The effects of phototherapy and melanocytes on keratinocytes

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Received June 22, 2016; Accepted April 28, 2017

DOI: 10.3892/etm.2018.5807

Abstract. Phototherapy is widely used in the treatment of vitiligo. Previous studies have focused on the effects of ultraviolet (UV) radiation on melanocytes; however, the biological effects of phototherapy and melanocytes on keratinocytes remain to be elucidated. To investigate and assess the effects of clinically doses of broad band (BB)-UVA, narrow band (NB)-UVB and melanocytes on human keratinocytes *in vitro*, clinical doses of BB-UVA or NB-UVB radiation and human melanoma cell A375 co-culture were performed as stress divisors to HaCaT cells. Cell proliferation, expression of protease-activated receptor-2 (PAR-2) and nuclear factor E2-related factor 2 mRNA, lipid peroxidation and intracellular antioxidant level of keratinocytes were analyzed. It was demonstrated that UV radiation inhibited the proliferation of cells apart from following exposure to low dose (1 J/cm^2) UVA. Medium dose (5 J/cm^2) UVA radiation had no adverse effects on lipid peroxidation and increased antioxidant levels in HaCaT cells. Medium (200 mJ/cm^2) and high (400 mJ/cm^2) doses of UVB radiation induced cellular damage due to increased lipid peroxidation as indicated by levels of malondialdehyde. Furthermore, A375 co-culture treatment induced a similar effect on the lipid peroxidation of HaCaT as with low dose UVB radiation. Therefore, the results of the present study determined that clinical doses of BB-UVA and NB-UVB radiation had varying effects on proliferation and related protein levels in HaCaT cells. Co-culture with A375 had similar effects as those of low dose UVA and UVB radiation, in which the PAR-2 expression was significantly upregulated.

Introduction

Vitiligo is characterized by depigmented patches of skin and is considered to be a depigmentary disorder. It affects 1-2% of the world's population and its incidence is increasing (1). The precise cause of vitiligo and its underlying mechanism of action remain unknown, thus the effective treatment of vitiligo remains challenging. Phototherapy is widely used as a second-line treatment to treat patients that fail local or systemic immunosuppressive therapy (2). Although the exact mechanism of action of vitiligo is poorly understood, continuous therapeutic trials have indicated that ultraviolet (UV) irradiation is able to promote the proliferation and availability of melanocytes and therefore weaken autoimmunity (3). Clinical trials have indicated that narrow band (NB)-UVB is a therapeutic option for vitiligo, as it increases the growth and migration of melanocytes and induces the expression of keratinocytic and melanocytic cytokines associated with repigmentation (4,5). However, a previous study by El Mofty *et al* (6) demonstrated that broad band (BB)-UVA may be an alternative therapeutic approach to treat vitiligo, as it results in a marked clinical improvement and induces few side effects.

Phototherapy irradiation is commonly used to treat vitiligo; however, UV radiation is considered to be the predominant factor that causes mutations in the skin. UVA and UVB affect the skin in different ways. Research into UVA has suggested that it is predominantly absorbed by cells in the basal layer of the epidermis (7). UVA frequently induces lesions via the accumulation of reactive oxygen species (ROS). Following UVA exposure, intracellular chromophores may generate ROS (8). UVB radiation also increases the generation of cellular ROS (9). ROS have a paradoxical effect on vitiligo as they promote depigmentation and increase pigmentation of the skin (10). The skin of patients with vitiligo contains high levels of superoxide dismutase (SOD) and low levels of catalase; this induces the transfer of H_2O_2 from keratinocytes to melanocytes. This transfer of H_2O_2 is considered to be one of the mechanisms by which vitiligo is induced (11,12).

Nuclear factor E2-related factor 2 (Nrf2) serves an essential role in coordinating the transcriptional induction of common antioxidant enzymes, including SOD, glutathione S-transferase (GST) and catalase. Nrf2 is a nuclear transcriptional activator that belongs to the nuclear factor E2 family of typical leucine zipper proteins (13). Under normal conditions, Nrf2 binds to kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm and has a high dissociation rate (14). Following stimulation by ROS, the Nrf2-Keap1 complex is disrupted and Nrf2 is rapidly translocated to the nucleus. In the nucleus, Nrf2 is combined with antioxidant response element (ARE) in a heterodimer that induces the phase 2 detoxification enzymes and antioxidant proteins (15). It has been demonstrated that

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Key words: broad band ultraviolet A, narrow band ultraviolet B, melanocytes, protease-activated receptors-2, nuclear factor E2-related factor 2, keratinocytes

Nrf2 is important in protecting against ROS and the cellular expression of Nrf2 may be the primary target in evaluating the intracellular antioxidant level.

