Quercetin Stimulates Melanogenesis in Hair Follicle Melanocyte of the Mouse

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Quercetin (3, 3', 4', 5, 7-pentahydroxylflavone) is one of the representative flavonoids and is present in many vegetables and fruits. We studied the effects of quercetin on melanin production in hair follicle tissues from the buccal region of C3H/HeN Jel mice. These follicle tissues synthesized larger amounts of melanin than control tissue, with the amount dependent on the concentration of added quercetin. Additionally, the expression of tyrosinase protein was significantly enhanced in proportion to increases in the concentration of quercetin added. Nevertheless tyrosinase mRNA expression was not changed. In addition, tyrosinase-related protein-2 (TRP-2), which is a melanogenic enzyme, was increased depending on the concentration of added quercetin but its mRNA expression was not altered. These results show that quercetin stimulates the synthesis of tyrosinase protein as well as TRP-2 protein, thereby enhancing melanin producibility in hair follicle tissues from the buccal region of C3H/HeN Jel mice.

Key words: Quercetin, hair follicle, tyrosinase, melanin, melanogenesis

INTRODUCTION

Flavonoid is a collective term for chemical compounds that have a structure of two phenyl groups (rings A and B) bound to one another via 3 carbon atoms [1–4]. Over 4000 kinds of flavonoids have been discovered, being classified into 5 groups: flavon, flavonol, flavanon, flavanonol and isoflavon. Flavonoids are known to possess various bioactive actions such as anti-oxidization, anti-inflammation and anti-tumor functions. Their useful pharmacological properties are now drawing attention.

The skin and hair of the mammal are colored by melanin pigment. Melanin is a generic name for eumelanin, pheomelanin, etc. which are synthesized from tyrosine via a series of enzymic/non-enzymic oxidative, decarboxylation and coupling reactions. Melanogenesis occurs in melanosomes harbored by melanocytes and tyrosinase participants in melanogenesis as is widely known [5, 6]. It is now known that two enzymes, which are collectively called tyrosinase-related protein (TRP) and belong to the tyrosinase gene family, play a pivotal role in melanogenesis in addition to tyrosinase.

Quercetin, which is one of the representative flavonoids, was reported to inhibit mushroom tyrosinase in an *in vitro* experimental system [1]. Meanwhile, it was unclear what actions the flavonoid exerted on tyrosinase and melanogenesis at the cellular level. We examined the effects of quercetin on tyrosinase activity and melanogenesis using a human melanoma cell line of vagina II (HMV-II) and a three-dimensional epidermal model [7, 8].

As a result, it was clarified that quercetin promotes melanogenesis at the cellular level. However, details of the melanogenic action of quercetin in the hair remained unclear. We therefore conducted this study to explore the stimulating effects of quercetin on melanogenesis and its mechanisms of action in hair follicle tissues from the buccal region of C3H/HeN Jel mice.

MATERIALS AND METHODS

Reagents

Quercetin and Williams medium E were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hydrocortisone and 0.01 M pH 6.8 phosphate-buffered saline (PBS) were purchased from the Kurabo Company (Osaka, Japan). Penicillin - streptomycin and trypsin-EDTA were obtained from Gibco (Carlsbad, CA, USA). Dimethylsulfoxide (DMSO) was purchased from Merck & Company (Darmstadt, Germany).

Experimental animals

Seventeen-day-old C3H/HeN Jrc mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). All animal experiments were approved by the Animal Experimentation Committee, Isehara campus (Tokai University, Kanagawa, Japan).

