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Specific accumulation of γ- and δ-tocotrienols in tumor and their antitumor effect in vivo

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Abstract

In contrast to extensive studies on tocopherols, very little is understood about tocotrienols (T3). We evaluated the antitumor activities of γ-T3 and δ-T3 in murine hepatoma MH134 cells in vitro and in vivo. We found that δ-T3 inhibited the growth of MH134 cells more strongly than γ-T3 by inducing apoptosis. In C3H/HeN mice implanted with MH134, it was found that γ-T3 and δ-T3 feeding significantly delayed tumor growth. On the other hand, both T3 had no significant effect on body weight, normal-tissue weight and immunoglobulin levels. Intriguingly, we found that T3 was detected in tumor, but not in normal tissues. These results, to our knowledge, are the first demonstration of specific accumulation of γ-T3 and δ-T3 in tumors and suggest that T3 accumulation is critical for the antitumor activities of T3.

Keywords: Tocotrienols; Antitumor; Accumulation; Murine hepatoma MH134 cells; Apoptosis

1. Introduction

Natural vitamin E consists of two classes of compounds: tocopherols (Toc) and tocotrienols (T3). The main difference between them is that T3 have an unsaturated phytyl chain attached to the 1-position on the chroman ring, whereas Toc have a saturated phytyl chain. In addition, Toc and T3 differ from each other by the number and position of methyl groups in the chroman ring.

Although both Toc and T3 have various effects such as antioxidative activities [1], T3 have been reported to have additional properties such as hypocholesterolemic and immunoregulatory activities [2–5]. Among T3 homologues, α-T3 has been reported to have a potent neuroprotective effect [6,7]. Moreover, T3 have also been reported to have antiproliferative activities against various cancer cells such as rat hepatoma dRLh-84 cells, human hepatoma Hep3B cells and human breast carcinoma MCF7 cells [8–10], and the effects are suggested to be due to apoptosis. However, little has been known about the antitumor effects of T3 in vivo. So far, γ-T3 feeding has been reported to decrease tumor weight and to prolong the survival rate of C57BL female mice transplanted with melanoma [11]. Furthermore, it has been reported that administration of T3 mixture (38% γ-T3, 22% α-T3 and 12% δ-T3) significantly suppresses liver and lung carcinogenesis in C3H/HeN male mice [12]. On the other hand, the antitumor effects of each T3 (especially δ-T3) in vivo are still elusive. On the distribution of T3, it has been reported that the amount of T3 is highly tissue-dependent [13]; namely, T3 was not detected at all in blood clots, brain, thymus, testes, vice-testes and muscles.

We previously reported that γ-T3 and δ-T3 have strong antiproliferative effects among T3 homologues [8,9]. Thus, in this study, we investigated the effect of γ-T3 and δ-T3 on the growth of murine hepatoma MH134 cells in vitro and in

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vivo. In addition, we examined the distribution of both T3 in normal tissues and tumors of C3H/HeN mice implanted with MH134 cells. We found that γ-T3 and δ-T3 exert antitumor activity and that both T3 specifically accumulate in tumors.

2. Materials and methods

2.1. Materials

γ-T3 and δ-T3 were kindly presented by Eisai Food and Chemical Co., Ltd. (Tokyo, Japan).

2.2. Cell culture

Mouse hepatoma MH134 cells were suspended in RPMI-1640 medium (Nissui, Tokyo, Japan) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin and 2% fetal calf serum (FCS; PAA Laboratories GmbH, Austria). The cells were cultured in 2% FCS/RPMI-1640 medium with γ-T3 or δ-T3 for the periods described in each experimental method. As vehicle, 0.1% of ethanol was added to control cells. The number of viable cells was counted using the trypan blue exclusion method.

