Induction of Apoptosis by Tocotrienol in Rat Hepatoma dRLh-84 Cells

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Abstract. *Our aim was to evaluate the antitumor activities of tocopherol (Toc) and tocotrienol (T3) derivatives. At first, we examined the effect of these vitamin E homologues on the proliferation of rat normal hepatocyte RLN-10 and hepatoma dRLh-84 cells and found that especially T3 inhibited cell proliferation in dRLh-84 cells. Then, we examined the effect of vitamin E homologues on apoptosis induction and found that T3 induced DNA fragmentation and stimulated a rise of caspase-3 activity. In addition, T3 stimulated a rise in caspase-8 activity, while a caspase-8 inhibitor suppressed apoptosis induction by T3. We also examined the incorporation of vitamin E homologues into dRLh-84 cells. T3 was incorporated more quickly, compared to Toc. These results indicated that T3 induces apoptosis in dRLh-84 cells and that caspase-8 is involved in this apoptosis induction. The difference in terms of apoptosis induction by vitamin E homologues seems to be related to their different rates of cellular incorporation.*

Tocopherol (Toc) and tocotrienol (T3) share a similar basic chemical structure, which is characterized by a long phytyl chain attached at the 1-position on the chroman ring. However, Tocs have a saturated phytyl chain, while T3s have an unsaturated phytyl chain. In addition, Toc and T3 homologues differ from each other in the number of methyl groups bound to their chroman ring. Vitamin E homologues have been reported to have antioxidative, hypocholesterolemic and immunoregulatory effects (1, 2). T3 has been reported to inhibit the proliferation of normal mouse mammary epithelial cells, human breast carcinoma MCF-7, human leukemia HL-60 and murine B 16 melanoma cells (3-5). T3 also has been

reported to inhibit the proliferation of skin cancer *in vivo* (5). It has been reported that tumor marker enzyme activities in rats chemically induced with cancer were moderated by α-Toc and γ-T3 supplementation (6).

Furthermore, T3 has also been reported to induce apoptosis in normal mouse mammary epithelial cells (3). When cells are induced to apoptosis, caspases are activated, they being considered to be the executive factor for apoptosis. Caspase-8, as one of the caspases, is activated when the death ligand directly binds the death receptor, such as the Fas and TNF receptor. Caspase-8 in turn activates caspase-3 (7, 8). When caspase-3 is activated, a DNA endonuclease is activated. This DNA endonuclease then fragments the genomic DNA and apoptosis occurs, so this characteristic DNA fragmentation is an indicator of apoptosis (9, 10). In this study, we compared the effect of T3 on proliferation and apoptosis induction in rat normal liver RLN-10 and rat hepatoma dRLh-84 cells.

Materials and Methods

Reagents. Tocol and vitamin E homologues, such as α-Toc, γ-Toc, α-T3 and γ-T3, were kindly presented by Eisai Co. (Tokyo, Japan). SYBR Green was purchased from Molecular Probes (Oregon, USA). The BCA assay protein assay reagent kit was purchased from PIERCE (Rockford, IL, USA). The substrate for synthesis of caspase-3, Ac-DEVD-MCA and the substrate for synthesis of caspase-8, Ac-IETD-AMC were purchased from Peptide Co. (Osaka, Japan) and BIOMOL Research Laboratories Inc. (PA, USA), respectively. The caspase-8 inhibitor, Z-IETD-FMK was purchased from KAMIYA BIOMEDICAL Co. (WA, USA).