Protease-activated receptor-2 (PAR-2), which belongs to the PAR family of G protein-coupled receptors (16), is also associated with vitiligo (17). PAR-2 is activated by trypsin-like serine proteases and is expressed by almost all cell types, particularly by keratinocytes (18). It has been demonstrated that PAR-2 is expressed predominantly in the granular layer of epidermis, suggesting that PAR-2 may be associated with epidermal mutations (19). PAR-2 is also associated with skin inflammation and cellular ROS generation. Increased levels of PAR-2 expression and distribution have been detected in the epidermal layers of lesions in atopic dermatitis and rosacea (20-22). Additionally, the regulation of PAR-2 expression by solar UV irradiation and its role in melanosome transfer has been determined (23). However, the association between PAR-2 and UVA/UVB remains to be elucidated.

During vitiligo treatment, keratinocytes adjacent to melanocytes contribute to UV-induced skin pigmentation (24,25); however, the precise functional effects of phototherapy and melanocytes on keratinocytes remain unknown. Therefore, the aim of the present study was to investigate the effects induced by clinical doses of BB-UVA, NB-UVB and melanocytes on human keratinocytes *in vitro*. The proliferation and expression of PAR-2 and Nrf2, and the lipid peroxidation and intracellular antioxidant levels in HaCaT cells were analyzed to evaluate these effects.

Materials and methods

Cell culture. HaCaT human immortalized keratinocyte cells and A375 human melanoma cells used in the present study were obtained from the American Type Culture Collection (Manassas, VA, USA). HaCaT and A375 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) and minimum essential medium (MEM; Hyclone), respectively, supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. In addition, a co-culture of HaCaT and A375 cells was also established, with an initial seeding ratio of 3:1. Co-cultured cells were maintained in culture dish with 3:1 DMEM to MEM (supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin). Cultures were maintained at 37˚C in a humidified atmosphere containing 5% CO_2.

BB-UVA and NB-UVB irradiation. Prior to irradiation, HaCaT cells were rinsed with PBS to avoid toxicity induced by UV exposure of the culture medium compounds. BB-UVA/NB-UVB irradiation was subsequently performed. The lid of the culture dish was replaced by a quartz plate and HaCaT cells were exposed to BB-UVA radiation at doses of 1, 5 or 10 J/cm^2 with an emission centered at 365 nm or NB-UVB radiation at doses of 100, 200 or 400 mJ/cm^2 with an emission centered at 311 nm. Emissions were based on the results of a previous study (26). Cells without any treatment were used as a negative control. Sigma SS-02 and SS-01 fluorescent lamps (Shanghai Sigma High-Tech Co., Ltd., Shanghai, China) were used as sources of UVA and UVB, respectively. Following irradiation, PBS was removed and HaCaT cells were maintained in DMEM culture medium at 37˚C and in 5% CO_2. Cell proliferation was analyzed at 0, 12, 24 and 48 h following UV irradiation. mRNA expression, lipid peroxidation and antioxidant levels were assessed at 48 h following UV irradiation.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total mRNA from HaCaT and co-cultured cells was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. cDNA was synthesized from 1 μg RNA using a RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). To evaluate the expression of PAR-2 and Nrf-2 in cells, qPCR was performed in an ABI-7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a SYBR-Green PCR kit (Fermentas; Thermo Fisher Scientific, Inc.). The thermocycling conditions were 10 min at 95˚C, 40 cycles of 15 sec at 95˚C and 45 sec at 60˚C, followed by 1 min at 60˚C, 15 sec at 95˚C and 15 sec at 60˚C. The primer sequences used were as follows: PAR-2, forward 5'-TGGCAC CATCCAAGGAAC-3' and reverse 5'-GGCAAACCCACC ACAAAC-3'; Nrf-2, forward 5'-CAAGTCCAGAAGCCA AAC-3' and reverse 5'-GATGCTGCTGAAGGAATC-3'; and GAPDH, forward 5'-AATCCCATCACCATCTTC-3' and reverse 5'-AGGCTGTTGTCATACTTC-3'. Experiments were repeated three times and GAPDH expression was used as an internal control. Gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (27).

