region.[14, 15]

Elastic fiber assembly has often been studied using primary cells isolated from fetal or neonatal auricular cartilage, aorta, or lung tissue because of the ability of chondroblasts, smooth muscle cells, and pulmonary fibroblasts to produce and assemble insoluble elastin *in vitro*.[16] We have developed an *in vitro* model of elastic fiber formation, enabling the quantitative analysis of recombinant (r)TE organization into a fibrillar matrix.[17] In this study, we investigated the involvement of ROS in photoaged skin including abnormal accumulation of TE induced by UVA irradiation using an *in vitro* model of elastic fiber formation.

MATERIALS AND METHODS

Cell Culture —— Human newborn dermal fibroblast cells (NB1RGB, RIKEN, Ibaraki, Japan) were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS; SIGMA, St. Louis, MO, U.S.A.). Cell viability was determined using a Coulter counter (Beckman Coulter, Tokyo, Japan) to count living cells. Confluent cells were gently washed with Hank's balanced solution before UVA irradiation. The cells were irradiated once with 2, 5, or 10 J/cm^2 of UVA using a solar simulator (WACOM WBS-85 × 130 -16, Tokyo, Japan) in Hank's balanced solution. Immediately after irradiation, the cultures were incubated with DMEM containing 10% FBS and 20 μg/ml rTE. Enhancer or scavenger regents of ROS were used in some experiments.

Recombinant Human TE —— V5-tagged human rTE was generated using an *Escherichia coli* expression system. An expression plasmid containing V5 and histidine-tagged protein was generated by cloning a polymerase chain reaction (PCR) fragment using the primer sequences: human exon 2 forward primer 5′-GGAGGGGTCCCTGGGGCCATTCC-3′ and human exon 36 reverse primer 5′-TCATTTTCTCTTCCGGCCACAAG-3′. PCR amplifications were performed and the construct was inserted into a bacterial expression pTrcHis-TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.). V5-tagged human rTE were obtained by over expressing the plasmid in these bacterial cells followed by purification with an Ni^{2+} resin column (Ni-NTA agarose, Qiagen K.K., Tokyo, Japan) as described previously.[17–19]

Immunofluorescence Staining and Semi-quantitative Assay for TE Deposition by ELISA —— Immnofluorescence staining and semi-quantitative assay for TE deposition by ELISA was performed as reported previously.[17–19] The NB1RGB cells were irradiated with UVA and cultured in a medium containing various concentrations of V5-tagged human rTE for 24 hr. The cells were washed with PBS and fixed with 2% paraformaldehyde in PBS. Nonspecific immunoreactivity was blocked with Block Ase (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) for 5 min at room temperature. The cell layers were then incubated with anti V5 polyclonal antibody (Invitrogen) or with anti fibrillin-1 antibody (Elastin Products Company, Inc., St. Louis, MO, U.S.A.) for 30 min at room temperature, followed by several washes with PBS and a second incubation with Alexa Fluor®488 goat anti-mouse IgG, Fluor®546 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, U.S.A.), or anti porseradish-peroxidase-linked mouse IgG (GE Healthcare UK Ltd., Buckinghamshire, U.K.). Fibrils were visualized using a microscope (KEYENCE, Osaka, Japan) with epifluoresence using 20 × phase/fluorescence objectives. V5-tagged human rTE deposition recognized by the antibodies was quantified with a colorimetric assay using the 3,3′,5,5′-tetramethylbenzidine-substrate reagent (Fermentas Inc., Hanover, MD, U.S.A.).

Statistical Analysis —— Data were statistically analyzed using unpaired *t*-tests. Results were considered statistically significant when the p value was < 0.05. All data are shown as the mean ± SEM.

RESULTS

Tropoelastin Deposition Increase by Treatment with UVA-irradiation

Skin fibroblast cells were treated with UVA-irradiation (10 J/cm^2) for understanding the relationship between deposition of tropoelastin and UVA-irradiation. When human rTE was added to the culture media, TE deposition was morphologically observed following the addition of rTE immediately after treatment with UVA-irradiation by immunofluorescence staining and semi-quantitative assay (Figs. 1 and 2B). We also confirmed that TE deposition following treatment with UVA-irradiation increased in a dose dependent manner (Fig. 2A).

