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GP91phox Play an Important Role in Long-term Ultraviolet a Irradiation-induced Photoaging-associated Changes of Collagen I and Metalloproteinase-1

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Abstract

Irradiation with long-term ultraviolet (UV) A induces photoaging. The mechanisms responsible for the skin structural changes induced by long-term UVA irradiation remain unknown. In this study, we targeted gp91phox and conducted analysis of the mechanism of the photoaging induced by UVA. Male C57BL/6j and gp91phox-knockout (gp91phox$^{-/-}$) mice were used in this study. The dorsal skin was locally exposed to UVA after covering the remaining body surface with aluminum foil at a dose of 110 kJ/m^2 using a FL20SBLB-A lamp for three times a week for 20 weeks. We unexpectedly found that the hypertrophy of a skin and the inovasion of the inflammatory cells were not induced in the UVA long-term radiation gp91phox$^{-/-}$ mice. The levels of plasma nitrogen oxide (NO_2/NO_3), tumor necrosis factor-α (TNF-α) and interleyukin-1 (IL-1) all increased following UVA irradiation in the C57BL/6j mice; however, there were no changes in the gp91phox$^{-/-}$ mice. Decreases in the expressions of collagen I and increases in the expression of metalloproteinase-1 (MMP-1) were observed following UVA irradiation in the C57BL/6j mice, whereas decreased expressions were not observed in the UVA-irradiated gp91phox$^{-/-}$ mice. In addition, the expression of the gp91phox on the surface of neutrophils was increased by UVA irradiation. Furthermore, the neutrophils-depleted mice exhibited strongly inhibited UVA-induced photoaging. These results clearly indicate that NADPH oxidase is activated by gp91phox, which is expressed on the surface in response to the increased expression of neutrophils induced by UVA irradiation, which subsequently simulates the generation of reactive oxygen species (ROS). This system may play an important role in photoaging; however, further studies are needed to confirm these findings.

Keywords: Ultraviolet A; Neutrophil; Gp91phox; Reactive oxygen species

Abbreviations: UV: Ultraviolet; TNF-α: Tumor necrosis factor-α; IL-1: Interleyukin-1; MMP-1: Metalloproteinase-1; ROS: Reactive Oxygen Species

Introduction

Skin aging, which causes wrinkling, sagging and roughness, can be divided into two distinct processes: intrinsic aging and photoaging. Photodamage and premature skin aging is caused by ultraviolet (UV) irradiation, which increases the synthesis of matrix metalloproteinases (MMPs) [1]. MMP upregulation in skin fibroblasts accelerates the degradation of dermal collagen, which confers tensile strength [2]. Skin aging is characterized by a reduction in the amount of type I collagen, the primary component of the extracellular matrix (ECM), which provides structural support to the skin dermis [3]. Therefore, natural compounds that decrease the MMP production and increase procollagen synthesis may have potential for preventing and treating photoaging. Most MMPs are secreted as inactive proenzymes (pro MMPs) that are primarily activated by proteolytic cascades catalyzed by natural proteinases. MMP-1 is primarily responsible for degrading the ECM [4].

Considerable evidence exists suggesting that reactive oxygen species (ROS) are involved in some of the deleterious effects of UV light on cutaneous cells and skin [5-7]. ROS have been clearly linked to processes of apoptosis [8,9]. ROS are believed to be involved in many inflammatory skin disorders in addition to skin cancer formation, phototoxicity and skin aging [10].

Recently, attention has increasingly been paid to a gene family that encodes the catalytic subunit of superoxide-producing nicotinamide adenine dinucleotide phosphate (NADPH) oxidases [11,12]. Gp91phox (glycoprotein 91 kDa phagocyte oxidase), the funding member of the phagocyte NADPH oxidase family, is predominantly expressed in phagocytes, such as neutrophils, and plays a crucial role in the host defense against microbial infection. Recently, NADPH oxidase has been shown to be involved in the generation of superoxide in the skin [13]. The role of gp91phox in UVA-exposed photoaging in skin, however, is unknown.

In this study, we investigated the role of phagocyte NADPH oxidase gp91phox in UVA-induced photoaging.

