Effect of Ultraviolet B Irradiation on Melanin Content Accompanied by the Activation of p62/GATA4-Mediated Premature Senescence in HaCaT Cells

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Abstract

Objective: To explore the effect and mechanism of ultraviolet B (UVB) on melanin synthesis and premature senescence in human immortalized keratinocytes (HaCaT) cells.

Methods: HaCaT cells were irradiated with 0, 20, 50, 80, 100, 150, and 200 mJ/cm² of UVB. NaOH method was used for melanin content assay, cellular tyrosinase (TYR) activity was determined by 3,4-Dihydroxy-L-phenylalanine (L-DOPA) oxidation to dopachrome, premature senescence was analyzed by senescence-associated beta-galactosidase (SA-β-gal) staining kit, and the levels of p21, p16, p62, and GATA4 proteins were detected by Western blotting. Premature senescence was inhibited by the inhibitors of ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3–related (ATR), and the p53 signaling pathway was activated by Nutlin-3. The mRNA levels of senescence-associated secretory phenotype (SASP) factors including tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor A (VEGF-A), and interleukin-8 (IL-8) were measured by real-time quantitative polymerase chain reaction in HaCaT cells after 80 mJ/cm² of UVB irradiation.

Results: The melanin level increased significantly with the elevation of irradiation dose (F = 28.19, 43.82, 143.60, P < .05), reaching the peak at the dose of 80 mJ/cm². The tyrosinase activity increased significantly (F = 84.50, P < .05), the percentage of premature senescence increased (F = 16.31, P < .05), the levels of p62 decreased, and the level of GATA4 increased obviously with the increase of UVB dose after irradiation. The UVB-induced promotion of GATA4 level was significantly inhibited by being treated with ATM or ATR inhibitor. However, this did not occur in the Nutlin-3-treated group. The mRNA and protein expression of TNF-α increased significantly at 72 h at 80 mJ/cm² of UVB irradiation.

Conclusions: Melanin contents increased first and decreased afterward with the increase of UVB irradiation. The decrease of p62-mediated selective autophagy was accompanied by the accumulation of GATA4 after different doses of UVB irradiation. Activation of this p62/GATA4 pathway depends on the ATM and ATR but is independent of p53, and the SASP factor was activated in HaCaT cells at 80 mJ/cm² of UVB irradiation.

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UVB, premature senescence, melanin content, p62, GATA4, tyrosinase (TYR)

Introduction
Ultraviolet rays are subdivided into UVA, UVB, and UVC according to the radiation wavelength. Long-term exposure to ultraviolet B (UVB) can facilitate skin aging and age-related melanogenesis. Tyrosinase (TYR) promotes the synthesis of melanin. Melanin can absorb UVB and dissipate them as heat, which protects the skin from the harmful effects of UVB. Excessive or deficient amounts of melanin causes abnormal skin pigmentation. Melanin is synthesized by melanosome, a lysosome-related organelle in melanocytes. Melanosomes can be transferred from melanocytes to keratinocytes. Declining autophagy activity in senescent cells resulted in decreased melanosomal degradation, which indicates the association of melanin synthesis with senescence.

Cellular senescence is classified into replicative senescence, oncogene-induced senescence, and premature senescence. Premature senescence is characterized by several physiological changes, including the decreased capacity of proliferation, increased bulk, increased number of lysosomes, and increased expression of senescence-associated genes, and is induced by various factors, such as DNA damage and oxidative stress. UVB can penetrate the epidermis and cause DNA damage in cells at the upper part of the dermis. If the DNA damage is not effectively repaired and persists, the cell stops dividing and ages in advance, namely premature senescence. Senescent cells secrete a group of factors that induce senescence in neighboring cells, a phenomenon termed senescence-associated secretory phenotype (SASP). GATA4 has a regulatory role in SASP through nuclear factor kappa-B (NF-kB). GATA4 is degraded by p62-mediated selective autophagy; once the equilibrium is destroyed, it leads to the accumulation of GATA4. GATA4 activates NF-kB and induces the secretion of SASP, finally resulting in premature senescence. Human immortalized keratinocytes (HaCaT) is a human keratinocyte line that has been widely used for studies of skin biology. This study aimed to investigate the effect of UVB on the mechanism of melanin synthesis and premature senescence in the HaCaT cell line.

