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ORIGINAL ARTICLE

Superoxide Dismutase 1 Inhibits Alpha-Melanocyte Stimulating Hormone and Ultraviolet B-Induced Melanogenesis in Murine Skin

Chang Taek Oh[1,4,*], Dohyun Lee[2,*], Kyotan Koo[2], Jay Lee[3], Ho Sang Yoon[5], Yoo Mi Choi[6], Tae-Rin Kwon[1,4], Beom Joon Kim[1,4]

[1]*Department of Dermatology, Chung-Ang University College of Medicine,* [2]*Nutrex Technology R&D Center, Seoul,* [3]*Biomedical Research Institute, MEDIPOST Co., Ltd., Seongnam,* [4]*Department of Medicine, Graduate School, Chung-Ang University,* [5]*MB Business Development Team, Pacificpharma,* [6]*Cosmeceutical Team, Pacificpharma, Seoul, Korea*

Background: Over the last decade, the incidence of ultraviolet B (UVB)-related skin problems has increased. Oxidative stress caused by UVB induces the secretion of melanocyte growth and activating factors from keratinocytes, which results in the formation of cutaneous hyperpigmentation. Therefore, increasing the antioxidant abilities of skin cells is thought to be a beneficial strategy for the development of sunscreen agents. Superoxide dismutase 1 (SOD1) is an antioxidant enzyme that is known to exhibit antioxidant properties. **Objective:** The purpose of this study was to investigate the effect of SOD1 on alpha-melanocyte stimulating hormone (α-MSH) and UVB-induced melanogenesis in B16F10 melanoma cells and HRM-2 melanin-possessing hairless mice. **Methods:** The inhibitory effect of SOD1 on tyrosinase activity was evaluated in a cell-free system. Additional experiments were performed using B16F10 melanoma cells to demonstrate the effects of SOD1 *in vitro*, and HRM-2 melanin-possessing hairless mice were used to evaluate the antimelanogenic effects of SOD1 *in vivo*. **Results:** We found that SOD1 inhibited melanin production in a dose-dependent manner without causing cytotoxicity in B16F10 melanoma cells. SOD1 did not inhibit tyrosinase activity under cell-free conditions. The results indicate that SOD1 may reduce pigmentation by an indirect, non-enzymatic mechanism. We also found that SOD1 decreased UVB-induced melanogenesis in HRM-2 melanin-possessing hairless mice, as visualized through hematoxylin and eosin staining and Fontana-Masson staining. **Conclusion:** Our results indicate that SOD1 has an inhibitory effect on α-MSH and UVB-induced melanogenesis, indicating that SOD1 may be a promising sunscreen agent. **(Ann Dermatol 26(6) 681~687, 2014)**

-Keywords-
Alpha-MSH, HRM-2, Superoxide dismutase 1, Skin pigmentation, Ultraviolet radiation

INTRODUCTION

Ultraviolet (UV) radiation is divided into three categories: UVC (<290 nm), UVB (290~320 nm), and UVA (320~400 nm). Short-wavelength UVC and a portion of UVB are absorbed in the ozone layer of the atmosphere and do not reach the earth's surface[1]. Although the UVB that does reach the surface represents only 4% of the total environmental UV light[2], it has a stronger phototoxic effect than does UVA because DNA shows an absorption maximum at about 260 nm with an absorption tail in the UVB region. As a result, UVB has many adverse effects on human skin[3], including cancer[4,5], immunosuppression[6], erythema[7], hyperpigmentation[8], and photoaging[9].

Skin susceptibility to UV depends on several factors, for

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*These authors equally contributed as first authors.

Corresponding author: Beom Joon Kim, Department of Dermatology, Chung-Ang University Hospital, 102 Heukseok-ro, Dongjak-gu, Seoul 156-755, Korea. Tel: 82-2-6299-1525, Fax: 82-2-811-1159, E-mail: beomjoon@unitel.co.kr

example, melanin, which plays a key protective role. Two main types of melanin have been defined: eumelanin, a characteristic of darkly pigmented skin, and pheomelanin, which is mainly present in the epidermis of fair-skinned subjects. Eumelanin acts as a filter against UV irradiation and possesses scavenger properties toward UV-induced free radicals[10,11]. Pheomelanin is a less effective filter against UV irradiation and apparently acts as an endogenous photosensitizer[12,13]. Since at least some of the adverse effects of UV irradiation are related to the generation of free radicals[14,15], antioxidant protection is clearly an important factor in protecting the skin[16,17].