Isolation and culture of mouse hair follicles

Hair follicles were isolated from C3H/HeN Jrc 17-day-old mice by the method of Green et al [9]. Skin was excised from the buccal region of the face and washed in phosphate-buffered saline (PBS), after which fat tissue was carefully removed from the skin

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under stereomicroscopy. Individual hair follicles were gently removed from the buccal region skin. Isolated hair follicle cells were cultured in William's E serum free medium supplemented with 10 μl/ml insulin, 10 ng/ml hydrocortisone, 100 unit/ml penicillin and 10 μl/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37℃ for either 7 or 14 days. After approximately 24 h, fresh medium and quercetin were added, and cells were harvested 7 or 14 days after drug was added. Tested quercetin was dissolved in dimethylsulfoxide (DMSO). The final DMSO concentration was 0.1% in all experiments (including control) and had no measurable effect on hair follicles.

Melanin assay

The melanin assay was performed using a procedure described previously [10]. After washes in PBS, hair follicles were detached by trypsin/EDTA (0.05%/0.02% in PBS). An aliquot was used for cell count. The remaining cells were sonicated and incubated overnight in 100 μl 1 M NaOH. Melanin concentration was calculated by comparison of the OD at 475 nm of unknown samples with a standard curve obtained with synthetic melanin.

Fontana-Masson silver stain

Fontana-Masson silver stain was performed as follows [11]. Fontana silver solution was made of 10% silver nitrate in aqueous solution and 28% ammonium hydrate, which was added drop by drop until a faint opalescence appeared. The ammoniated silver nitrate solution was mixed with distilled water and filtered. Paraffin embedded hair follicle sections were deparaffinized with xylene and rehydrated in ethanol. The ammoniated silver nitrate solution was applied to the deparaffinized sections, which were stored overnight at room temperature in the dark. After washing with distilled water, the sections were fixed with 0.25% sodium thiosulphate for 5 min.

Immunoblotting

Cells were scraped into 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100, 1 mM PMSF, 1 mg/ml aprotinin, and 10 μg/ml leupeptin. Homogenates were vortexed for 1 h at 4℃ and centrifuged at 12,000g for 15 min, and the total protein content of the supernatant was determined by a protein assay kit (Bio-Rad, Hercules, CA, USA). Samples (50 μg protein) were boiled in sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 10% 2-mercaptoethanol) and separated by SDS-PAGE (10% polyacrylamide gels) and then electro-transferred to nitrocellulose membranes. Membranes were blocked with 2% Normal Goat Serum (NGS), and 0.05% Tween 20 in 0.01 M PBS for 15 min at room temperature. After 3 washes with PBS containing 0.05% Tween 20 (T-PBS), membranes were incubated with anti-tyrosinase-MAT-1 monoclonal antibody (POLA R&D Lab, Kanagawa, Japan), TRP-1 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and TRP-2 goat polyclonal antibody (Santa Cruz) overnight at 4℃. After 8 washes with T-PBS, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Amersham Bioscience Co., Piscataway, NJ, USA) or HRP-conjugated anti-goat IgG (Jackson Immuno Research, West Grove, PA, USA) in T-PBS, for 1 h at room temperature. After 8 washes with T-PBS, bands were detected by the enhanced chemiluminescence method (Amersham). Quantitative analysis was performed with Scion image software (Scion Corporation, Frederick, MD, USA).

Immunohistochemistry

Hair follicle samples were fixed with 4% paraformaldehyde in PBS at 4℃ for 12 h and embedded in paraffin. 5 μm paraffin-embedded hair follicle sections were deparaffinized with xylene and rehydrated in ethanol. To facilitate antigen retrieval, sections were incubated in 2N HCl at room temperature for 5 min. After blocking with 2% NGS in PBS for 15 min, sections were incubated with anti-tyrosinase-MAT-1 monoclonal antibody (POLA R&D Lab) overnight at 4℃. Sections were then incubated with alkaline phosphatase-linked anti-mouse envision (DAKO, Carpinteria, CA, USA) for 30 min at room temperature, after which they were incubated with avidin-biotin peroxidase complex (Vector Laboratories, Peterborough, UK) for 30 min and stained with 5-Bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride (BCIP/NBT) (Sigma). The alkaline phosphatase liquid-chromogen system generates an insoluble blue-purple reaction product that is easily distinguishable from the brown/black melanin granules seen in melanocytes.

