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Delta-Tocotrienol Causes Decrease of Melanin Content in Mouse Melanoma Cells

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We examined the effect of δ-tocotrienol on melanin content in mouse melanoma B16 cells. Melanin content was significantly reduced in cells treated with 50 and 100 µM δ-tocotrienol, but not 10 µM δ-tocotrienol. The activity and amount of tyrosinase also significantly decreased in cells treated with 10, 50, and 100 µM δ-tocotrienol. Furthermore, the mRNA level of tyrosinase as measured using real-time PCR was significantly decreased compared to controls in cells treated with 100 µM δ-tocotrienol, but not 10 or 50 µM δ-tocotrienol. These results indicated that at first δ-tocotrienol caused tyrosinase degradation, and then caused a further decrease in the tyrosinase protein level via both tyrosinase degradation and a decrease in the mRNA level of tyrosinase. We conclude that the decrease of melanin content in the cells by δ-tocotrienol was the result of the decrease of the protein level of tyrosinase (tyrosinase degradation is more important than the decrease of mRNA).

Key words —— tocotrienol, tyrosinase, melanin, melanosome, mouse melanoma

INTRODUCTION

In nature, eight substances have been found to have vitamin E activity: α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol.[1] Tocotrienols and tocopherols can be subdivided into four isomers with regards to the numbers and positions of methyl groups on their chromanol ring, and differ only in possessing a farnesyl or saturated phytyl side chain, respectively. Tocotrienols possess powerful neuroprotective, anti-cancer, and cholesterol-lowering properties that are often not exhibited by tocopherols.[2–11] δ-Tocotrienol is the strongest suppressor of vascular endotherial growth factor (VEGF) secretion from HepG2, especially under hypoxic conditions, as compared with other isomers.[12] Also, γ- and δ-tocotrienol cause degradation of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, which is a cholesterol biosynthetic rate-limiting enzyme, whereas α- and β-tocotrienol do not.[11] The nutritive value of tocopherols and tocotrienols in food products emanates from their well-known antioxidant capacity, which helps prevent oxidative damage to polyunsaturated fatty acids.[13] Tocotrienols are also thought to have more potent antioxidant properties than α-tocopherol.[14, 15]

Melanin is synthesized (in an oxidative enzymatic reaction) by tyrosinase or other enzymes that use tyrosine in melanosomes. Melanosome biogenesis involves four stages of maturation with distinct morphological and biochemical characteristics that reflect the biogenesis process of structural and enzymatic proteins (tyrosinase family proteins), which is followed by structural organization and melanin deposition.[16]

In general, it is thought that the antioxidant properties of tocotrienol decrease melanin levels by inhibiting oxidative enzymatic reactions, including tyrosinase or other enzymes in melanosomes. Therefore, tocotrienol may be an effective component in whitening and/or lightening cosmetics. However, there is no experimental evidence that tocotrienol causes a decrease in melanin content. In the present study, we examined whether the melanin content decreases by δ-tocotrienol administration (δ-tocotrienol has more actions/effects than other isomers) using mouse melanoma B16 cells.

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MATERIALS AND METHODS

Materials —— δ-Tocotrienol was kindly provided

by Eisai Food & Chemical Co., Ltd. (Tokyo, Japan). Dulbecco's modified Eagle's medium (D-MEM) was obtained from Gibco (Tokyo, Japan). B16 cells were obtained from RIKEN (Ibaraki, Japan). Goat anti-lactate dehydrogenase (LDH)-A (N-14) IgG and Goat anti-tyrosinase (C-17) IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Rabbit anti-goat IgG conjugated to horseradish peroxidase was obtained from American Qualex Antibodies (San Clemente, CA, U.S.A.). Quick Gene RNA cultured cell kit S was obtained from Fujifilm (Tokyo, Japan). SYBR Ex Script reverse transcription (RT)-PCR kit was obtained from TaKaRa (Tokyo, Japan). All other chemicals were of reagent grade, and purchased from various commercial sources.