2.3. Immunoblot analysis for poly (ADP-ribose) polymerase cleavage

MH134 cells were treated with γ-T3 and δ-T3 for 24 h. Then cells were collected, washed with phosphate-buffered saline (PBS) three times and lysed in cell lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton-X 100, 1 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM phenylmethylsulfonyl fluoride, 2.0 μg/ml aprotinin and 1 mM pervanadate. Whole-cell lysate was incubated at 4°C for 30 min and then centrifuged at 15,000×*g* for 30 min. The supernatant was mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The mixture was loaded onto 8% SDS-PAGE gel, and electrophoresis was performed under reducing conditions. The sample was then electrotransferred onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The blotted nitrocellulose was probed for rabbit anti-human poly (ADP-ribose) polymerase (PARP) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody used was horseradish-peroxidase-conjugated anti-rabbit immunoglobulin (Ig) G, and detection was performed using Enhanced Chemiluminescence Reagent Advance Western Blotting Detection kit (GE Healthcare, UK).

2.4. Animal experiments

Four-week-old male C3H/HeN mice were obtained from Kyudo Co. Ltd. (Tosu, Japan). They were kept at the Biotron Institute of Kyushu University in a 12-h light/12-h dark cycle (lights on, 0800–2000 h) in an air-conditioned room (20°C and 60% humidity under specific pathogen-free conditions). This experiment was carried out in accordance with the guidelines for animal experiments of the Faculty of Agriculture and the Graduate Course of Kyushu University, and law no. 105 and notification no. 6 of the Japanese government. After preliminary breeding for 1 week, the mice were divided into three groups and allowed free access to experimental diets and water. Diets were prepared according to the recommendations of the American Institute of Nutrition, AIN-93G (10.0% safflower oil, 10.0% sucrose, 13.2% α-cornstarch, 20% casein, 36.7% β-cornstarch, 5% cellulose, 1.0% vitamin mix, 3.5% mineral mix, 0.25% choline bitartrate, 0.3% L-cystine and 0.0014% *t*-butylhydroquinone). In addition, 0.1% γ-T3 and δ-T3 were added to AIN-93G, respectively. After 4 weeks of feeding, the mice were fasted for 10 h and then killed by drawing blood from the abdominal aorta under light anesthesia with diethyl ether. The heart, lungs, liver, kidney, spleen, epididymal adipose and renal adipose were immediately excised. Immediately after excision, each tissue was weighed, and the lymphocytes were isolated from the spleen. Serum was obtained by centrifugation at 1000×*g* for 15 min at 4°C and stored at −80°C until use.

2.5. Tumor cell inoculation and tumor growth

Murine hepatoma MH134 cells (5×10^6 cells) were injected into the subcutaneous tissue in the back of mice. Tumors were measured using vernier calipers every other day, and volumes were calculated according to the equation: volume (mm^3)=length (mm)/2×width^2 (mm^2).

2.6. Measurement of vitamin E levels

Vitamin E levels in vitro and in vivo were determined using the method of Burton et al. [14]. The levels of each T3 were determined by comparing their peaks with the peak of tocol, which was added as internal standard.

2.7. Measurement of Ig levels

Measurement of Ig concentration in mice sera was performed using sandwich enzyme-linked immunosorbent assay. Rabbit anti-mouse IgA (Zymed, San Francisco, CA), goat anti-mouse IgG (H+L; Zymed) and rabbit anti-mouse IgM (μ-chain-specific; Zymed) were used to fix each Ig. These antibodies were diluted using 1% bovine serum albumin (BSA)–PBS, added to a 96-well plate and incubated for 1 h at 37°C. Then, 300 L of 1% BSA–PBS was added and kept at 37°C for 2 h, and samples (50 μl) were added to each well for 1 h at 37°C. Each well was treated with a solution of either peroxidase (POD)-conjugated goat anti-mouse IgA (Zymed), POD-conjugated goat anti-mouse IgG (H+L; Zymed) or POD-conjugated rabbit anti-mouse IgM (Zymed) to detect the respective Ig and incubated for 1 h at 37°C. The plates were rinsed with PBS containing 0.5 ml/L polyethylene sorbitan monolaurate (Nacalai Tesque, Kyoto, Japan) between each step.

Then a 10:9:1 mixture of 1.8 mM H_2O_2 in 0.2 M citrate buffer (pH 4.0), H_2O and 11.7 mM 2,2′-azinobis (3-ethylbenzothiazoline sulfonic acid) was added. Finally, absorbance at 415 nm was measured after the addition of 160 mM oxalic acid to stop the coloring reaction.