Materials and Methods

Animals

All animals were treated according to the animal care regulations of Osaka City University Medical School. Specific pathogen-free, 8-week-old male C57BL/6j mice (SLC, Hamamatsu, Aichi, Japan), and C57BL/6j gp91phox knockout (gp91phox$^{-/-}$) mice (Jackson Laboratories, Bar Harbour, ME) (the difference in suppliers had no effect on the results)

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were used in the experiments. UVA irradiation was performed as described in a previous study [14]. Under light Nembutal anesthesia, the dorsal skin was locally exposed to UVA (wavelength of 320 to 400 nm), after covering the remaining body surface with aluminum foil, at a dose of 110 kJ/m^2 using a FL20SBLB-A lamp (wavelength: 300-400 nm, peak emission: 352 nm, Toshiba Co., Tokyo, Japan). The dose of UVA administered was confirmed using an UV radiometer (Topcon Co., Tokyo, Japan) filtered through a glass filter sheet. The dorsal fur of the mice was shaved with electric clippers. In the control experiments, the dorsal skin was irradiated with visible light (400 to 700 nm). The dorsal skin was exposed three times a week for 20 weeks in total. In addition, each time, we are analyzing the genotype by PCR using the protocol by Jackson lab.

Anti-lymphocyte antigen 6G (Ly6G) MAb treatment

Neutrophils were depleted using anti-Ly6G MAb (eBioscience Inc., San Diego, CA). The anti-Ly6G MAbs were administered via i.p. injection throughout the experimental period. The injections comprised 500 μg of antibodies. The treatment controls were treated with PBS.

Preparation and staining of the dorsal skin

For the histological studies, the mice were sacrificed six hours after the final exposure. The dorsal skin specimens were fixed in phosphate-buffered paraformaldehyde (4%), embedded in frozen Tissue Tek, OCT compound and cut into 5-μm-thick sections. Thin sections were stained with hematoxylin-eosin (HE) according to the standard procedure.

For the analysis of the expressions of collagen I, MMP-1, gp91phox and Ly6G, the sections of dorsal skin were washed in PBS and subsequently incubated overnight at 4°C with rabbit anti-collagen type I (1:40) polyclonal antibodies (END Chemicals Inc. Darmstadt, Germany), rabbit anti-MMP-1 (1:50) polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti-gp91phox (1:100) polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or rat anti-Ly6C (1:50) monoclonal antibodies (AbD Serotec, Oxford, UK). The sections were then washed in PBS and incubated at room temperature for two hours with FITC-conjugated anti-rabbit immunoglobulin, TRITC-conjugated anti-rabbit immunoglobulin or FITC-conjugated anti-rat immunoglobulin (1:30) (Dako Cytomation, Glostrup, Denmark). The expressions of collagen I, MMP-1, gp91phox and Ly6C were evaluated immunohistochemically using a fluorescence microscope.

Western blot analysis of the dorsal skin

The dorsal skin samples were homogenized in a lysis buffer containing 0.5% Nonidet P-40, 10% glycerol, 137 mM NaCl, 2 mM ethylenediamine tetraacetic acid and 50 mM Tris–HCl buffer (pH 8.0). After centrifugation at 8000×g for 10 min, the supernatant fractions were separated and stored at -80°C until use. The stored dorsal skin specimens were subjected to 10% polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% SDS. The electrophoresed proteins in the gel were transferred to an Immobilon membrane (Millipore, Bedford, MA). The membrane was blocked with 5% skim milk at 4°C for over-night and subsequently incubated with primary antibodies against collagen I or MMP-1 (1:1000) polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 25°C for 1 h and then with horseradish peroxidase-conjugated secondary antibody (DakoCytomation, Glostrup CA). Immune complexes thus formed were detected with Enhanced chemiluminescence (ECL) regents (GE Healthcare Bio-Sciences, Piscataway, NJ).

Quantification of NO_2/NO_3, interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) using an enzyme-linked immunosorbent assay (ELISA)

Blood samples were obtained from the heart six hours after UVA irradiation, and the plasma was then fractionated. The plasma levels of NO_2/NO_3, IL-1 and TNF-α were determined using a commercial ELISA kit (NO_2/NO_3; DOJINDO, Kumamoto, Japan; IL-1 and TNF-α; Thermo Scientific, Rockford, IL) according to the manufacturer's instructions.

Statistical Analysis

All data are presented as the means ± SD of the values of 10 animals. The results obtained from the two animal groups were analyzed with either Student's *t*-test or an ANOVA using a computer software package. Differences were considered to be significant for values of $p<0.05$.