Cell proliferation. HaCaT and co-cultured cells were seeded into 96-well plates to evaluate cell proliferation. Following UVB irradiation, the effect of UVB exposure on cell proliferation was examined using the Cell Counting Kit-8 (CCK-8; 7 Sea Biotech, Shanghai, China) at 0, 12, 24 and 48 h according to the manufacturer's protocol. Briefly, 10 μl CCK-8 was added to each well and cells were maintained in the dark at 37˚C and 5% CO_2 for 2 h. Absorbance was measured at a wavelength of 450 nm to calculate relative proliferation.

Lipid peroxidation assay. Lipid peroxidation was assessed using a thiobarbituric acid (TBA) reactive substances assay (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) (28). Malondialdehyde (MDA), which is a product of lipid oxidative degradation, reacts with TBA, yielding red complexes that are absorbent at 532 nm. HaCaT cells and co-cultured cells were washed twice with PBS, incubated with TBA (2.8% w/v) at 95˚C for 40 min and centrifuged at 4˚C, 2,500 x g for 10 min. The relative total protein was determined by bicinchoninic acid (BCA) assay following the manufacturer's protocol (cat. no. A045-4; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The amount of reactive complexes was measured using a spectrophotometer at 532 nm.

Antioxidant level assay. SOD, total antioxidant capacity (TOAC), and protein content were measured in cells using SOD, TOAC and BCA kits (cat. nos. A003-1, A015 and A045-4, respectively; Nanjing Jiancheng Bioengineering Institute)

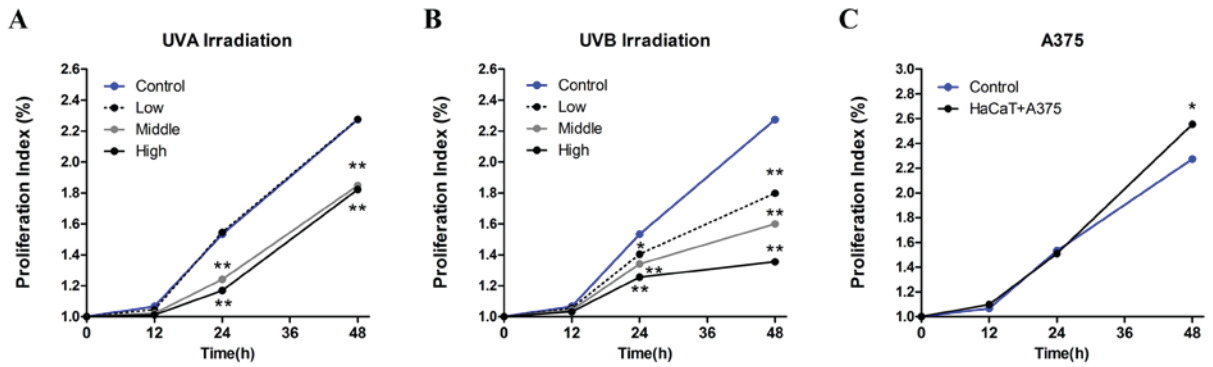

Figure 1. Effect of UVA or UVB irradiation, or A375 co-culture on HaCaT cell proliferation over 48 h (n=3). Proliferation index of HaCaT cells treated with low, middle or high dose (A) UVA and (B) UVB radiation. (C) Proliferation index of co-cultured HaCaT and A375 cells. Values are relative to that of the HaCaT cells maintained for 0 h after radiation. *P<0.05 and **P<0.01 vs. control. UV, ultraviolet.

according to the manufacturer's protocols. At 24 h following UVB/UVA irradiation and A375 co-culture, HaCaT cells were homogenized in PBS (pH 7.4) and centrifuged at 4˚C and 2,500 x g for 10 min. The resulting supernatant was used for the subsequent assays. A xanthine oxidase assay was used to measure SOD activity detected at 450 nm using a microplate reader, which monitors the inhibition of reducing the nitro blue tetrazolium in samples. TOAC was analyzed using the ferric reducing-antioxidant power method and measured at 520 nm with the spectrophotometer. Protein content was analyzed using a BCA assay; absorbance was monitored at 562 nm using a microplate reader.