Methods

Cell Culture
HaCaT cells were cultured in MEM/EBSS medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) and 1% penicillin-streptomycin solution (Beyotime Biotechnology, Shanghai, China) at 37°C in 5% CO2. The complete culture medium was changed every 2 days until the cells reached 90% confluence. HaCaT cells were purchased from the Cell Resource Center, IBMS, CAMS/PUMC.

Cell Irradiation
HaCaT cells in the logarithmic growth phase were irradiated with an UVB (311-313 nm) lamp (model: SH4B-T UV, SIGMA, Shanghai, China) at 0, 20, 50, 80, 100, 150, and 200 mJ/cm² of UVB. The homogeneous irradiated field was 10×15 cm, and the field was uniform within 1%. After UVB irradiation, the cells were cultured in a constant temperature incubator at 37°C with 5% CO2.

Melanin Content Assay
HaCaT cells were collected at 72 h after UVB irradiation, washed twice with phosphoric acid buffer salt solution (PBS), and mixed with 1 mol/L NaOH. The mixture was seeded into 96-well plates at 100 μL per well, and was incubated in a constant temperature incubator at 37°C for 60 min. The melanin content is evaluated by measuring the absorbance at 492 nm (Multiskan MK3, Thermo Electron Corporation, MA, USA).

Measurement of Tyrosinase Activity
HaCaT cells were seeded in 96-well plates, and 100 μL of 1% Triton X-100 solution was added to each well at 72 h after UVB irradiation, and then plates were shaken for 15 min. 100 μL of 1% 3, 4-Dihydroxy-L-phenylalanine (L-DOPA) solution was added to each well, and the 96-well plates were incubated at 37°C for 2 h. The tyrosinase activity is evaluated by measuring the absorbance of L-DOPA at 492 nm (Multiskan MK3, Thermo Electron Corporation, MA, USA).

Senescence-Associated Beta-Galactosidase (SA-β-Gal) Activity Assay
HaCaT cells were stained with SA-β-gal Staining Kit (Beyotime Biotechnology, Shanghai, China) following the manufacturer’s protocol. Briefly, cells were rinsed with PBS, fixed with the fixative solution for 15 min at room temperature, and then incubated at 37°C with a staining solution overnight. The results were analyzed at 200 magnifications with optical microscope in three random fields. Positive cells stained in blue were counted. Finally, the percentage of the SA-β-gal-positive was calculated using Image Pro Plus 6.0 software (Media Cybernetics, Silver Spring, USA).

Western Blotting Analysis
HaCaT Cells were lysed at 4°C using RIPA buffer (Beyotime Biotechnology, Shanghai, China) supplemented with
phosphatase inhibitors and protease inhibitors (Roche, Basel, Switzerland) for 30 min. Cell lysates were centrifuged at 12000 r/min for 10 min. Loading buffer (Dingguo Biotechnology, Beijing, China) added and boiled at 100°C for 10 min to denature the protein. The protein concentration in the supernatant was determined using the Bicinchoninic acid (BCA) protein quantitative assay kit (Thermo Fisher Scientific, Waltham, MA, USA). 50 μg extracted protein was separated by vertical electrophoresis with polyacrylamide gel (SDS-PAGE). The proteins were transferred onto polyvinylidene difluoride (PVDF) membrane, and the blots were blocked with 5% non-fat dry milk in TBS containing 1% Tween-20 for 1 h at room temperature. The blots were then incubated overnight with primary antibodies at 4°C. Anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase (HRP) were used at concentrations according to the manufacturer’s instructions for 1 h at room temperature. Protein bands were visualized using Super Signal West Pico Plus Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA), and complementary deoxyribose nucleic acid (cDNA) was synthesized using PrimeScript reverse transcriptase cDNA Synthesis Kit (TaKaRa, Tokyo, Japan). RT-qPCR was performed using SYBR® green supermix (Bio-Rad Laboratories, Hercules, CA, USA). Antibodies against GATA4, p62, p16, and p21, and TNF-α were purchased from Abcam (Cambridge, USA), antibodies against β-actin were purchased from Proteintech (Chicago, USA). Secondary antibodies, including anti-rabbit IgG, and anti-mouse IgG were purchased from ZSGB (Beijing, China).

**Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)**

Total RNA was extracted with TRIzol reagent (Ambion, Austin, TX, USA), and complementary deoxyribose nucleic acid (cDNA) was synthesized using PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Tokyo, Japan). RT-qPCR was performed using SYBR® green supermix (Bio-Rad Laboratories, USA) in triplicate. All targets gene were normalized to the level of β-actin mRNA by the ΔΔCT method. Primer sequences are as follows: interleukin-8 (IL-8), 5'-CACTGCAGCCAACAGAAAT-3' (forward), 5'-TTCTCTAGCCCTTCTCAAAAAACTTC-3' (reverse); tumor necrosis factor alpha (TNF-α), 5'-CACAGTGAAATTGTGGGGAAC-3' (forward), 5'-AGGAAGGCTAGTTGGCCT-3' (reverse); vascular endothelial growth factor A (VEGF-A), 5'-CTGGGACTGTGCTGTTCTG-3' (forward), 5'-GGAGGCTCAGG GCATGAG-3' (reverse); β-actin, 5'-ATGACATGGGCAACTGAGGC-3' (forward), 5'-TTGAAGGTATTTGCGAGATG-3' (reverse).

**Statistical Analysis**

The statistical analysis and graphs were generated using GraphPad Prism 9.0 software (San Diego, CA). Comparisons for multi-group were carried out using one way ANOVA. Student’s t-test was used for two-group comparison. Differences were considered statistically significant at \( P < .05 \).

**Results**

Melanin contents in HaCaT cells shows first increased and afterward decreased trend with the increasing dose of UVB irradiation, which be accompanied by the activation of tyrosinase (TYR).

To explore the effect and mechanism of UVB on melanin content in HaCaT cells, we detected the melanin level and tyrosinase activity in HaCaT cells after 0, 20, 50, 80, and 100 mJ/cm² of UVB irradiations. The results showed that melanin contents increased dose-dependent manner at 24, 48, and 72 h until the radiation dose exceeded 80 mJ/cm² (\( F = 28.19, 43.82, 143.60, P < .05 \)) (Figure 1A). In addition, the tyrosinase activities increased significantly in a dose-dependent manner (\( F = 84.50, P < .05 \)) (Figure 1B).

These results suggested that UVB induces the increase of melanin contents, accompanied by the activation of TYR in HaCaT cells. However, there is a clear dose threshold of 80 mJ/cm² below which UVB irradiation can promote the melanin contents in HaCaT cells.

The decrease of p62 is accompanied by the accumulation of GATA4 in HaCaT cells after being irradiated with UVB of different doses.

Previous study revealed that 2 major pathways in HaCaT cells mediate premature senescence after UVB irradiation: the
p53 pathway and p16-pRB pathway, which functions by arresting the cell cycle.\textsuperscript{15} It is well known that the third 1 of the new branch senescence regulatory pathway is mediated by p62/GATA4, which acts on regulating SASP, finally resulting in premature senescence. In order to clarify whether the p62/GATA4 pathway is activated by UVB irradiation, the percentage of premature senescence and the levels of p62 and GATA4 proteins were measured in HaCaT cells at 72 h after 0, 20, 50, 80, and 100 mJ/cm\textsuperscript{2}. The results showed that the percentage of the SA-β-gal-positive senescent cells was significantly increased with the increase of radiation dose ($F = 16.31$, $P < .05$) (Figure 2A and 2B). The levels of p62 decreased significantly, and the levels of GATA4 increased obviously with the increase of radiation dose at 72 h after irradiation with 0, 20, 50, 80, and 100 mJ/cm\textsuperscript{2} of UVB (Figure 2C and 2D).