Cells and cell culture. Rat normal hepatocyte RLN-10 and hepatoma dRLh-84 cells derived from Donryu rat were purchased from the Japan Cancer Research Resources Bank (Tokyo, Japan). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin and 5% fetal bovine serum (FBS) (Biosource, CA, USA). The cells were cultured in 5% FBS / DMEM with or without vitamin E homologues for the appropriate time described in each experimental method. Then, the attached cell number was counted after trypsinization (0.2% trypsin

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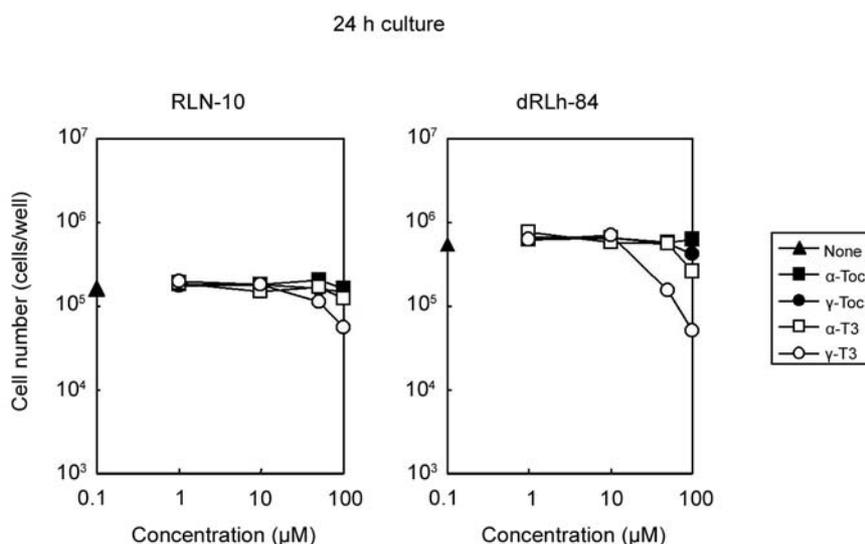
24 h culture

Figure 1. *Dose-dependent effect of vitamin E homologues on the proliferation of RLN-10 and dRLh-84 cells. These cells were inoculated at 5 x 10^4 cells/well and cultivated in DMEM supplemented with 5% FBS for 24 h with 0, 1, 10, 50 and 100 μM vitamin E homologues.*

with 0.025% ethylene diamine tetraacetic acid) in PBS using a cell counter (Sysmex, Kobe, Japan).

Detection of DNA fragmentation. dRLh-84 cells were treated with α- or γ- Toc at 100 μM, α-T3 at 50 μM and γ-T3 at 25 μM for 24 h. Then cells were collected by centrifugation at 200 x g for 5 min, washed with PBS and then suspended in 200 μl PBS. After centrifugation at 300 x g for 10 min, the cell pellet was suspended in 100 μl lysis buffer (10 mM Tris-HCl buffer, 10 mM EDTA and 0.5% Triton X-100) for 10 min at 4°C. The lysates were centrifuged at 15,000 x g for 5 min. The pellet was treated with 10 mg/ml RNase A for 1 h at 37°C and then with 10 mg/ml proteinase K for 30 min at 5°C. DNA was precipitated with 0.5 M NaCl and 50% isopropanol at –20°C overnight. Then the solution was centrifuged at 13,000 x g for 15 min. The DNA fragmentation pattern was visualized after electrophoresis using 2% agarose gel containing SYBR Green and photographed under UV light.

Determination of caspase activity. dRLh-84 cells were treated with α- or γ- Toc at 100 μM, α-T3 at 50 μM or γ-T3 at 25 μM for 24 h for measuring caspase-3 activity or 18 h for measuring caspase-8 activity. Then, the cells were collected and washed with PBS. The cell pellet was suspended in the extraction buffer containing 25 mM Hepes, 5 mM EDTA, 5 mM EGTA, 1 mM MgCl$_2$, 2 μg/ml aprotinine, 5 μg/ml phenylmethylsulfonyl fluoride (PMSF) and 100 μM dithiothreitol, and kept on ice for 30 min. The cells were sonicated for 30 sec and the lysate was centrifuged at 15,000 x g for 20 min at 4°C. The protein content of the supernatant was measured using the BCA assay protein assay reagent kit before the analysis of caspase-3 or caspase-8 activity. The supernatant was mixed with the assay buffer containing 25 mM Hepes, 10% sucrose, 0.1% CHAPS and 100 μM dithiothreitol and then incubated for 10 min at 37°C. The reaction was started with 10 μM Ac-DEVD-MCA for caspase-3 and Ac-IETD-AMC for caspase-8, and the amount of MCA was determined using RF-1500 spectrofluorophotometer (Shimadzu, Kyoto, Japan). Then

