Cytotoxicity of α-Tocopheryl Succinate, Malonate and Oxalate in Normal and Cancer Cells In Vitro and Their Anti-Cancer Effects on Mouse Melanoma In Vivo

Kentaro KOGURE, Sachie MANABE, Ichiro SUZUKI, Akira TOKUMURA and Kenji FUKUZAWA*

Graduate School of Pharmaceutical Sciences, University of Tokushima,
Shomachi-1, Tokushima 770-8505, Japan

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Summary α-Tocopheryl succinate (TS), which is known to induce apoptosis selectively in cancer cells, has attracted attention as a chemotherapeutic agent. Recently, we found that α-tocopheryl malonate (TM) and α-tocopheryl oxalate (TO), among the α-tocopheryl esters tested, have high apoptogenic activity as well as TS. In this study, we investigated the characteristics of their cytotoxicity on normal cells and cancer cells in vitro, and their anti-cancer effects on mice inoculated with melanoma B16-F1 cells in vivo. The order of in vitro cytotoxicity was TO ≥ TM ≥ TS in all cell lines examined. Addition of exogenous superoxide dismutase (SOD) and the antioxidant N-acetyl cysteine (NAC) inhibited TS- and TM- but not TO-induced cell deaths. A selective cytotoxic effect on cancer cells was observed with TS but not with TM or TO. c-Jun N-terminal kinase (JNK) inhibitor II prevented cell death induced by TS but did not prevent cell deaths induced either by TM or TO. Intravenous administration of vesiculated TS and TM to mice inoculated with melanoma B16-F1 cells prevented tumor growth and enhanced the mean survival time, but TO administration killed the mice due to its acute high toxicity. From these results, we discussed the characteristics of their selective cytotoxicity toward tumor cells in vitro and anti-cancer effects in vivo.

Key Words α-tocopheryl succinate, anti-cancer effect, reactive oxygen species, c-Jun NH2-terminal kinase

α-Tocopheryl succinate (TS), a succinyl ester of α-tocopherol, has been reported to have various biological activities, such as enhancement of nitric oxide production induced by lipopolysaccharide/interferon-γ in rat vascular smooth muscle cells (1), suppression of the growth of various cancer cells (2–7) and induction of apoptosis in various cell lines (8–12). With a view to examine its anti-cancer effect, research on TS-induced cytotoxicity has been progressing (13–21).

Birringer et al. (16) examined apoptogenic activities of novel synthesized α-tocopherol derivatives containing its esters in various cancer cell lines, and concluded that the apoptogenic activity is dependent on the length and charge of the ester moiety. Recently, we investigated the structural characteristics of the terminal dicarboxylic moiety of α-tocopheryl esters required for their apoptogenesis (22), and observed higher activity in the newly synthesized α-tocopheryl malonate (TM) and α-tocopheryl oxalate (TO) than in TS, of which only difference is the length of the terminal carboxylic moiety, carbon numbers of which are 4, 3 and 2 for TS, TM and TO, respectively (Fig. 1). In this study, we investigated their cytotoxicity, mechanisms and specificity to normal and cancer cells in vitro. Furthermore, we examined their inhibiting effects on the growth of B16-F1 melanoma cells inoculated into the backs of mice when intravenously administered and their survival times. Then we discuss their possibilities as anti-cancer drugs from the results in vivo and in vitro.

MATERIALS AND METHODS

Materials. Superoxide dismutase (SOD), N-acetyl cysteine (NAC) and RRR-α-tocopheryl succinate (TS) were purchased from Sigma-Aldrich Co. (St. Louis, MO). RRR-α-Tocopheryl malonate (TM) and RRR-α-tocopheryl oxalate (TO) were synthesized as previously described (22). JNK inhibitor II was purchased from Calbiochem-Novabiochem Co. (San Diego, CA). SF6847 was kindly provided by Prof. Terada (Tokyo University of Science). A normal mouse breast cell line, NmuMG, a mouse fibroblast cell line, 3T3/Swiss, a mouse breast cancer cell line, C127L, and a mouse melanoma cell line, B16-F1, were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan).

Treatment of cells with α-tocopheryl esters. Cells (1.0×10⁶ cells) were seeded into 35 mm dishes and cultured for 24 h in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum in a CO2-incubator at 37°C. Then, the medium containing serum was removed, and the cells were washed with phosphate-buffered saline (PBS). Next, 1 mL of serum-free medium containing

*To whom correspondence should be addressed.
E-mail: fukuzawa@ph.tokushima-u.ac.jp
various concentrations of α-tocopheryl esters was added to the dishes. NAC and JNK inhibitor II, which were dissolved in ethanol, and SOD, which was dissolved in the medium, were added simultaneously with the α-tocopheryl esters to the medium (the final concentration of ethanol was less than 0.1%). After 24 h, the cells were subjected to various assays.