Results

Effects of UVA irradiation on the dorsal skin in the gp91phox$^{-/-}$ mice

Under Nembutal anesthesia, the dorsal skin was irradiated topically with UVA three times a week for 20 weeks for one hour per day, and dorsal skin specimens were obtained after the 60th day of UVA irradiation. The chronic UVA exposure resulted in an increase in the epidermal thickness of the dorsal skin in the C57BL/6j mice compared to that observed in the unexposed dorsal skin in the HE-stained skin specimens. In contrast, this change was not observed in the gp91phox$^{-/-}$ mice (Figure 1).

Effects of UVA irradiation on collagen I and MMP-1 in the dorsal skin

We observed about the expression of MMP-1 which is the degradative enzyme of a collagen I and the collagen I which causes wrinkles in photoaging. Localized UVA irradiation of the dorsal skin increased the MMP-1 expression in the skin of the UVA-irradiated mice. In addition, the collagen I expression was decreased in the skin of the UVA-irradiation mice. However, the expressions of MMP-1 and collagen I did not change in the UVA-irradiated gp91phox$^{-/-}$ mice (Figures 2).

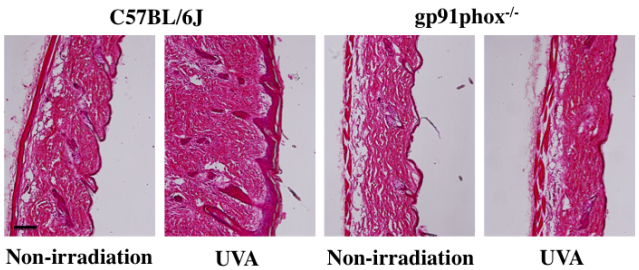

Figure 1: Effects of UVA irradiation. Six hours after the final irradiation of the dorsal skin in the C57BL/6j and gp91phox$^{-/-}$ mice, skin specimens were obtained and fixed with 4% PFA. Thin sections of the skin specimens were stained with hematoxylin-eosin (Scale bar=100 μm).

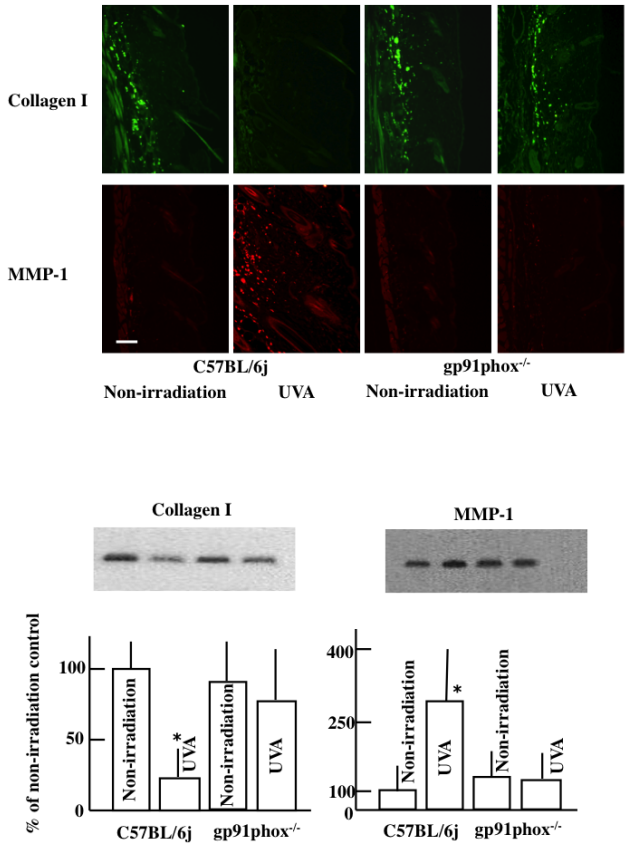

Figure 2: Effects of UVA irradiation on the collagen I and MMP-1 expressions in the skin. Six hours after the final UVA irradiation of the dorsal skin in the C57BL/6j and gp91phox$^{-/-}$ mice, the animals were sacrificed, and dorsal skin specimens were frozen, cut into thin sections and stained according to routine histological methods. The data show one typical experiment from 10 animals (Scale bar=100 μm).