Melanogenesis has many important physiological functions, including photoprotection of human skin from UV irradiation[18]. Melanin synthesis[19] is stimulated by various molecules such as alpha-melanocyte stimulating hormone (α-MSH)[20] and cyclic adenosine monophosphate-elevating agents (including forskolin, glycyrrhizin, and isobutylmethylxanthine), UVB, and the placental total lipid fraction[21]. Melanin synthesis occurs in melanocytes and melanoma cells in an enzymatic process catalyzed by tyrosinase. Tyrosinase catalyzes the oxidation of L-tyrosine to 3,4-dihydroxyphenyl-L-alanine (L-DOPA), followed by the oxidation of L-DOPA to dopaquinone; oxidative polymerization of several dopaquinone derivatives then produces melanin. Thus, tyrosinase inhibitor is a candidate for reducing melanogenesis[22]. It has been reported that superoxide dismutase (SOD) is one of the key factors that reduces melanin production caused by UV irradiation[23].

We studied the inhibitory effects of copper- and zinc-dependent SOD1 on α-MSH-induced melanogenesis in B16F10 melanoma cells and in a UVB-treated melanin-possessing hairless mouse model. We show that SOD1 leads to antimelanogenesis effects through α-MSH-induced melanogenesis and UVB-induced melanogenesis in HRM-2 melanin-possessing hairless mice.

MATERIALS AND METHODS

Compounds and reagents

SOD1 was provided by Nutrex Technology Corporation (Seoul, Korea). Mushroom tyrosinase, α-MSH, L-DOPA, and phenylthiourea were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), trypsin-ethylenediaminetetraacetic acid, phosphate-buffered saline, and penicillin/streptomycin were purchased from WelGENE Biopharmaceuticals (Daegu, Korea). Fetal bovine serum (FBS) was purchased from Life Technologies (Gibco; Life Technologies, Grand Island, NY, USA).

Cell culture

B16F10 murine melanoma cells (CRL-6475) were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in 5% CO_2 at 37°C. Cells were passaged every 3 days until a maximum passage number of 20 was achieved.

Animals and skin color determination

Male, 4-week-old, HRM-2 melanin-possessing hairless mice (total = 9) were obtained from Hoshino Laboratory Animals (Saitama, Japan) and housed in an air-conditioned room maintained at 24°C ± 2°C and 55% ± 15% humidity with a 12-hours light-dark cycle. The mice were placed individually in separate cages and fed *ad libitum*. All procedures involving animals were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chung-Ang University in Korea (IRB Number: 13-0013). After a 1-week stabilization period, mice were randomly divided into three groups of three animals each. The mice were anesthetized with Zoletil 50 (50 mg/kg) and xylazine (10 mg/kg). SOD1 was dissolved in a vehicle consisting of ethanol and propylene glycol at a ratio of 3 : 7, and then topically applied to a designated site on the dorsal skin of the back of the animal once a day. Mice were exposed to UVB using an NB-UVB lamp (Dermalight Psoracomb UV-B-311nm-narrowband; National Biological Corp., Twinsburg, OH, USA) and a UV-B Narrowband PL-L/PL-S lamp (Philips, Eindhoven, Netherlands) at 190 mJ/cm^2. The experiment schedule is shown in Table 1[24,25]. The color of the skin sites was measured using a spectrophotometer (CR-10; Konica Minolta Sensing Inc., Osaka, Japan), and the colors are

Table 1. The experimental schedule of UVB irradiation in HRM-2 melanin-possessing hairless mice

	Day																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
SOD1	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
UVB (mJ/cm^2)			190		190		190		190		190		190		190	190	190

SOD1: superoxide dismutase 1, UVB: ultraviolet B.

described by L-values according to the Commission Internationale de l'Eclairage color system. The mice were killed and their skin was quickly removed and immediately frozen in liquid nitrogen and stored at −80°C.