Cell survival assay. Two fluorescent dyes, acridine orange and ethidium bromide, were used to discriminate living cells. The former stains living cells green, and the latter stains dead cells orange. After removal of the medium containing α-tocopheryl esters, the cells were treated with a mixture of acridine orange (0.05 mg/mL) and ethidium bromide (0.05 mg/mL) for 15 min. The stained cells were observed using a fluorescent microscope (OLYMPUS IMT-2). Percentages of surviving cells were determined as the number of cells stained green relative to the total number of cells.

Estimation of destabilization of mitochondrial membranes. Destabilization of mitochondrial membranes was estimated by treatment with rhodamine123, which is taken up by mitochondria in proportion to their membrane potential. After removal of the medium containing α-tocopheryl esters from a culture dish, the cells were washed with fresh medium. Then, the cells were incubated with 2 mL of the medium containing 10 μM rhodamine123 for 30 min at 37°C. After the incubation, the medium was removed, and 1 mL of a fresh medium was added to the cell dish. Mitochondria of the cells stained by rhodamine123 were observed by confocal laser scanning microscopy (LSM5 Pascal-V2.8, Carl Zeiss, Germany). Treatment with 10 μM SF6847, an uncoupling agent of mitochondria, completely destabilized mitochondrial membranes. The membrane destabilization percentage was calculated as the ratio of cells containing destabilized mitochondria to the total number of cells.

Assay of anti-cancer effect of α-tocopheryl esters in vivo. To create an in vivo cancer model, a cultured melanoma cell suspension (5.0×10⁶ cells) was inoculated under the skin on the backs of 5-wk-old male hairless mice (Hos:HR-1 strain). Tumor volume was determined by caliper measurements according to the formula, Tvol=length×width×depth×0.5236 (23). α-Tocopheryl esters were administered by i.v. injection of 200 μL of 50 mm vesiculated α-tocopheryl ester suspension (24). Vesicles of α-tocopheryl esters were prepared by the method of Jizomoto et al. (25), i.e., α-tocopheryl esters were suspended in PBS by treatment with NaOH and sonication using a bath type sonicator (Sakura, Tokyo, Japan). The diameters of the TS, TM and TO vesicles were 204, 207 and 281 nm, respectively. The diameter of the vesiculated samples was measured by means of electrophoretic light scattering apparatus (NICOMP Model380ZLS, Particle Sizing Systems, Santa Barbara, CA).

Statistical analysis. Statistical analysis of data was performed by ANOVA with the Bonferroni method. Data are expressed as the means±standard deviation of at least three independent experiments.

RESULTS

Cytotoxic effects of α-tocopheryl esters on mouse melanoma B16-F1 cells

Figure 2 shows the treatment time- and concentration-dependent cytotoxic effects of α-tocopheryl esters on mouse melanoma B16-F1 cells. The three α-tocopheryl esters caused cell death in a treatment time- and concentration-dependent manner. TO was more potent in inducing cell death than TS and TM. About 50% of cells were dead at 12 h after addition of TO, but TS and TM required approximately 24 h for 50% cell death. The number of cell deaths increased with the increase of concentration of α-tocopheryl esters, and the order of their cytotoxicity was TO>TM>TS at the same concen-
Fig. 3. Effect of SOD and NAC on (A) cell death and (B) mitochondrial membrane destabilization induced by TS, TM and TO in B16-F1 cells. B16-F1 cells were treated with 50 μM of the α-tocopheryl esters for 24 h. The concentrations of SOD and NAC were 5,000 units/mL and 5 mM, respectively. Values are means±SD.

* p<0.01, ** p<0.05
toxopheryl esters destabilized mitochondrial membranes. The order of their destabilization abilities was TO>TM>TS, which is the same as the order of their cytotoxic abilities, as shown in Fig. 3A. The preventive effects of SOD and NAC on the destabilization of mitochondrial membranes induced by α-tocopheryl esters were also similar to those on cytotoxicity induced by α-tocopheryl esters, i.e. TS- and TM-induced destabilizations were prevented by SOD and NAC but TO-induced destabilization was not.