Statistical analysis. At least three independent duplicates were performed for each experiment. Statistical analysis was performed by GraphPad Prism software, version 5 (GraphPad Software, Inc., La Jolla, CA, USA) using one-way analysis of variance followed by Tukey's post-hoc test. Data are presented as the mean ± standard deviation and P<0.05 was considered to indicate a statistically significant difference.

Results

Proliferation analysis. To determine the effect of UVA and UVB radiation, and melanocytes on keratinocytes, HaCaT cells were exposed to 1, 5 or 10 J/cm^2 BB-UVA, or 100, 200 or 400 mJ/cm^2 NB-UVB, and co-cultured with A375 cells. Cell proliferation was measured at 0, 12, 24 and 48 h following UV irradiation and co-culture, according to the aforementioned procedure. As presented in Fig. 1, a significant inhibition in HaCaT cell proliferation compared with control cells was induced by UV irradiation (P<0.01), apart from by low dose UVA. Furthermore, the inhibition of cellular proliferation took place progressively over a 48 h period. The most significant decrease in the proliferation index was observed 24 h following UVA exposure (P<0.01; Fig. 1A), whereas, the decrease in the proliferation index following UVB radiation was greatest at 48 h (P<0.01; Fig. 1B). Co-culture with A375 cells induced a significant increase in cell proliferation at 48 h (P<0.05; Fig. 1C).

RT-qPCR assessing PAR-2 expression in UVA/UVB/A375-treated HaCaT cells. The keratinocyte receptor PAR-2 is a key molecule associated with inflammation and pigment transfer. In the present study, PAR-2 mRNA expression in UVA/UVB/A375-treated keratinocytes was determined using RT-qPCR. As presented in Fig. 2A, PAR-2 expression was significantly upregulated following low dose UVA (P<0.01) and UVB irradiation (P<0.05). A marked decrease in PAR-2 mRNA expression was observed following exposure to medium dose UVA and UVB radiation compared with low dose treatment and the expression of PAR-2 was significantly decreased following exposure to medium dose UVB compared with the control (P<0.05). High dose UVA and UVB radiation treatment had no significant impact on PAR-2 mRNA expression. PAR-2 mRNA expression in co-cultured HaCaT and A375 cells was significantly increased compared with HaCaT cells cultured alone (P<0.01; Fig. 2B).

Expression of Nrf2 in HaCaT cells treated with UVA/UVB/A375. Nrf2 serves a key role in anti-inflammatory and antioxidant response of cells to UV irradiation. To determine the effect of UVA/UVB/A375 treatment on the expression of Nrf2 mRNA, RT-qPCR was performed. The expression of Nrf2 mRNA was significantly decreased compared with the control following low dose UVB (P<0.05), whereas no significant change was observed following low dose UVA exposure (Fig. 3A). However, following medium dose irradiation, UVA and UVB significantly elevated Nrf2 expression compared with the controls (each, P<0.01). Furthermore, a significant increase in Nrf2 expression was observed in cells following high dose UVA irradiation, whereas there was no difference in Nrf2 expression treatment following high dose UVB treatment compared with the control. As presented in Fig. 3B, the expression of Nrf2 mRNA in co-culture cells was significantly inhibited, compared with HaCaT cells. This indicates that A375 may inhibit Nrf2 expression in HaCaT.

Lipid peroxidation assay in UVA/UVB/A375-treated HaCaT cells. It has been demonstrated that lipid peroxidation may induce the breakdown of cell membranes and cell death (29). MDA is a key indicator of lipid peroxidation; therefore the concentration of intracellular and supernatant MDA was examined 24 h after the exposure of HaCaT cells to different doses of UVA or UVB irradiation, or following co-culture with A375 cells. As presented in Fig. 4A, similar responses

