— **Rapid Communication** —

Reduction of Mevalonate Pyrophosphate Decarboxylase in Mouse Melanoma Cells Treated with δ-Tocotrienol Is Not Associated with Reduction of Cholesterol Content or Release of Lysosomes and Melanosomes

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We previously reported that a decrease in the melanin content of mouse melanoma cells (B16 cells) treated with δ-tocotrienol was the result of a decrease in the level of tyrosinase activity and protein. Use of δ-tocotrienol as a whitening agent, may therefore have side effects. In the present study, we examined whether δ-tocotrienol caused side effects (the release of lysosomes from and a decrease in the cholesterol content of cells). We also examined the release of melanosomes (lysosome-related organella). Neither of lysosomes nor melanosomes were released from cells treated with δ-tocotrienol, since β-glucuronidase (melanosomal and lysosomal enzyme) activity, melanin content (melanosomal marker), and tyrosinase (melanosomal enzyme) activity did not increase in the cell culture medium. Although mevalonate pyrophosphate decarboxylase (MPD; an enzyme of cholesterol biosynthesis) was significantly reduced in the cells treated with δ-tocotrienol, cholesterol content was not. Thus, δ-tocotrienol might be useful as a therapeutic or preventive drug for hyperpigmentation and as a component of whitening and/or lightening cosmetics not causing severe side effect (reduction of cholesterol content and release of lysosomes/melanosomes), although δ-tocotrienol cause a decrease of MPD.

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

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INTRODUCTION

Vitamin E is a generic term for eight naturally occurring forms of lipophilic compounds called tocopherols and tocotrienols. Tocopherols and tocotrienols share a polar chromanol ring that is linked to an isoprenoid-derived hydrocarbon side chain. Tocotrienols and tocopherols can be subdivided into four isomers (α, β, γ, and δ) with regards to the numbers and position of methyl groups on their chromanol ring, and differ only in possessing a farnesyl or saturated phytyl side chain, respectively.[1] The nutritive value of tocopherols and tocotrienols in food products emanates from their well-known antioxidant capacity, which helps to prevent oxidative damage to polyunsaturated fatty acids.[2]

Tocotrienols, but not tocopherols, have been linked to additional beneficial therapeutic properties that include antithrombotic and neuroprotective activities and the ability to inhibit proliferation of breast cancer cells and lower serum cholesterol levels when administered in the diet of chickens, swine, rats, and hypercholesterolemic patients.[3–9] Early studies revealed an association between the hypocholesterolemic activity of tocotrienol-rich extracts and decreased levels of hepatic 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (rate-limiting enzyme of cholesterol biosynthesis) activity.[7] Subsequent structure-activity studies revealed that δ- and γ-tocotrienols were the most potent suppressors of HMG-CoA reductase in primary rat hepatocytes and cultured $HepG_2$ cells.[10,11] Furthermore, it was reported that δ-tocotrienol stimulates ubiquitination and degradation of reductase and blocks processing of sterol regulatory element-binding proteins (SREBPs), an-

other sterol-mediated action of Insigs.[12)]

We previously reported that the exocytosis of mature enzymes from lysosomes into the medium was caused by a lovastatin (HMG-CoA reductase inhibitor)- and/or lipoprotein-deficient serum (LDS)-induced decrease in the cholesterol content in B16F10 mouse melanoma cells.[13)] Melanosomes contain several lysosomal enzymes such as β-glucuronidase,[14, 15)] suggesting that melanosomes are lysosome-related organelles.[16)] Therefore, it was suggested that the release of not only lysosomes but also melanosomes was caused by the decrease in cholesterol content.

We previously reported that a decrease in the melanin content of mouse melanoma cells (B16 cells) treated with δ-tocotrienol was the result of a decrease in the level of tyrosinase activity and protein.[17)] Use of δ-tocotrienol as a therapeutic or preventive drug for hyperpigmentation or as a component of whitening and/or lightening cosmetics, may result in the release of lysosomes or melanosomes from and a decrease in the cholesterol content of cells as side effects.

In the present study, we examined the effect of δ-tocotrienol on the release of lysosomes and melanosomes into cell culture medium and the cholesterol content of cells.