2.8. Statistical analysis

Student's *t* test was utilized to analyze the antiproliferative effects of γ-T3 and δ-T3 in vitro. The data of animal experiments were tested by one-way analysis of variance, followed by Tukey–Kramer test. Differences were considered significant at $P<05$.

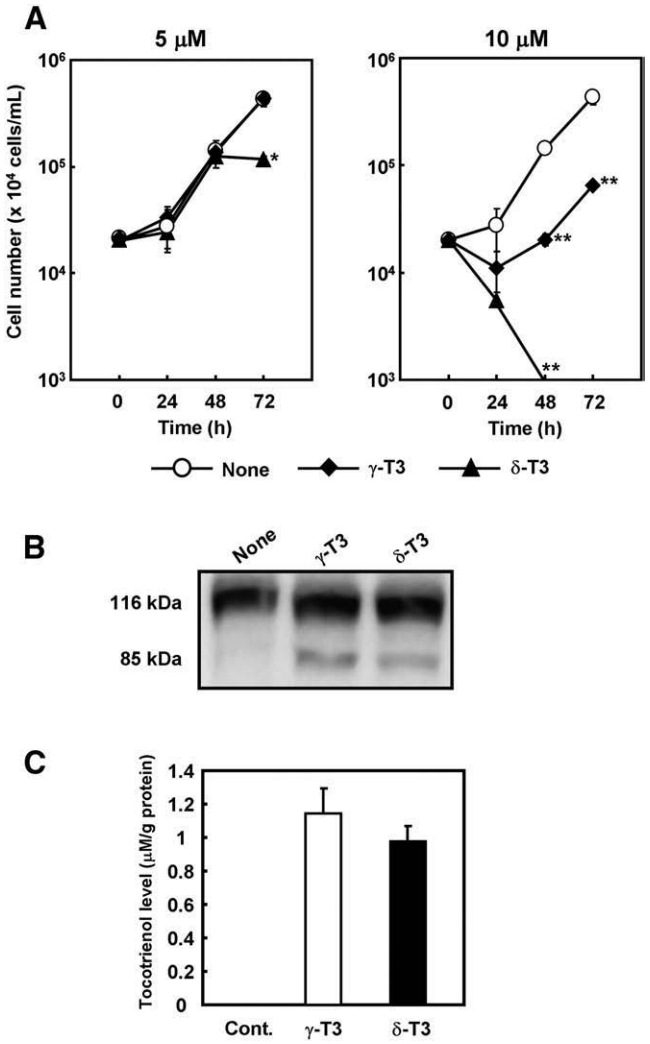

Fig. 1. Effect of γ-T3 and δ-T3 on the proliferation of MH134 cells. (A) Cells were inoculated at 2×10^4 cells/ml and cultivated in RPMI-1640 medium supplemented with 2% FCS and 0, 5 and 10 μM γ-T3 or δ-T3 for the indicated time periods. Data shown are presented as mean±S.D. for three samples. Data marked with asterisk are significantly different from values in controls (*$P<.01$ and **$P<.001$; Student's *t* test). (B) Cleavage of PARP protein induced by γ-T3 and δ-T3. PARP protein was detected after treatment with 20 μM γ-T3 or 10 μM δ-T3 for 24 h. (C) Incorporation of γ-T3 or δ-T3 into MH134 cells. Cells were inoculated at 1×10^6 cells/ml and cultivated in RPMI-1640 medium supplemented with 2% FCS and 10 μM γ-T3 or δ-T3 for 8 h. Data shown are presented as mean±S.D. for three samples.

Table 1
Effect of γ-T3 and δ-T3 on body weight and tissue weight

	Control	γ-T3	δ-T3
Body weight (g)			
Initial	16.7±0.5	16.6±0.5	16.6±0.5
Final	26.7±1.2	25.4±0.9	24.2±1.0
Tissue weight (g)			
Heart	0.08±0.01	0.10±0.00	0.08±0.00
Lungs	0.12±0.01	0.11±0.01	0.12±0.00
Liver	1.20±0.04	1.18±0.04	1.07±0.06
Kidney	0.31±0.00	0.32±0.01	0.30±0.01
Spleen	0.20±0.02	0.18±0.01	0.19±0.02
Epididymal adipose	0.05±0.01	0.09±0.02	0.04±0.01
Renal adipose	0.03±0.00	0.03±0.01	0.02±0.00

Date are presented as mean±S.E. (n=3–5).

