Vitamin E δ -Tocotrienol Levels in Tumor and Pancreatic Tissue of Mice after Oral Administration

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Key Words

δ -Tocotrienol • δ -Tocotrienol, mice • Pancreatic cancer • Pharmacokinetics • Tissue distribution

Abstract

Tocotrienols are natural vitamin E compounds that are known to have a neuroprotective effect at nanomolar concentration and anti-carcinogenic effect at micromolar concentration. In this report, we investigated the pharmacokinetics, tumor and pancreatic tissue levels, and toxicity of δ -tocotrienol in mice because of its anti-tumor activity against pancreatic cancer. Following a single oral administration of δ -tocotrienol at 100 mg/kg, the peak plasma concentration (C_{max}) was $57 \pm 5 \mu\text{mol/l}$, the time required to reach peak plasma concentration (T_{max}) was 2 h and plasma half-life ($t_{1/2}$) was 3.5 h. The δ -tocotrienol was cleared from plasma and liver within 24 h, but delayed from the pancreas. When mice were fed δ -tocotrienol for 6 weeks, the concentration in tumor tissue was $41 \pm 3.5 \text{ nmol/g}$. This concentration was observed with the oral dose (100 mg/kg) of δ -tocotrienol which inhibited tumor growth by 80% in our previous studies. Interestingly, δ -tocotrienol was 10-fold more concentrated in the pancreas than in the tumor. We observed no toxicity due to δ -tocotrienol as mice gained normal weight with no histopathological changes in tissues. Our data sug-

gest that bioactive levels of δ -tocotrienol can be achieved in the pancreas following oral administration and supports its clinical investigation in pancreatic cancer.

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Introduction

Tocotrienols are natural vitamin E compounds. They exist in four chemical forms (α -, β -, γ -, and δ -tocotrienol) and are abundant in cereal grains, including soybeans, oats, rice bran and palm oil [1, 2]. Recent studies have demonstrated protective effects of tocotrienols against neurodegenerative diseases, atherosclerosis and cancer besides antioxidant properties [2–6]. Tocotrienol-rich fraction has been demonstrated to lower the cholesterol levels in humans as well as in animals [6–8]. A nanomolar concentration of α -tocotrienol has been shown to protect against neurodegeneration [5, 9, 10], whereas a micromolar concentration of γ -tocotrienol has anti-cancer effects [4, 11, 12]. There has been intense interest in the role of nutritional and dietary factors in the prevention of pancreatic cancer [13]. Several reports show that individuals who consume more cereal grains have a lower cancer risk [14–16]. Recently, we have observed that δ -tocotrienol is the most potent tocotrienol in the induc-

tion of apoptosis and inhibition of growth of pancreatic cancer cells [17]. However, the concentration of δ -tocotrienol in tumors and in pancreatic tissue after consumption of δ -tocotrienol is unknown. The purpose of this study was to determine the level of δ -tocotrienol in tumor tissue which is associated with tumor growth inhibition and to compare this to the level of tocotrienol accumulation in pancreatic tissue. Furthermore, we investigated the pharmacokinetics and toxicity of δ -tocotrienol in mice fed purified δ -tocotrienol from palm oil.

Materials and Methods

Chemicals

Chemicals such as acetonitrile, ethanol, hexane, 2-propanol, tetrahydrofuran, and HPLC water were obtained from Fisher Scientific Co. (Fair Lawn, N.J., USA). Pancreatic cancer cell line MIA PaCa-2 was obtained from ATCC (Manassas, Va., USA). Dulbecco's modified minimal essential medium (DMEM), trypsin-EDTA, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Invitrogen (Carlsbad, Calif., USA). Purified δ -tocotrienol (97%) was obtained from Davos Life Ltd. (Singapore).

