Combination Effect of δ-Tocotrienol and γ-Tocopherol on Prostate Cancer Cell Growth

Chiaki Sato1, Saki Kaneko1, Ayami Sato2, Nantiga Virgona2, Kozue Namiki1 and Tomohiro Yano1,3,4,*

1 Graduate School of Food and Nutritional Sciences, and 2 Research Institute of Life Innovation, Toyo University, Gamma 374–0193, Japan
3 Graduate School of Science and Engineering, Saitama University, Saitama 338–8570, Japan

(Received April 6, 2017)

Summary Tocotrienols (T3s) and tocopherols (Tocs) are both members of the vitamin E family. It is known that δ-tocotrienol (δ-T3) has displayed the most potent anti-cancer activity amongst the tocotrienols. On the other hand, γ-tocopherol (γ-Toc) is reported to have a protective effect against prostate cancer. Therefore, we investigated whether the combination of γ-Toc and δ-T3 could strengthen the inhibitory effect of δ-T3 on prostate cancer cell growth. In this study the effect of combined δ-T3 (annatto T3 oil) and γ-Toc (Tmix, γ-Toc-rich oil) therapy was assessed against human androgen-dependent prostate cancer cells (LNCaP). We found that combined treatment of δ-T3 (10 μM) and γ-Toc (5 μM) resulted in reinforced anti-prostate cancer activity. Specifically, cell cycle phase distribution analysis revealed that in addition to G1 arrest caused by the treatment with δ-T3, the combination of δ-T3 with γ-Toc induced G2/M arrest. Enhanced induction of apoptosis by the combined treatment was also observed. These findings indicate that combination of δ-T3 and γ-Toc significantly inhibits prostate cancer cell growth due to the simultaneous cell cycle arrest in the G1 phase and G2/M phase.

Key Words combination, δ-tocotrienol, γ-tocopherol, prostate cancer, prevention

Prostate cancer is a common malignancy and the second major cause of cancer death among men in developed countries (1.1 million new cases in 2012 resulting in 307,000 deaths) (1). In Japan, recently, both the prevalence and the mortality rate of prostate cancer have increased significantly. It is predicted that prostate cancer will be the most common cancer in Japanese men, with the incidence overtaking that of gastric cancer (2). At an early stage, prostate cancer responds to androgen deprivation therapy (3). However, the effect of the therapy does not continue and the cancer finally becomes aggressive with severe chemoresistance, referred to as castration-resistant prostate cancer (3). Thus, an effective preventive strategy for prostate cancer should be urgently established.

Many reports have demonstrated that vitamin E can be a candidate for the adjuvant treatment of cancer and it plays an important role in the prevention of cancer (4). Vitamin E occurs naturally in two classes of compounds: tocopherols (α, β, γ and δ-Toc) and tocotrienols (α, β, γ and δ-T3). There are structural differences between the two in that tocopherols have a saturated phytyl side chain attached to their chroman rings, and tocotrienols possess an unsaturated isoprenoid side chain. Both Tocs and T3s have substantial antioxidant activity as well as possessing several functions irrespective of their antioxidant property (4, 5). Recent studies have identified T3s as having more potent anticancer potential than the well-established Tocs (5), and are speculated to be more bioavailable than the other isomers, making T3s more favorable as potential anticancer candidates (6, 7). From in vitro studies it has been shown that the various forms of T3s possess different anticancer effects, although they differ from each other only in the location of methyl groups on their chromanol rings (4). To date, of the T3s, δ-T3 has demonstrated both in vitro and in vivo the most potent antiproliferative activities against various cancers, including prostate cancer (7). In addition, T3s were found to accumulate in pancreatic tissue after oral administration (8), and in xenografted prostate tumors after intraperitoneal injection (9).

Due to the long latency period, and slow disease progression, prostate cancer is considered an ideal candidate for the use of vitamin E as a chemoprotective agent, which provides an opportunity to intervene before malignancy (10). Support for the possible use of vitamin E came from a study that demonstrated reduced risk of prostate cancer in increased consumption of γ-Toc (11). Experimental and epidemiological studies have suggested that γ-Toc may be superior to the commonly tested α-Toc as a chemopreventive agent due to its more potent anti-nitrative and anti-inflammatory activities (5, 11). Since δ-T3 and γ-Toc have different target points to induce each anti-cancer effect (5, 12), it is assumed that the δ-T3-induced anti-prostate cancer...
activity may be enhanced in the combination of ϒ-Toc with δ-T3. In this context, this study was undertaken to clarify this hypothesis. Given that tocopherol-free annatto T3 oil (annatto T3) and ϒ-Toc-rich oil (Tmix) are readily available and abundant sources of δ-T3 and ϒ-Toc, as well as cost effective for future clinical application, we utilized the two oils as sources of δ-T3 and ϒ-Toc.