Cell viability assay

B16F10 melanoma cells were seeded in 96-well plates (2×10^4 cells/well). After 24 hours incubation at 37°C, the medium was replaced with media containing SOD1 diluted to the appropriate concentration. Control cells were treated with distilled water (DW) at a final concentration of 0.1%. After 24 hours, the media containing the compounds or DW were replaced with media containing 10% EZ-CyTox (Daeil Lab Service, Seoul, Korea). The cells were then incubated at 37°C for 30 minutes, and the absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) reader (VERSAMax; Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 450 nm. All assays were performed in triplicate. The cytotoxic effect of each treatment was expressed as a percentage of cell viability relative to the untreated control cells.

Cell-free enzymatic assay for tyrosinase activity

Tyrosinase activity was assayed as a function of DOPA oxidase activity. A total of 70 μl of 0.1 M phosphate buffer (pH 6.8) containing SOD1 was mixed with 20 μl of 10 μg/ml mushroom tyrosinase in a well of a 96-well plate, and 10 μl of 10 mM L-DOPA was added. Absorbance values were measured every 10 minutes for at least 1 hour at 475 nm using an ELISA reader at an incubation temperature of 37°C. The quantity of dopachrome formed in the reaction mixture was determined against a blank (solution without enzyme) at 475 nm in the ELISA reader. The percentage of tyrosinase activity was calculated as follows: tyrosinase activity (%) = [(A − B)/(C − D)] × 100, where A is the absorbance of the reaction mixture containing the test sample and mushroom tyrosinase, B is the absorbance of the blank sample containing the test sample but no mushroom tyrosinase, C is the absorbance of the reaction mixture without the test sample and with mushroom tyrosinase, and D is the absorbance of the well lacking both the test sample and mushroom tyrosinase (L-DOPA alone).

Measurement of melanin content

B16F10 melanoma cells were incubated at a density of 1×10^5 cells in 6-well plates overnight. The α-MSH (1 μm) was then added, and the cells were treated with increasing concentrations of substance in phenol red-free DMEM for 3 days. Two hundred-microliter aliquots of medium were then placed in 96-well plates, and the optical density of each culture well was measured using an ELISA reader at 405 nm. The data were normalized to the protein content of the cell lysates. The cell lysates were subsequently processed for determining the protein concentration using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Melanin production was expressed as the percentage of living cells compared to the controls.

Histological analysis

Skin samples were fixed in 4% paraformaldehyde overnight at room temperature and stained for melanin using a Fontana-Masson staining kit (American Mastertech Inc., Lodi, CA, USA) according to the manufacturer's instructions. Briefly, sliced skin samples were stained with ammoniacal silver solution for 60 minutes at 60°C, followed by incubation in 0.1% gold chloride and then in 5% sodium thiosulfate. The tissue was also stained with hematoxylin and eosin for morphological analyses. Staining was examined under a phase-contrast microscope (Eclipse TS100; Nikon Instruments Inc., Melville, NY, USA).

Statistical analysis

Data are presented as mean ± standard deviation, and statistical comparisons between treated groups and the untreated group were performed using one-way ANOVA followed by *post hoc* Tukey tests for direct comparison between specific groups (PASW Statistics ver. 18.0; IBM Co., Armonk, NY, USA). $p<0.05$, $p<0.01$, and $p<0.001$ were considered statistically significant.

RESULTS

Effect of superoxide dismutase 1 on cell viability in B16F10 melanoma cells

To determine if SOD1 could inhibit melanogenesis, B16F10 melanoma cells were treated with the SOD1 before α-MSH stimulation. We first tested the cytotoxicity of SOD1 in B16F10 melanoma cells using the EZ-CyTox assay. B16F10 melanoma cells were treated with SOD1 at concentrations of 1 to 1,000 ng/ml for 24 hours. SOD1 was found to have no cytotoxic effect on B16F10 melanoma cells at the concentrations used (Fig. 1).

Effects of superoxide dismutase 1 on tyrosinase activity in B16F10 melanoma cells

To investigate the direct effects of SOD1 on tyrosinase activity, we measured the tyrosinase activities of mushroom tyrosinase in a cell-free system, as described in the Materials and Methods. However, SOD1 was found to have no direct inhibitory effect on mushroom tyrosinase activity at concentrations ranging from 1 to 1,000 ng/ml (Fig. 2).