RT-PCR

Total RNA was extracted from hair follicles using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA). cDNA was synthesized from the total RNA using Ready-To-Go™ T-Primed First-Strand Kit (Amersham). cDNA for tyrosinase, TRP-2 and G3PDH were amplified by PCR with specific primers. The sequences of the sense and antisense primers for tyrosinase were: 5'-GGGCCCAAATTGTAGAGAGA-3' and 5'-ATGGGTGTTGACCCATTGTT-3', respectively. Sequences of the sense and antisense primers for TRP-2 were: 5'-AGCAGACGGAACACTGGACT-3' and 5'-GCATCTGTGGAAGGGTTGTT-3', respectively. Sequences of the sense and antisense primers for G3PDH were: 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3', respectively.

RESULTS

Effects of quercetin on the amount of melanin synthesized in mouse follicular tissues

The amount of melanin synthesized in mouse follicular tissues to which quercetin was added increased enormously in comparison with control tissue (Fig. 1). The increments in melanin were dependent on the concentration of added quercetin.

Observation of melanin pigment in quercetin-treated mouse follicular tissues with the use of Fontana-Masson staining

We performed Fontana-Masson staining to observe the deposition of melanin pigment in quercetin-treated mouse follicular tissues and found that Fontana-Masson-positive cells were dramatically increased in quercetin-treated mouse follicular tissues in compari-

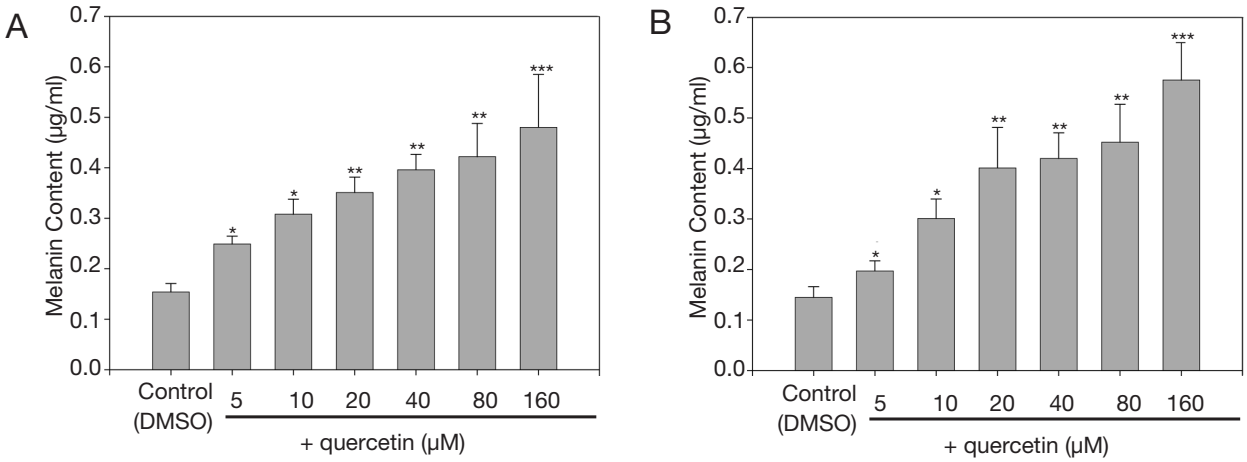

Fig. 1 Quercetin increased the melanin content in mouse hair follicles. (A) Melanin content was determined by measuring absorbance at 475 nm of hair follicles treated with various concentrations of quercetin for 7 days, as described in Materials and Methods. (B) Hair follicles were treated with various concentrations of quercetin for 14 days. Each value of melanin content is the means ± SD of 5 determinations. Significant differences were determined by Student's t-test; *P < 0.01, **P < 0.001, ***P < 0.0001, ****P < 0.00001.