dRLh-84 cells were treated for 24 h with α-T3 or γ-T3 at 10, 25, 50 and 100 μM and caspase-8 inhibitor, Z-IETD-FMK. Attached cells were trypsinized with 0.2% trypsin containing 0.025% ethylene diamide tetraacetic acid (EDTA) and the number of viable cells was counted using the trypan blue exclusion method.

Detection of vitamin E homologues. dRLh-84 cells were treated with 5 μM vitamin E homologues for 12, 24, 48 and 72 h. Then, the cells were trypsinized, suspended in PBS and sonicated. One ml of 0.05 M SDS, 2 ml of ethanol and 1ml of hexane were added to 1 ml of the lysate. The solution was mixed for 1 min and then centrifuged at 1,000 x g for 1 min. After drying under N$_2$ gas flow, the residue was dissolved in 100 μl of 985 : 10 : 5 (v / v / v) mixture of hexane, 1,4-dioxiane and 2-propanol containing 25 μg/ml of tocol. Twenty μl of each sample was analyzed by high-performance liquid chromatography (HPLC), using Shimadzu SPD-10AV (Tokyo, Japan) equipped with Wakosil 5sil 4.6 mm x 250 mm ODS column (Wako, Osaka, Japan). The mobile phase used was a 985 : 10 : 5 (v / v / v) mixture of hexane, 1,4-dioxiane and 2-propanol.

Results

Figure 1 shows the dose-dependent effect of vitamin E homologues on the number of RLN-10 and dRLh-84 cells after a 24-h cultivation. α- or γ-Toc had no effect at concentrations between 1-100 μM. On the other hand, α-T3 induced a slight decrease of cell number at 100 μM, while γ-T3 strongly decreased cell number at 50 and 100 μM in a dose-dependent manner.

Figure 2 shows the growth curves of the cells in the presence of 50 μM vitamin E homologues. Proliferation of RLN-10 cells was not affected by the addition of α-Toc, γ-

50 μM addition

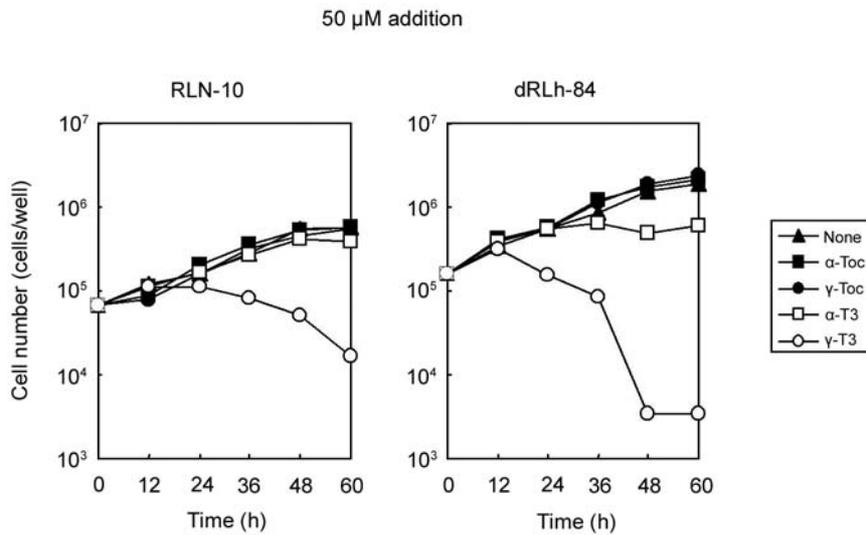Figure 2. *Growth curves of RLN-10 and dRLh-84 cells in the presence of 50 μM vitamin E homologues. These cells were inoculated at 5 x 10⁴ cells/well and cultivated in DMEM supplemented with 5% FBS for 0, 12, 24, 36, 48 and 60 h with 50 μM vitamin E homologues.*