Cultured Melanoma Cell Line —— B16 cells were diluted to 1.5×10^6 per 35-mm tissue culture dish with D-MEM containing 10% fetal bovine serum (FBS), and then incubated in humidified air containing 5% CO_2 at 37°C for 24 hr. In some experiments, the cells were shifted to D-MEM containing 10% FBS in the absence or presence of 10–100 µM δ-tocotrienol [dissolved in 2 µl dimethyl sulfoxide (DMSO)] for 24 hr.

Preparation of Sample —— B16 cells incubated on 35-mm dishes were washed several times in cold Hanks' buffer. On the day of the experiment, the medium was aspirated from the culture dishes, and cells were then washed twice with 1 ml ice-cold phosphate-buffered saline (PBS), and 750 µl of homogenate buffer containing 1% triton X-100, 0.5 mM Phenylmethylsulfonyl fluoride (PMSF), 10 mM 2-mercaptoethanol, 1 mM EDTA, and protease inhibitors (1 µM leupeptin, 1 µM pepstatin A, 1 µM chymostatin, and 1 µM antipain) was added to the cells, which were then scraped off using a rubber policeman. The solution of B16 cells was homogenized with five strokes in a Teflon homogenizer. After centrifugation for 5 min at $1000 \times g$, the post-nuclear supernatant (PNS) was used to assay tyrosinase activity, β-G activity, cholesterol content, and for immunoblotting.

Protein Assay —— Protein levels were measured by the method of Lowry *et al*. using bovine serum albumin (BSA) as the standard.[17]

Tyrosinase Activity —— Two hundred microliters of 0.3% dopa solution was added to the 100 µl of obtained supernatant (PNS) and incubated at 37°C for 20 min. Tyrosinase activity was measured with a spectrophotometer at 475 nm.[18]

Melanin Content —— Cells dissolved in 1 ml of alkaline solution (1 N NaOH) and incubated at 80°C for 2 hr were used to measure the protein concentration and melanin. The amount of melanin was measured with a spectrophotometer at 420 nm.[19]

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting —— SDS-PAGE was performed on 10% slab gels according to the method of Laemmli.[20] Proteins on the SDS-slab gel were transferred to a nylon membrane (NEN) by electrophoresis, using a modified version of the procedure of Towbin *et al*.[21] Positive bands were visualized using ECL Western blotting detection kits (Amersham Pharmacia, Amersham, U.K.) that contained a sensitive chemiluminescent substrate for horseradish peroxidase.

Real-time PCR —— Total RNA (50 µl) was isolated from cells using Quick Gene RNA cultured cell kit S (total RNA extract kit) and Gene-810 (Nucleic Acid Isolation System; Fujifilm). The concentration of total RNA was calculated by a Qubit™ fluorometer (Invitrogen). Two-microgram samples of total RNA from each group of cells were subjected to reverse transcription (RT) using reverse transcriptase in a 50-µl reaction volume. After the RT reaction, the cDNA template was amplified by polymerase chain reaction with a SYBR Ex Script RT-PCR kit. SYBR Green was used for the real-time PCR analysis of tyrosinase. Real-time PCR was performed using an ABI 7500 system (Applied Biosystems Japan, Tokyo, Japan). Relative gene expression was quantified using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. The primer pairs (GAPDH, F-TGTGTCCGTCGTGGATCTGA and R-TTGCTGTTGAAGTCGCAGGAG; Tyrosinase, F-CAAGTACAGGGATCGGCCAAC and R-GGTGCATTGGCTTCTGGGTAA) were designed using the primer Select program of TaKaRa. The cDNA products generated by RT-PCR were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining under UV light.

Statistics —— Statistical analysis was carried out using Student's *t*-test. Data are presented as the means ± S.D.