Animals and Treatments

Female athymic nude mice (4–5 weeks old, 20–25 g) were obtained from Charles River (Wilmington, Mass., USA) and kept in the institute's animal facility for 1 week for quarantine. For the pharmacokinetic study, mice ($n = 22$) were fed by oral gavage with PBS/ethanol-extracted olive oil (vehicle control) and δ -tocotrienol (dissolved in ethanol-extracted olive oil) at a dose of 100 mg/kg. The animals from the control and δ -tocotrienol-treated groups were euthanized by CO₂ inhalation after 0.5, 1, 2, 4, 8, 12 and 24 h post-treatment. The blood was collected in heparinized-capped vials and plasma was separated by centrifugation of blood at 10,000 rpm for 10 min and stored at -80°C . Liver and pancreas were isolated, immediately immersed in liquid nitrogen and stored at -80°C .

For repeated dose experiments, athymic nude mice ($n = 18$) were injected with 1 million MIA PaCa-2 cells in a 50- μl PBS solution into the spleen. These cell lines were previously grown in monolayers with DMEM supplemented with 10% FBS and were cultured at 37°C in a humidified atmosphere of 5% CO₂. After 1 week, when the tumor volume attained 100 mm³, the mice were randomized to be orally fed with PBS/ethanol-extracted olive oil (vehicle control) or δ -tocotrienol (dissolved in ethanol-extracted olive oil) at a dose of 50 and 100 mg/kg. The mice were dosed twice daily 5 times a week and once a day on the weekends for up to 6 weeks. Animals were monitored daily and body weights were recorded. At the end of 6 weeks, the animals were euthanized by CO₂ inhalation. The blood was collected in EDTA containing capped vials and plasma was separated by centrifugation of blood at 10,000 rpm for 10 min and stored at -80°C . Liver, heart, kidney, pancreas and tumors were isolated. Part of the tissues were fixed in buffered formalin and the remaining tissues were immediately immersed in liquid nitrogen and stored at -80°C . The care and

use of the animals reported in this study were approved by the Institutional Laboratory Animal Care and Use Committee and as per the guidelines of the National Institute of Health.

Preparation of Plasma and Tissue Samples

The extraction of δ -tocotrienol was performed according to the method described earlier [18, 19]. Stock standards and quality control samples (QCS) were prepared in 1 ml of plasma. The appropriate amount of δ -tocotrienol working solution was added to the prelabeled tubes to create stock plasma solutions, resulting in final plasma concentrations of 0, 5, 10, 50, 100, 500, and 1,000 ng/ml for the standards and 8, 80, 800 ng/ml for the QCS. A solution of 5 ml of acetonitrile:tetrahydrofuran (3:2) was added to 250 μl to precipitate proteins and extract δ -tocotrienol. Aliquots of 500 μl of the precipitation and extraction solvent were added to each sample, vortexed for 5 min and centrifuged at 12,500 rpm for 5 min at 4°C . Aliquots of 600 μl of the organic layer were transferred to another set of prelabeled microcentrifuge tubes and supernatant was evaporated to dryness under purified air at 40°C for 15 min at 4 psi. The pellets were reconstituted with 110 μl of hexane, mixed for 20 s and centrifuged for 30 s. The hexane was transferred into a limited volume glass insert within an amber vial. Frozen tissue (100 mg) was thawed and 400 μl of ethanol was added. For standards or QCS, the appropriate amount of δ -tocotrienol working solution was added to the prelabeled vials containing blank tissue to create controls that reflect nanogram amounts per 100 mg of tissue. These samples were homogenized for 30 s, and 500 μl of HPLC water was then added to all samples and homogenized for 15 s. Then, 500 μl of hexane was added and homogenized for 15 s. The samples were centrifuged at 3,500 rpm for 5 min at 4°C . The organic layer was evaporated to dryness under purified air at 40°C for 7 min at 4 psi. The pellets were reconstituted with 110 μl of hexane, mixed for 20 s and centrifuged for 30 s.

