Melanogenesis Inhibition by an Oolong Tea Extract in B16 Mouse Melanoma Cells and UV-Induced Skin Pigmentation in Brownish Guinea Pigs

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To investigate the new physiological functions of oolong tea, the effects on melanogenesis were studied. An oolong tea extract inhibited melanogenesis without affecting cell growth in B16 mouse melanoma cells. However, the oolong tea extract hardly showed any inhibitory effect on mushroom tyrosinase in a cell-free system. The effects of an oolong tea extract on the intracellular tyrosinase level in B16 cells were therefore studied. All the levels of activity, protein and mRNA were decreased in the oolong tea extract-treated cells. We also investigated the inhibitory effects of oolong tea on the pigmentation induced by ultraviolet B (UVB) by using brownish guinea pigs in vivo. The number of 3,4-dihydroxyphenylalanine (DOPA)-positive melanocytes increased by UVB was repressed by an oral administration of oolong tea. These results imply that oolong tea might be effective in whitening and that its inhibitory effect on melanogenesis was involved in the decrease of intracellular tyrosinase at the mRNA level.

Key words: oolong tea; melanogenesis; melanin; tyrosinase; polyphenol

Tea is the most popular beverage in the world.1) It can be categorized into three types, depending on the level of fermentation, i.e., green tea (unfermented), oolong tea (partially fermented) and black tea (highly to fully fermented). The difference in manufacturing process causes changes in the ingredients. Tea is a good beverage not only for its good smell and taste but also for health maintenance. In particular, tea is known to have physiological functions, and many studies have been focused on tea polyphenols. For instance, (−)-epicatechin-3-gallate (ECG), (−)-gallocatechin-3-gallate (GCG) and (−)-epigallocatechin-3-gallate (EGCG) which showed strong inhibitory effects,15) the effect of oolong tea is not known.

We investigated in this study the effect of oolong tea on melanogenesis in cultured B16 mouse melanoma cells and brownish guinea pigs irradiated by UVB in vivo.

Materials and Methods

Oolong tea extract. Leaves of Oolong tea (shui xian) from Fujian, China were supplied by Suntory Ltd. (Osaka, Japan). The leaves of oolong tea (84 g) were...
soaked in 700 ml of hot water (90°C) for 5 min. The extracted water was collected by filtration and lyophilized. The oolong tea extract (13 g) was obtained and used for the test on mushroom tyrosinase inhibition and cultured B16 mouse melanoma cells. In the animal study, the extracted water was obtained by the same method and diluted to give 1% oolong tea.

**Materials.** Mushroom tyrosinase was purchased from Sigma Chemicals (MO, USA) or Wako Pure Chemicals (Osaka, Japan). 1-3,4-dihydroxyphenylalanine (L-DOPA) was purchased from Sigma, and l-ascorbic acid (AsA) was purchased from Wako Pure Chemical (Osaka, Japan). The antibody against tyrosinase (M-19) and horseradish peroxidase-conjugated bovine anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Cell culture.** B16 mouse melanoma cells were obtained from Riken Cell Bank (Tsukuba, Japan). The cells were cultured in Eagle’s minimum essential medium containing 10% fetal bovine serum, 2 mM l-glutamine, 50 units/ml of penicillin and 50 µg/ml of streptomycin in a humidified atmosphere containing 5% CO₂ in air at 37°C.

**Measurement of melanin synthesis.** The B16 cells were seeded into 60-mm dishes at 4.5 × 10⁵ cells per dish. After 24 h of incubation, the medium was changed to one containing the oolong tea extract or AsA. The B16 cells were then cultured for 3 days. After being detached from the dishes, the number of viable cells was counted by the trypan blue exclusion method. The cells were rendered soluble in 1 N NaOH at 80°C for 30 min, the absorbance then being measured at 472 nm. The melanin content was calculated by using an authentic standard of synthetic melanin, and the results are expressed as the melanin content per cell (µg/10⁴ cells).

**Tyrosinase activity assay.** The tyrosinase inhibition in a cell-free system was tested by mushroom tyrosinase. Pre-incubation was conducted with 0.5 ml of mushroom tyrosinase in a 0.1 M phosphate buffer at pH 6.8 (190 units/ml) and then in 0.5 ml of the phosphate buffer with the oolong tea extract or AsA for 10 min at 37°C. After adding 0.5 ml of L-DOPA, the reaction mixture was incubated for another 10 min. The tyrosinase activity was measured by OD 475 nm in the reaction mixture and is expressed as a percentage of the control value (= 100%).