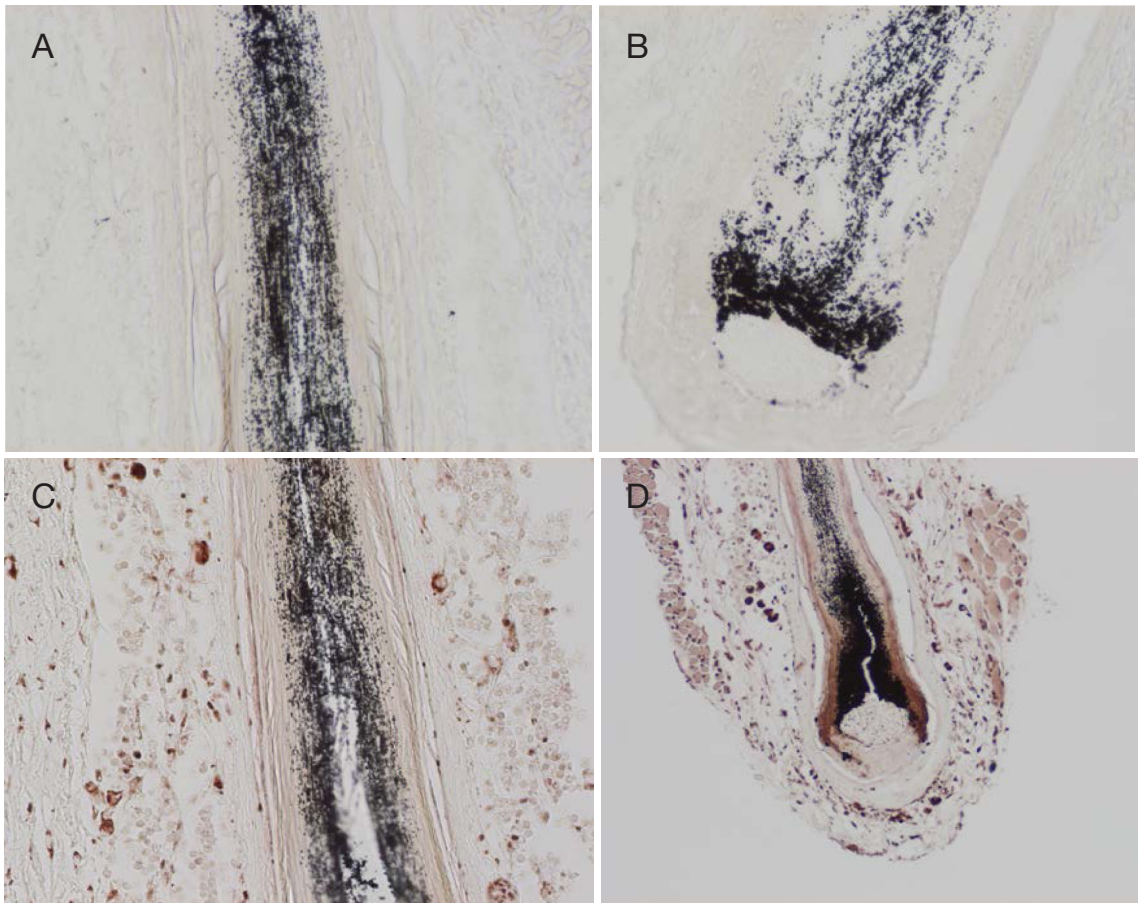

Fig. 2 Quercetin-induced melanin content in hair follicle observed after Masson-Fontana staining. (A, B) Control hair follicles and (C, D) quercetin-treated hair follicles stained with Masson-Fontana stain. Upper panel shows hair follicle stem and lower panel the hair follicle bulb.

son with control tissues (Fig. 2). It was clear from these results that quercetin caused the accumulation of melanin pigment in follicular tissues.

Analysis of expression of proteins related to melanogenesis in quercetin-treated mouse follicular tissues

In mouse follicular tissues to which quercetin was added, the expression of tyrosinase as well as TRP2 protein was enhanced but that of TRP1 protein was not affected (Fig. 3).

