Melanin is the predominant contributing to skin pigmentation, and is also found in the hair, eyes, ears, and brain. A primary function of melanin is to counteract skin damage caused due to ultraviolet radiation (UVR) (Moon et al., 2012). Since UVR exposure causes intense and extensive damage to the skin, the function of melanin is very important. Melanin acts as a shield for our skin yet its abundance in the body can be carcinogenic and could potentially lead to malignancy. In recent years, resolving problems caused by UV irradiation has become imperative.

Melanin is produced by melanocytes in the basal layer of the epidermis. Melanocytes contain granules within the cytoplasm called melanosomes. These melanosomes have melanin pigments, which can be transferred out to adjacent keratinocytes in the epidermis (Riley, 1997). Melanin synthesis, or melanogenesis, is affected by many enzymes and is induced by the α-melanocyte-stimulating hormone (α-MSH) and UVR. Tyrosinase is the predominant enzyme in melanogenesis, as it is involved in two melanin synthesis processes. The first step is the conversion of tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA), which is subsequently converted to dopaquinone (Gillbro and Olsson, 2011). Melanin is a polymer synthesized by tyrosinase-related protein (TRP)-2 that converts DOPAchrome to 5,6-dihydroxyindole 2-carboxylic acid (DHICA). One of another enzymes, TRP-1, can oxidize DHICA to indole-5,6-quinone carboxylic acid. Thus, they regulate the synthesis of brownish 5,6-dihydroxyindole-2-carboxylic acid-rich melanins. These proteins are activated by microphthalmia-associated transcription factor (MITF), the key transcriptional regulator in melanogenesis (Bertolotto et al., 1998; Lin et al., 2002). In this way, the melanin synthesis-related enzyme tyrosinase, TRP-1, TRP-2 and its transcription factor MITF are necessary for
regulating melanogenesis. Therefore, efforts have increased to find the regulatory agents of these melanogenic proteins (Kobayashi et al., 1994).

Resveratrol (3',4',5'-trihydroxy-trans-stilbene) is a polyphenolic compound found in various natural products such as grapes and berries and has been reported to possess biological activities such as antioxidation (Goldberg, 1996), anticancer (Song et al., 2010; Del Folio-Martinez et al., 2013), anti-inflammation (Gatson et al., 2013), cardioprotection (Osman et al., 2013), and anti-aging (Marchal et al., 2013). Moreover, degemination effects of resveratrol has recently also been demonstrated in human melanocytes by transcriptional regulation of melanogenic genes (Newton et al., 2007). Notably, resveratrol inhibits mRNA expression of tyrosinase, TRP-1, MITF, and DCT in human melanocytes. However, it is unknown whether topical application of resveratrol to the dorsal skin of brownish guinea pigs in vivo prevents ultraviolet B (UVB)-induced hyperpigmentation. To make sure the whitening effects of resveratrol, our study was conducted to focus on animal experiments using UVB-irradiated brownish guinea pigs in vivo models. In addition, the whitening efficacy of resveratrol was confirmed in α-MSH-stimulated B16 melanoma cell line.

MATERIALS AND METHODS

Cell culture

B16F10 mouse melanoma cells were cultured in Dulbecco’s modified eagle medium (DMEM) with 10% fetal bovine serum and penicillin/streptomycin in air containing 5% CO2 at 37°C.

Measurement of melanin contents

The melanin content was determined according to the modified methods of Hosoi et al. (1985) (Hosoi et al., 1985). B16F10 cells were cultured at 5×10^4 cells/well in 48-well plates. After 24 h, the cells were stimulated with α-MSH 100 ng/ml. At the same time, various concentrations of resveratrol (1, 10, and 100 μM) treated for 48 h. After washing with phosphate buffered saline (PBS), the cells were harvested by trypsinization. The cell pellet was solubilized in 200 μl of 1 N NaOH. The absorbance of each well was measured at 405 nm using a spectrophotometer.

Measurement of cell viability

The cell proliferation assay was carried out using 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide (MTT). B16F10 cells were cultured in 5×10^4 cells/well in 48-well plates. After 24 h, the cells were treated with various concentrations of samples (1, 10, and 100 μM) for 48 h. At the end of the incubation, 50 μl of MTT solution (1 mg/ml in PBS) was added to each well. After incubation at 37°C for 1 h, the medium was gently removed and 150 μl of dimethyl sulfoxide (DMSO) was added. The absorbance of each well was measured at 570 nm using a spectrophotometer.