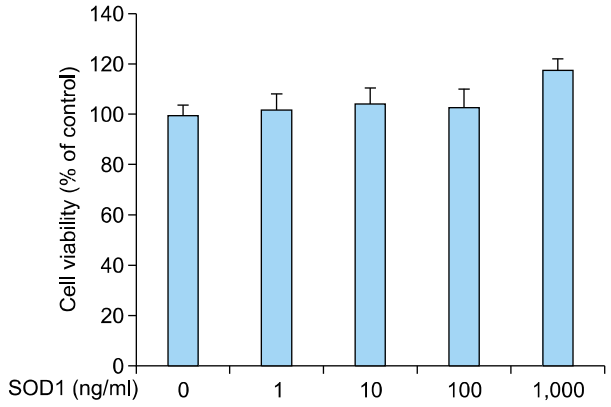

Fig. 1. Cell viability of B16F10 melanoma cells treated with superoxide dismutase 1 (SOD1) at different concentrations. Cells were treated with SOD1 at 1 to 1,000 ng/ml for 24 hours. Cell viability was determined using EZ-CyTox assays. Results are expressed as the percent viability relative to controls. Most of the B16F10 melanoma cells were viable at concentrations in the range of 1 to 1,000 ng/ml. Each measurement was made in triplicate and data represent the mean±standard deviation.

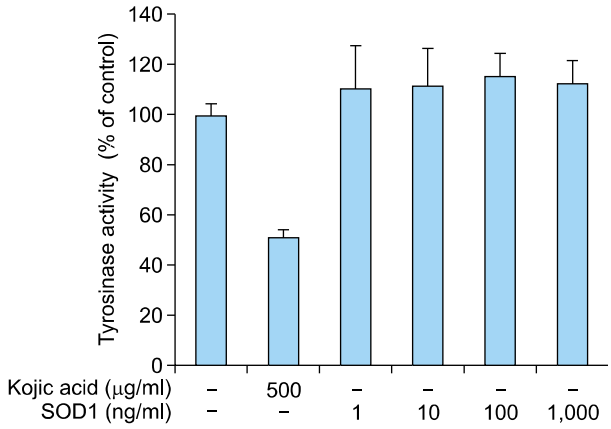

Fig. 2. Inhibitory effects of superoxide dismutase 1 (SOD1) on tyrosinase activity in cell-free assays. Mushroom tyrosinase was treated with SOD1 at 1 to 1,000 ng/ml. SOD1 did not appear to inhibit mushroom tyrosinase activity. Each measurement was made in triplicate and data shown represent the mean±standard deviation.

Effect of superoxide dismutase 1 on melanogenesis in B16F10 melanoma cells

The effect of SOD1 on the melanogenesis of B16F10 melanoma cells was examined. To determine whether SOD1 inhibits α-MSH-induced melanin synthesis in B16F10 melanoma cells, we measured the α-MSH-induced melanin production of B16F10 melanoma cells after 3 days of SOD1 treatment at 10 to 1,000 ng/ml. SOD1 considerably inhibited melanin synthesis when used at concentrations greater than 10 ng/ml (Fig. 3). SOD1 treatment also reduced α-MSH-induced melanin release in a dose-dependent manner.