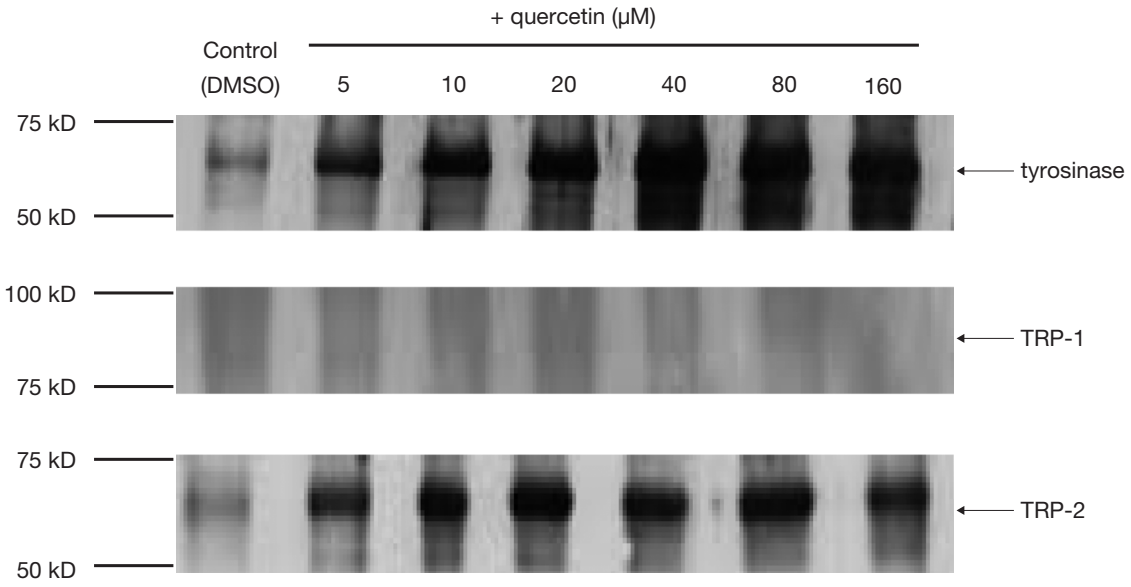

Fig. 3 Immuno-blotting analysis for tyrosinase, TRP-1 and TRP-2. Hair follicles were treated with quercetin (5, 10, 20, 40, 80 and 160 μM) for 7 days. Lane 1, DMSO-treated hair follicles; lanes 2, 3, 4, 5, 6, 7 show hair follicles treated with 5, 10, 20, 40, 80 and 160 μ M quercetin for 7 days, respectively.

