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The Enhancing Effect of γ-Cyclodextrin Inclusion on γ-Tocotrienol-dependent Negative Growth Control of Mesothelioma Cells in a Xenograft Model

[1,] *Yano T, [1] Sato A, [1] Sekine M, [2] Kashiwagi K, [1] Ota M.

[1]Research Group of Nutritional Sciences, Faculty of Life Sciences, Toyo University, Gunma, 374-0193, Japan; [2]Department of Experimental Pathology, Yokohama City University Graduate School of Medicine, Kanagawa, 315-0345, Japan

*Corresponding Author: Tomohiro Yano, PhD, Professor, Research Group of Nutritional Sciences, Faculty of Life Sciences, Toyo University, Gunma, 374-0193, Japan. Phone number: +81-274-82-9143

Submission date: October 19, 2011; Acceptance date: December 11, 2011; Publication date: December 30, 2011

ABSTRACT

Background: Malignant mesothelioma is an aggressive cancer with no effective treatment options. Of phytochemicals, tocotrienol (T3), a member of vitamin E, is one of the most potent anti-mesothelioma agents, but the effectiveness *in vivo* is quite limited, due to its low bioavailability. In this study, we investigated if the oral treatment of γ-T3 inclusion with γ-cyclodextrin (CD) could improve the bioavailability and anticancer activity of the T3.

Findings: Using nude mice bearing MSTO-211H cells (a human malignant mesothelioma cell line), the effect of γ-T3 inclusion with γ-CD on γ-T3 level in tumor tissues, tumor growth, and its related mRNA levels were examined. The difference of tumor growth between the two groups had no statistical significance, but the latter showed a lower tendency compared with the former. In linked with this observation, the level of vascular endothelial growth factor mRNA required for *in vivo* tumor growth in γ-T3 inclusion with γ-CD group was lower than that in γ-T3 group, on the contrary, the level of γ-T3 level showed an opposite tendency.

Conclusion: Our study demonstrated that the bioavailability of γ-T3 was improved by an oral administration of a novel γ-T3 inclusion complex with CD. Furthermore, the improvement of

the bioavailability contributed to the increase of anticancer activity of γ-T3 *in vivo*.

Key words: Anti-cancer agent, bioavailability, cyclodextrin, mesothelioma, tocotrienol.

FINDINGS: Malignant mesothelioma from the serosal membranes of the body cavities is a particularly aggressive cancer, which is characterized by rapid progression, late metastases, and poor prognosis [1]. Although surgery, radiotherapy, chemotherapy, and/or their combinations have been used as therapeutic modalities, median patient survival is 8–18 months [2]. Malignant mesothelioma cells exhibit resistance to many chemotherapeutic agents, including doxorubicin and cisplatin, which are, nevertheless, widely used to treat Malignant mesothelioma [3]. A recent report of a phase III study showed that the combination of pemetrexed and cisplatin is more effective than cisplatin alone, with differences in response rates of 41.3%, versus 16.3% [4]. However, most of the patients relapsed within a year after starting the treatment. Therefore, new therapeutic approaches are urgently needed for malignant mesothelioma patients.

Vitamin E is one of the most promising lipophilic antioxidants, consisting of eight naturally occurring forms, known as tocopherols (Ts), and tocotrienols (T3s). Ts carry a saturated phytyl group that is derived from homogentisic acid and phytyl pyrophosphate, whereas T3s are thought to arise from the condensation of homogentisic acid and geranylgeranyl pyrophosphate [5]. Ts and T3s can be subdivided into four isomers (α, β, γ and δ) relating to the numbers and the position of the methyl groups on their chromanol ring. The major source of vitamin E is plant-derived oils. T3s are the major vitamin E component of palm oil. Significant amounts are also found in barley, oats, and rice bran [6,7]. Since T3s differ from Ts by the presence of three trans double bonds in the hydrocarbon tail, T3s are thought to assume a unique conformation. Therefore, members of the natural vitamin E family possess overlapping and unique functional properties that could be due to their structural difference. Among these, T3s, but not Ts, have been linked to additional beneficial therapeutic properties, such as lowering serum cholesterol levels when administered in the diet of hypercholesterolemic humans [8]. Moreover, recent studies also revealed their beneficial biological properties, such as anti-angiogenesis, and anticancer activities [9]. Therefore, T3s are one of the attractive groups, not only as a functional food constituent but also as a clinical therapeutic agent. Especially, γ-T3 is the most potent anti-cancer isoforms of T3 isforms, so this isform is as promising as a clinical therapeutic agent.