RESULTS

Rate of Survival of Mouse Melanoma Cells Treated with δ-Tocotrienol

We estimated the rate of survival of δ-

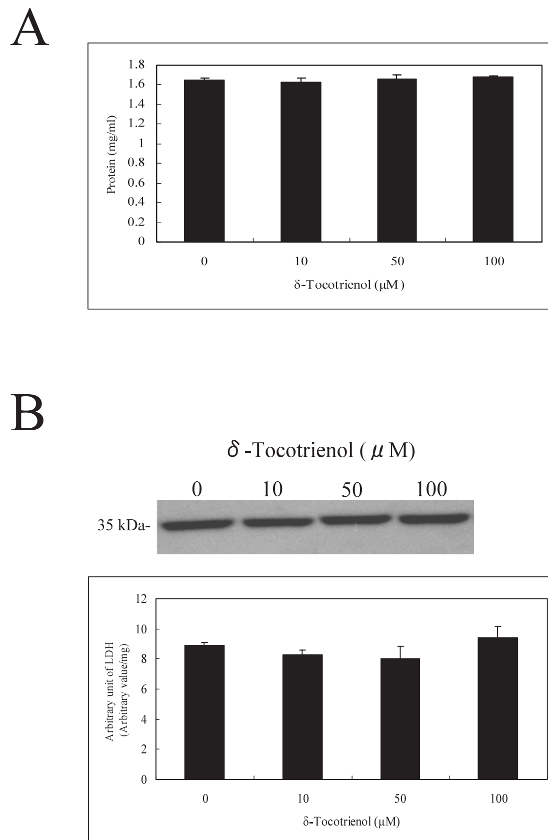

Fig. 1. LDH Activity and Amount of Protein in Cells Treated with δ-Tocotrienol

B16 cells were cultured in the absence or presence of 10, 50, and 100 μM δ-tocotrienol for 24 hr, and cells were homogenized with 750 μl of homogenate buffer. After centrifugation at $1000 \times g$ for 5 min, PNS was obtained. A: Protein contents in PNS were measured as described in Materials and Methods. B: PNS (30 μg) was subjected to immunoblotting using anti-LDH antiserum, and these signals were then measured using an Intelligent Quantifier. Data are the means of three identical experiments.

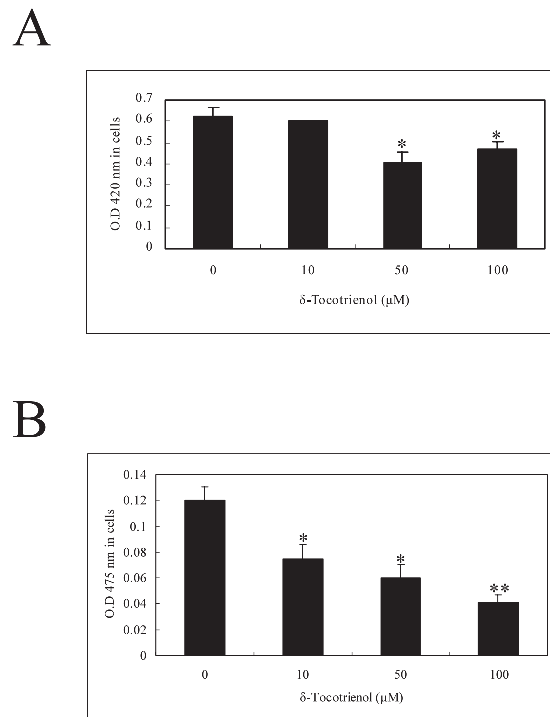

Fig. 2. Melanin Content and Tyrosinase Activity in Cells Treated with δ-Tocotrienol

The preparation (sample used for melanin content and tyrosinase activity of cells) in Fig. 2 was similar to that in Fig. 1. Melanin content (A) in cells and tyrosinase activity (B) in PNS were measured as described in Materials and Methods. Data are the means of three identical experiments. Significant differences: $^{*}p < 0.05$, $^{**}p < 0.01$

tocotrienol-treated and untreated cells by measuring the amount of protein and LDH. As shown in Fig. 1, the amount of protein and LDH in cells treated with 10–100 μM δ-tocotrienol for 24 hr was similar to that in untreated cells. These results indicated that almost all δ-tocotrienol-treated cells survived.