The intracellular tyrosinase activity was measured after culturing the B16 cells with the oolong tea extract or AsA for 3 days as just described. After the medium had been removed, the cells were washed twice with PBS. The cells were then collected with a cell scraper and sonicated in 0.5% sodium deoxycholate in distilled water. The collected cell solution was centrifuged at 11,000 rpm for 20 min at 4°C, the supernatant being used for the measurement of tyrosinase activity. The cell supernatant (0.5 ml) was mixed with 1 ml of pre-incubated 0.05% L-DOPA in the 0.1 M phosphate buffer at pH 6.8, and the reaction was measured at OD 475 nm every 1 min for 10 min at 37°C. The tyrosinase activity is shown as OD 475 nm/µg of protein/min and is expressed as a percentage of the control value (= 100%).

**Western blot analysis.** B16 cells were cultured with the oolong tea extract for 3 days as already described. After the medium had been removed, the cells were washed twice with PBS and lysed in Nonidet P-40/SDS (1% Nonidet P-40, 0.01% SDS, 0.1 M Tris–HCl at pH 7.2, 100 µM PMSF, and 1 µg/ml of aprotinin). Each cell lysate was centrifuged at 11,000 rpm for 20 min at 4°C, and the supernatant was collected. Twenty micrograms of total protein from each cell extract was resolved by SDS–PAGE on 7.5% acrylamide gel and then transferred to a nitrocellulose membrane. Blocking was performed in Tris-buffered saline containing 2% skim milk powder. The membrane was incubated with the anti-tyrosinase antibody (1:200 dilution) and further incubated with the horseradish peroxidase-conjugated bovine anti-goat antibody (1:5000 dilution). The proteins were visualized with an ECL Plus western blotting detection system (Amersham Biosciences, Piscataway, NJ, USA).

**Real-time reverse transcription-polymerase chain reaction (RT-PCR).** B16 cells were seeded into 60-mm dishes at 4.5 × 10⁵ cells per dish. After 24 h of incubation, the medium was changed for a fresh one. After the B16 cells had reached the sub-confluent stage, the medium was changed to one containing the oolong tea extract. The B16 cells were then cultured for 24 h. Total RNA was extracted from the B16 cells by using Isogen (Nippon Gene Corp., Toyama, Japan) according to the manufacturer’s instructions. RNA samples were reverse-transcribed by using random primers (Promega Corp., Madison, WI, USA) and TaqMan® reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed on a GeneAmp® 5700 sequence detection system (Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The primers for PCR were as follows: for tyrosinase, 5’-GTCCTACAACCTGAAAATCCTAA-CT-3’ (forward) and 5’-CATGCCATAAAACCTGAT-GGC-3’ (reverse); for β-actin, 5’-ACTATGGCACAAG-AGCGGTT-3’ (forward) and 5’-ATGGATGCCACAG-GATTCCA-3’ (reverse).

**Protein assay.** The protein concentration was measured by the Lowry method,¹⁶ using bovine serum albumin as a standard.

**Animal study on brownish guinea pigs with UVB-induced pigmentation.** Three-week-old male brownish...
results indicate that the inhibitory effect of the oolong tea extract for 3 days is shown in Fig. 2B. The oolong tea extract showed an inhibitory effect on cellular tyrosinase activity in B16 cells cultured with the oolong tea extract or AsA (50 µg/ml) for 3 days. The tyrosinase activity was dose-dependently decreased by adding the oolong tea extract or AsA compared with the control. However, the oolong tea extract was much less effective on inhibiting the mushroom tyrosinase activity than AsA.

**Effect of the oolong tea extract on the cellular tyrosinase activity**

Tyrosinase is a rate-limiting enzyme in melanin synthesis. Many melanin synthesis inhibitors reduce melanogenesis by directly inhibiting the tyrosinase activity. We examined the direct effect of oolong tea extract on tyrosinase activity by using mushroom tyrosinase, because the inhibitory effect of the oolong tea extract on melanin synthesis was greater than that of AsA. Figure 2A shows the change in mushroom tyrosinase activity when the oolong tea extract or AsA was added in the range of 0–500 µg/ml (a final concentration of 0–167 µg/ml). The tyrosinase activity was dose-dependently decreased by adding the oolong tea extract or AsA compared with the control. However, the oolong tea extract was much less effective on inhibiting the mushroom tyrosinase activity than AsA.