MATERIALS AND METHODS

Materials —— The δ-tocotrienol was obtained from Eisai (Tokyo, Japan). Cholesterol E-test Wako kits from Wako (Osaka, Japan), Dulbecco's modified Eagle medium (D-MEM) was obtained from Gibco (Tokyo, Japan), B16 cells from RIKEN (Ibaraki, Japan), goat anti-lactate dehydrogenase (C-17) IgG from Santa Crutz Biotechnology, Inc. (Santa Crutz, CA, U.S.A.), rabbit anti-goat IgG conjugated to horseradish peroxidase from Invitrogen Corporation (San Clemente, CA, U.S.A.), goat anti-rabbit IgG conjugated to horseradish peroxidase from Invitrogen Corporation (Carlsbad, CA, U.S.A.). 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 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 D-MEM. On the day of the experiment, the medium was aspirated from the culture dishes, and cells were then washed twice with 1 ml of ice-cold phosphate buffered saline (PBS). Next, 750 μl of homogenate buffer containing 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluolide (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 5 strokes in a Teflon homogenizer. After centrifugation for 5 min at 1000 $\times$ g, the post-nuclear supernatant (PNS) was used to assay β-glucronidase (β-G) activity, to measure 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.[18)]

β-G Activity —— β-G activity was assayed as described by Robins *et al.*[19)]

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

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 levels of protein and melanin. The amount of melanin was measured with a spectrophotometer at 420 nm.[21)]

Cholesterol Content of Cells —— Two hundred microliters of PNS was mixed with 5 ml of Folch extract (chloroform [2] : methanol [1]), and the mixture was incubated for 10 min at 37°C with shaking. After the mixture was centrifuged at 3000 $\times$ g for 10 min, 3 ml of the supernatant was evaporated dry by boiling at 100°C and then dissolved in 200 μl of isopropylalchol containing 1% Triton X-100. The cholesterol content of the solution was determined using the Cholesterol E-test Wako kit.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting —— SDS-PAGE was performed on 10% slab gels according to the method of Laemmli.[22)] 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.*[23)] Pos-

itive bands were visualized using ECL Western blotting detection kits (Amersham Pharmacia, Amersham, U.K.) that contained a sensitive chemiluminescent substrate for horseradish peroxidase.

Antibody Mevalonate Pyrophosphate Decarboxylase (MPD) —— MPD was purified from rat liver, as described by Michihara *et al.*, and a rabbit polyclonal antiserum raised against the rat MPD was used.[24)]

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

RESULTS

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

We estimated the rate of survival of δ-tocotrienol-treated and untreated cells by measuring the amount of protein and Lactate dehydrogenase (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 the Release of β-G, Tyrosinase, and Melanin into the Medium

To examine whether δ-tocotrienol caused the release of lysosomes or melanosomes from cells, we measured levels of β-G (a lysosomal and melanosomal enzyme) activity, melanin (a melanosomal marker), and tyrosinase (a melanosomal marker enzyme) activity in the culture medium of cells treated with 10, 50, or 100 μM δ-tocotrienol. As shown in Fig. 2A, levels of β-G activity did not increase significantly in either the medium or cytoplasm of the treated cells (Fig. 2A). The melanin content inclined to increase in the medium of treated cells compared to that of untreated cells, but the difference was not significant (Fig. 2B). The melanin contents significantly decreased by 33 or 25% in cells, 22 or 15% in medium plus cells by treatment with 50 or 100 μM δ-tocotrienol, respectively, compared with the control cells. Tyrosinase activity in the medium did not decrease significantly (Fig. 2C). The tyrosinase activity significantly decreased by 34, 42 or 58% in cells, 27, 33 or 40% in medium plus cells by treatment with 10, 50 or 100 μM δ-tocotrienol, respectively, compared with the control cells. These results suggested that δ-tocotrienol did not cause the release of lysosomes or melanosomes, although it did cause a decrease in melanin contents and tyrosinase activity in cells as described previously.

