Flavonoids Enhance Melanogenesis in Human Melanoma Cells

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INTRODUCTION

Flavonoids are pigmentary compounds existing widely in plants. We have reported that quercetin (3, 3', 4', 5, 7-pentahydroxylflavone), one of the typical flavonoids, strongly promotes melanogenesis by melanocytes. Meanwhile, there are 8000 or more flavonoids having a chemical structure different from each other in the natural world. Their distinctive chemical properties suggest that they may be different in melanogenic actions. In the present study, the melanogenic actions of 14 flavonoids were analyzed to correlate their chemical structures with melanogenic actions. To evaluate the effects of flavonoids on melanogenesis, the HMV II cell line derived from human malignant melanoma was used. Flavonoids including quercetin, kaempferol, rhamnetin and fisetin, flavones including apigenin, luteolin and chrysirin, and isoflavones including genistein showed melanogenesis-promoting actions but rutin, robinetin, myricetin, iripiflavone, epigallocatechin gallate (EGCg) and naringin did not. From analyses of the relationships between the chemical structures of flavonoids and their melanogenesis-promoting actions, it was inferred that a hydroxyl group bound to the phenyl group plays an important role in stimulating melanogenesis. From the above results, 8 flavonoids were identified as melanogenesis promoters. Also, correlations were established between the melanogenesis-promoting actions of flavonoids and their chemical structures.

Key words: Flavonoid, tyrosinase, melanin, melanogenesis, HMVII cell

MATERIALS AND METHODS

Chemicals and reagents

Mushroom tyrosinase, melanin, L-dopa, quercetin, rutin, kaempferol, robinetin, rhamnetin, fisetin, epigallocatechin gallate (EGCg), myricetin, chrysin, iripiflavone, genistein and naringin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Apigenin was purchased from Indofine (Somerville, NJ). Luteolin was purchased from Extrasynthese (Lyon, France). Ham's F12 medium and trypsin/EDTA were obtained from GIBCO (Carlsbad, CA, USA). Dimethylsulfoxide (DMSO) was purchased from Merck & Company (Darmstadt, Germany).

Cell culture

HMVII is a human melanoma cell line that was established from a black-brown malignant melanoma in the vaginal wall of woman [11]. Human melanoma HMVII cells were kindly provided by the RIKEN Cell Bank. HMVII cells were cultured in Ham's F12 medium containing 10% fetal bovine serum (FBS) in a humidified 37°C atmosphere consisting of 5% CO2 and 95% air. Cell plating densities were arranged so that cells were in the log phase of growth for the duration of incubation with drug. Subcultures of cells were plat-
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ed at a density of $4 \times 10^4$ cells/cm$^2$. Approximately 24 hr later, fresh medium and flavonoids were added, and cells were harvested 7 days after drug addition. For testing, flavonoids were dissolved in DMSO (dimethylsulfoxide). The final DMSO concentration was 0.1% in all experiments (including control) and had no measurable effect on HMVII cells.

**Measurement of melanin content**

The melanin content was determined as previ-
ously described [12]. After washes in PBS, cells were detached by short incubation in trypsin/EDTA (0.05%/0.02% in PBS). An aliquot was used for cell counts. The remaining cells were sonicated and incubated overnight in 500 μl 1 M NaOH. Melanin concentration was calculated by comparing the OD at 475 nm of unknown samples with a standard curve obtained with synthetic melanin.

**Tyrosinase activity**

Cellular tyrosinase activity using L-DOPA as the substrate was assayed by the method of Maeda and Fukuda [13]. 1 × 10⁵ cells were washed with 10 mM phosphate-buffered saline (PBS) and lysed with 45 μl of 1% Triton X-100-PBS. After sonication, 5 μl of 20 mM L-DOPA were added to the wells. The 96 well plates were incubated at 37°C for 1 hr, and the absorbance was measured at 475 nm in the model SPECTRAmax 250 microplate reader (Molecular Device Co). The absorbance values were compared with a standard curve obtained with purified mushroom tyrosinase. The standard curve was linear within the range of experimental values.

**RESULTS**

**Effects of individual flavonoids on melanogenesis**

The effects on melanogenesis by 14 flavonoids differing from each other in chemical structure were tested in HMVII cells (Fig. 1). The 5 classes of flavonoids and compounds tested were 1) flavonol: quercetin, rutin, kaempferol, robinetin, rhamnetin, fisetin, and myricetin; 2) flavone: apigenin, luteolin, and chrys; 3) isoflavone: ipriflavone, genistein, (4) flavan: epigallocatechin gallate (EGCg); and 5) naringin: naringin. The concentration of the tested flavonoids was 20 μM DEMSO, which was a solvent for the tested flavonoids. The absorbance values for DMSO-treated control cells, at each of the flavonoid concentrations, respectively:

- kaempferol to 1.21-, 1.44-, 1.54- and 3.30-fold;
- apigenin to 1.20-, 1.75-, 2.08-, 5.53-fold;
- luteolin to 1.49-, 2.76-, 4.64-, 8.90-fold;
- chrys to 1.35-, 1.89-, 2.27-, 4.17-fold;
- fisetin to 1.11-, 1.36-, 1.39-, 2.94-fold;
- rhamnetin to 0.84-, 0.87-, 1.43-, 3.50-fold;
- genestein to 1.43-, 1.87-, 2.89-, 4.46-fold

As a result, concentration-dependent promotion of melanogenesis was demonstrated in all of the 7 flavonoids.

**DISCUSSION**

It has now been clarified that 8 flavonoids, including quercetin, kaempferol, rhamnetin, fisetin, apigenin, luteolin, chrysin, and genistein, are promoters of melanin production in HMVII cells whose tyrosinase activity was stimulated by the flavonoids. Tyrosinase is a key enzyme for the melanin synthesis pathway of melanin-producing cells [14]. With these results, we established that these 8 flavonoids promoted melanin production via enhancing intracellular tyrosinase activity. We reported previously that quercetin, which was among the flavonoids in the present study, enhanced tyrosinase activity in HMVII cells and murine buccal follicular tissues through induction of tyrosinase protein expression [8–10]. It strongly suggested that the other 7 flavonoids might share the same mechanism of tyrosinase activity stimulation with quercetin.
is therefore inferred that a hydroxyl group at position R2 of ring B of the flavonoid skeleton is essential for the melanogenic action. Robinetin, myricetin and epigallocatechin gallate, although having a hydroxyl group at position R2 of ring B, did not show a melanogenesis-promoting action. These flavonoids are characterized by the presence of additional hydroxyl groups at positions R1 and R3 near R2 of ring B. Thus, the structure where hydroxyl groups are bound to R1, R2, and R3 of ring B may suppress a melanogenesis-stimulating action. Other flavonoids are not melanogenesis promoters, such as rutin and naringin which have a glycoside bound to ring A, and ipriflavone which has a similarly bound isopropoxy side chain. These side chains may suppress melanogenesis-stimulating actions.

Seven flavonoids from the present study have
been added to the list of melanogenesis-promoting flavonoids that hitherto included only quercetin. It was also shown that flavonoid chemical structures have correlations with melanogenesis-stimulating actions. Flavonoids are expected to improve acquired depigmentation of skin and hair like vitiligo and white hair, respectively. Hopefully, the results of the present study will contribute to the development of more powerful...
melanogenesis-stimulating agents.

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