The Effect of δ-Tocotrienol on Melanin Content and Tyrosinase Activity in Cells

We examined the effect of δ-tocotrienol on the melanin content in cells. As shown in Fig. 2A, the melanin content significantly decreased by treatment with 50–100 μM δ-tocotrienol. The melanin content decreased by 35 or 28% by treatment with 50 or 100 μM δ-tocotrienol, respectively, compared to control cells. Next, we examined the effect of δ-tocotrienol on tyrosinase activity in cells. As shown in Fig. 2B, tyrosinase activity significantly decreased by treatment with 10–100 μM δ-tocotrienol. Tyrosinase activity decreased by 37, 50, or 66% by treatment with 10, 50, or 100 μM δ-tocotrienol, respectively, compared to control cells. These data suggest that the decrease of melanin content in the cells by δ-tocotrienol was caused by a decrease in tyrosinase activity.

The Effect of δ-Tocotrienol on Protein and mRNA Levels of Tyrosinase in Cells

To examine whether the decrease of tyrosinase activity by δ-tocotrienol was caused by a decrease in the tyrosinase protein level, we measured the amount of tyrosinase using immunoblot analysis. As shown in Fig. 3A, the tyrosinase protein level significantly decreased by treatment with 10–100 μM δ-tocotrienol, similar to the decrease in activity as shown in Fig. 2B. Tyrosinase levels decreased by 45, 46, or 67% by treatment with 10, 50, or 100 μM δ-tocotrienol, respectively, compared with control cells. These data suggest that the decrease of tyrosinase activity in cells by δ-tocotrienol was caused by a decrease of the tyrosinase protein level. Next, to examine whether the decrease in the protein level of tyrosinase by δ-tocotrienol was