Toc, or α-T3, while γ-T3 not only strongly suppressed proliferation, but also decreased the number of cells at and after 36 h cultivation. Proliferation of dRLh-84 cells was not affected by the addition of α- or γ-Toc, but ceased to proliferate at and after 24 h cultivation in the presence of α-T3. γ-T3 inhibited proliferation of the cells more strongly than α-T3 and it again exerted a cytotoxic effect. These results suggest that T3 suppresses proliferation of these cells more strongly than Toc and that γ-T3 has a stronger suppressive effect than α-T3.

Figure 3 shows the effect of vitamin E homologues on DNA fragmentation in dRLh-84 cells. When the cells were treated with α- or γ-Toc at 100 μM for 24 h, DNA fragmentation was not detectable. On the other hand, it was detected in the cells treated with α-T3 at 50 μM or γ-T3 at 25 μM for 24 h.

Figure 4 shows the effect of vitamin E homologues on caspase-3 and caspase-8 activities in dRLh-84 cells. When the cells were treated with α- or γ-Toc at 100 μM for 24 h, the activity of caspase-3 was not affected. However, an increase of caspase-3 activity was observed when the cells were treated with α-T3 at 50 μM or γ-T3 at 25 μM. When the cells were treated with α- or γ-Toc at 100 μM for 18 h, the activity of caspase-8, like caspase-3, was unaffected. On the other hand, caspase-8 activity was increased when the cells were treated with α-T3 at 50 μM or γ-T3 at 25 μM.

Then, we examined the effect of a caspase-8 inhibitor on the viability of the cells to clarify the relationship between the induction of caspase activities and apoptosis in dRLh-84 cells by T3. Figure 5 shows the effect of the caspase-8 inhibitor, Z-IETD-FMK, on the viability of dRLh-84 cells treated with the vitamin E homologues. The cells were treated with α- or γ-
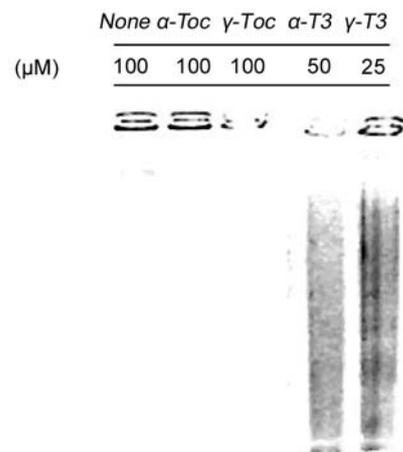

Figure 3. *Effect of vitamin E homologues on DNA fragmentation in dRLh-84 cells. DNA fragmentation was examined after the cells had been inoculated at 5 x 10⁴ cells/well and cultivated in DMEM supplemented with 5% FBS for 24 h with α- or γ-Toc at 100 μM, α-T3 at 50 μM and γ-T3 at 25 μM.*

T3 at 10, 25, 50 and 100 μM with or without Z-IETD-FMK for 24 h. In the presence of α- or γ-T3 at concentrations over 50 μM, the inhibition of Z-IETD-FMK improved the viability of the cells. This result suggests that caspase-8 activity is involved in induction of apoptosis by T3.