3. Results

3.1. Effect of γ-T3 and δ-T3 on the proliferation of MH134 cells and PARP fragmentation

The effect of γ-T3 and δ-T3 on the proliferation of MH134 cells was investigated. On γ-T3, the proliferation of MH134 cells was not suppressed at 5 μM. On the other hand, the antiproliferative effect of δ-T3 at 5 μM was found after 72 h of incubation, and 10 μM δ-T3 exerted an antiproliferative effect stronger than that of γ-T3 each time (Fig. 1A).

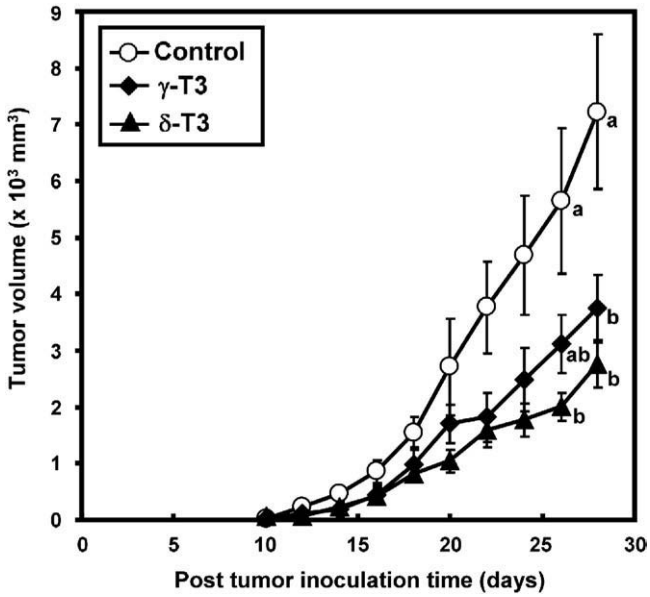

Fig. 2. Effect of γ-T3 and δ-T3 on the tumor growth of C3H/HeN mice. Murine hepatoma MH134 cells (5×10^6 cells) were injected into the subcutaneous tissue in the back of mice. Ten days after injection, a tumor was observed and measured using vernier calipers. All mice were fed a powdered AIN-93G diet and treated for 28 days. Then mice were killed by drawing blood from the abdominal aorta under light anesthesia with diethyl ether. Data are presented as mean±S.E. (n=3–5), and values without a common superscript letter are significantly different at $P<05$.

Because T3 has been known to induce apoptosis in various cells [8,9], we examined the effect of T3 on the PARP fragmentation of MH134 cells by Western blot analysis. PARP is a 116-kDa protein in intact form and is decomposed to an 85-kDa fragment by induction of apoptosis. When the cells were treated with 20 μM γ-T3 or 10 μM δ-T3 for 24 h, the 85-kDa fragment was detected in the T3-treated cells (Fig. 1B). These results suggest that both γ-T3 and δ-T3 induce apoptosis in MH134 cells.

Then we examined the incorporation of γ-T3 and δ-T3 into MH134 cells to clarify the difference in the activities of both T3. When the cells were cultured for 8 h, there was no significant difference between γ-T3 and δ-T3 levels (Fig. 1C).