We also examined the effect of the oolong tea extract on the cellular tyrosinase activity in B16 cells. The tyrosinase activity in B16 cells cultured with the oolong tea extract for 3 days is shown in Fig. 2B. The oolong tea extract showed an inhibitory effect on cellular tyrosinase activity in a dose-dependent manner. These results indicate that the inhibitory effect of the oolong tea extract on melanin synthesis was related to the decrease in cellular tyrosinase activity rather than to the direct inhibitory effect on tyrosinase activity.
Effect of the oolong tea extract on the tyrosinase protein and mRNA levels in B16 cells

Since the foregoing results suggested that the oolong tea extract decreased the melanin content by decreasing the cellular tyrosinase activity, we examined the cellular tyrosinase protein level by using a Western blotting analysis. As shown in Fig. 3, the tyrosinase protein level was dose-dependently decreased by the oolong tea extract.

Furthermore, we examined the cellular tyrosinase mRNA level by an RT-PCR analysis. B16 cells were cultured with the oolong tea extract or AsA at the indicated concentrations for 3 days. Cellular tyrosinase activity in B16 cells was measured as described in the Materials and Methods section. These experiments were carried out in triplicate. Each value is expressed as the mean ± SD. *p < 0.05 compared to the control.

**Effect of the oolong tea extract on the tyrosinase protein and mRNA levels in B16 cells**

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Furthermore, we examined the cellular tyrosinase mRNA level by an RT-PCR analysis. B16 cells were exposed to the oolong tea extract in a concentration range of 0–100 µg/ml for 24 hours, and the tyrosinase mRNA level in the cells was measured. The tyrosinase mRNA level was decreased by the oolong tea extract (Fig. 4). These results indicate that the decrease in cellular tyrosinase activity by treating with the oolong tea extract was caused by the decrease in the cellular tyrosinase protein and mRNA levels.

**Effect of the oolong tea intake on pigmentation induced by UVB in the guinea pigs**

The inhibitory effect of the oolong tea extract on melanogenesis was apparent in cultured B16 mouse melanoma cells. To further study this phenomenon, we examined the effect of oolong tea on the pigmentation in vivo.

The oolong tea intake by the oolong tea group was an average of 52 ml/day throughout the experimental period. The polyphenol content in this daily oolong tea intake was about 138.4 mg/day. The body weight of the oolong tea group was lower than that of the control group, a significant difference being found at 6 and 7 weeks (Fig. 5). The food intake by the oolong tea group (average 40 g/day) was lower than that by the control group (average 50 g/day), but no side effect
from oolong tea drinking was seen during the experiment period.

We measured the L*/C3 value for the back skin of the guinea pigs before and one week after UVB irradiation to evaluate the degree of pigmentation. The L*/C3 value after UVB irradiation was significantly lower, although no significant difference in L*/C3 value was found between the control group and oolong tea group (data not shown). The result of counting the number of DOPA-positive melanocytes is shown in Fig. 6. Figure 6A shows the number of DOPA-positive melanocytes in the non-irradiated and irradiated skin. Figure 6B shows the number of DOPA-positive melanocytes in the irradiated skin when the number of DOPA-positive melanocytes in the non-irradiated skin is expressed as 100% for each guinea pig. The number of DOPA-positive melanocytes was higher in the irradiated skin than in the non-irradiated skin. In the control group, the number of DOPA-positive melanocytes was increased to about 3 times by UVB irradiation (from 80 to 250 cells/mm²). On the other hand, the number of DOPA-positive melanocytes was increased to about 1.4 times (from 110 to 150 cells/mm²) in oolong tea group. Altogether, the DOPA-positive melanocytes induced by UVB irradiation were inhibited in the oolong tea group.

**Discussion**

Oolong tea is a traditional Chinese tea and has recently been reported to have physiological functions such effects as atherogenic, cancer, obesity and antioxidant actions. We paid attention in this study to melanogenesis as one of the new physiological functions of oolong tea and investigated the effects of oolong tea on melanogenesis by using mushroom tyrosinase in a cell-free system, cultured B16 melanoma cells and guinea pigs irradiated by UVB in vivo.