Animals

Five-week-old male guinea pigs (KIWA:A1) (weight, 272-297 g; n=4) were obtained from Japan Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan). The experimental protocol for this study was approved by the Institutional Animal Care and Use Committee of Korea Conformity Laboratories (IA13-00229). The guinea pigs were housed in a temperature- and humidity-controlled room (22 ± 1°C, 60 ± 5% humidity) with 12 h light/dark cycles. After 1 week of quarantine, the guinea pigs were acclimated to individual cages. During the experimental period, food and water were given ad libitum.

Ultraviolet B irradiation-induced hyperpigmentation

The UV source was supplied by a closely spaced array of five Sankyo Denki sun lamps, which have peak irradiance at 310 nm (Kanagawa, Japan). Bulbs were positioned 15 cm above the guinea pigs. The irradiation (0.1 mW/cm²) was measured with an IL1700 Research Radiometer (International Light, Inc., Newburyport, MA, USA) equipped with a UVB sensor. The guinea pigs were exposed to 390 mJ/cm² UVB radiation three times per week for two weeks. UVB irradiation was applied to the dorsal skin of the guinea pigs after hair removal.

Sample treatment in guinea pigs

Resveratrol was dissolved in a mixture of ethanol and propylene glycol (3:7, v/v). The sample solution was applied topically to the dorsal skin once a day for 12 days after the final UVB treatment. Post treatment, 1% resveratrol (200 μl; 10 mg/ml) was applied to 2 cm² of skin.

Fontana-Masson stain

To analyze changes in the melanin content of the skin tissue, the guinea pigs were sacrificed after the final treatment and biopsies were obtained from the dorsal skin, which were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Sections, approximately 4 μm thick, were stained with Fontana-Masson staining solution (IHC WORLD, GA, USA). The stained slides were examined under a light microscope.
Western Blot analysis

Changes in the levels of proteins related to melanin production, including tyrosinase, TRP-1, TRP-2, and MITF in the B16F10 cells and skin of guinea pigs were evaluated by Western blotting. Prepared samples were homogenized and lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors and was centrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was collected and assayed for protein concentration using the protein assay kit (Bio-Rad, CA, USA). Lysates containing 40 μl of protein were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% resolving and 3% acrylamide stacking gel and were transferred to nitrocellulose sheets. Blocking was performed in tris-buffered saline containing 5% skim milk powder and 0.1% Tween-20. The primary antibodies used for Western blotting include mouse anti-α-tubulin antibody, goat anti-tyrosinase, and goat anti-TRP-1 and -TRP-2 (Santa Cruz Biotechnology Inc., CA, USA), and mouse anti-MITF (Neomarkers, CA, USA). Finally, immune complexes were detected with a ChemiDocXRS+ imaging system (Bio-Rad, CA, USA).

Statistical analysis

Data were expressed as mean ± SD from at least three independent experiments. Student's t-test was used for statistical analyses, *p<0.05 and **p<0.01 indicate statistically significant differences compared to the normal. *p<0.05, **p<0.01 and ***p<0.001 indicate statistically significant differences compared to the control.

RESULTS

Resveratrol decreases melanin production in cultured B16F10 cells

In Fig. 1B, the melanin contents were increased in α-MSH (100 ng/ml)-stimulated control cells as compared to the non-stimulated normal cells. However, resveratrol treatment significantly decreased melanin production by 28.2 ± 1.9% without cell toxicity at 100 μM after 48 h treatment in α-MSH stimu-
Resveratrol down-regulates the expression of melanogenic proteins in B16F10 cells

Chen et al. reported that resveratrol inhibits melanogenesis in B16 melanoma cells (Chen et al., 2013). However, this paper focused on regulation of skin cancer such as melanoma rather than melanogenesis. They studied effects of resveratrol on α-MSH signal transduction involving Wnt/β-catenin, c-Kit, and MITF. Melanogenesis mainly depends on regulation of melanogenic proteins such as tyrosinase, TRP-1, and TRP-2. Therefore, we performed Western blot analysis to determine whether the inhibitory effects of resveratrol are related to the regulation of melanogenesis-related proteins. As shown in Fig. 2, the protein expression of tyrosinase, TRP-1, TRP-2, and MITF were increased by α-MSH treatment. But, resveratrol led to a significant decrease in α-MSH-induced melanogenic protein levels at 100 μM. Based on data shown in Fig. 2, we confirmed the inhibitory effects of resveratrol on melanin synthesis via regulation of melanogenic proteins such as tyrosinase, TRP-1, TRP-2, and MITF by Western blot analysis in skin tissue. As seen in Fig. 4, the expression of tyrosinase, TRP-1, TRP-2, and MITF were increased by UVB exposure. However, the protein levels of tyrosinase, TRP-1, TRP-2, and MITF were decreased by resveratrol treatment. Thus, we confirmed that resveratrol regulated melanogenesis-related protein in the dorsal skin of UVB-irradiated guinea pigs. In addition, we assessed melanin production in the skin tissues by the Fontana-Masson staining technique. Melanin synthesis of skin epidermis was increased by UVB exposure. Based on the histological study in Fig. 5, resveratrol treatment decreased melanin production, but not to the levels observed for the normal group.