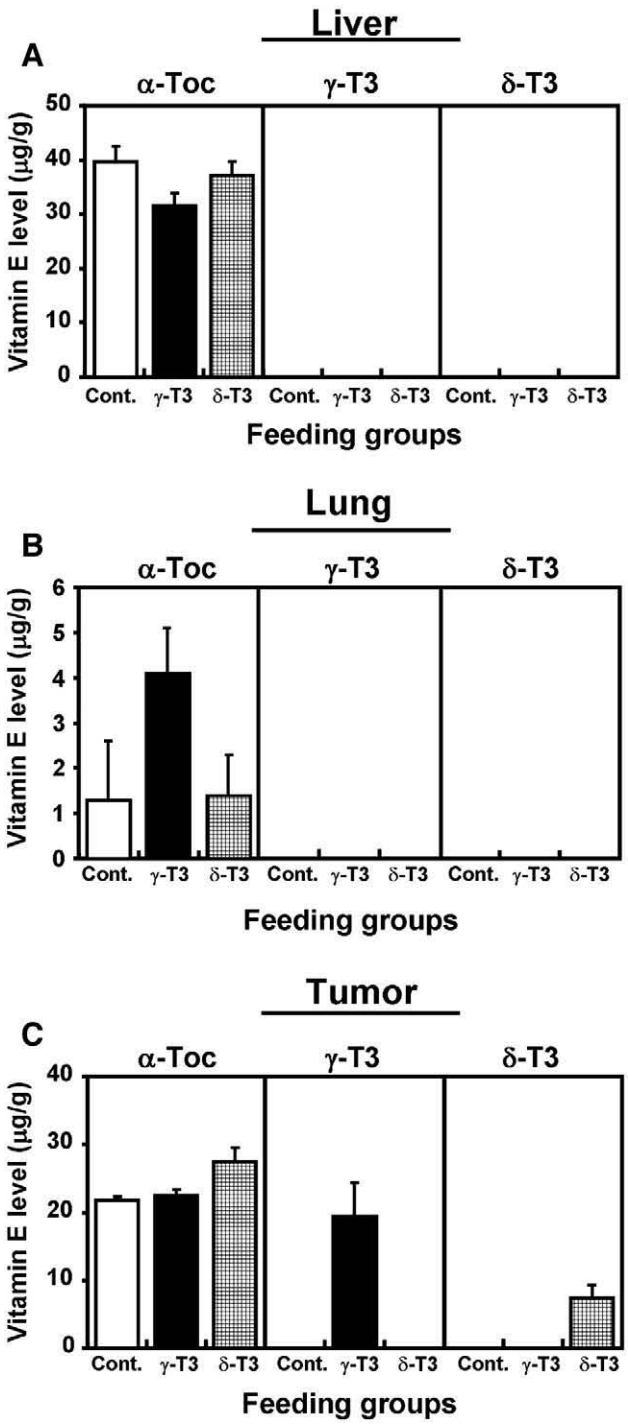

Fig. 3. Effect of dietary γ-T3 and δ-T3 on vitamin E levels in normal tissues and tumors. The vitamin E levels in the liver (A), lung (B) and tumor (C) were measured with high-performance liquid chromatography. The levels were determined by comparing their peaks with the peak of tocol, which was added as internal standard. Data are presented as mean±S.E. (n=3–5).

Table 2
Total serum Ig levels in C3H/HeN mice fed γ-T3 or δ-T3

	Control	γ-T3	δ-T3
IgA (mg/ml)	0.17±0.03	0.24±0.07	0.13±0.01
IgG (mg/ml)	2.03±0.29	2.33±0.51	1.81±0.29
IgM (mg/ml)	2.49±0.27	2.37±0.26	2.63±0.13

Data are presented as mean±S.E. (n=3–5).

3.2. Effect of γ-T3 and δ-T3 feeding on body weight and tissue weight

We investigated the effect of γ-T3 and δ-T3 feeding on body weight and normal-tissue weight in mice. As shown in Table 1, there was no significant difference in body weight and tissue weight during the study. These results suggest that both T3 diets did not affect body weight and tissue weight in mice.

3.3. Effect of γ-T3 and δ-T3 on tumor volume

We next investigated the effect of γ-T3 and δ-T3 diets on tumor volume. Ten days after the inoculation of MH134 cells in the subcutaneous tissue in the back of mice, a tumor was observed in each group. The growth of the tumor was significantly delayed in both γ-T3 and δ-T3 feeding groups compared with the control group (Fig. 2). In addition, δ-T3 tended to exert a stronger antitumor effect than γ-T3 in the latter stage, although there was no statistical difference.