The p62/GATA4 pathway is regulated by the DDR kinases both ATM and ATR in HaCaT cells after UVB irradiation at 80 mJ/cm\textsuperscript{2}. Under normal conditions, p62/GATA4 pathway is regulated by the DNA damage repair (DDR) kinases ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3–related). To clarify whether ATM and ATR also play a role in GATA4 accumulation after UVB irradiation, the level of GATA4 was observed after being treated with the inhibitor of ATM (ATMi) or ATR (ATRi) and UVB irradiation. The results showed that the level of GATA4 obviously decreased at 72 h after being treated with ATMi or ATRi and UVB irradiation (Figure 3A).

**Figure 2.** Changes in the percentage of premature senescence and the levels of p62 and GATA4 in HaCaT cells at 72 h after different doses of UVB irradiation. (A, B) The percentage of premature senescence was measured by a SA-β-gal staining kit and quantified by Image Pro Plus. (C, D) Protein expression of p62 and GATA4 in HaCaT cells at 72 h after being irradiated with UVB of different doses (0, 20, 50, 80, and 100 mJ/cm\textsuperscript{2}). The optical density for each ladder was calculated by ImageJ software. Data were obtained from three independent experiments. Values shown are means±SD (n = 3). * means $P < .05$, compared with 0 mJ/cm\textsuperscript{2} group; # means $P < .05$, compared with 20 mJ/cm\textsuperscript{2} group; and means $P < .05$, compared with 50 mJ/cm\textsuperscript{2} group; $\#$ means $P < .05$, compared with 80 mJ/cm\textsuperscript{2} group; $n = 3$.

**Figure 3.** Changes of p16, p21, and GATA4 protein levels after being treated with ATM, ATR, and p53 inhibitors in UVB irradiated HaCaT cells. (A) Protein expression of p16, p21 and GATA4 after being treated with 10 μM ATM inhibitors (KU55933, ATMi), ATR inhibitors (VE821, ATRi). (B) Nutlin-3 (p53 agonist) in UVB irradiated HaCaT cells. DMSO acts as a negative control. Representative images were acquired from 3 different experiments.
The GATA4 pathway acts independently on the p53 in HaCaT cells after UVB irradiation at 80 mJ/cm².

To examine the relationship between GATA4 and p53 in regulating the premature senescence after UVB irradiation, we tested the effect of p53 agonist Nutlin-3 on GATA4 level. The results showed that the levels of p21 after being treated with ATMi, and p16 after being treated with ATMi or ATRi decreased obviously, compared with DMSO at 72 h after UVB irradiation (Figure 3A). The result also demonstrated that the level of p21 was obviously decreased after being treated with Nutlin-3 compared with the DMSO at 72 h after UVB irradiation. However, there was no significant change in the level of GATA4 after being treated with Nutlin-3 relative to the control (Figure 3B). The result indicated that the GATA4 pathway acts independently of the p53 in HaCaT cells after UVB irradiation at 80 mJ/cm², and agreed with experimental results in references.16–20

UVB Irradiation Highly Increase SASP-Related Gene Expression in HaCaT Cells

It has been reported previously that accumulated GATA4 initiates SASP, finally induces premature senescence.21–23 The expression change of SASP factors was detected after UVB irradiation, the results showed that the expressions of TNF-α, VEGF-A, and IL-8 mRNA had an increasing trend compared with the control group (Figure 4), especially the expressions of TNF-α mRNA increased significantly ($t = 7.67$, $P < .05$) (Figure 4A), and the level of TNF-α protein also increased significantly compared with control group (Figure 4D) at 72 h after UVB irradiation.