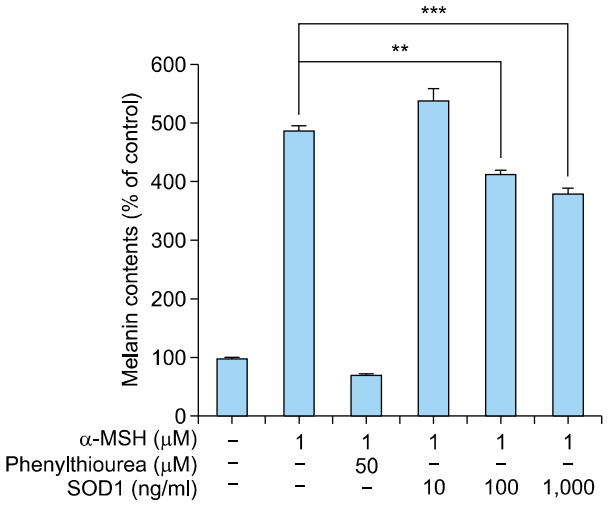

Fig. 3. Effect of superoxide dismutase 1 (SOD1) on melanin content in B16F10 melanoma cells. SOD1 was tested at 10, 100, and 1,000 ng/ml in B16F10 melanoma cells. B16F10 melanoma cells were co-cultured with alpha-melanocyte stimulating hormone (α-MSH) (1 μm) and SOD1 at 10 to 1,000 ng/ml for 72 hours, and compared with untreated and α-MSH-stimulated control cells. Levels of inhibition of melanogenesis are expressed as percentages of the control. The results are averages of three independent experiments and the data are expressed as mean±standard deviation. Inhibition of melanin content was related to exposure to SOD1 in a dose-dependent manner. $^{**}p<0.05$, $^{***}p<0.001$ as compared with α-MSH-treated controls.

Inhibitory effect of superoxide dismutase 1 on ultraviolet B-induced melanogenesis in HRM-2 melanin-possessing hairless mice

The effect of SOD1 on skin pigmentation was examined *in vivo* in HRM-2 melanin-possessing hairless mice treated according to the schedule shown in Table 1. Treatment of animals with SOD1 dissolved in vehicle did not cause any noticeable skin irritation. Exposure of mice to UVB led to decreased △L (lightness) values, which represented a pigmentation effect. The UVB-induced changes in △L (lightness) values were significantly reversed in SOD1-treated animals, and the color properties of the skin of SOD1-treated animals were comparable to those of the control groups (Fig. 4). The pigmentation pattern was photographed using a Dermoscope (DermLite Pro, California, USA) and a Folliscope (LeedM, Seoul, Korea) (Fig. 4). In addition, SOD1 was found to have a protective effect on UVB-induced epidermal and dermal changes (Fig. 5). To visualize the melanin, the skin samples were stained with Fontana-Masson stain and examined. As shown in Fig. 6, the amount of melanin granules was significantly increased by UVB irradiation (arrows in Fig. 6B). Topical