3.4. Vitamin E distribution in liver, lung and tumor in mice

Fig. 3 shows the α-Toc, γ-T3 and δ-T3 levels in the liver (A), lung (B) and tumor (C) of mice fed both diets. α-Toc was detected in normal tissues and tumors from all groups; in particular, the increase in α-Toc level in the lungs was observed in the γ-T3 feeding group, although the difference was not significant.

Table 3
Total Ig productivity of spleen lymphocytes in C3H/HeN mice fed γ-T3 or δ-T3

	Control	γ-T3	δ-T3
IgA (ng/ml)	40.9±1.2	35.3±6.0	43.7±5.8
IgG (μg/ml)	0.10±0.01	0.09±0.01	0.08±0.00
IgM (μg/ml)	1.65±0.10	1.24±0.12	1.47±0.07

Data are presented as mean±S.E. (n=3–5).

On the other hand, γ-T3 and δ-T3 were not detected in the liver and lungs. However, in tumors, γ-T3 was detected in the γ-T3 feeding group, and δ-T3 was detected in the δ-T3 feeding group. There was no significant difference between γ-T3 and δ-T3 levels in tumors.

3.5. Effect of γ-T3 and δ-T3 on immune function in mice

Table 2 shows the effect of dietary γ-T3 and δ-T3 on serum Ig levels. IgA and IgG levels showed an increasing tendency in the γ-T3 group and a suppressing tendency in the δ-T3 group, although the significant difference was not marked. IgM level showed a suppressing tendency in the γ-T3 group and an increasing tendency in the δ-T3 group, but there was no significant difference either.

In splenocytes, IgA level was slightly lower in the γ-T3 group and higher in the δ-T3 group. In addition, IgG and IgM levels showed a suppressing tendency in both groups. However, no significant difference was observed in all immune functions (Table 3).

4. Discussion

Anticancer activity has been reported in various food components such as tea catechin [15], capsaicin [16], polyunsaturated fatty acids [17,18] and dietary fibers [19]. It has also been reported that lipophilic vitamins such as vitamins A, D and K have antitumor effects [20–22].

We showed here that γ-T3 and δ-T3 inhibited the proliferation of murine hepatoma MH134 cells in a dose-dependent and time-dependent manner. We also found that the antiproliferative effect of δ-T3 occurred in a lower level as compared with γ-T3. These results agree with previous reports that δ-T3 exerts stronger antiproliferative effects than γ-T3 against human hepatoma Hep3B cells [9] and HepG2 cells [12]. Compared with these results, the proliferation of MH134 was suppressed at lower concentrations, suggesting that each kind of cell line has its own sensitivity to T3. In addition, there was no significant difference at the intracellular level between γ-T3 and δ-T3, suggesting that the difference in both T3 activities is not due to difference in the speed of incorporation of T3 into the cells.

In human hepatoma Hep3B cells, γ-T3 induces apoptosis by cleaving PARP through the caspase-8/caspase-9 activation pathway [9]. In our study, PARP fragmentation was induced with γ-T3 and δ-T3 treatments, suggesting that γ-T3 and δ-T3 induced apoptosis in MH134 cells. In contrast, it has been reported that although γ-T3 obviously induced apoptosis, fragmentation of PARP is not detected in human breast cancer MDA-MB-231 cells [23]. These results suggest that the pathway of apoptosis used by T3 is cell-line-dependent. Further study will be needed to clarify the mechanism of T3-induced apoptosis in MH134 cells.

In vivo, we found that γ-T3 and δ-T3 significantly delayed tumor growth, although both T3 had no effect on the body weight and tissue weight of mice. We also found that part of the inside of the tumor was decayed by T3 (data not shown). These results suggest that γ-T3 and δ-T3 inhibit tumor growth without harming normal tissues. In previous reports, it has been reported that γ-T3 decreased tumor weight in mice implanted with B16 mouse melanoma cells [11]. In addition, T3 mixture (38% γ-T3, 22% α-T3 and 12% δ-T3) significantly suppressed liver and lung carcinogenesis [12]. In the present study, it is suggested that δ-T3 tends to inhibit tumor growth more strongly than γ-T3 in vitro. Considering these results, it is suggested that each T3 has a different efficiency in inducing apoptosis in vivo.