DISCUSSION

Recently, interest in the various activities of resveratrol has risen along with the well-being trend. Resveratrol is widely present in natural sources such as grapes, berries, and nuts and possesses various activities including anticancer properties, extension of lifespan (Baur and Sinclair, 2006), and anti-hyperpigmentation (Wu et al., 2012). Depigmentation agents commonly have similar phenol-based structures, which can inhibit tyrosinase activity (Newton et al., 2007). Resveratrol also has a phenol-based structure, and several in vitro studies have reported that resveratrol originated from various natural products, which inhibit melanin synthesis (Ohguchi et al., 2003; Yanagihara et al., 2012). Shin et al. reported that oxyresveratrol, found in natural sources such as Morus alba L., suppressed the dopa oxidase activity of tyrosinase (Shin et al., 1998). Moreover, various resveratrol analogs demonstrated tyrosinase inhibitory activities (Franco et al., 2012). In addition, resveratrol suppresses not only melanin production but also cell viability and invasiveness of melanoma (Chen et al., 2013). However, Chen et al. focused on inhibition of skin cancer yet this study did not investigate changes in melanogenesis-related proteins such as tyrosinase, TRP-1, and TRP-2. Therefore, in this study we investigated the effects of resveratrol on melanogenesis-related proteins by Western blot analysis. Our study shows that resveratrol significantly suppresses expression of all melanogenic protein such tyrosinase, TRP-1, TRP-2, and MITF in α-MSH stimulated B16F10 melanoma cells. Although it has been previously reported that resveratrol inhibits overexpressed MITF level by α-MSH stimulation, we additionally confirmed that resveratrol reduced the expression of tyrosinase, TRP-1, and TRP-2, which are typical proteins involved in melanogenesis. We found that this process involves a decrease in intracellular melanogenic enzymes at the protein level.

Although previous studies have reported the inhibitory role of resveratrol in vitro, depigmentation effects of resveratrol in vivo, UVB-induced brownish guinea pigs model, one of the most authentic animal models, have not yet been determined. In addition, there is no report on the effect of resveratrol on the
expression of melanogenesis-related proteins in the dorsal skin tissue by western blotting analysis. In this study, UVB-induced dorsal skin of guinea pigs visually changed from black to brown color, and pigmentation values were increased from 44.9 ± 0.8 to 58.1 ± 4.4 after the final tanning treatment. After UVB exposure, the color change value of the dorsal skin was gradually increased for up to 9 days, possibly because UVB stimulated-melanocytes continued to produce melanin (Fig. 3B). However, melanin production was steadily suppressed by resveratrol treatment. Moreover, there were no side effects such as erythema, edema, and itching during all the experiment. As shown in vitro, the molecular mechanisms of resveratrol for depigmentation involve inhibition of the expression of melanogenesis-related proteins. Melanin production was also decreased in the skin tissue, as shown by a histological study (Fig. 5). These findings seem that resveratrol directly affects melanocytes located in the epidermis after penetration. Given the in vivo efficacy of resveratrol, although resveratrol

Fig. 4. Effects of resveratrol on the expression of melanogenesis-related proteins in the skin of guinea pigs. Forty micrograms of total protein from the skin tissue lysates were used for Western blot analysis (A). For Western blot analysis, the data are represented as the relative density of tyrosinase (B), TRP-1 (C), TRP-2 (D) and MITF (E) bands normalized to α-tubulin.

Fig. 5. Results of Fontana-Masson stain for melanin in brown guinea pig skins. (A) UVB negative group, (B) UVB positive group, (C) UVB positive and 1% resveratrol treated group. Arrow heads indicate melanin.
is relatively polar substance, it seems that resveratrol is somewhat absorbed by the skin. All data indicate that resveratrol potentially decreases melanin synthesis via regulation of expression of melanogenic proteins in the skin exposed to UVB irradiation by Western analysis. Our study suggests that resveratrol significantly inhibits melanin synthesis in vitro and in vivo. These results also support the fact that resveratrol may be a potential skin whitening agent for pharmaceutical and cosmetic use. Further studies are required for clinical and toxicological investigation of resveratrol in the field of skin depigmentation.

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