We examined the incorporation of vitamin E homologues into dRLh-84 cells to clarify the difference in the activity of vitamin E homologues. Figure 6 shows the intracellular levels of vitamin E homologues when the cells were cultured with 5 μM of each vitamin E homologue for 72 h. When the
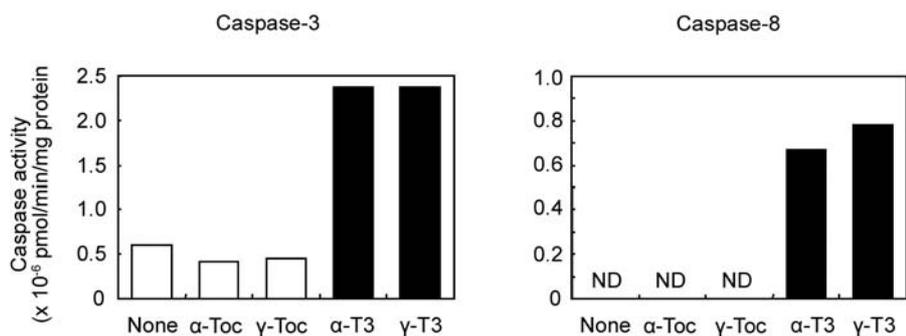
Figure 4. *Effect of vitamin E homologues on caspase-3 and caspase-8 activities in dRLh-84 cells. Caspase-3 and caspase-8 activities were measured after the cells had been inoculated at 5 x 10⁴ cells/well and cultivated in DMEM supplemented with 5% FBS for 24 h to measure caspase-3 activity, or 18 h to measure caspase-8 activity, with α- or γ-Toc at 100 μM, α-T3 at 50 μM and γ-T3 at 25 μM. ND; not detected.*
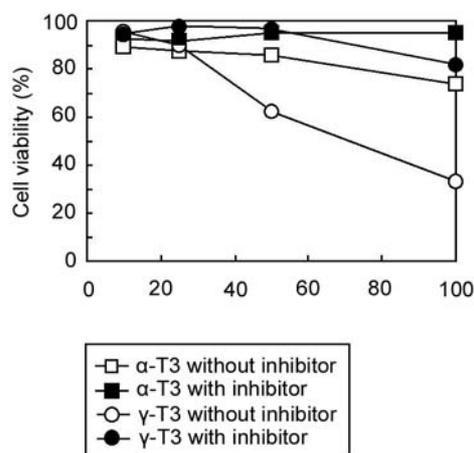

Figure 5. *Effect of caspase-8 inhibitor, Z-IETD-FMK on the viability of the cells treated with vitamin E homologues. The cells were inoculated at 5 x 10⁴ cells/well and cultivated in DMEM supplemented with 5% FBS for 24 h with α-T3 or γ-T3 at 10, 25, 50 and 100 μM and Z-IETD-FMK.*

cells were cultured with α-Toc, it was not detected in the cells. When the cells were cultured with γ-Toc, it was detected in the cells at and after a 24-h cultivation. In the presence of T3 derivatives, they were detected in the cells at and after a 12-h cultivation, while γ-T3 demonstrated the highest intracellular level. The levels of γ-Toc and α-T3 showed a maximum value at 48 h, while the levels of γ-T3 reached a plateau at 24 h. These results suggested that the difference of cellular incorporation was involved in the difference in apoptotic induction by vitamin E homologues.

Discussion

Anticancer activity has been reported for various foods components, such as tea catechin (11), capsaicin (12), polyunsaturated fatty acids (13, 14) and dietary fibers (15).

It has also been reported that lipophilic vitamins such as vitamin A, D and K have an antitumor effect but the mechanism of expression remains unknown (16-18).

Among the vitamin E homologues, T3 has been reported to inhibit the proliferation of normal mouse mammary epithelial cells, human breast carcinoma MCF-7, human leukemia HL-60 and murine B 16 melanoma cells (3-5). In addition, it has been reported that they inhibit the development of skin cancer *in vivo* (5). We showed here that T3 derivatives exert a stronger cytotoxic effect against rat hepatoma dRLh-84 cells than Toc derivatives. It has also been reported that RRR-α-tocopheryl succinate (VES), an esterified derivative of RRR-α-tocopherol, can inhibit the proliferation of tumor cells more strongly than α-tocopherol (19-21). These results suggest that Toc has a weaker anticancer activity than T3 and that some chemical modification is necessary for the expression of strong anticancer activity in Toc. Furthermore, we showed that γ-T3 suppressed proliferation of the cells more strongly than α-T3. In this study, the degree of proliferation suppressive activity is as follows: γ-T3 > α-T3 > γ-Toc > α-Toc.