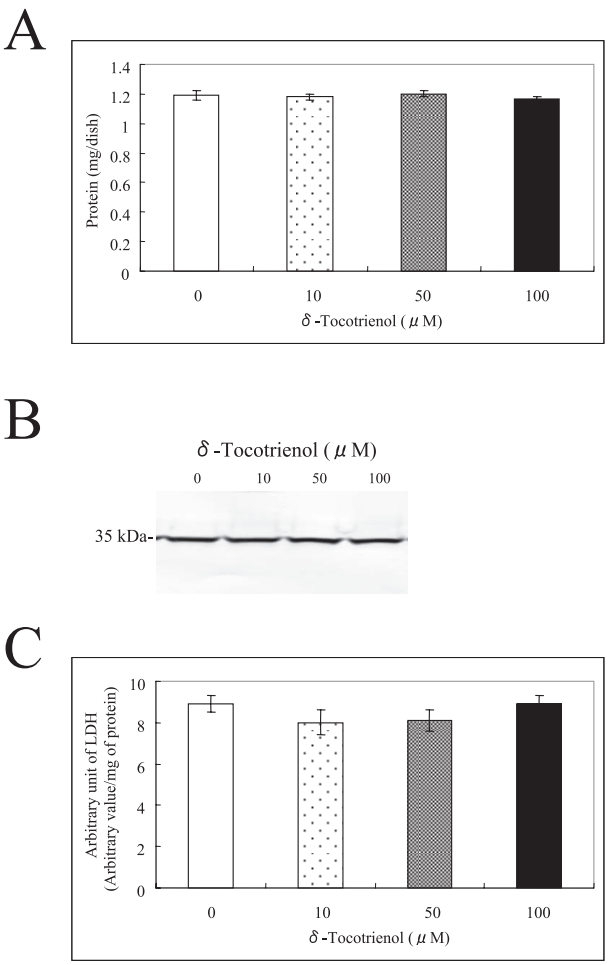

Fig. 1. Amount of Protein and LDH Activity in Cells Treated with δ-Tocotrienol

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 × *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 antibody, and these signals were then measured using an Intelligent Quantifier. Data are the means of three identical experiments.

The Effect of δ-Tocotrienol on Cholesterol Biosynthetic Enzymes and Cholesterol Content in the Cell

To examine whether levels of enzymes involved in cholesterol biosynthesis other than HMG-CoA reductase decreased with δ-tocotrienol treatment, the amount of MPD in treated and untreated cells was measured by immunoblot analysis. One of the first steps in the biosynthesis of cholesterol from acetic acid is catalyzed by MPD. As shown in Fig. 3A, the amount of MPD significantly de-

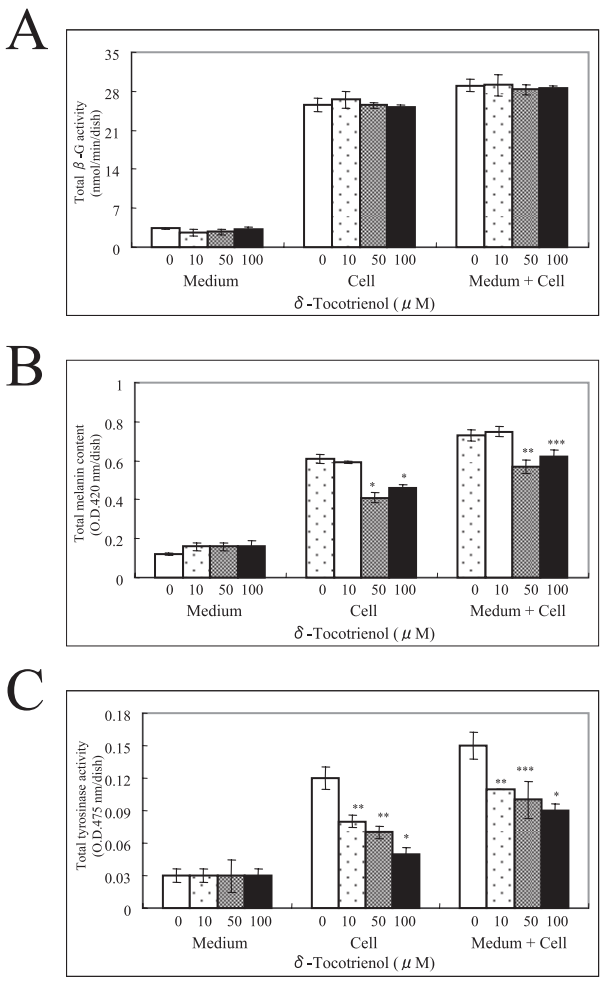

Fig. 2. Melanin Content and β-G Activity in Media of Cells Treated with δ-Tocotrienol

β–G activity (A), melanin content (B) and tyrosinase activity (C) in media (1 ml) and cells (750 μl) of dish were measured as described in Materials and Methods. Data are the means for three identical experiments. Significant differences: $^{*}p < 0.001$, $^{**}p < 0.005$, $^{***}p < 0.05$.