Determination of δ -Tocotrienol by HPLC

Analysis of δ -tocotrienol was performed according to the method reported earlier [18, 19]. An aliquot of 100 μl was injected into the HPLC system (Agilent 1100 series liquid chromatographic instrument coupled to an Agilent 1100 fluorescence detector). The pump was run at a flow rate of 1.0 ml/min and the mobile phase was composed of hexane:2-propanol (99:1). The total run time was 14 min and chromatographic separation was carried out by a Zorbax Rx-Sil normal phase column (5 μm , 4.6 mm $\times$ 15 cm). The eluate was monitored for peaks by fluorescence detector set at an excitation wavelength of 296 nm and emission wavelength of 330 nm. Calibrations were linear for both plasma and tissue samples for the determination of δ -tocotrienol.

Histologic Evaluation

Liver, heart, kidney and pancreas mice tissues were fixed in 10% neutral buffered formalin for 6 h. After fixation, the tissue samples were processed into paraffin blocks. Four-micrometer-thick tissue sections were obtained from the paraffin blocks and stained with hematoxylin and eosin (Richard-Allan Scientific, Kalamazoo, Mich., USA) using standard histologic techniques. 6–8 sections (100 μm apart) of tissues from the control group ($n = 6$) and the treated group ($n = 6$) were examined under a light microscope (Olympus BX51) by a board-certified pathologist with expertise in mice pathology (D.C.). The tissues were qualita-

Fig. 1. Chemical structure of δ -tocotrienol.

tively assessed for pathologic evidence of tissue toxicity. The pathologist was blinded as to the control versus the tocotrienol-treated mice.

Statistical Analysis

The data were expressed as mean $\pm$ SEM. The data were analyzed statistically using unpaired *t* tests or one-way analysis of variance (ANOVA) where appropriate. ANOVA was followed by Duncan's multiple range tests using SAS statistical software for comparison between different treatment groups. Statistical significance was set at $p < 0.05$.

Results

Determination of δ -Tocotrienol Pharmacokinetics in Mice

First, we standardized the HPLC conditions for δ -tocotrienol (fig. 1) with fluorescence detection sensitivity in the linear range. The δ -tocotrienol peak appeared in the chromatogram at a retention time of 7.4 min. The mean absolute recovery values were about 98–99%, while the within-day and between-day relative standard deviation and percent error values of the assay method were $<2.0\%$. The calibration curve for δ -tocotrienol was found linear in the range of 5–1,000 ng/ml of plasma and 5–1,000 ng/100 mg of tissues.

The plasma concentration-time curve of δ -tocotrienol after the oral administration of a single 100 mg/kg dose to mice is depicted in figure 2. The peak plasma concentration ($C_{\max}$) of δ -tocotrienol was $57 \pm 5 \mu\text{mol/l}$ and the time required to reach peak plasma concentration ($T_{\max}$) was 2 h. The plasma half-life ($t_{1/2}$) of δ -tocotrienol was 3.5 h. The δ -tocotrienol was cleared from plasma within 24 h. The peak tissue concentration ($C_{\max}$) of δ -tocotrienol was $14 \pm 2 \text{ nmol/g}$ in the liver and $32 \pm 4 \text{ nmol/g}$ in the pancreas. The time required to reach peak tissue concentration ($T_{\max}$) was 2 h in the liver and 8 h in the pancreas (fig. 3). The δ -tocotrienol was cleared from liver within 24 h, but did not completely clear from the pancreas.

Fig. 2. Plasma concentration-time curve of δ -tocotrienol after oral administration (100 mg/kg) in athymic nude mice. Each point represents mean $\pm$ SEM ($n = 3-5$). The peak plasma concentration ($C_{\max}$) of δ -tocotrienol was $57 \pm 5 \mu\text{mol/l}$ and the time required to reach peak plasma concentration ($T_{\max}$) was 2 h. The plasma $t_{1/2}$ of δ -tocotrienol was 3.5 h. The δ -tocotrienol was cleared from plasma within 24 h.

Fig. 3. Tissue concentration-time curve of δ -tocotrienol after oral administration (100 mg/kg) in athymic nude mice. Each point represents mean $\pm$ SEM ($n = 3-5$). The peak tissue concentration ($C_{\max}$) of δ -tocotrienol was $14 \pm 2 \text{ nmol/g}$ in the liver and $32 \pm 4 \text{ nmol/g}$ in the pancreas. The time required to reach peak tissue concentration ($T_{\max}$) was 2 h in the liver and 8 h in the pancreas. The δ -tocotrienol was cleared from the liver within 24 h, but had delayed clearance from the pancreas.