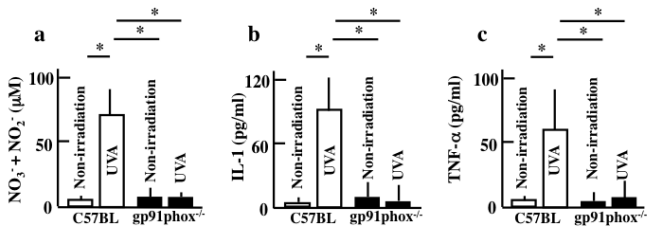

Figure 3: Effects of UVA irradiation on the plasma concentrations of NO_2/NO_3, IL-1 and TNF-α. Six hours after the final UVA irradiation of the dorsal skin in the C57BL/6j and gp91phox$^{-/-}$ mice, the plasma concentrations of NO_2/NO_3, IL-1 and TNF-α were determined. The values indicate the mean ± SD derived from 10 animals. *p<0.05 compared with the control mice.

Effects of UVA irradiation of the dorsal skin on the plasma levels of NO_2/NO_3, IL-1 and TNF-α

Localized UVA irradiation of the dorsal skin induced significant increases in the concentrations of NO_2/NO_3 (a), IL-1 (b) and TNF-α (c) in the plasma of the C57BL/6j mice. However, the plasma NO_2/NO_3, IL-1, and TNF-α levels did not increase in the UVA-irradiated gp91phox$^{-/-}$ mice (Figure 3).

Effects of UVA irradiation on the expressions of neutrophils and gp91phox in the C57/6j mice

It is known well that gp91phox is high-revealed to the neutrophil in a skin. To understand the pathological significance of UVA irradiation, we analyzed potential factors associated with the induction of inflammation by examining the number of expression of neutrophils and gp91phox. An immunohistochemical analysis revealed that UVA irradiation increased the levels of neutrophils and gp91phox (Figure 4). In addition, in the UVA-irradiated skin, the gp91phox expression was associated with the neutrophil expression. This indicates that the expression of gp91phox increases on the surface of neutrophils.

Effects of UVA irradiation on epidermal thickness and the expression of gp91phox in the dorsal skin following anti-Ly6G MAb treatment

Since the expression of gp91phox in the skin was mainly a neutrophil, we checked the expression of gp91phox using the neutrophil deficient mice. UVA irradiation induced epidermal thickness in the dorsal skin. However, treatment with anti-Ly6G MAb (this antibody strongly depletes neutrophils) abrogated the UVA-induced increase in epidermal thickness. In addition, the expression of gp91phox was not affected by UVA irradiation in the anti-Ly6G MAb-treated mice (Figure 5).

Discussion

He et al. have reported that the oxidant stress and the apoptosis of a keratinocyte are induced by short-term radiation of UVA in the cultured cell of the keratinocyte of gp91phox$^{-/-}$ mice origin [15]. In this research, the alteration of the thickness of the epidermis by the early time of a UVA irradiation was not seen. This reason has the low amount of radiations of UVA in this study, and the amount of energy which does not give damage to an epidermis is used for it by the single irradiation. Therefore, with early time, the thickness of a skin does not change by UVA irradiation, but the damage of UVA is accumulated over a long period of time, and it is thought that the tylosis of the epidermis was induced as a result.

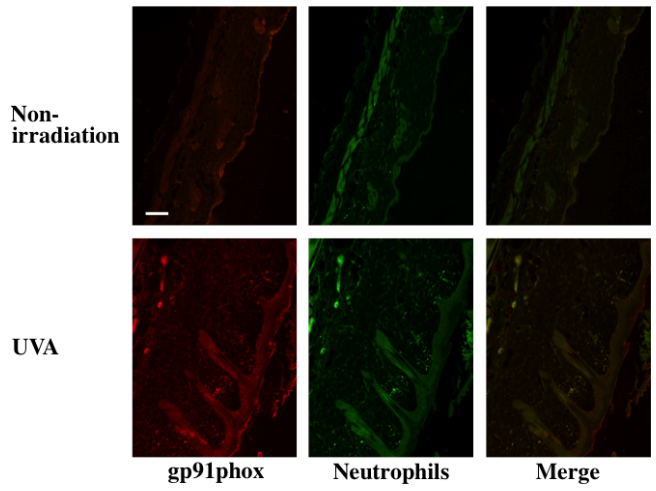

Figure 4: Effects of UVA irradiation on the gp91phox and neutrophil expressions in the dorsal of skin. Six hours after the final UVA irradiation of the dorsal skin in the C57BL/6j mice, the mice were sacrificed, and dorsal skin specimens were frozen, cut into thin sections and stained according to routine histological methods. The data show one typical experiment from 10 animals. Scale bar=100 μm.