In addition, we showed here that the levels of vitamin E homologues incorporated into dRLh-84 cells were in the following order: γ-T3 > α-T3 > γ-Toc > α-Toc. The same result has been reported in human breast cancer cells (3). We showed here that γ-T3 has the most potent cytotoxic activity and was incorporated at the highest level in dRLh-84 cells. It has been reported that the amount of vitamin E homologues are highly tissue-dependent when Toc and T3 were administered (22). Namely, T3 was not detected at all in blood clots, brain, thymus, testes, vice-testes and muscle. The level of T3 was maintained or increased for 24 h after its administration in the epididymal adipose and brown adipose tissues and in the heart. T3 levels were highest 8 h after administration in the serum, mesenteric lymph node, spleen, lungs and liver, and then 32.1 μg/g of α-T3 and 12.1 μg of γ-T3 were detected in liver. These levels of T3 are similar
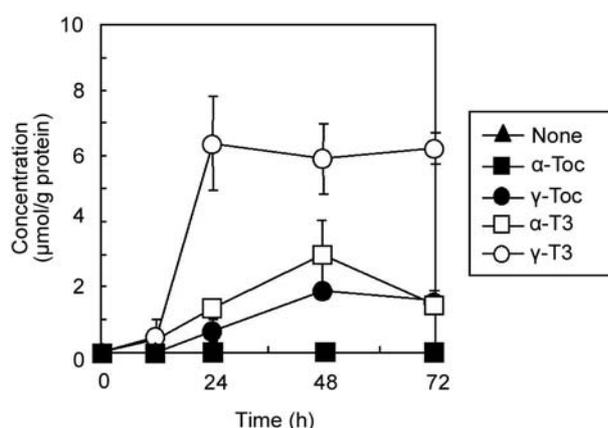
Figure 6. *Incorporation of vitamin E homologues into dRLh-84 cells. The cellular accumulation of each vitamin E homologue measured after the cells had been inoculated at 5 x 10^4 cells/well and cultivated in DMEM supplemented with 5% FBS for 0, 12, 24, 48 and 72 h with 5 μM vitamin E homologues. Data are means±SE for three samples.*

to the concentrations of T3 used in this experiment. It has also been reported that T3 can accumulate in the adipose tissue of hamsters (23) and in the skin of rats (24). These results suggest that the effect of T3 shows tissue specificity. Thus the expression of antitumor activation needs to consider the target tissue and the mode of administration and transport *in vivo*.

Cell death is classified into necrosis and apoptosis. The characteristics of apoptosis are induction of chromosomes condensation and fragmentation of nuclei or DNA (9, 10). We showed here that T3 induced DNA fragmentation but Toc did not. In normal mouse mammary epithelial cells, it has also been reported that T3 induces fragmentations of nuclei and DNA (3). In addition, RRR-α-tocopheryl succinate (VES) has been shown to induce apoptosis in tumor cells (21). In the induction of apoptosis, activation of caspases has been reported (7, 8). Among them, caspase-8 was first activated and then it in turn activated caspase-3. In this study, the rise in caspase-3 and caspase-8 activities was detected in the cells treated with T3. In addition, we showed that a caspase-8 inhibitor did indeed inhibit the induction of apoptosis by T3. The participation of caspase-8 has been reported in human gastric cancer SGC-7901 cells treated with RRR-α-tocopheryl succinate (VES) (21, 25). These results suggested that the expression of apoptosis induced by T3 is involved in the induction of caspase-8 activity. However, we need to further study this T3-induced apoptosis to fully elucidate its mechanism.

Acknowledgements

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