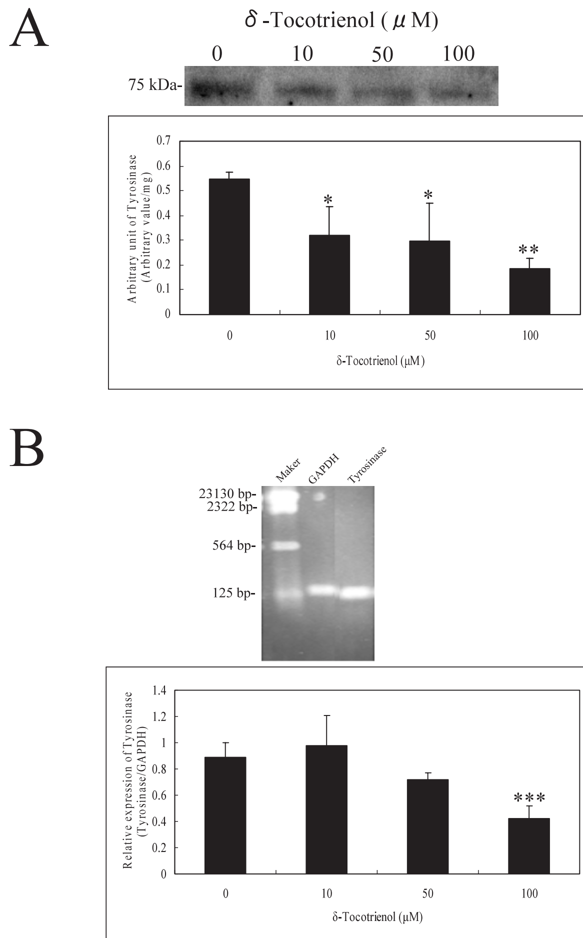

Fig. 3. Protein and mRNA Level of Tyrosinase in Cells Treated with δ-Tocotrienol

The preparation (sample used for protein and mRNA level of tyrosinase of cells) in Fig. 3 was similar to that in Fig. 1. A: PNS (30 µg) was subjected to immunoblotting using anti-tyrosinase antibody, and the signals were measured using an Intelligent Quantifier. B: RT-PCR was performed using primer pairs for tyrosinase or GAPDH from total RNA in cells treated with 10–100 µM δ-tocotrienol or non-treated cells as described in Materials and Methods. cDNA products (1 µl) were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining under UV light. After we recognized that each band [GAPDH (150 bp) or tyrosinase (125 bp)] generated by RT-PCR was a single band, real-time PCR was performed as described in Materials and Methods. Data are the means of three identical experiments. Significant differences: $^{*}p < 0.005$, $^{**}p < 0.0001$, $^{***}p < 0.001$.

caused by an increase in tyrosinase degradation or a decrease in the tyrosinase mRNA level, we measured the tyrosinase mRNA level using real-time PCR for cells treated with 10–100 µM δ-tocotrienol. As shown in Fig. 3B, the mRNA level of tyrosinase significantly decreased by treatment with 100 µM δ-tocotrienol, but not 10 or 50 µM δ-tocotrienol. The tyrosinase mRNA level increased by 10% with treatment with 10 µM δ-tocotrienol, and decreased by 25% or 54% by treatment with 50 or 100 µM δ-tocotrienol, respectively. Although the melanin content and protein level of tyrosinase significantly decreased by treatment with 50 µM δ-tocotrienol, the mRNA level of tyrosinase did not; however, it significantly decreased with 100 µM δ-tocotrienol (Figs. 2 and 3). These data indicated that at first δ-tocotrienol causes tyrosinase degradation, and then causes a further decrease in the protein level of tyrosinase by both tyrosinase degradation and a decrease in the mRNA level of tyrosinase. Overall, the results suggest that the decrease of melanin content in cells caused by δ-tocotrienol treatment was mediated by a decrease in the protein level by tyrosinase degradation rather than a decrease in the mRNA level.

DISCUSSION

In the present study, we found for the first time that a decrease in melanin content in cells is caused by a decrease in the protein level of tyrosinase caused by δ-tocotrienol. Recent advances in molecular biology and genomic techniques have led to the discovery of a novel effect of tocotrienol. δ-Tocotrienol suppresses hypoxia-induced VEGF and interleukin-8 (IL-8) expression in a cancer cell line (human colorectal adenocarcinoma cells) at both the mRNA and protein level by reducing hypoxia-inducible factor-1α (HIF-1α).[12] HIF-1 induces the transcription of >70 genes, including VEGF, IL-8, and cyclooxygenase 2 (COX-2). In addition, δ-tocotrienol does not affect hypoxia-induced COX-2 mRNA expression; however, δ-tocotrienol tends to suppress hypoxia-induced COX-2 protein expression, implying a possible post-translational mechanism for δ-tocotrienol action.[12] Furthermore, δ-tocotrienol stimulates ubiquitination of HMG-CoA reductase and degradation by proteasomes, and blocks the processing of sterol regulatory element binding proteins, which are transcription factors of cholesterol biosynthetic enzymes, including HMG-CoA reductase.[11] From these reports and our results, we propose that at first δ-tocotrienol causes tyrosinase degradation by proteasomes, and then causes a further decrease in the tyrosinase protein level by both tyrosinase degradation and a decrease of the mRNA level of tyrosinase by reducing tyrosinase transcriptional factors. The decrease of transcriptional factors of tyrosinase may be caused by a decrease of their mRNA level, an increase in degradation, or blockage of processing. Further study is necessary to understand the mechanism of tyrosinase mRNA and protein suppression by δ-

tocotrienol.

In conclusion, we found that δ-tocotrienol inhibited melanin biosynthesis in mouse melanoma B16 cells via a decrease in the protein level of tyrosinase. As a result of this and future research, δ-tocotrienol might be a useful as therapeutic or preventive drug for treating hyperpigmentation and as an effective component in whitening and/or lightening cosmetics.

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