Discussion

It has been demonstrated that the synthesis and distribution of melanin were mediated by TYR after UVB irradiation in melanocytes and HaCaT cells in a previous study.24–26 Ultraviolet radiation is a significant inducer of vitiligo. It is widely known that vitiligo is the result of reducing melanin content. On the other hand, Narrow Bound-UVB (NB-UVB) irradiation is clinically effective and safe in vitiligo treatment. However, the relationship between skin color and UVB radiation has not been clarified. Therefore, different doses of UVB irradiation were set in this study to explore the dose range which induces the 2 opposite effects on melanin content. The results have shown that UVB induced the increase of melanin levels in HaCaT cells. Significantly, a dose of 80 mJ/cm² was identified as the threshold value below UVB irradiation can promote melanin content in HaCaT cells. Therefore, theoretical hypotheses are formulated that the proportion of premature senescence in HaCaT cells was too large to synthesize melanin beyond the threshold of 80 mJ/cm², and resulting in reduced melanin levels.

It has been found that UVB-induced premature senescence in melanocytes was highly correlated with melanin content.27–29 Our result showed that premature senescence was induced by different doses of UVB irradiation in HaCaT cells, and the percentage of premature senescence increased with the irradiation dose increasing. In 2015, the premature senescence pathway mediated by p62/GATA4 was proposed first. Kang et al.30 found that the p62/GATA4 activates NF-κB to induce the secretion of SASP factors, finally resulting in premature senescence. P62, also known as ubiquitin-binding protein, is involved in autophagy regulation. Studies have reported that premature senescence was promoted via the inhibition of p62-mediated autophagy.31,32 Similarly, our results showed that the decrease of p62-mediated selective autophagy was accompanied by the increase of premature senescence and accumulation of GATA4.

ATM and ATR how to regulate the GATA4 was measured in HaCaT cells after UVB irradiation. Significantly, our results suggested that the levels of p62 decreased after being treated with the inhibitor of ATM and ATR. Normally, GATA4 is degraded by p62-mediated selective autophagy; once the equilibrium is destroyed, it leads to the accumulation of GATA4.33 Similar to these reports, our results showed that the accumulation level of GATA4 induced by UVB irradiation decreased significantly after being treated with the inhibitor of ATM and ATR. The p62/GATA4 activates NF-κB to induce SASP secretion.16–20 Following this, our results show that the accumulation of GATA4 induces the secretion of TNF-α, one of the SASP factors. These indicated that the GATA4 pathway acts independently of the p53 in HaCaT cells after UVB irradiation at 80 mJ/cm². Therefore, we can draw a conclusion that UVB-induced premature senescence is accompanied by the GATA4 accumulation and the increasing of TNF-α in HaCaT cells.
The melanin contents in HaCaT cells were regulated by UVB irradiation. Further study found that the premature senescence was induced by UVB irradiation, and p62-mediated selective autophagy promoted premature senescence by GATA4 accumulation and the secretion of SASP factors with increased expression of TNF-α after UVB irradiation at 80 mJ/cm² in HaCaT cells. The activation of the p62-GATA4 pathway depends on the DNA damage response regulators ATM and ATR, but independently on the p53 pathway after UVB irradiation. Therefore, it is speculated that UVB affects ATM and ATR, but independently on the p53 pathway after UVB irradiation doses were beyond the threshold of 80 mJ/cm², HaCaT cells was too large to synthesize melanin when the irradiation was accompanied by the activation of p62/GATA4-mediated premature senescence melanin content after UVB irradiation was induced by UVB irradiation, and p62-mediated selective autophagy promoted premature senescence by p62-mediated selective autophagy.

In summary, these findings indicated that regulation of melanin content after UVB irradiation was accompanied by the activation of p62/GATA4-mediated premature senescence in HaCaT cells. The proportion of premature senescent in HaCaT cells was too large to synthesize melanin when the irradiation doses were beyond the threshold of 80 mJ/cm², resulting in reduced melanin levels. UVB is the inducer of vitiligo, which perhaps can be explained by the view.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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