Effects of SOD and NAC on (α-tocopheryl ester)-induced death of B16-F1 cells

We previously reported that superoxide (O2−) generated by NADPH-oxidase is responsible for TS-induced apoptosis in rat vascular smooth muscle cells (12). Figure 3A shows the effects of the antioxidative enzyme SOD and the chemical antioxidant NAC on the cell death induced by the three α-tocopheryl esters in B16-F1 cancer cells. TS- and TM-induced cell deaths were prevented by SOD and NAC but TO-induced cell death was not. These results suggested that O2− generation and the free-radical reactions derived from it, such as lipid peroxidation, are responsible for TS- and TM-induced cytotoxicities but not for TO-induced cytotoxicity.

Effects of SOD and NAC on destabilization of mitochondrial membranes induced by α-tocopheryl esters

TS has been known to cause apoptosis associated with modulation of mitochondrial membranes (13). To characterize the cell death induced by α-tocopheryl esters, we examined their destabilization effect on mitochondrial membranes. As shown in Fig. 3B, three α-tocopheryl esters destabilized mitochondrial membranes. The order of their destabilization abilities was TO>TM>TS, which is the same as the order of their cytotoxic abilities, as shown in Fig. 3A. The preventive effects of SOD and NAC on the destabilization of mitochondrial membranes induced by α-tocopheryl esters were also similar to those on cytotoxicity induced by α-tocopheryl esters, i.e. TS- and TM-induced destabilizations were prevented by SOD and NAC but TO-induced destabilization was not.

Effects of JNK inhibitor II on (α-tocopheryl ester)-induced death of B16-F1 cells

Activation of c-Jun N-terminal kinase (JNK) has been reported to cause TS-induced apoptosis (26). Therefore, we examined the effect of JNK inhibitor II on the cell death of B16-F1 cells induced by α-tocopheryl esters. As shown in Fig. 4, TS-induced cell death was significantly prevented by JNK inhibitor II, but TM- and TO-induced cell deaths were not affected by JNK inhibitor II.

Cytotoxicity of α-tocopheryl esters to normal and cancer cell lines

We previously reported that cancer cells were more susceptible to the cytotoxicity of TS than normal cells (27). Therefore, we compared the cytotoxic activity of TS, TM and TO on normal cell lines (Swiss3T3 and NMuMG) and cancer cell lines (B16-F1 and C127I). As shown in Fig. 5, the susceptibilities of normal cells to TM and TO differed from their susceptibility to TS. The cancer cell lines C127I and B16-F1 were more susceptible to TS-induced cytotoxicity than the normal cell lines Swiss3T3 and NMuMG, but these cancer cell lines and normal cell lines showed similar susceptibilities to TM- and TO-induced cytotoxicities.

Effect of α-tocopheryl esters on tumor growth in vivo

We further studied the anti-cancer effect of α-tocopheryl esters in vivo to confirm their effects in vitro. The anti-cancer effect was assayed by measuring the inhibition of growth of mouse melanoma cells B16-F1 inoculated into the backs of male hairless mice in vivo. α-Tocopheryl esters were vesiculated by the method
Fig. 5. Comparison of the sensitivity to TS-, TM- and TO-induced cytotoxicities of normal cell lines Swiss 3T3 and NMuMG and cancer cell lines B16-F1 and C1271. The cells were treated with various concentrations of the α-tocopheryl esters for 24 h. Values are means±SD. *p<0.01, **p<0.05.

Fig. 6. Effect of TS and TM on the growth of mouse melanoma B16-F1 inoculated into the backs of hairless mice in vivo. Administration points of the vesiculated α-tocopheryl esters are indicated by arrows. The dose of TS and TM per administration was 10 μmol/mouse. ○, control group (n=10); ●, TS group (n=13); ■, TM group (n=5). Values are means±SD.

reported previously (24) and administered i.v. to tumor-inoculated mice every 3 d from day 7 to day 19 after inoculation of melanoma cells (Fig. 6). Melanoma B16-F1 cells inoculated into the backs of hairless mice grew, and the relative volume of tumors 28 d after the inoculation was about 400-fold that on day 7 (Figs. 6 and 7). Administration of PBS did not affect tumor growth (data not shown). Treatment i.v. with TS almost completely prevented tumor growth in all mice even by day 28, although the administration was stopped on day 19 (Figs. 6 and 7). Treatment with TM also remarkably prevented tumor growth, but its effect was slightly weaker than that of TS (Figs. 6 and 7). The inhibition percentages of tumor growth at day 28 in the TS and TM groups were 84 and 65%, respectively. In contrast, all mice in the TO group died during the administration period although the TO dose was the half the concentration of the TS and TM doses.