Singlet Oxygen Does Not Effect on Abnormal TE Deposition Induced by UVA-irradiation

Singlet oxygen, an electronically excited form of molecular oxygen, is a primary mediator of biological UVA-irradiation effects. Cells were treated with UVA-irradiation in the presence of histidine, a singlet oxygen quencher, or deuterium oxide (D_2O), an enhancer of singlet oxygen lifetime for understanding the effect of singlet oxygen on abnormal TE deposition induced by UVA-irradiation. Our data showed that histidine or D_2O do not inhibit or

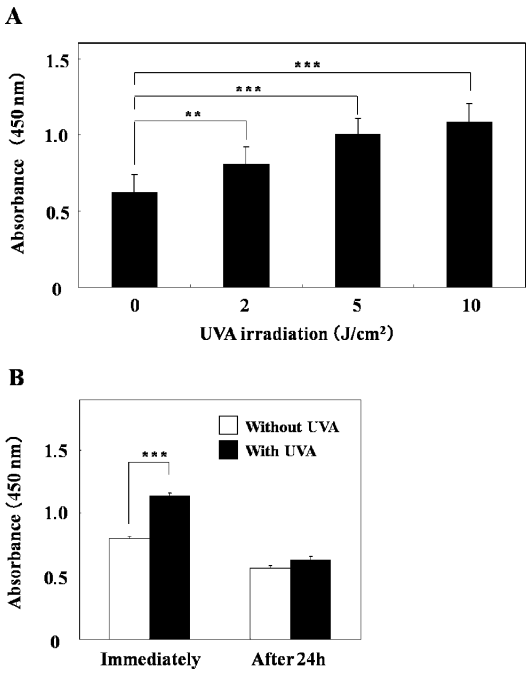

Fig. 2. UVA-irradiation Increases Amount of TE Deposition

NB1RGB cells were incubated with 20 μg/ml rTE immediately after treatment with 0, 2, 5, or 10 J/cm^2 (A) or with 10 J/cm^2 (B) of UVA-irradiation for 24 hr. The deposited rTE was detected with MAB2503. The plates were read at a wavelength of 450 nm. The bar indicates means ± SEM, $n = 3$ $^{**}p < 0.01$ or $^{***}p < 0.001$ *vs.* without UVA-irradiation.

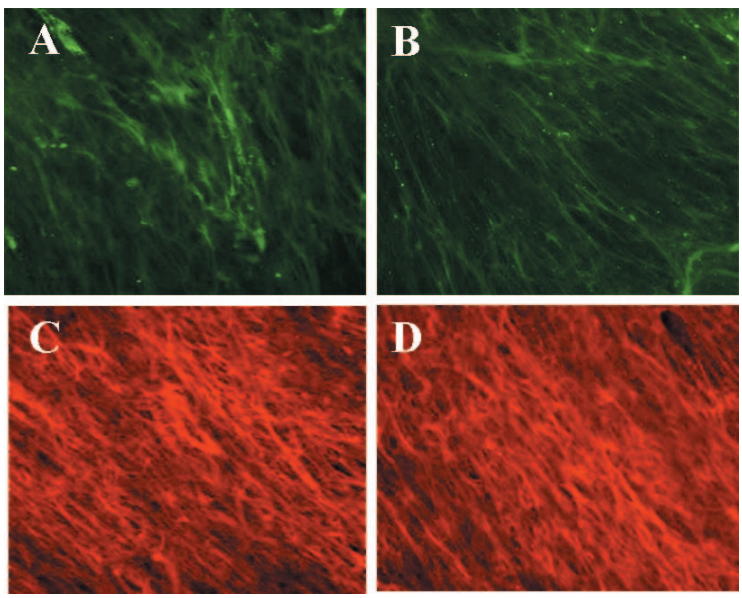

Fig. 1. UVA-irradiation Induces Morphological Change of TE Deposition

NB1RGB cells were incubated for 24 hr with 20 μg/ml rTE immediately (A, C) or 24 hr (B, D) after treatment with 10 J/cm^2 of UVA-irradiation. The deposited TE and fibrillin-1 fibers in the extracellular matrix were detected by indirect immunofluorescence with anti V5 monoclonal antibody (A, B) or with anti fibrillin-1 polyclonal antibody (C, D). Magnification, × 200.

