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 (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.

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.
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% CO₂ at 37°C 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 protein assay kit (Bio-Rad, Hercules, CA, USA). Samples were centrifuged at 12,000g for 15 min, and the total protein content of the supernatant was determined by a protocol described previously [10].

**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 thiosulfate 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°C 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) overnight at 4°C. Sections were then incubated with alkaline phosphatase-linked anti-mouse enzyme (DAKO, Carpenteria, 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'-GGGCGCGAATTTGAGAGAGA-3' and 5'-ATGGGTGTGGGACCACTATTGT-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'-GGCAGGCAATTTGAGAGAGA-3' and 5'-ATGGGTGTGGGACCACTATTGT-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 com-
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).
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 resulting 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, Valérie et al. reported that melanogenesis was remarkably enhanced in normal human melanocytes by
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 isobutylmethylxanthine (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.

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 G3PDH 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.

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 C3H10T1/2Cs 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.

ACKNOWLEDGMENTS

The authors thank to Johbu Itoh and Hideaki Hasegawa, the Education and Research Support Center, Tokai University for excellent technical advice and assistance.

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