We investigated the inhibitory effects of the oolong tea extract with AsA used as a positive control. AsA has been reported to inhibit melanogenesis by inhibiting tyrosinase and reducing dopaquinone to DOPA. The melanin content in the oolong tea extract-treated cells was significantly reduced, the inhibitory effect being greater than that of AsA. However, in the tyrosinase inhibition experiment using mushroom tyrosinase in the cell-free system, the inhibition effect of the oolong tea extract was much weaker than that of AsA. These results indicate that the oolong tea extract was an effective inhibitor of melanogenesis, but that this effect was not achieved by the direct inhibition of tyrosinase activity. We suggest that the oolong tea extract inhibited melanogenesis by a different mechanism from that of AsA.

We therefore examined the effect of the oolong tea extract on the cellular tyrosinase level. B16 cells treated with the oolong tea extract showed the cellular tyrosinase activity to be significantly decreased. The results of Western blotting and the real-time RT-PCR analysis showed the tyrosinase protein and mRNA levels to be decreased by the oolong tea extract. We suggest from the foregoing results that the oolong tea extract inhibited melanogenesis at the transcription level.

Tyrosinase gene expression is regulated by the microphthalmia-associated transcription factor (MITF). MITF is involved in the pigmentation, proliferation, and survival of melanocytes and activates melanogenic gene (tyrosinase, TRP-1 and TRP-2).
TRP-2) promoters, thereby increasing their expression, which results in increased melanin synthesis. EGCG has been reported to reduce melanin synthesis via decreased MITF production. Although we did not examine the effect of the oolong tea extract on the MITF level and its inactivation, the oolong tea extract might also have decreased cellular tyrosinase and melanogenesis by a similar mechanism to that of EGCG.

We also examined the effect of oolong tea on pigmentation in vivo. Most animal experiments have examined the whitening effect by a topical application, experiments involving an oral administration being scarce. To evaluate the whitening effect of oolong tea, brownish guinea pigs were administered with the drinking form of oolong tea in this study. In the oolong tea group, the increase of DOPA-positive melanocytes by UV irradiation was lower than that in the control group. These results indicate that oolong tea might have prevented the progression of UVB-induced pigmentation. However, we were not able to support the difference in pigmentation between the control group and oolong tea group by the difference in L* values shown before and after UVB irradiation. Melanin generated in melanocytes is transferred to the neighboring keratinocytes and forms the skin color, so it takes time until it can be recognized as the skin color. Moreover, a difference in the increase of DOPA-positive melanocytes by UVB irradiation was found in this study, hence extra time might be required to confirm the difference in pigmentation between groups.

UV irradiation has been reported to increase tyrosinase activity and expression. UV irradiation can increase melanogenesis and the proliferation of melanocytes by directly or indirectly acting on melanocytes through the release of keratinocyte-derived factors. UV irradiation also increases the generation of reactive oxygen species (ROS) in the skin, and generated ROS assists melanogenesis. It has been reported that UV irradiation-induced melanogenesis was reduced by the topical application of such antioxidants as vitamins C and E to the skin. We have also reported the effect of an oral administration of vitamin C, L-cysteine and vitamin E. As oolong tea has an antioxidative action, we suggest the possibility that oolong tea inhibited UV-induced melanogenesis by scavenging UV-induced ROS. We investigated in this study the TBARS level, an oxidation stress marker, induced by UV irradiation in the skin, although no significant difference was found (data not shown). These results suggest that the inhibition of UVB-induced pigmentation by oolong tea drinking was not a result of any antioxidative action. On the other hand, the results for the B16 cells suggest that the oolong tea extract inhibited melanogenesis by inhibiting tyrosinase expression, therefore the inhibition of UVB-induced pigmentation by the oral administration of oolong tea to guinea pigs might have been caused by the inhibition of tyrosinase expression induced by UV irradiation.

In conclusion, it is suggested that the oolong tea extract was a melanogenesis inhibitor in B16 mouse melanoma cells and brownish guinea pigs, and that the mechanism might involve the decrease of intracellular tyrosinase at the mRNA level. The concentration of oolong tea used in this animal study was acceptable for use in a human beverage, even though about five times stronger than that in normal beverages in the market. We therefore suggest that drinking oolong tea might help whitening.

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References

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