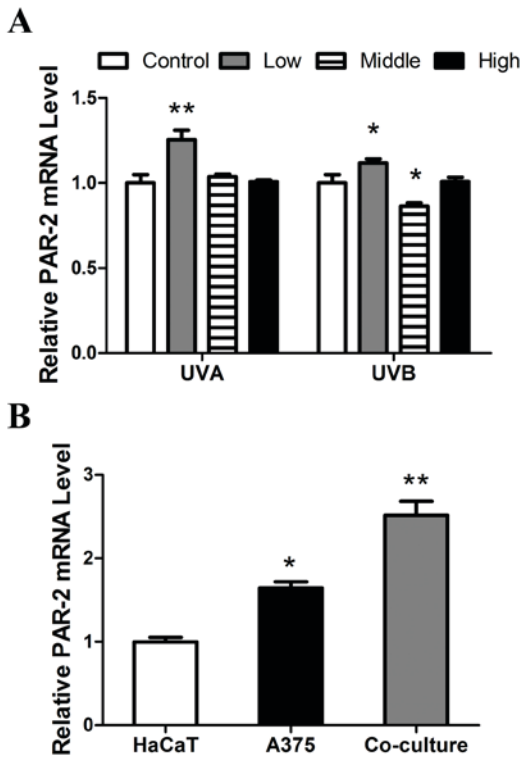

Figure 2. Expression of PAR-2 mRNA of HaCaT cells at 24 h following UVA/UVB/A375 treatment, determined by reverse transcription-quantitative polymerase chain reaction (n=3). (A) Expression of PAR-2 mRNA in cells at 24 h following low/middle/high dose UVA and UVB irradiation. *P<0.05 and **P<0.01 vs. control. (B) Expression of PAR-2 in HaCaT, A375 and co-cultured cells. Values are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. HaCaT. PAR-2, protease-activated receptor-2; UV, ultraviolet.

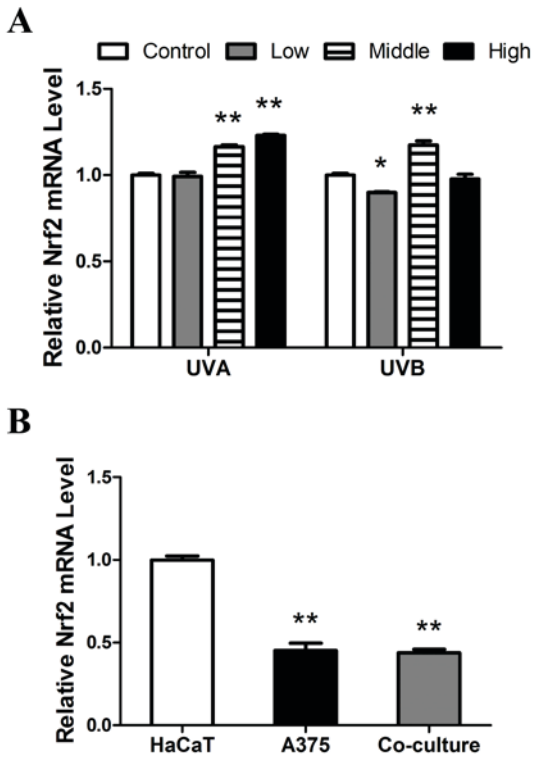

Figure 3. Expression of Nrf2 mRNA of HaCaT cells 24 h following UVA/UVB/A375 treatment determined by reverse transcription-quantitative polymerase chain reaction (n=3). (A) Expression of Nrf2 mRNA in cells at 24 h following low/middle/high dose UVA and UVB irradiation. *P<0.05 and **P<0.01 vs. control. (B) Expression of Nrf2 in HaCaT, A375 and co-cultured cells. Values are presented as the mean ± standard deviation. **P<0.01 vs. HaCaT. Nrf2, nuclear factor E2-related factor 2; UV, ultraviolet.

in intracellular MDA were detected following exposure to UVA and UVB. Following treatment with high doses of UVA and UVB, the concentration of cellular MDA increased significantly (P<0.01). However, MDA levels were significantly inhibited compared with controls following treatment with medium doses of UVA (P<0.05). MDA levels were significantly increased following low dose UVB treatment (P<0.05). As presented in Fig. 4B, all doses of UVB irradiation, as well as low and high doses of UVA irradiation significant increased the concentration of supernatant MDA compared with controls (P<0.05). However, medium doses of UVA radiation did not increase supernatant MDA levels (Fig. 4B). Furthermore, the concentration of intracellular and supernatant MDA in HaCaT cells co-cultured with A375 cells was significantly higher than in HaCaT cells (P<0.01; Fig. 4C and D). These results indicate that medium doses of UVA or UVB treatment did not affect lipid peroxidation, whereas low and high dose irradiation and A375 co-culture increased lipid peroxidation levels.