Materials and Methods
Reagents. All cultures and chemicals were obtained from Nacalai Tesque, Inc. (Kyoto, Japan), unless otherwise indicated. Fetal bovine serum (FBS) was purchased from Sigma-Aldrich (St. Louis, MO). Annatto T3 containing 90% δ-T3 and 10% ϒ-T3, and Tmix (GammaBright90) containing more than 90% ϒ-Toc were supplied by CycloChem (Kobe, Japan) and Eisai Food & Chemical Co., Ltd. (Tokyo, Japan), respectively. High-performance liquid chromatography (HPLC)-purified δ-T3 and ϒ-Toc were from Tama Seikagaku (Tokyo, Japan). WST-1 reagent was purchased from Roche Diagnostics, K.K. (Tokyo, Japan).

Cell culture and treatment. LNCaP cells, a representative human androgen-dependent prostate cancer cell line (ATCC, Manassas, VA), were routinely grown in RPMI1640 supplemented with 10% FBS, 50 IU/mL penicillin, and 50 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO2. LNCaP cells were plated on culture plates and cultured for more than 24 h to permit the cells to adhere and reach the exponential growth phase. After attachment, the cells were cultured in RPMI1640 supplemented with 2% FBS containing each reagent (annatto T3 and Tmix) for each indicated period, and subsequently each assay was performed. In this study, where the cells were treated with either annatto T3 or Tmix, the descriptions of treatment groups were indicated as either δ-T3 or ϒ-Toc, respectively. The exact concentrations of δ-T3 contained in annatto T3 and ϒ-Toc contained in Tmix were described when treated with each agent. Cell viability was determined by trypan blue dye exclusion.

Cell growth and apoptosis. To determine cell growth, WST-1 assay was utilized. Cell cycle analysis and apoptosis were evaluated with a Muse Cell Cycle kit, and a Muse Annexin V and Dead Cell kit (EMD Millipore, Darmstadt, Germany), respectively.

Analysis of δ-T3 and ϒ-Toc levels. δ-T3 and ϒ-Toc levels in LNCaP cells were determined by HPLC with fluorescence detection. δ-T3 and ϒ-Toc were extracted from the cells as described previously (13). HPLC was performed using a Cosmosil Packed Column (4.6×250 nm, Nacalai Tesque, Inc.) and Cosmosil Guard Cartridge (4.6×10 nm, Nacalai Tesque, Inc.) with a mobile phase of methanol/water (9 : 1) at a flow rate of 0.7 mL/min. δ-T3 and ϒ-Toc were detected with a FP-2025 Plus Intelligent Fluorescence Detector (excitation 298 nm, emission 325 nm; Jasco Co., Tokyo, Japan).

Statistical analysis. Differences among groups were analyzed by one-way ANOVA followed by the Tukey-Kramer test, and differences between two groups were analyzed by one-way ANOVA followed by Student’s t-test. All statistical analyses were performed using EKuseru-Toukei software (Social Survey Research Information Co., Ltd., Tokyo, Japan). p values of 0.05 or less were considered significant.

Results and Discussion
The slight differences between vitamin E deriva-
Combination of δ-Tocotrienol and γ-Tocopherol in Prostate Cancer Cells

Combination of δ-Tocotrienol and γ-Tocopherol in Prostate Cancer Cells

Given by individual treatments. Combined treatment of compounds may exert a greater effect. In this context, we first assessed the effect of δ-T3 and γ-Toc either alone or in combination on the viability of LNCaP cells. As shown in Fig. 1, treatment of LNCaP cells with δ-T3 concentrations of 5 and 10 μM produced a significant cell growth inhibition. Exposure of LNCaP cells to 5 and 10 μM γ-Toc showed similar findings, but with a lower degree of cell growth inhibition. These observations suggest that prostate cancer cells respond to δ-T3 more effectively than to γ-Toc. The inhibition of cell growth in vivo and in vitro by δ-T3 on various prostate cancer cells has been reported (14–16). From these studies, it appears evident that T3 possesses antioxidant efficacy as well as potent suppression of cancer functions that are independent of its antioxidant properties and its efficacy is superior to that of γ-Toc (14, 16). Furthermore, of the four T3 isomers, δ-T3 has the most potent anti-cancer activity (14, 16).