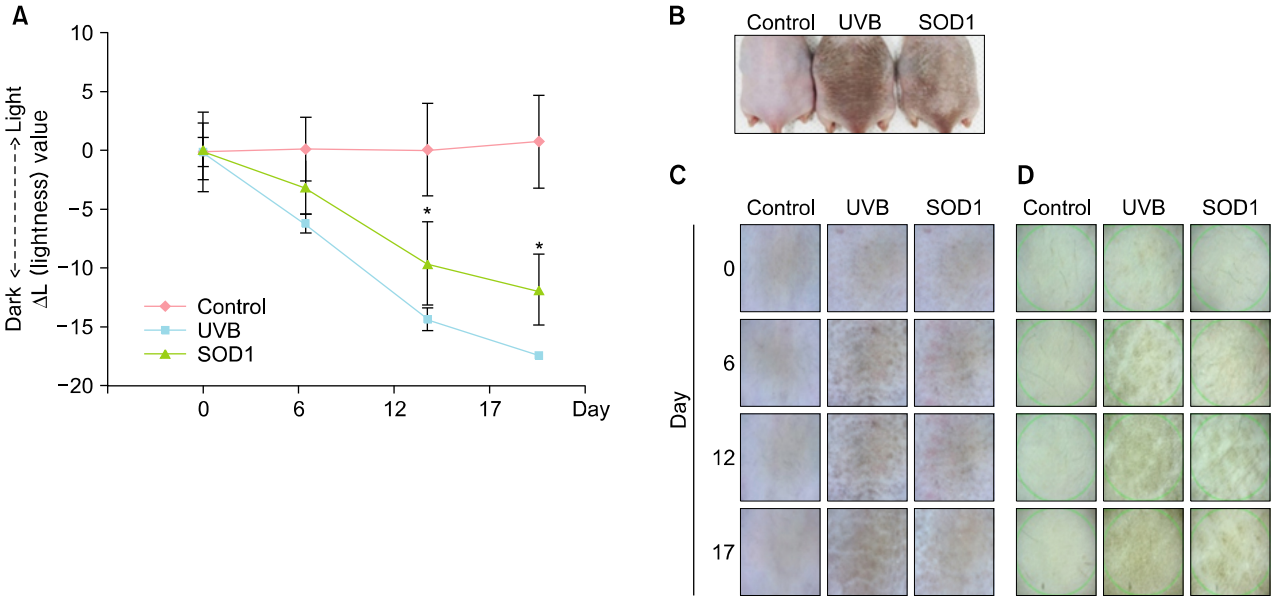

Fig. 4. Effect of superoxide dismutase 1 (SOD1) on the level of ultraviolet B (UVB)-induced melanogenesis in HRM-2 melanin-possessing hairless mice. HRM-2 melanin-possessing hairless mice were pretreated with vehicle or SOD1 on a designated site on the dorsal skin and then UVB-irradiated according to the indicated schedule. The picture represents the dorsal skin of the HRM-2 melanin-possessing hairless mice after treatment. For statistical analysis, △L (lightness) values of each animal were calculated as the average values after UVB exposure (day 17) minus the average baseline values before any treatment (day 0). Values represent the mean±standard deviation of five experiments. For statistical analysis, results of one-way ANOVA were used: $*p<0.05$, as compared with UVB-treated controls. (A) △L (lightness) values. (B) D3200. (C) Dermoscope. (D) Folliscope.

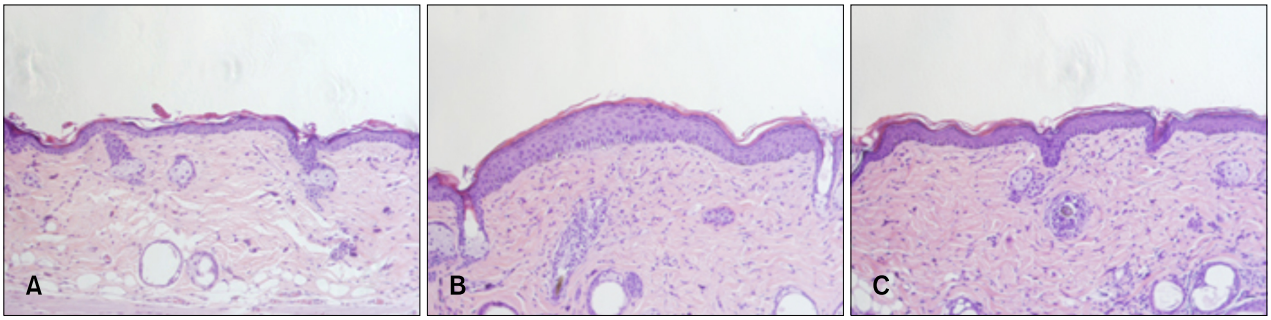

Fig. 5. Effect of superoxide dismutase 1 (SOD1) on ultraviolet B (UVB) irradiation-induced histopathological changes in skin tissue. Change in histopathological features of the skin after repeated treatment with SOD1. Representative histopathological images of UVB-irradiated skin lesions stained by H&E (×200). (A) Control. (B) UVB 190 mJ/cm^2. (C) 1,000 ng/ml SOD1+UVB 190 mJ/cm^2.