Effect of three α-tocopheryl esters on survival of tumorigenic mice

Figure 8 shows the number of survival days of the tumorigenic mice treated with and without TS, TM and TO vesicles. The percentage survival of tumor-inoculated control mice decreased from day 42, and on day 65 all mice in the control group were dead. The average of survival in the control group was 50.2 d. Survival time was extended in mice treated with TS and TM. The mean survivals were 64.2 d (1.28-fold the control) in the TS-group and 63.8 d (1.27-fold the control) in the
TM-group. On the other hand, the TO-treated mice died after the first or second administration of TO.

**DISCUSSION**

We compared the in vitro cytotoxic effects and in vivo preventive effects on tumor growth of the α-tocopheryl esters TS, TM and TO. In all cultured normal and tumor cells examined, TO showed the highest cytotoxic activity, and TM was more potent than TS. TS- and TM-induced cell deaths were prevented, but TO-induced cell death was not, by the O$_2^-$ scavenging enzyme SOD and the antioxidant NAC in the mouse melanoma cell line B16-F1. In the breast cancer cell line C1271, SOD showed a similar effect (22). These results indicate that the cytotoxic mechanism of TS and TM was different from that of TO. We supposed that O$_2^-$ and free radical reactions such as lipid peroxidation are involved in the mechanisms of TS- and TM-induced cell deaths of B16- F1 and C1271 cells.

It is known that the activities of anti-oxidative enzymes such as SOD and catalase are lower in various cancer cells of mouse, rat and human origin than in normal cells (28), indicating that cancer cells are more susceptible to reactive oxygen species than their normal counterparts. Recently, Huang et al. (29) suggested SOD as a target for the selective killing of cancer cells by inducing apoptosis, and the combination of SOD inhibitors with an O$_2^-$ producing agent as a promising approach for clinical cancer treatment. Considering these reports, we previously assumed that the high susceptibility of C1271 cancer cells to TS-induced apoptosis is due to failure of their antioxidant defense systems because SOD strongly prevented TS-induced apoptosis (27). In this study, however, TS-induced cytotoxicity was not, although both TS- and TM-induced cell deaths were inhibited by SOD. This suggested that the high susceptibility of cancer cells to TS-induced cytotoxicity is not necessarily due to their lowered antioxidative defense, and/or that TM has, but TS does not, characteristics which strongly induce cytotoxicity in both normal and cancer cells.

Yu et al. (26) reported that JNK inhibitor II prevented the TS-induced Bax conformation change involved in TS-induced apoptosis. They considered that activation of JNK, which translocates Bax to mitochondria and increases permeability of mitochondrial membrane resulting in activation of caspase-9 and -3, is a critical event in TS-induced apoptosis. In this study, we observed that TS-induced cell death was significantly inhibited by JNK inhibitor II, but TM-induced cell death was not. These results suggest that the JNK activation pathway was involved in TS-induced cytotoxicity on cancer cells but was not involved in TM-induced cytotoxicity. Taking this result into consideration, we assumed that activation of the JNK pathway participates in the TS-induced selective cytotoxicity on cancer cells. It is very interesting that the difference of only one or two carbon units in the length of dicarboxylate moiety causes a different cytotoxic mechanism and a difference in susceptibility to death between cancer cells and normal cells.

In in vivo experiments, TS and TM showed similar and significant preventive effects on tumor growth and life prolonging activities. It is unknown why TS and TM showed similar anti-cancer activity in vivo, although the toxicity of TS was selective but that of TM was not selective to cancer cell lines in vitro. Some side effects are supposed to have occurred in mice treated with TM because of its unsuitable cytotoxicity. Further study is necessary to confirm the in vivo toxicity of TM for its utilization as an anticancer drug.

In conclusion, we found that TS, TM and TO show potent cytotoxicity on the mouse melanoma cell line B16-F1, and that antioxidative reagents SOD and NAC inhibited the cell deaths induced by TS and TM, but did not inhibit the cell death induced by TO. We further found that JNK inhibitor II prevented cell death induced by TS but not that by TM or TO. These results indicate that their cytotoxic mechanisms are individually different. Cytotoxic effect on cancer cells was only observed in TS, suggesting that activation of JNK is related to the cancer selective cytotoxicity of TS. Moreover, we observed that TM showed anti-tumor activity that was as effective as TS in vivo. Thus, we concluded that TS is most suitable as an anti-cancer drug. TM is also a likely anti-cancer drug but fear of its secondary harmful effects on normal tissues in vivo remains.

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