Studies on the Biological Activity of Tocotrienols

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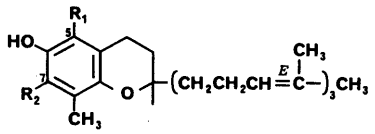
Tocotrienols were evaluated for activity against transplantable murine tumors inoculated i.p. into mouse, and the activities of two tocotrienols and α-tocopherols were compared. When the compounds were injected i.p., α- and γ-tocotrienols were effective against sarcoma 180, Ehrlich carcinoma, and IMC carcinoma, and γ-tocotrienol showed a slight life-prolonging effect in mice with Meth A fibrosarcoma, but the tocotrienols had no antitumor activity against P388 leukemia at doses of 5—40 mg/kg/d. On the other hand α-tocopherol had only a slight effect against sarcoma 180 and IMC carcinoma. The antitumor activity of γ-tocotrienol was higher than that of α-tocotrienol. Tocotrienols showed growth inhibition of human and mouse tumor cells when the cells were exposed to these agents for 72 h *in vitro*, whereas tocopherol did not show any marked cytotoxic activity. Alpha- and γ-tocotrienols had inhibitory effects on lipid peroxidation of murine microsomes by adriamycin.

Keywords tocotrienol; murine tumor; cytotoxicity; microsome; antitumor activity; antioxidant activity

Alpha-tocopherol (vitamin E) has many kinds of physiological activity. In the field of cancer chemotherapy, α-tocopherol enhanced the cytotoxic effect of adriamycin *in vitro*[1] and provided protection against adriamycin-induced side effects.[2,3] In contrast, the physiological effects of tocotrienols (Fig. 1) have not been studied[4] much. Recently, during a search for new physiological activities of tocotrienols isolated from rice bran oil, we found that α-tocotrienol showed antitumor activity against mouse transplantable IMC carcinoma.[5] Thereafter, we successfully isolated large amounts of α- and γ-tocotrienols from palm oil. This report describes the effects of α- and γ-tocotrienols on murine tumors and on lipid peroxidation *in vitro* in a system consisting of rat liver microsomes, reduced nicotinamide adenine dinucleotide phosphate (NADPH) and adriamycin.



	R_1	R_2
α-tocotrienol:	CH_3	CH_3
β-tocotrienol:	CH_3	H
γ-tocotrienol:	H	CH_3
δ-tocotrienol:	H	H

Fig. 1. Structure of Tocotrienols

Materials and Methods

Animals Female ICR, ddY, CDF_1 and BALB/c mice, 6 weeks of age, were purchased from the Shizuoka Laboratory Animal Center, Hamamatsu. Seven mice were used in each group.

Agents Tocotrienols were isolated from palm oil in our laboratory using high performance liquid chromatography (HPLC) (multicolumn system, Soken Chemical and Engineering Co., Ltd., Tokyo). Alpha- and γ-tocopherols were obtained from Sigma (T 3251), U. S. A. and Eisai Co., Ltd., Tokyo, respectively. Agents were dissolved in a small amount of MeOH and Tween 80, and diluted with saline solution before use in animal studies. For *in vitro* tests, agents were dissolved in a small amount of MeOH and diluted with culture medium.

Tumor Cell Lines and Antitumor Activity Tumor cells were maintained in ascitic form by serial i.p. passaging in mice. Tumor cell lines and mice used in the present experiment are listed in Table I. In all tumor models, the agents were administered i.p. as ten doses on days 1—5 and 7—11 after tumor inoculation. Animals were observed daily and experiments were terminated on day 60.

TABLE I. Tumors and Mice Used

Tumor	Mouse	Inoculum Size[a]	Site
Ehrlich carcinoma	ddY	2.5×10^5	i.p.
Sarcoma 180	ICR	3.0×10^5	i.p.
IMC carcinoma	CDF_1	1.0×10^6	i.p.
P388 leukemia	CDF_1	3.0×10^4	i.p.
Meth A fibrosarcoma	BALB/c	1.0×10^5	i.p.

a) Cells/mouse.

Evaluation of Antitumor Activity Antitumor activity of the samples on ascitic tumors was evaluated in terms of the increase in life span (ILS); $(T/C-1) \times 100(\%)$, where T is the mean survival time in days (MSD) of the treated group and C is the MSD of the control group. Survival of the mice was scored at 60 d after inoculation with the tumors, and mice remaining alive after the period of observation were considered as cured. For the statistical analysis of the results, Student's t test was used.