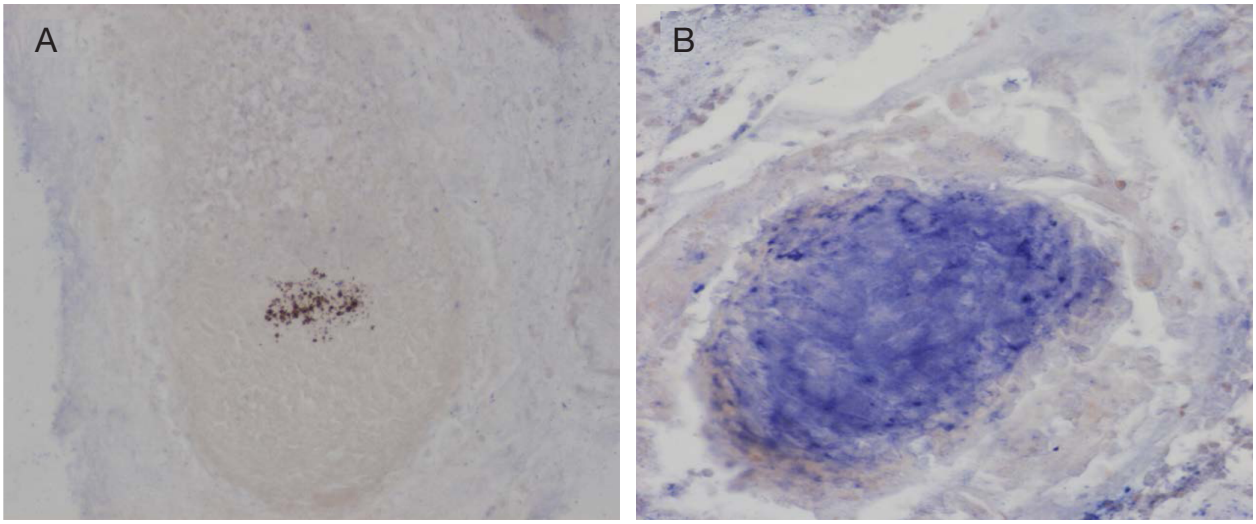

Fig. 4 Immunohistochemical observation of tyrosinase.
(A) DMSO-treated hair follicle and (B) quercetin-treated (160 μM) hair follicle were stained for anti-tyrosinase antibody (MAT-1). Immuno-stained product is observed as a blue deposit.

Immunohistochemical analysis of tyrosinase in quercetin-treated mouse follicular tissues

Tyrosinase expression was enhanced in quercetin-treated mouse follicular tissues in comparison with control tissues (Fig. 4).

Analysis of expression of mRNA encoding melanogenesis-related factors in quercetin-treated mouse follicular tissues

The expression of tyrosinase and TRP2 protein was enhanced in follicular tissues in which melanogenesis was enhanced by quercetin. Therefore, we observed the expression of mRNA encoding tyrosinase and TRP2, both of which play an important role in melanogenesis, with the use of RT-PCR. We found that quercetin did not affect TRP2 mRNA expression (Fig. 5).

DISCUSSION

In follicular tissues from the buccal region of C3H/HeN Jcl mice, melanogenesis was enhanced by quercetin in a concentration-dependent manner. It was therefore suggested that quercetin enhanced melanogenesis through increased production of tyrosinase in those tissues. We previously reported that quercetin enhanced tyrosinase activity resulted in the stimulation of melanogenesis both in normal melanocytes as well as in HMV-II cells derived from malignant melanoma [7, 8]. There was, however, no change in tyrosinase expression in these quercetin-treated cells. In 1996, Valèrie *et al.* reported that melanogenesis was remarkably enhanced in normal human melanocytes by