Antioxidant levels in UVA/UVB/A375-treated HaCaT cells. Antioxidants serve an essential role in balancing the production of ROS by mitochondria. To detect the effect of UVA or UVB radiation, or A375 co-culture on HaCaT cellular antioxidant level, intracellular SOD activity and TOAC levels were measured following treatment. Notably, a significant increase in SOD activity was observed following exposure to medium and high doses of UVA radiation (P<0.05), with a non-significant increase detected following treatment with low doses of UVA. A significant increase in SOD activity was observed following treatment with low and high doses of UVB, however, there was a significant decrease in SOD activity following treatment with medium doses of SOD (Fig. 5A). Additionally, the data presented in Fig. 5B indicate that there was a dose-dependent increase in TOAC levels following UVA irradiation, with significant differences compared with controls at medium and high doses (P<0.05). Furthermore, the change in TOAC levels following UVB exposure exhibited an analogous trend to that of SOD activity.

Following co-culture with A375 cells, cellular SOD activity and TOAC levels in HaCaT cells significantly increased (P<0.01; Fig. 5C and D). A 93.5 and 106.15% increase were observed in SOD activity and TOAC levels compared with sole HaCaT cells, respectively (Fig. 5C and D). The results of the present study are summarized in Table I.

Discussion

UV irradiation is the 'gold standard' of therapies to treat patients with vitiligo. Phototherapy has been used to treat vitiligo since the 1800s and NB-UVB is the most frequently used method (30). It has been demonstrated that BB-UVA may be an alternative treatment method (6). Melanocytes are important in therapy for vitiligo and may be affected

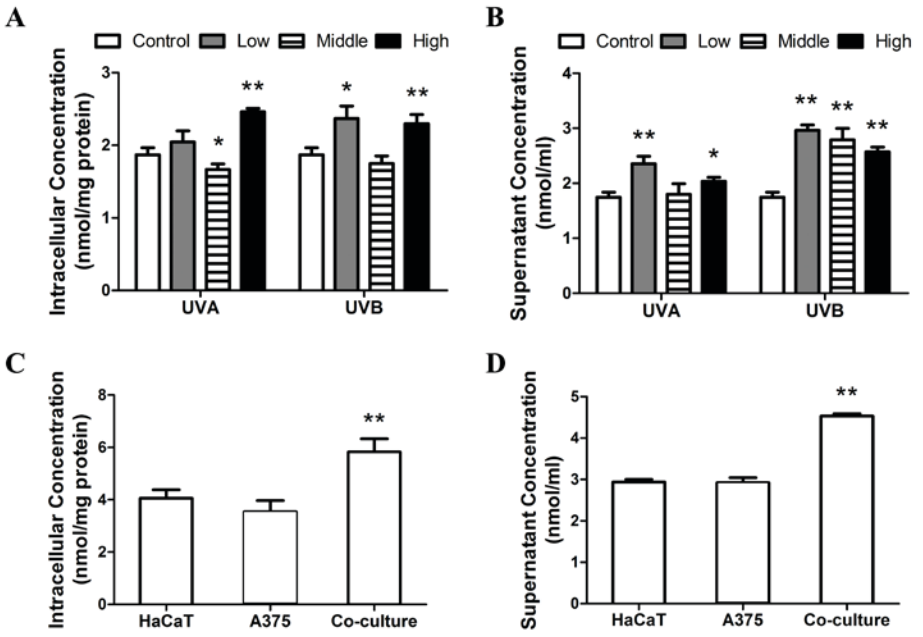

Figure 4. Effects of UVA/UVB/A375 treatment on the concentration of intracellular and supernatant MDA (n=3). (A) Intracellular MDA content at 24 h following low/middle/high doses of UVA and UVB irradiation. (B) Supernatant concentration of MDA 24 h following low/middle/high doses of UVA and UVB treatment. *P<0.05 and **P<0.01 vs. control. The concentration of (C) cellular and (D) supernatant MDA in HaCaT, A375. Values are presented as the mean ± standard deviation. **P<0.01 vs. HaCaT. UV, ultraviolet; MDA, malondialdehyde.