Cytotoxicity to Tumor Cells Tumor cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and kanamycin (50 μg/ml) at 37 °C. To determine the cytotoxicity of tocotrienols, HeLa (4×10^4 cells), P388 (1×10^5 cells) or H69 human lung carcinoma (5×10^4 cells) in 1.5 ml of medium were placed in a tissue culture plate (Falcon, 24 wells) and incubated in a 95% air–5% CO_2 atmosphere at 37 °C for 24 h. To each culture well, 0.5 ml of fresh medium containing a different concentration of tocotrienol was added. After further incubation for 72 h under the same conditions described above, P388 and HeLa (trypsinized to make a single cell suspension) cells were treated with 0.5% trypan blue and counted with a hemocytometer.

For determination of the combined effects of tocotrienol and adriamycin, HeLa cells (8×10^3) in 0.2 ml were placed in a 96-well microculture plate and incubated at 37 °C for 72 h. To each culture well, 10 μl of a mixture of adriamycin and tocotrienol was added. After further incubation for 72 h, cell growth was determined by MTT assay as described by Alley *et al.*[6]

Preparation of Microsomes A mouse was decapitated and allowed to exsanguinate. The liver was quickly excised and washed in saline solution, and 20% (w/v) liver homogenate was prepared in 1.15% KCl. This homogenate was centrifuged at 10000 *g* for 30 min. The mitochondrial supernatant fraction was centrifuged at 78000 *g* for 1 h. The resulting microsomal pellet was rehomogenized and diluted to 1.7 mg protein/ml in 150 mM KCl–50 mM Tris–HCl buffer, pH 7.5.

Incubation Conditions for Microsomal Lipid Peroxidation The reaction mixture consisted of 1.0 ml of microsome suspension, 0.1 ml of 42.5 mM NADPH, 0.052 ml of 2 mg/ml of adriamycin, 0.1 ml of tocopherol or

tocotrienol and 0.475 ml of 150 mM KCl–50 mM Tris–HCl buffer, pH 7.5. Incubations were carried out at 37 °C for 1 h in a water bath with agitation.

Determination of Lipid Peroxidation After incubation as described above, 0.5 ml of reaction mixture was transferred to a tube and lipid peroxidation was determined by the method of Uchiyama and Mihara.[7]

Results and Discussion

Tocotrienols were evaluated for activity against transplantable murine tumors, and the activities of two tocotrienols and α-tocopherol were compared. As shown in Tables II and III, α- and γ- tocotrienols were effective against sarcoma 180, Ehrlich carcinoma, and IMC carcinoma. A slight life prolonging of γ-tocotrienol was observed in mice with Meth A fibrosarcoma, but tocotrienols had no antitumor activity against P388 leukemia. On the other hand α-tocopherol showed only a slight effect on sarcoma 180 and IMC carcinoma. The antitumor activity of γ-tocotrienol was higher than that of α-tocotrienol. The cytotoxicity of tocotrienols against tumor cells *in vitro* was determined and the results are summarized in Table IV. Tocotrienols showed growth inhibitory activity on these cell lines when the cells were exposed to the agents for 72 h, whereas α-tocopherol did not show any marked cytotoxic activity.

A discrepancy was observed between *in vitro* cytotoxicity and *in vivo* activity of tocotrienols against P388 leukemia. Since P388 leukemia, unlike the other tumors used in this experiment, readily metastasized systemically in the i.p. inoculated mice, it appeared that tocotrienol was not distributed systemically in sufficient amounts to show cytotoxicity. No toxic symptoms appeared in mice which received α-tocotrienol or α-tocopherol at a dose of 400 mg/kg, while body weight loss was observed in mice which received γ-tocotrienol at the same dose (Tables II and III). Inhibitory effects of α- and γ-tocotrienols and α- and γ-tocopherols on lipid peroxidation of murine microsomes by the addition of adriamycin are shown in Table V. The effects of tocotrienols were superior to those of tocopherols.

The structural difference between α- and γ-tocotrienols is a methyl group at the C-5 position as shown in Fig. 1. There are four kinds of natural tocotrienols, and it would also be of interest to investigate the antitumor activities of the other two compounds. The reaction mechanism of the antitumor activity of tocotrienols is not well understood. However, the following possible mechanisms have been suggested: a) direct cytotoxic activity as shown in this report; and b) stimulation of the host immune system, since tocopherol can potentiate the host immune system.[8]

Tocopherol has also been proposed to provide protection against the cardiotoxicity caused by adriamycin.[9] The mechanisms of this protection are unknown, but it has been postulated that membrane stabilization and antioxidation are involved.[10,11] Tocotrienol showed a greater antioxidant activity than that of α-tocopherol. Tocopherol was reported