Fig. 4. Tissue distribution of δ -tocotrienol (nmol/g) in the liver, pancreas and the tumor after oral administration (100 mg/kg) twice daily 5 times a week and once a day on the weekends for up to 6 weeks in athymic nude mice with pancreatic tumor xenograft or treated with vehicle (PBS/olive oil) for 6 weeks. Animals were sacrificed >12 h after the last dose. Each point represents mean $\pm$ SEM (n = 3–5). * Significant compared to vehicle control (p < 0.001). ** Significant compared to vehicle control (p < 0.05); the data were analyzed statistically using ANOVA followed by Duncan's multiple range test.

δ-Tocotrienol Levels in Tumor and Pancreatic Tissue

The tissue distribution after 100 mg/kg oral dose of δ -tocotrienol for 6 weeks in mice is depicted in figure 4. In this experiment, the mice in the control group (n = 6) and the treated group (n = 6) were sacrificed 12 h after the last dose and tissues collected. The concentration of δ -tocotrienol was significantly increased in the liver (p < 0.05), pancreas (p < 0.001) and tumor (p < 0.05) compared to vehicle. It is interesting to note that δ -tocotrienol was 10-fold more concentrated in the pancreas than in the tumor or liver tissues after repeated feeding in mice.

The dose response of δ -tocotrienol after 50 and 100 mg/kg repeated oral administration to mice for 6 weeks is depicted in figure 5. In this experiment, the mice in the control group (n = 6) and the treated group (n = 6) were sacrificed 6 h after the last dose and tissues collected. There was a dose-dependent and significant increase in δ -tocotrienol concentration in the pancreas and in the tumor compared to vehicle-treated control mice (p < 0.01 and p < 0.001). However, the dose response was not ob-

Fig. 5. Dose response of δ -tocotrienol concentration (nmol/g) in the liver, pancreas and the tumor after oral administration (50 and 100 mg/kg) twice daily 5 times a week and once a day on the weekends for up to 6 weeks in athymic nude mice with pancreatic tumor xenograft or treated with vehicle (PBS/olive oil) for 6 weeks. Animals were sacrificed 6 h after the last dose. Each point represents mean $\pm$ SEM (n = 3–5). * Significant compared to vehicle control (p < 0.01). ** Significant compared to vehicle control (p < 0.001); the data were analyzed statistically using ANOVA followed by Duncan's multiple range test.

served in the xenografted tumor tissue, suggesting differences in drug metabolism, tissue microenvironment, size and composition between tumor and normal pancreatic tissue.

Toxicity of δ-Tocotrienol

There was no significant change in body weight observed following oral administration of δ -tocotrienol to mice for 6 weeks. Pathologic evaluation of the liver, heart, kidney and pancreas of mice treated with δ -tocotrienol showed normal histology and was similar to the vehicle-treated group (fig. 6).

Discussion

This study addresses the changes in tissue distribution, pharmacokinetics and toxicity of natural bioactive vitamin E compound δ -tocotrienol in nude mice after a single and repeated dose for 6 weeks. Simple HPLC meth-

Fig. 6. Histological changes in the liver, kidney, heart and pancreas of athymic nude mice with pancreatic tumor xenograft following oral δ -tocotrienol administration (50 and 100 mg/kg) twice daily 5 times a week and once a day on the weekends for up to 6 weeks or treated with vehicle (PBS/olive oil) for 6 weeks. No significant structural changes observed in tissues following oral administration of δ -tocotrienol to mice.