Subsequent cell viability tests using δ-T3 in combination with γ-Toc at varying doses of 5 and 10 μM were examined. The growth inhibition of cells affected by combinations of δ-T3 and γ-Toc was greater than that given by individual treatments. Combined treatment of either 5 or 10 μM δ-T3 with 5 μM γ-Toc was found to have a greater significant effect, as evidenced by marked reduction of cancer cell proliferation. However, combination of 5–10 μM δ-T3 with 10 μM γ-Toc produced no further enhancement in growth inhibition when compared to the combined dose containing 5 μM γ-Toc. Mixtures of about 70% T3 and 30% α-Toc, referred to as tocotrienol-rich fraction (TRF), can also exhibit significant synergistic effects by inhibiting cellular proliferation and accelerating apoptotic events in prostate cancer cells (17). Similarly, T3s have shown inhibition of prostate tumorigenesis in mice (8). Furthermore, anticancer effects of δ-T3 are synergistic by co-administration with γ-Toc in human colorectal adenocarcinoma cells (18). This is consistent with our observations, which strongly suggests that δ-T3 may provide significant benefit in the inhibition of prostate cancer cell growth when used in combination with γ-Toc. In addition, a previous study has shown that 50 μM treatment of γ-Toc has no influence on normal prostate epithelial cells (19). Overall, the combination of δ-T3 with γ-Toc may be an effective preventive strategy for prostate cancer with less toxicity.

It is quite interesting that the anticancer effects of δ-T3 are enhanced by co-treatment with γ-Toc; however, increase in the co-treatment dose of γ-Toc from 5 to 10 μM did not show a dose-responsive further reduction of LNCaP cell viability. Based on this finding, a combination dosage of 10 μM for both δ-T3 and γ-Toc was chosen to investigate the cellular uptake of δ-T3 and γ-Toc. The HPLC analysis results, as illustrated in Fig. 2, revealed that the cellular uptake of both δ-T3 and γ-Toc were significantly reduced in the presence of the combination treatment. Of importance, it is interesting to note, however, that δ-T3 showed a higher cellular uptake than γ-Toc, being approximately twofold more concentrated. Studies have shown that, in highly malignant mammary epithelial cells, the relative cellular accumulation of individual T3 and Toc isoforms was not equal and was characterized as δ-T3 > γ-T3 > α-T3 > δ-Toc > γ-Toc > α-Toc (6). The same relative order was also observed for the anti-proliferative effect exerted by T3 and Toc. It is believed that T3s are more easily accumulated, or taken up, resulting in higher concentrations of T3 at intracellular sites of action, and are thereby able to induce a greater biological response (6). The present uptake results also demonstrated that δ-T3 is more effectively incorporated into LNCaP cells, similar to observations from in vitro studies when δ-T3 was used in combination with α-Toc (13, 18). In human umbilical vein endothelial cells (HUVECs), the proliferation inhibitory potency of δ-T3 is likely due to its correlation with greater incorporation into HUVECs than α-Toc’s (13). Co-administration of α-Toc decreased uptake of δ-T3 into human colorectal adenocarcinoma cells (DLD-1) and suppressed both cytotoxicity and induction of apoptosis, which suggested that cellular δ-T3 content solely determines the intensity of δ-T3-induced cytotoxicity (18).

