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Exposure of human melanocytes to UVB twice and subsequent incubation leads to cellular senescence and senescence-associated pigmentation through the prolonged p53 expression

Suh-Yeon Choi[1], Bum-Ho Bin[1], Wanil Kim, Eunkyung Lee, Tae Ryong Lee, Eun-Gyung Cho*

Basic Research and Innovation Division, R&D Unit, AmorePacific Corporation, Yongin-si, Gyeonggi-do, 17074, Republic of Korea

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ABSTRACT

Background: Ultraviolet radiation (UVR) is a well-known factor in skin aging and pigmentation, and daily exposure to subcytotoxic doses of UVR might accelerate senescence and senescence-associated phenomena in human melanocytes.
Objective: To establish an *in vitro* melanocyte model to mimic the conditions of repeated exposure to subcytotoxic doses of UVB irradiation and to investigate key factor(s) for melanocyte senescence and senescence-associated phenomena.
Methods: Human epidermal melanocytes were exposed twice with 20 mJ/cm^2 UVB over a 24-h interval and subsequently cultivated for 2 weeks. Senescent phenotypes were addressed morphologically, and by measuring the senescence-associated β-galactosidase (SA-β-Gal) activity, cell proliferation capacity with cell cycle analysis, and melanin content.
Results: The established protocol successfully induced melanocyte senescence, and senescent melanocytes accompanied hyperpigmentation. Prolonged expression of p53 was responsible for melanocyte senescence and hyperpigmentation, and treatment with the p53-inhibitor pifithrin-α at 2-weeks post-UVB irradiation, but not at 48 h, significantly reduced melanin content along with decreases in tyrosinase levels.
Conclusion: Melanocyte senescence model will be useful for studying the long-term effects of UVB irradiation and pigmentation relevant to physiological photoaging, and screening compounds effective for senescence-associated p53-mediated pigmentation.

1. Introduction

Skin aging is induced both intrinsically and extrinsically, with intrinsic aging reflecting the genetic background and dependent upon the passage of time, whereas extrinsic aging is caused by environmental factors, such as ultraviolet radiation (UVR), smoking, alcohol abuse, and poor nutrition [1,2]. Among these environmental factors, UVR contributes to up to 80% of the acceleration of skin aging by inducing DNA damage (photoaging) or by activation of diverse oncogenes [3]. To prevent DNA damage by UVR, skin cells harbor well-coordinated systems, including those associated with cell cycle checkpoints, DNA repair, apoptosis, and premature senescence. Once unrepairable and extensive DNA damage occurs, cells terminate proper division and enter a cell-senescent state. Senescent cells are no longer capable of dividing, but remain viable and metabolically active for long periods of time. A potential role in aging by the accumulation of senescent cells in skin and other tissues was recently demonstrated, where the clearance of p16Ink4a-positive cells attenuated age-related changes in several organs and extended healthy lifespan [4,5].

UVR is mostly absorbed in the epidermis and predominantly affects epidermal cells by generating severe oxidative stress, resulting in transient and permanent genetic damage and consequent regulation of signaling pathways related to growth,

Abbreviations: UVR, ultraviolet radiation; UVsen, UVB-induced senescence; SAP, senescence-associated pigmentation; SA-β-Gal, senescence-associated β-galactosidase; TPA, tetradecanoyl phorbol acetate; CT, cholera toxin; pTpT, thymine dinucleotides; HEMn, human epidermal melanocyte neonatal; CDKN1A, cyclin dependent kinase inhibitor 1A; PFTα, pifithrin-α; TYR, tyrosinase; TYRP1, tyrosinase-related protein 1; POMC, pro-opiomelanocortin; α-MSH, α-melanocyte-stimulating hormone; KITLG, c-KIT ligand; HGF, hepatocyte growth factor.

* Corresponding author at: Basic Research and Innovation Division, R&D Unit, AmorePacific Corporation, 1920 Yonggu-daero, Giheung-gu,Yongin-si, Gyeonggi-do, 17074, Republic of Korea.
E-mail address: egcho@amorepacific.com (E.-G. Cho).
[1] These authors equally contributed to this work.

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differentiation, and senescence [6]. Upon exposure to UVR, epidermal melanocytes produce melanin that is transferred to neighboring keratinocytes, allowing their absorption of UVR to protect the cells from UVR-induced damage, thereby displaying photoprotective and thermoregulatory roles [7]. Similar to other cells, the proliferative potential of adult melanocytes is reduced relative to those of fetal and neonatal melanocytes *in vitro* [8] and eventually 10%–20% of melanocytes are lost every decade after 30 years of age [9,10]. Additionally, melanocytes from patients with a premature-aging disorder show reduced proliferative potential [11]. Although physiological aging-related increases in cell senescence is possibly associated with a tumor-suppressive mechanism [12,13], the emergence of age-related senescent melanocytes might be related to uneven skin tone and uncontrolled pigmentation, resulting in pigmented spots, heterogeneity in the skin epidermis, and pigmentary disorders. This speculation is partly supported by the fact that senescent melanocytes are frequently observed in human naevi [14–16]. UVR-induced melanocyte senescence is characterized by growth-arrested and flattened cells [17,18]; however, *in vitro* assays using a single dose of UV irradiation targeting melanocytes are often intended to activate melanocytes to stimulate melanogenesis rather than to induce senescence. Currently, mitogenic supplements, such as tetradecanoyl phorbol acetate [commonly referred to as TPA or phorbol-12-myristate-13-acetate (PMA)] and cholera toxin (CT), are senescence-regulating factors used to increase melanocyte growth rate in culture [17]. Given that repeated exposure to UVR naturally occurring in the skin epidermis represents a direct cause of skin-cell photoaging, it will be useful to develop a senescence model, especially in melanocytes, relevant to mimicking *in vivo* circumstances associated with repeated exposure to UVR over time.