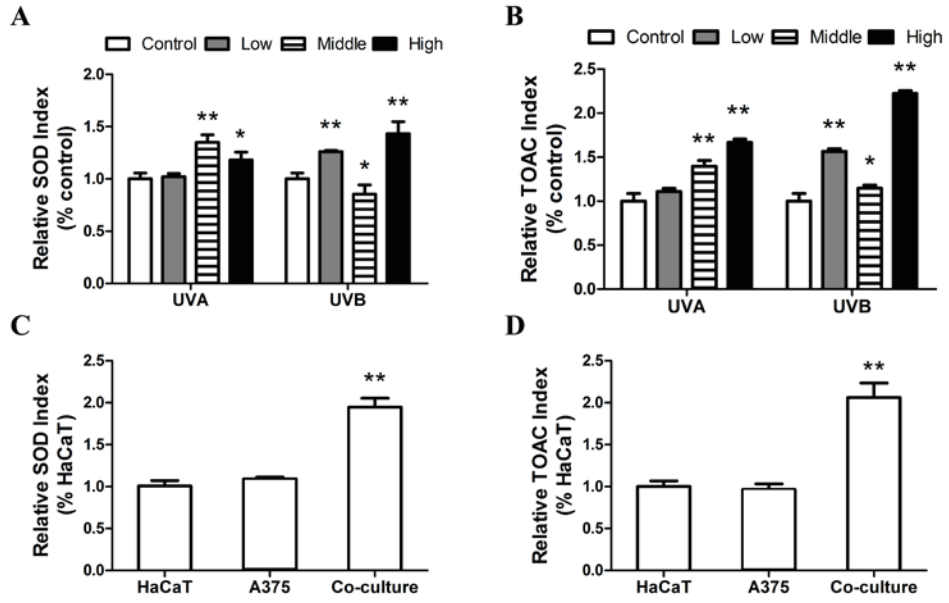

Figure 5. Effects of UVA/UVB/A375 treatment on the concentration of intracellular antioxidant level (n=3). (A) Intracellular SOD activities at 24 h following low/medium/high doses of UVA and UVB irradiation. (B) Cellular TOAC at 24 h following low/medium/high doses of UVA and UVB treatment. *P<0.05 and **P<0.01 vs. control. (C) SOD and (D) TOAC in HaCaT, A375 and co-cultured cells. Values are presented as the mean + standard deviation. **P<0.01 vs. HaCaT. UV, ultraviolet; SOD, superoxide dismutase; TOAC, total antioxidant capacity.

by various factors, including UV light, oxidation and keratinocytes (31,32). The association between melanocytes and keratinocytes is essential during the pathogenesis of vitiligo; however, few studies have focused on the effects of melanocytes on healthy keratinocytes. In the present study, keratinocyte HaCaT cells were treated with different clinical doses of BB-UVA or NB-UVB radiation and co-cultured with melanocyte A375 cells. The expression of PAR-2, Nrf2 and cellular antioxidant levels were examined to evaluate the effects of UV light and melanocytes on HaCaT. The present results demonstrated that UV radiation was able to inhibit cell proliferation, apart from low doses of UVA radiation. Medium doses (5 J/cm^2) of UVA radiation increased intracellular antioxidant levels in HaCaT cells and did not affect lipid peroxidation. However, medium or high dose UVB radiation promoted lipid peroxidation. Furthermore, treatment with

Table I. Effects of UVA and UVB irradiation, and A375 co-culture on keratinocytes.

	UVA			UVB			
Relative factors	Low	Med	High	Low	Med	High	Co-culture A375
Proliferation	0	-	-	-	-	-	+
PAR-2	+	0	0	+	-	0	+
Nrf2	0	+	+	-	+	0	-
Lipid peroxidation (supernatant)	+	0	+	+	+	+	+
SOD	0	+	+	+	-	+	+
TOAC	0	+	+	+	-	+	+

+, significant promotion vs. control; -, significant inhibition vs. control; 0, no significant difference vs. control. $P<0.05$ was considered to indicate a statistically significant difference. UV, ultraviolet; PAR-2, protease-activated receptor-2; Nrf2, nuclear factor E2-related factor 2; SOD, superoxide dismutase; TOAC, total antioxidant capacity; med, medium.

A375 co-culture induced a similar effect on lipid peroxidation in HaCaT cells as low dose UVB radiation.