Table II. Effect of Tocotrienols on Ehrlich Carcinoma and Sarcoma 180

Sample	Dose (mg/kg/d)	Ehrlich carcinoma MSD±S.D.[a]	ILS (%)[b]	Body weight[c]	Sarcoma 180 MSD±S.D.	ILS (%)	Body weight
Saline		17.9± 4.9	0	35.1	11.7±1.9	0	38.1
α-Tocotrienol	40	29.6±13.2	65.4	28.9	21.3±3.4[e]	82.1	28.7
	20	16.6± 1.8	0	34.6	18.3±5.7[d]	56.4	32.3
	10	16.9± 2.3	0	34.8	11.8±2.5	0.9	34.4
	5	20.4± 3.2	14.0	36.8	10.5±2.4	0	31.7
γ-Tocotrienol	40	34.4± 8.9[e]	92.2	25.0	26.4±7.5[d]	125.9	28.2
	20	31.9±14.1[d]	78.2	30.8	25.4±4.2[e]	117.1	28.9
	10	19.0± 3.9	6.1	34.1	15.7±4.2[d]	34.2	33.1
	5	18.8± 5.0	5.0	35.5	18.4±6.7[d]	57.3	33.6
α-Tocophenol	40	19.7± 3.0	10.1	33.3	16.4±2.2[d]	40.2	35.0
	20	18.6± 3.6	3.9	34.1	9.2±1.3	0	27.0
Mitomycin C	0.5	41.9± 8.0[e]	134.1	25.7	32.0±8.1[e]	173.5	27.9

a) Mean survival days. *b*) Increase in life span. *c*) Mean body weight (g) on day 10. *d*) $p<0.05$; *e*) $p<0.001$.

Table III. Effect of Tocotrienols on IMC Carcinoma and Meth A Fibrosarcoma

Sample	Dose (mg/kg/d)	IMC carcinoma MSD±S.D.[a]	ILS (%)[b]	Body weight[c]	Meth A fibrosarcoma MSD±S.D.	ILS (%)	Body weight
Saline	—	13.4±0.8	0	27.9	18.7±3.8	0	19.2
α-Tocotrienol	40	19.6±1.3[f]	46.3	25.7	14.5±2.2	0	21.1
	20	18.0±1.0[f]	34.3	25.2	17.3±2.8	0	21.7
	10	16.6±0.5[f]	23.9	28.2	—	—	—
	5	18.3±2.2[e]	36.6	27.7	—	—	—
γ-Tocotrienol	40	26.4±8.9[f]	97.0	16.2	25.7±3.9[e]	37.4	14.5
	20	21.9±3.1[e]	63.4	26.7	25.3±4.9	35.3	17.8
	10	16.4±1.9[e]	22.4	29.4	—	—	—
	5	16.7±1.7[f]	24.6	28.3	—	—	—
α-Tocophenol	40	18.1±2.2[e]	35.1	25.3	16.5±1.5	0	21.1
	20	18.6±1.3[f]	38.8	26.3	19.0±3.6	1.6	20.3
Mitomycin C	0.5	>60±8.5[f]	>348 (4)[d]	21.3	30.9±4.9[f]	65.2	18.1

a—*c*) See Table II. *d*) Number of cured mice out of 7 mice. *e*) $p<0.05$; *f*) $p<0.001$.

TABLE IV. IC_{50} Values (μg/ml) of Tocotrienols

Cell	α-T3	γ-T3	α-T
H69	47	47	1000
P388	47	23	1000
HeLa	47	47	1000

T3, tocotrienol; T, tocopherol.

TABLE V. Inhibition of Malonaldehyde Generation

Concentration (μg/ml)	α-T3	α-T	γ-T3	γ-T
100	100	87	100	100
20	100	25	100	41
4	92	2	91	4
0.8	42	0	1	3
0.16	0	0	0	0

Amount of malonaldehyde in the control group was 8.86 nmol. T3, tocotrienol; T, tocopherol.

TABLE VI. Effect of α-Tocotrienol and α-Tocopherol on Cytotoxicity of Adriamycin

Sample	Conc. (μg/ml)	Conc. of adriamycin (ng/ml) 625	156	39	10	2.5	0.63
—	—	78[a)]	52	46	15	12	0
α-Tocotrienol	25	80	60	42	24	22	0
	6.3	81	55	45	15	8	3
α-Tocopherol	25	77	63	49	26	12	0
	6.3	88	58	58	26	13	0

a) Percent growth inhibition.

to enhance the growth inhibitory effect of adriamycin on a variety of cancer cells *in vitro*.[1)] In a preliminary experiment, α-tocotrienol did not enhance the effect of adriamycin on HeLa cells, but at least did not decrease the cytotoxicity of adriamycin (Table VI). Thus, tocotrienols not only have antitumor activity but may also act as antioxidants. They could play an important role in the treatment of tumors due to their variety of physiological activities.

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