Overall results (Fig. 1) indicated that the combination of δ-T3 and γ-Toc showed impressively significant growth inhibition. However, the results, as shown in Figs. 1 and 2, indicated that the cellular γ-Toc level might be a critical factor to determine the synergistic inhibition on LNCaP cell growth. We therefore evaluated the effects of δ-T3 and/or γ-Toc on cell cycle progression in the cells. As shown in Fig. 3A, treatment with 10 μM δ-T3 caused LNCaP cells to accumulate in the G1 phase with a concomitant decrease in the number of cells in the S-phase. This result suggested that δ-T3 induces G1-phase cell cycle arrest in LNCaP at the expense of a decrease in the S-phase population. On the other hand, 10 μM γ-Toc administration induced cell cycle arrest in LNCaP cells.
cycle arrest in the G2/M phase. The combined treatment of 10 μM δ-T3 and γ-Toc simultaneously induced cell cycle arrest in both the G1 and G2/M phases, and a lower percentage in the S phase. The identification of the early and late apoptotic cells (Fig. 3B) showed that LNCaP cells treated with δ-T3 exhibited an increase in the number of apoptotic cells and the combination of δ-T3 and γ-Toc resulted in the onset of an especially massive apoptotic cell death. It is well known that anti-cancer activities of most anti-cancer agents mainly depend on cell cycle arrest and subsequent induction of apoptosis (20). The loss of cell cycle control in G1 has been implicated in tumor development and proliferation in prostate cancer cells (21). Additionally, our previous work reported that the occurrence of apoptosis in cancer cells could be reinforced by simultaneous cell cycle arrest in the G1 and G2/M phases (21). Thus, it is considered that the simultaneous accumulations of the LNCaP cells in G1 and G2/M phases act as a stronger apoptotic signal and lead to synergistic inhibition on the cells by the combination. The regulation of G2/M transition could be an effective target to control the growth and proliferation of prostate cancer cells and facilitate their apoptotic death, and is considered to be a potential anticancer mechanism (22). In fact, it has been reported that G2/M arrest sensitizes prostate cancer cell death through the apoptotic pathway (23). The induction of apoptosis by γ-Toc is shown to be dependent on the cellular microenvironment (6), or alternative independent pathways (19, 24). Another study (25) showed that Toc inhibits mitosis of the cells in a xenograft mouse model. The treatment dose and plasma level were 50 μM and 1 μM, respectively (25). These reports suggest that cell cycle arrest caused by γ-Toc is different depending on its exposure level. These data also suggest that the synergistic efficacy of δ-T3 and γ-Toc at 10 μM doses, observed in the present study, is at physiologically achievable concentrations.

Based on recent studies, we can speculate a possible mechanism on the co-treatment effect. Signals for cell growth, survival, and other physiological processes are transmitted across the plasma membrane through discrete regions known as lipid rafts (26). In many types of cancer cells, including prostate, oncogenic receptors involved in aberrant signaling are concentrated in these lipid rafts (26, 27). Lipid raft disruption affects homeostasis by altering cellular survival and apoptotic pathways (28). A recently published paper (29) demonstrated that γ-T3 selectively accumulates within the lipid rafts and interferes with receptor tyrosine kinase...
dimerization. The disruption of lipid rafts by γ-T3 inhibits Her-2 activation, leading to inhibition of proliferation in human breast cancer cells. It has been reported that LNCaP cells contain altered signaling caused by oncogenic mutations in the ErbB family of receptor tyrosine kinases (e.g. HER1 and HER2), found in lipid-rafts (3, 4, 26). Thus, the probable mechanism of T3’s inhibition of LNCaP growth may occur in a similar manner to that of human breast cancer (29). Additionally, it is well known that T3 exhibits an inhibitory effect on HMG-CoA reductase (4, 5) causing an inability to prenylate Ras, inhibiting its activation. Ras is a downstream target of receptor tyrosine kinases in the signal transduction pathway involved in cell growth and proliferation. It is possible, then, that δ-T3 has a dual anti-cancer effect on LNCaP cells through disruption of both ErbB and Ras signaling (5, 26).

In this study, it was also revealed that G2/M accumulation was induced in LNCaP cells by γ-Toc, and we further observed that this cell cycle alteration is associated with changes in the expression level of a cell cycle regulatory protein, cyclin B (data not shown). It has been reported that a balance between cyclins, cyclin-dependent kinases and phosphatases controlled cell cycle progression and cell proliferation (23). Therefore, this suggests that the effect of γ-Toc on G2/M arrest of LNCaP cells may not depend on the disruption of lipid rafts; however, the exact mechanism is still unclear.

Thus, in order to clarify a possible mechanism on the combination effect of γ-Toc and δ-T3, further study is needed before the results of this study can be applied to clinical use.

Acknowledgements
This study was supported by JSPS KAKENHI, Grand-in-Aid for JSPS Fellows (15K12329), and by the Inoue Enryou Memorial Foundation of Toyo University.

REFERENCES