ods for the separation, determination and detection of vitamin E compounds (tocopherols and tocotrienols) have been used previously [18, 20]. Among three detection methods such as ultraviolet, evaporative light scattering and fluorescence, the best results were obtained with fluorescence detectors [19, 21]. Using HPLC methodology coupled with fluorescence detection, we were able to detect δ -tocotrienol and quantified it in small amounts (ng) in a linear range. The detection limit for δ -tocotrienol by the HPLC system used in the present study is comparable and consistent with those reported earlier [18–22]. Although earlier reports demonstrated the distribution of mixed tocotrienols in various tissues of mice, this is the first report to focus on the pharmacokinetics of pure δ -tocotrienol and its concentration in tumor tis-

sue and pancreas. The objective of this study is to determine the bioactive levels of δ -tocotrienol in a potential target tissue such as the pancreas. At a dose of 100 mg/kg twice a day, δ -tocotrienol significantly inhibited the tumor growth of human pancreatic cancer cell lines MIA PaCa-2 and AsPc-1 xenografted in mice [17, 23]. The concentration in the tumor when δ -tocotrienol was administered 6 h before the extraction was higher (fig. 5) compared to δ -tocotrienol dosing >12 h before the extraction (fig. 6). These data suggest that tumor tissue clears δ -tocotrienol similar to clearance in the liver tissue, although this appears to be slower. The plasma $t_{1/2}$ of δ -tocotrienol was 3.5 h and complete clearance within 24 h in mice after a single oral dose that is comparable with humans ($t_{1/2} = 2.3$ h) [24]. Interestingly, δ -tocotrienol was cleared

from liver >80% at 8 h. In contrast, in pancreatic tissue, it peaked at 8 h (fig. 4). It is important to note that most of the earlier pharmacokinetic studies either used mixed tocotrienols or a tocotrienol-rich fraction [18–22, 24]. In contrast, our study used pure δ -tocotrienol (97%). Our finding of similar pharmacokinetics of δ -tocotrienol to previous reports of other tocotrienols confirms that purified δ -tocotrienol also has rapid clearance in plasma and liver. Taken together, our data suggest that targeting tumor with δ -tocotrienol or the pancreas will require twice-a-day dosing. This underlies the significance of determination of the drug levels in the target tissue. The slow clearance and accumulation of δ -tocotrienol in tumor and pancreatic tissue may be due to the lipophilic nature of δ -tocotrienol and the high lipid content of tumor and pancreas. The mammalian pancreas is rich in lipids [25, 26], but has lower drug-metabolizing enzyme activity compared to the liver, which is the main site of drug metabolism [27, 28]. Our findings are consistent with earlier studies that reported tocotrienol accumulation in adipose tissues and skin of animals and humans [29–31]. Hayes et al. [32] have shown that hamsters fed tocotrienols from palm oil had an accumulation of tocotrienols mainly in adipose tissue as well as a high concentration in the heart and muscle. They found little accumulation of tocotrienols in the brain, kidney, liver, and spleen. Podda et al. [33] have shown that large amounts of α - and γ -tocotrienols are present in the skin of hairless mice fed a commercial diet containing a small amount of tocotrienols. Ikeda et al. [34] reported α - and γ -tocotrienol accumulation in the adipose tissue and skin, but not in the plasma or other tissues of rats fed a tocotrienol-rich fraction extracted from palm oil or rice bran tocotrienol con-

centrate [35]. Moreover, the dose response of δ -tocotrienol was observed in pancreas, but not in tumor tissue. Tumor tissue has been reported to contain more drug-metabolizing enzymes than control pancreatic tissue [36, 37]. On the other hand, there are alterations and differences in tumor tissue microenvironment, size and composition compared to normal tissue, which may lead to alterations of the pharmacokinetics of the drug.

Our data further indicate no observed toxicity or side effects of δ -tocotrienol administration, as evidenced by normal weight gain of the mice with no mortality as well as no observed histopathological changes in liver, heart, kidney and pancreas. This finding is consistent with another study that has reported no toxicity in other animals such as rats [31]. It is also important to note that clinical trials conducted in humans resulted in no toxicities up to 800 mg/day of chronic administration of δ -tocotrienol for several months' duration [24]. Therefore, our results suggest that bioactive levels of δ -tocotrienol can be delivered safely to pancreatic tissue and support the investigation of this natural vitamin E compound for pancreatic cancer chemoprevention and treatment.

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