UV radiation may induce intracellular mutations, which may in turn induce malignant transformation. The results of previous studies have suggested that environmentally relevant doses of UVA (>20 J/cm^2) irradiation may induce highly pernicious transformation, including anchorage-independent growth, the hypersecretion or overexpression of carcinogenic factors, and alterations in the morphology and apoptosis of keratinocytes (33,34). However, in the present study, clinical doses of UVA irradiation negative influences in HaCaT cells; they promoted the expression of Nrf2 and cellular antioxidant levels. Lehmann *et al* (35) previously identified that a 5 J/cm^2 dose of UVA did not impair cellular viability or DNA mutations. It has also been demonstrated that UVA irradiation promotes the expression of various cytoprotective genes, including HO-1 and Nrf2. Nrf2 mRNA expression and the intracellular antioxidant level exhibited a dose-dependent increase following UVA irradiation. This may be responsible for the generation of ROS following UVA radiation, which may mediate Nrf2 activation and its accumulation in the nucleus (36). Previous studies have demonstrated that increased Nrf2 mRNA expression occurs due to UVA-induced oxidative stress (37,38). Additionally, Marrot *et al* (39) elucidated that UVA radiation promotes the expression of phase2 enzymes, particularly heme oxygenase 1, in keratinocytes. These phase 2 enzymes are the main method by which cells inhibit ROS generation.

NB-UVB phototherapy is frequently used to treat vitiligo and is considered to be an effective method of treatment (40). However, previous studies have demonstrated that environmental doses of UVB radiation exposure may induce various cutaneous disorders (41,42). In the present study, various effects on keratinocytes were observed following exposure to UVB radiation. For example, the expression of Nrf2 mRNA was inhibited by low doses of UVB, but was significantly promoted by medium doses of UVB. However, levels of cellular antioxidants exhibited an opposing trend. Increased antioxidant levels may be a result of ROS generation induced by UVB radiation, whereas the antioxidant inhibition following exposure to medium doses of UVB may be associated with DNA damage. UVB has been demonstrated to cause the irreversible damage of DNA due to the formation of cyclobutane pyrimidine dimers, pyrimidine (6-4) pyrimidine photodimers and 8-hydroxy-2'-deoxyguanosine, which efficiently activates the p53 pathway (43). Faraonio *et al* (44) demonstrated that p53 is able to compete with Nrf2 on ARE-containing promoters, which inhibits the transcription of antioxidant response genes. Furthermore, the high expression of Nrf2 may be regulated by nuclear factor-κB, which may be activated by UVB irradiation (45).

Following co-culture with A375 cells, marked mutation was detected in HaCaT cells, which may be induced by the association between the two cell lines. The expression of PAR-2 mRNA was significantly elevated and Nrf2 expression was decreased, whereas the intracellular antioxidant levels were significantly increased. These results suggest that melanocytes may affect the regulation of oxidative stress in keratinocytes, and that the high expression of PAR-2 may promote the regulation of pigmentation and the phagocytosis of melanosomes. Joshi *et al* (46) previously demonstrated that a 'ligand-receptor' type interaction exists between melanocytes and keratinocytes and that this interaction may regulate pigment transfer by triggering intracellular calcium signaling in keratinocytes. Melanin is considered to be a key factor in protecting cells against the oxidative stress caused by UV irradiation. The accordant phenomena have also been identified following low dose UVA and UVB treatment in keratinocytes. Previous studies have demonstrated that low dose UV radiation is able to upregulate the expression of PAR-2 (47,48), which may be relevant to the overexpression of cytokines including interleukin-1 and tumor necrosis factor-α, caused by exposure to UV radiation (47). Therefore this pigment may affect the cellular secretion of cytokines or chemokines.

It was also demonstrated in the present study that the expression of PAR-2 mRNA was positively associated with MDA levels in the supernatant. This result illustrates that PAR-2 may regulate the permeability of the cellular membrane. Previous studies on PAR-2 have determined that PAR2 activation increases intracellular Ca^+ concentrations (49-51). Furthermore, melanocytes increase pigment transfer in keratinocytes by triggering intracellular calcium signaling (37). Therefore, PAR-2 may upregulate the absorption of melanin

in keratinocytes via a previously unknown method, which differs from Rho-dependent mediating phagocytosis in keratinocytes (52). Further studies are therefore required to further elucidate the association between PAR-2 and melanin.

In conclusion, the results of the present study demonstrate that clinical doses of BB-UVA and NB-UVB radiation induce varying effects on the proliferation of HaCaT cells and the expression of Nrf2 and PAR2. Co-culture with A375 induced similar effects as those of low dose UVA and UVB radiation. Therefore, the present study may provide novel therapeutic targets for the treatment of vitiligo; however further *in vitro* and *in vivo* studies are required.

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