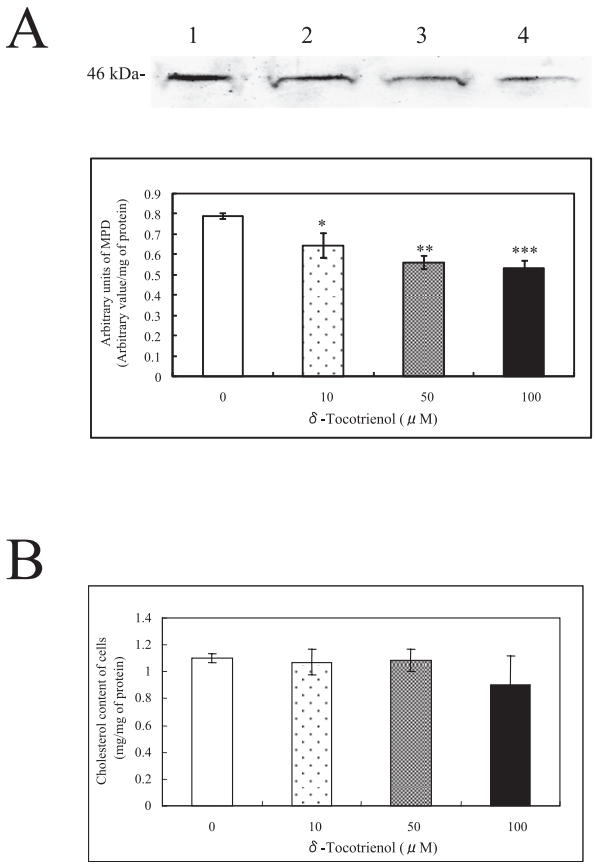

Fig. 3. Amount of MPD and Cholesterol Content in Cells Treated with δ-Tocotrienol

Samples from the same PNS as those used in experiments shown in Fig. 1 were subjected to immunoblot analysis for MPD and cholesterol content. A: Signals for MPD in the immunoblot analysis were quantified using an Intelligent Quantifier. B: Cholesterol content was measured under the above conditions. Data are the means for three identical experiments. Significantly different: $^{*}p < 0.05$, $^{**}p < 0.0005$, $^{***}p < 0.001$.

creased in a dose-dependent manner on treatment with δ-tocotrienol. The MPD level decreased by 20, 31, and 35% on treatment with 10, 50, and 100 μM δ-tocotrienol, respectively, compared with the level in control cells. These results suggested that δ-tocotrienol lowered levels of cholesterol biosynthetic enzymes other than HMG-CoA reductase. However, the cholesterol content of cells treated with 10–100 μM δ-tocotrienol for 24 hr did not decrease significantly (Fig. 3B).

DISCUSSION

Results of the present study show that δ-tocotrienol caused a decrease in the level of a cholesterol biosynthetic enzyme (MPD) other than HMG-CoA reductase (Fig. 3A). It was reported that δ-tocotrienol effectively blocks cleavage and translocation to the Golgi of SREBP.[25] δ-Tocotrienol may have caused the decrease in MPD by promoting the ubiquitination and degradation of HMG-CoA reductase or inhibiting processing of SREBP-2. However, there was no change in the cholesterol content in cells treated with δ-tocotrienol (Fig. 3B). These results suggested that B16 cells have a mechanism to maintain the appropriate cholesterol level despite the decrease in amount of cholesterol biosynthetic enzymes by δ-tocotrienol, although the mechanism remains unclear. Therefore, δ-tocotrienol does not appear to decrease the cholesterol contents.

We previously reported that the exocytosis of mature enzymes from lysosomes into the medium was caused by a lovastatin- and/or LDS-induced de-

crease in the cholesterol content in B16F10 mouse melanoma cells.[13] We suggested that lysosomal exocytosis promotes the rapid repair of wounds in plasma membranes ruptured by a loss of cholesterol, as cholesterol is a major constituent of the plasma membrane. Therefore, it was suggested that not only lysosomes but also lysosome-related melanosomes were released by the decrease in cholesterol. However, the release of lysosomes and melanosomes was not caused by δ-tocotrienol (Fig. 2), because δ-tocotrienol did not decrease the cholesterol contents in the cells. Thus, δ-tocotrienol does not mediate side effect cause the release of lysosomes and melanosomes.

In conclusion, δ-tocotrienol may be useful as a therapeutic or preventive drug for hyperpigmentation and as a component of whitening and/or lightening cosmetics without side effects (reduction of cholesterol content and release of lysosomes/melanosomes), although δ-tocotrienol cause a decrease of MPD.

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