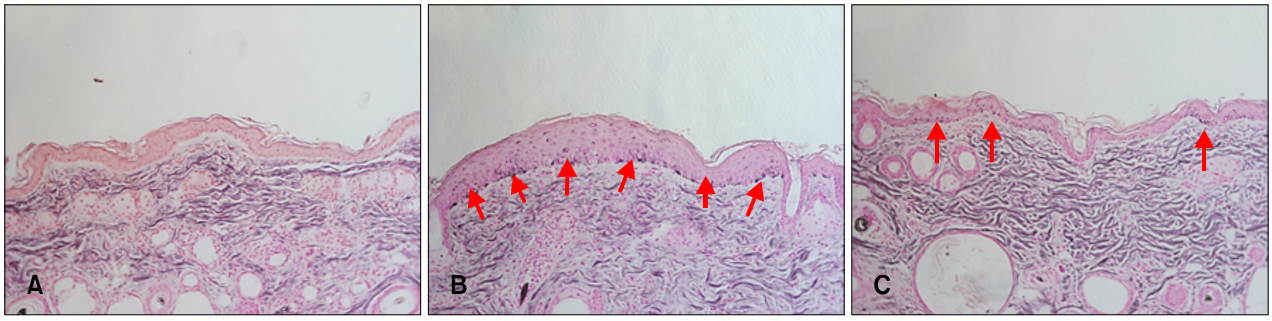

Fig. 6. Effect of superoxide dismutase 1 (SOD1) on ultraviolet B (UVB) irradiation-induced hyperpigmentation changes in skin tissue. Change in histopathological features of the skin after repeated treatment with SOD1. Representative histopathological images of UVB-induced skin pigmentation stained by Fontana and Masson (×200). Melanin pigments (arrows) are stained black. (A) Control. (B) UVB 190 mJ/cm^2. (C) 1,000 ng/ml SOD1+UVB 190 mJ/cm^2.

application of SOD1 inhibited the UVB-induced increase in melanin generation (Fig. 6C).

DISCUSSION

Exposure of skin to UV irradiation generates reactive oxygen species that damage DNA, membranes, mitochondria, and proteins. To protect against such damage, skin cells have evolved antioxidant enzymes, including SOD1, glutathione peroxidase, mitochondrial manganese-dependent superoxide dismutase (SOD2), and catalase. It has been reported that SOD1 is significantly suppressed by a single dose of UVB irradiation[26], whereas it is induced by repeated exposure to UVB in response to chronic photooxidative stress[27]. The UVB-mediated melanogenic response of melanocytes and melanoma cells is complex and remains unclear. While melanin is believed to exert photoprotective effects on human skin under physiological conditions, overstimulation of melanin production could be harmful and could possibly contribute to melanoma development; the biological implication of melanogenesis should thus be taken into account[28]. Since excess melanin under UVB-induced oxidative stress may become pro-oxidant and carcinogenic, blocking the aggravation of melanogenesis might therefore serve as a potential target for the prevention of hyperpigmentation and melanoma development induced by UVB[29].

Hyperpigmentation is characterized by an abnormal increase in melanin in the skin[30,31]. Recently, much effort has been focused on understanding the mechanisms underlying melanogenesis in order to develop new therapeutic agents against skin pigmentation abnormalities. Tyrosinase is a key enzyme in melanogenesis and plays a regulatory role in the production of melanin[32]. In general, assays for mushroom tyrosinase have been applied to screening for putative depigmenting agents by assessing their abilities to inhibit tyrosinase-mediated $_{L}$-DOPA oxidation[33]. SOD1 did not directly inhibit tyrosinase activity, indicating its involvement in alternative mechanisms (Fig. 2). These results suggest that reduced pigmentation by SOD1 might be attributed to the effect of SOD1 upon the signaling pathways regulating tyrosinase.

Melanin pigments produced in human melanocytes are classified into two categories: black-colored eumelanin and reddish-yellow pheomelanin. Stimulation of melanocytes with α-MSH, one of several melanogenic factors, has been reported to enhance eumelanogenesis to a greater degree than pheomelanogenesis, which contributes to hyperpigmentation. This study demonstrates the potent inhibitory effect of SOD1 on melanogenesis in α-MSH-induced B16F10 melanoma cells (Fig. 3). The inhibitory effects of SOD1 were dose-dependent and did not incur significant cytotoxicity (Fig. 1).

This study is valuable in its discovery that SOD1 exhibited consistent antimelanogenic activity in both *in vitro* and *in vivo* models. We used a murine skin model, since it is difficult to test the antimelanogenic effects of novel compounds *in vivo* in humans. The HRM-2 melanin-possessing hairless mouse, which was used in our study, was generated from a cross between HR-1 hairless mice, which have an albino phenotype, and the C3H/He strain produced by the Hoshino laboratory in 2007. The HRM-2 melanin-possessing hairless mouse is a widely used animal research model that can be used for the measurement of skin color, wrinkle formation, and damage, without requiring hair removal in long-term experiments.

In conclusion, our data showed that SOD1 suppressed α-MSH-induced melanogenesis. We propose that SOD1 prevents abnormal hyperpigmentation induced by UVB and may be a promising sunscreen agent for preventing esthetic problems related to hyperpigmentation.

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