In this study, we developed a melanocyte model involving exposure of cells to subcytotoxic doses of UVB twice and cultivation for 2 weeks. This treatment resulted in features of cell senescence in human melanocytes accompanied by hyperpigmentation. Additionally, we identified p53 as a key factor for UVB-induced melanocyte senescence and senescence-associated hyperpigmentation through its increased expression and sustained high levels following UVB exposure. Furthermore, we observed reversals of UVB-induced p53-mediated effects following treatment with a p53 inhibitor.

2. Material and methods

2.1. Cell culture and UVB irradiation

Human epidermal melanocytes derived from neonatal foreskin of moderately pigmented donors (African American males) were purchased (HEMn-MP; Cascade Biologics, Portland, OR, USA) and cultured in a humidified incubator containing 5% CO_2 in Medium 254 supplemented with human melanocyte growth supplement (HMGS; Life Technologies, Carlsbad, CA, USA). The cells of passages two through five were used for the following experiments. At 24 h after plating, the supernatant was replaced with a thin layer of phosphate-buffered saline (PBS) with calcium and magnesium (Corning Life Science, Corning, NY, USA) and exposed to UVB radiation after lids were removed. Immediately after irradiation, the PBS was removed and replaced with Medium 254 supplemented with HMGS. Irradiation using various doses of UVB was performed twice at a 24-h interval using a UV irradiator (Bio-Sun; Vilber Lourmat, Marne-la-Vallée, France), and the irradiated cells were cultivated for 2 weeks. Control cells were kept in the same culture conditions without UVB exposure. During cultivation after UVB irradiation twice, the cells were treated with PFTα (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Cell-proliferation assay

Cell proliferation was measured using cell-proliferation reagent WST-1 (Roche Applied Science, Penzberg, Upper Bavaria, Germany). Cells (7×10^3) were placed in each well of 12-well plates and irradiated by different doses of UVB (11, 20, or 30 mJ/cm^2) twice in a 24-h interval. Cell viability was tested at 48 h, 1 week, and 2 weeks after irradiation according to manufacturer instructions.

2.3. SA-β-Gal assay

The mammalian β-Gal assay kit was purchased from Thermo Fisher Scientific (#75707; Waltham, MA, USA). At 2 weeks after UVB exposure twice, cells were collected in PBS and extracted using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific). After centrifugation at 13,000 rpm for 15 min, the supernatant was collected, and 50 μL of β-Gal assay reagent was added to 50 μL of the supernatant. After enzymatic reaction for 30 min at 37 °C, the absorbance at 405 nm was measured using a Synergy H2 microplate reader (BioTek, Winooski, VT, USA). SA-β-gal staining was performed using a β-gal staining kit (#K1454-01; Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. Briefly, cells were washed with 1 × PBS, fixed with fixative solution for 10 min at room temperature, and incubated in the staining solution for 2 h at 37 °C. Pictures of five to 10 random fields were obtained under an optical microscope (200×), and SA-β-gal$^+$ cells were counted.

2.4. Flow cytometry

Cells were re-suspended in 300 μL PBS, and 700 μL of pre-chilled 100% ethanol was gradually added to fix cells without clumping. Fixed cells were incubated with 1 μg/mL propidium iodide (Sigma-Aldrich) for 10 min at room temperature. Stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) for cell cycle and granularity analyses.

2.5. Melanin assay

Cells were lysed by sonication in radioimmunoprecipitation assay (RIPA) buffer [0.1 M Tris-HCl (pH 7.2), containing 1% Nonidet P-40, 0.01% sodium dodecyl sulfate (SDS) and a protease-inhibitor cocktail] (Roche Applied Science) and centrifuged at 15,000*g* for 10 min. The supernatant containing proteins was used for protein quantification by BCA protein assay kit (#23227; Pierce; Thermo Fisher Scientific), and the pellets containing melanin were dissolved in 1 N NaOH and incubated for 30 min at 60 °C. Protein and melanin content was determined by measuring the absorbance at 562 nm and 450 nm, respectively, using the Synergy H2 microplate reader (BioTek). Three independent experiments were performed.

2.6. ddPCR analysis

ddPCR was performed to assess the number of mRNA molecules. Total RNA was prepared using Trizol reagent (Thermo Fisher Scientific) according to manufacturer instructions. Total RNA (1 μg) was reverse transcribed using a RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific) with MS2 RNA spike-in (Roche, Indianapolis, IN, USA) according to manufacturer instructions. Synthesized cDNA was diluted to allow 10 ng of RNA to be analyzed for each ddPCR reaction in a 20-μL volume. A QX200 droplet generator and droplet reader (Bio-Rad, Hercules, CA, USA) were used to quantitatively partition and analyze cDNA. The number of mRNA copies was further normalized according to

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