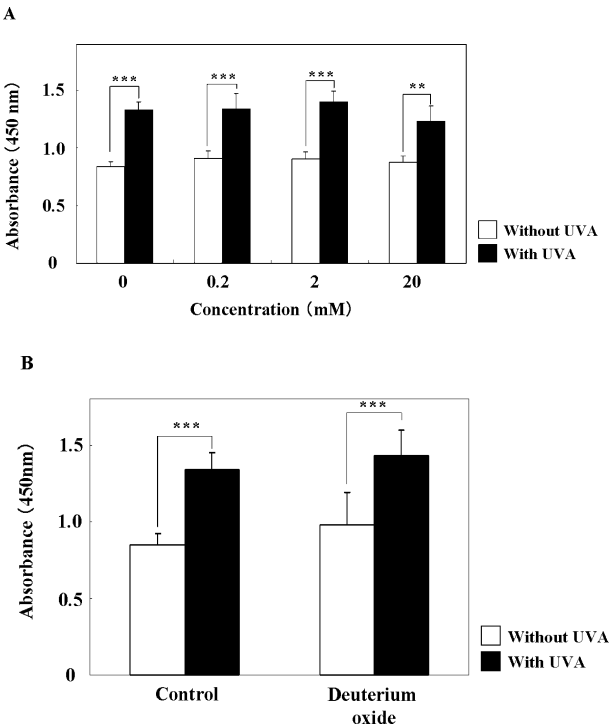

Fig. 3. Effect of Histidine and D_2O on the Abnormal Deposition of TE Induced by UVA-irradiation

NB1RGB cells were incubated with 20 μg/ml rTE immediately after treatment with 10 J/cm^2 of UVA-irradiation in the presence of histidine (A) or D_2O (B) for 24 hr. The deposited rTE was detected with MAB2503. The plates were read at a wavelength of 450 nm. The bar indicates means ± SEM, $n = 3$ $^{**}p < 0.01$ or $^{***}p < 0.001$ *vs.* without UVA-irradiation.

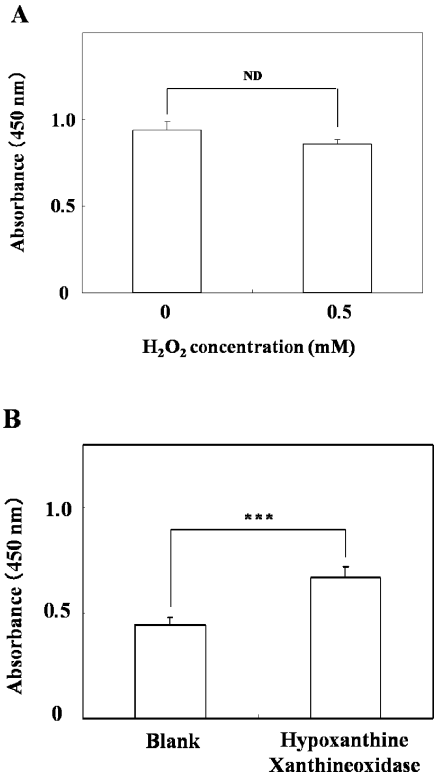

Fig. 4. Effect of Superoxide Radical or Hydroxyl Radical on the Deposition of TE

NB1RGB cells were incubated with 20 μg/ml rTE in the presence of hydroxyl oxygen (A) or hypoxanthine-xanthine oxidase (B) for 24 hr. The deposited rTE was detected with MAB2503. The plates were read at a wavelength of 450 nm. The bar indicates means ± SEM, $n = 3$ $^{***}p < 0.001$ *vs.* control.

enhance, respectively, the abnormal TE deposition induced by UVA-irradiation (Fig. 3).