In the present study, we demonstrated for the first time that γ-T3 and δ-T3 accumulated only in tumors, although α-Toc accumulated not only in tumors but also in the liver and lungs. Of note, the increase in α-Toc level in the lungs was observed in the γ-T3 feeding group. On the other hand, the α-Toc level in the lungs of Sprague–Dawley rats fasted for 10 h was decreased by T3 mixture (36.5% γ-T3, 21.4% α-T3 and 8.6% δ-T3) feeding [3]. Taken together, it is suggested that the composition of T3 diets plays an important role in α-Toc accumulation in tissues. According to previous reports, T3's metabolism is different manner from that of α-Toc. It has been reported that the retention time of α-T3 in vital organs is shorter than that of α-Toc [24]. In addition, α-Toc is detected in many tissues, whereas T3 accumulate in parts of tissues such as adipose, skin and mesenteric lymph node [25,26]. Moreover, the distribution and metabolism of T3 in the rats vary considerably among different tissues. In some tissues including the liver and lungs, T3 levels are highest 8 h after T3 administration [13]. Considering these results, T3 are metabolized in a tissue-dependent manner. It is known that both Toc and T3 are metabolized to carboxyethyl-hydroxychroman (CEHC) through the same pathway (namely, ω-oxidation followed by β-oxidation of the side chain) [27]. Prostate cancer cells have been reported to transform vitamin E homologues into CEHC exclusively as a detoxification mechanism that is useful in maintaining their malignant properties [28]. In addition, cytochrome P450 is regarded as a critical determinant of this metabolism [29]. It has been reported that α-Toc transfer protein (α-TTP) has a strong affinity for α-Toc and a relatively weak affinity for the other vitamin E homologues [30]. Moreover, due to the high affinity of α-Toc for α-TTP, α-Toc is preferentially transferred from the liver to very-low-density lipoproteins in the circulation via α-TTP. Therefore, α-Toc is not metabolized by cytochrome P450 in the liver to the same extent as other vitamin E homologues [31]. Kawakami et al. have suggested that γ-T3 found in adipose tissues may have been transferred directly from chylomicrons by lipoprotein lipase, rather than passing through the liver. Kawakami et al. [32] and Ikeda et al. [33] have also suggested that the distribution of T3 is based on the cytochrome P450 expression level in each organ. Taken together, it is suggested that direct T3 transfer from chylomicrons and P450 expression level in tumors are involved in T3 accumulation. T3 has been shown to accumulate in rat

hepatoma dRLh-84 cells, human T-leukemia Jurkat E6-1 cells and intestinal epithelial Caco2 cells [8,34,35], suggesting that Toc and T3 have different metabolic speeds between normal tissues and tumors. It is also probable that the T3 metabolic mechanism does not work properly in tumors. In humans, the postprandial metabolic fate of T3-rich vitamin E differs significantly from that of α-Toc [36]. In addition, δ-T3, as well as γ-T3, exerted antitumor activity, although there was a tendency for the level of δ-T3 in the tumor to be lower than that of γ-T3. In the present study, we showed that there was no significant difference in intracellular levels between γ-T3 and δ-T3, suggesting that cellular incorporation is not involved in the level of each kind of T3 in tumors. It is probable that, in tumors, δ-T3 is metabolized to other derivatives faster than γ-T3. It is also probable that δ-T3 is transported to tumors more slowly than γ-T3.

On immune function, we found that neither γ-T3 nor δ-T3 significantly changed serum Ig levels and Ig productivity of the spleen. These results suggest that immune function may be not involved in T3-induced tumor-suppressing effects and that the antitumor effect may be due to the direct effect of T3 on tumor cells. Under normal conditions, T3 mixture (36.5% γ-T3, 21.4% α-T3 and 8.6% δ-T3) feeding has been reported to significantly increase Ig productivity in spleen lymphocytes of young Brown Norway rats [4]. In addition, it has been reported that T3 mixture feeding significantly increased serum IgA level in young Sprague–Dawley rats [5]. Taken together, it is suggested that the effect of T3 on immune function depends on the composition of T3 diets.

In conclusion, our results suggested that accumulation is critical for the antitumor activity of T3.

Acknowledgments

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