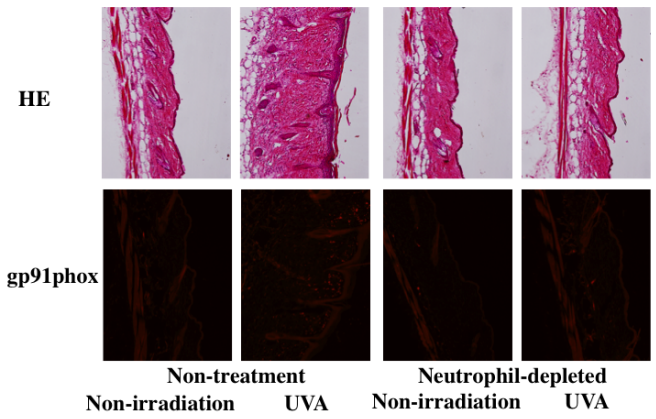

Figure 5: Effects of anti-Ly6G MAb on UVA-induced photoaging and the expression of gp91phox. Throughout the experimental period, anti-Ly6G MAbs were injected intraperitoneally into the C57BL/6j mice. Six hours after the final UVA irradiation of the dorsal skin, the mice were sacrificed, and skin specimens were frozen, cut into thin sections and stained with hematoxylin and eosin in addition to being stained using routine histological methods. The data show one typical experiment from 10 animals (Scale bar=100 μm).

UV irradiation induces the generation of ROS, direct and/or indirect DNA damage, and inflammatory responses and damage to the integrity of the extracellular matrix [16]. UV-induced ROS generation stimulates cell surface cytokines, growth factor receptors, and mitogen-activated protein kinases (MAPKs), such as p38 kinase, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK), which in turn regulate activator protein-1 (AP-1). An increased AP-1 activity downregulates type I procollagen and upregulates MMP-1. The transcriptional activity of AP-1, a heterodimer composed of c-Jun and c-Fos, is dependent on the degree of c-Jun phosphorylation and the c-Fos expression [17,18]. UV irradiation also alters transforming growth factor β (TGF-β)/Smad signaling, which modulates extracellular matrix metabolism and tissue genesis via the production of type I collagen. The actions of TGF-β are enhanced by Smad3 and antagonized by Smad7 [19,20]. These mechanisms have been shown to be related to collagen production and breakdown, as well as inflammatory cytokine (IL-1 and IL-6) production.

This study demonstrated the role of neutrophil NADPH oxidase (gp91phox) induced by UVA irradiation in gp91phox$^{-/-}$ mice. In the gp91phox$^{-/-}$ mice, no decreases in NOx or epidermal thickening due to long-term UVA irradiation were observed. In addition, decreases in NOx and epidermal thickening were not seen in the neutrophil-deficient mice. Furthermore, gp91phox exhibited a high expression on the surface of neutrophils following long-term UVA irradiation. Therefore, the accumulation of a certain level of UVA irradiation may be required to stimulate the release of ROS through the actions of neutrophils and subsequently induce photodamage.

The generation of ROS results in the production of superoxide anion radical (O_2^-) and singlet oxygen ($^1O_2$). O_2^- and $^1O_2$ are also produced by increased neutrophils in photodamaged skin that contribute to an overall prooxidant state. Superoxide dismutase converts O_2^- to hydrogen peroxide (H_2O_2). It has been reported that inflammasomes are activated by H_2O_2, thereby inducing inflammation [21]. EBBP/TRIM16 interacts with proIL-1β and may present this compound to inflammasomes through its interactions with these proteins [22]. Active caspase-1 then cleaves proIL-1β and caspase-1, ASC, EBBP, NLRP1 and NLRP3 are secreted independently of the ER/Golgi, together with IL-1β. IL-1β secretion induces inflammation *in vivo* [23]. However, in this study, long-term irradiation resulting in UVA-induced photoaging was not observed in the gp91phox$^{-/-}$ mice, thus suggesting that the ROS derived from NADPH oxidase play an important role in inducing photoaging. NADPH oxidase is activated by gp91phox, which is expressed on neutrophils following stimulation by long-term UVA irradiation, which subsequently increase the generation of ROS. The ROS then activate inflammasomes, which stimulate the production of caspase-1, which increases the expression of IL-1β, thereby inducing photoaging. Further studies are required to better elucidate the full cascade of signal transmission that occurs between long-term UVA stimulation and the actions of neutrophils.

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