Abnormal TE Deposition Induced by UVA-irradiation is Caused by Superoxide Radical

To classify which ROS were mediators of abnormal TE deposition induced by UVA-irradiation in human skin fibroblast cells, these cells were treated with hypoxanthine (100 mM)-xanthine oxidase (50 mU/ml), which generates superoxide radical, or hydroxyl oxygen, which generates hydroxyl radical. Our data revealed that superoxide radical, but not hydroxyl radical, stimulated an increase in TE deposition (Fig. 4). Furthermore, when cells were treated with UVA-irradiation in the presence of Cu/Zn SOD, a superoxide radical scavenger, TE deposition was not stimulated by UVA-irradiation (Fig. 5). These results suggest that superoxide radical is closely related to the increase in TE deposition.

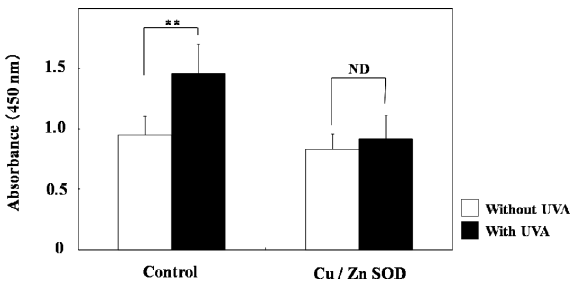

Fig. 5. Effect of Cu/Zn SOD on the Abnormal Deposition of TE Induced by UVA-irradiation

NB1RGB cells were incubated with 20 μg/ml rTE immediately after treatment with 10 J/cm^2 of UVA-irradiation in the absence or presence of Cu/Zn SOD for 24 hr. The deposited rTE was detected with MAB2503. The plates were read at a wavelength of 450 nm. The bar indicates means ± SEM, $n = 3$ $^{**}p < 0.01$ *vs.* without UVA-irradiation. ND: no difference.

DISCUSSION

The morphological appearance of photoaged skin is invariably reflected by the biochemical alterations in dermal connective tissue metabolism including collagen or elastin.[8, 20] In the present

study, we showed that ROS generated from UVA-irradiation caused abnormal TE deposition in cultured human skin fibroblast cells. We subsequently found cellular evidence showing ROS-induced morphological changes in TE deposition.

The influence of environment, notably solar UV irradiation, is of considerable importance for skin aging.[21–24] Histologically, photoaging and chronological skin aging have been considered distinct entities. Although, the typical appearance of photoaged and chronologically aged human skin can be readily distinguished, recent evidence indicates that chronologically aged and UV-irradiated skin share important molecular features, including connective tissue damage. UV irradiation of skin increases hydrogen peroxides and other ROS as well as decreases anti-oxidant enzymes,[23, 25–27] which is also observed in chronologically aged human skin.[21, 28] In both cases, increased ROS production alters gene as well as protein structure and function leading to skin damage. This suggests that UV irradiation accelerates many key aspects of the chronological aging process in human skin.

Singlet oxygen, an excited state molecule and established mediator of skin photodamage, is formed by direct energy transfer between the excited sensitizer and ground state triplet oxygen.[29–31] The formation of superoxide radical as a precursor of hydroxyl peroxide occurs via electron transfer and the production of a sensitizer radical cation, or after intermediate reduction of the sensitizer by a substrate with subsequent single electron reduction of oxygen. Hydroxyl peroxide can then be formed by spontaneous or enzyme catalyzed dismutation of superoxide radical.[32, 33]

In conclusion, our present data suggests that superoxide radical is a candidate for the morphological changes and increased TE deposition induced by UVA-irradiation in human skin fibroblast cells. UVA-generated ROS, especially singlet oxygen and superoxide radical, cause cross-linking of proteins and oxidation of sulfydryl groups cause disulfide cross-links.[34, 35] These TE aggregations are an important step for TE deposition. Considering a report indicating that TE globules deposited increase in size with time and form large fibrillar structures, the development of TE deposition depends on the fibrillar/aggregate sizes of TE, and polymerization of fibrillar formed TE facilitates the expansion to package of fibrillar bundles. Taken together, we speculate that superoxide radical promotes TE aggregation, such as TE cross-linking, and possibly contributes *in vivo* to the abnormal TE deposition during photoaging of exposed skin. The present study has provided a better understanding of the mechanisms of abnormal elastic fiber assembly and provided a basis for the development of a prophylactic agent for photoaged skin.

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