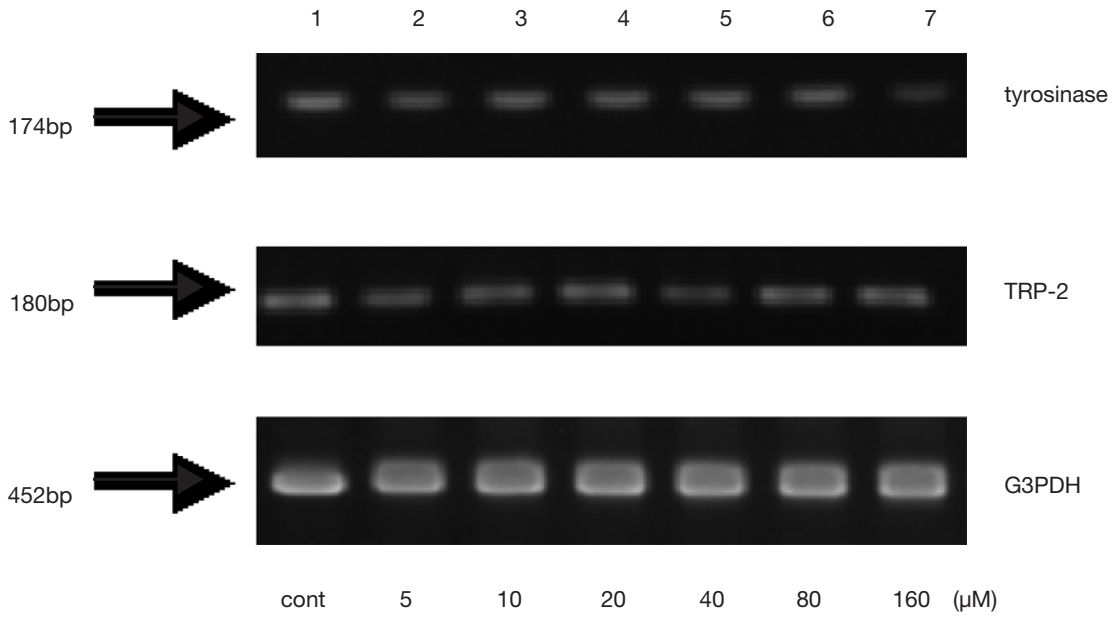

Fig. 5 Expression of tyrosinase mRNA after quercetin treatment in mouse hair follicles. Hair follicles were treated with quercetin (5, 10, 20, 40, 80 and 160 μM) for 7 days and analyzed for expression of tyrosinase, TRP-1 and TRP-2 mRNA. Housekeeping gene G3DPH was used as a control. Lane 1, DMSO-treated cells; Lanes 2, 3, 4, 5, 6, and 7 cells treated with 5, 10, 20, 40, 80 and 160 μM quercetin, respectively, for 7 days.

applying the PUVA method, which is generally used to stimulate melanogenesis [12]. They found no change in the expression of tyrosinase mRNA despite a striking increase in tyrosinase protein expression. In accordance with this observation, Naeyaert *et al.* reported that melanogenesis-promoting isobuthylmethylxanthine (IBMX), when added to culture medium of human melanocytes, enhanced melanogenesis in a time-dependent manner with no change in the expression of tyrosinase mRNA [13]. These results suggested that the expression of tyrosinase critical for melanogenesis is regulated by the modulation of tyrosinase protein.

According to Mengeaud *et al.*, PUVA caused human melanocytes to remarkably promote melanogenesis after 24 h with an increase in tyrosinase protein but with no change in the expression of tyrosinase mRNA [12]. These investigators explained this discrepancy between the enzyme protein and mRNA by the assumption that human melanocytes increased mRNA expression during several minutes to several hours of PUVA treatment, with the mRNA expression returning to a normal level at 24 h. In this study, we found that the expression of tyrosinase mRNA in the mouse follicular tissues 7 days after the addition of quercetin was at a normal level. Taking into account that the expression of tyrosinase protein was enhanced, it was possible that mRNA expression was increased immediately after quercetin addition, then returned to the normal level at 7 days.

Flavonoids including quercetin are well known to be anti-oxidants that scavenge active oxygen species. Vitamins C and E and β-carotene are likewise effective anti-oxidants [14, 15]. In 1997, Postaire *et al.* reported that vitamins C and E and β-carotene induced pigmentation when applied to the skin of mice [16]. It was reported by Jimenez-Cervantes *et al.* that hydrogen peroxide inhibited melanogenesis in melanoma cells [17]. The occurrence of active oxygen species, including lipid peroxide and free radicals, is supposed to be responsible for depigmentation in vitiligo and other pigmentation disorders [18]. Accordingly, there is a possibility that quercetin induces melanin deposition through its eliminating and reducing action on active oxygen species in melanin-producing cells.

Nylander *et al.* reported that UV irradiation increased the expression of wild type p53, followed by activation of tyrosinase in an in vivo experimental system [19]. It is generally acknowledged that quercetin induces apoptosis of C3H10T1/2C8 cells by arresting the cell cycle and then enhances the expression of wild type p53. The role of wild type p53 in the stimulating effect of quercetin on melanogenesis provokes much interest.

Results of the present study clarified that quercetin enhances the expression of tyrosinase protein, leading in turn to the promotion of melanogenesis. It is expected that quercetin will be put to practical use in the management of vitiligo and acquired pigment loss like gray hair.

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