Cyclodextrins (CDs) are polysaccharides built from six to eight (α=6, β=7, γ=8)

D-glucose units. The α-CD, β-CD and γ-CD are widely used natural CDs. The D-glucose units are covalently linked at the carbon atoms C1 and C4 to form a macrocycle [10,11]. CDs can generally form inclusion complexes with a number of lipophilic substances, and thus have been used for improving their water solubility, stability, diffusibility, and bioavailability [12,13]. Previous studies have shown that the bioavailability of coenzyme Q10 was improved by CD inclusion [14]. Among the three natural CDs, γ-CD shows the highest water solubility and is the only form digested in the intestinal tract. Therefore, much attention has recently been given to the usage of γ-CD as a "host" molecule of the inclusion complex in oral bioavailability experiments and as a promising nutrition delivery system.

The aim of this study was to examine the effect of γ-T3 inclusion with γ-CD on its bioavailability. Further, we evaluated anti-mesothelioma activity of this inclusion. A T3-rich fraction (TRF) was prepared by extraction from rice bran, which is a readily available and abundant source of γ-T3.

METHODS:

A TRF or a TRF inclusion complex with γ-CD (TRF/CD). TRF (Oryza tocotrienol 72), γ-CD, and TRF/CD were provided by Oryza Oil & Fat Chemical Company (Aichi, Japan), and Cyclochem (Kobe, Japan). TRF contained vitamin E as follows: 0.5% α-T, 0.4% β-T, 2.8% γ-T, 0.5% δ-T, 1.8% α-T, 0.4% β-T, 65.0% γ-T3 and 4.6% δ-T3. All animal experiments were approved by the local animal ethics committee and performed according to guidelines for the care and use of laboratory animals at the University. Small fragments of tumor (2 × 2 × 2 mm) established from MSTO-211H cells (a human malignant mesothelioma cell line) were transplanted subcutaneously into the right flank region of 4 to 5-week-old female athymic CAnN.Cg-Foxn1^{nu}/CrlCrlj mice (Charles River Laboratories Japan Inc., Yokohama, Japan). After 3 days, the mice received oral treatment of TRF, TRF/CD or CD (control), three times a week for 4 consecutive weeks. The mice were weighed and the tumor was measured every 3 or 4 days. Tumor volume was calculated as $(\pi/6) \times$ large diameter $\times$ (small diameter)^2. On day 33, tumors were carefully removed after death, and stocked at −80 °C for isolation of RNA or T3 analysis. The following equivalent doses of TRF and γ-CD were used for oral administration on Days 28, 30 and 32: TRF, 2.79 mg in 200 μl of corn oil; TRF/CD, 14.5 mg in 200 μl of corn oil; γ-CD, 11.71 mg in 200 μl of corn oil; or corn oil, 200 μl as control. Amounts of infused total T3s were 2.00 and 1.99 mg per mouse in TRF and TRF/CD, respectively. Similarly, amounts of infused γ-T3 were 1.81 and 1.74 mg per mouse in TRF and TRF/CD, respectively. The level of γ-T3 in tumors was determined by HPLC as

described previously [15]. Total RNA was isolated using QIAshredder (Qiagen, Valencia, CA, USA) and the RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. cDNA was synthesized. Real-time PCR was performed by using an ABI Prism 7000 Sequence Detection System (Applied Biosystems Japan Ltd. Tokyo, Japan) and SYBR Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan), according to the manufacturer's instructions. The primers were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), accession number (BC023632), sense (nucleotides 737–756), antisense (nucleotides 916–897); vascular endothelial growth factor (VEGF), accession number (NM_001025368), sense (nucleotides 1163–1182), antisense (nucleotides 1370–1390). Data is expressed as the mean±SD and analyzed by one-way ANOVA followed by Dunnett's *t*-test or Student's *t*-test only. $P<0.05$ was considered as showing a significant difference between means.

RESULTS:

We investigated the effect of γ-T3 inclusion with γ-CD on tumor development. Eight days after the inoculation of MSTO-211H cells in the subcutaneous tissue in the back of mice, a tumor was observed in each group (data not shown). The growth of the tumor was significantly suppressed in both TRF and TRF/CD groups compared with γ-CD group (Figure 1). In addition, TRF/CD tended to exert a stronger anti-mesothelioma effect than TRF in the last stage, although there was no statistical difference.γ-T3 was detected in tumors from TRF and TRF/CD but not CD, in particular, the increase in γ-T3 level in the tumors was observed in TRF/CD group, although the difference was not significant (Figure 2). Also, γ-T3 level in plasma from each group showed the same tendency (data not shown). Furthermore, TRF/CD and TRF treatment in tumor tissues significantly reduced mRNA level of VEGF (the major molecule necessary for angiogenesis) compared with γ-CD treatment (Figure 3). These results indicate that the inclusion with γ-CD is effective to enhance anti-mesothelioma effect which T3 has.

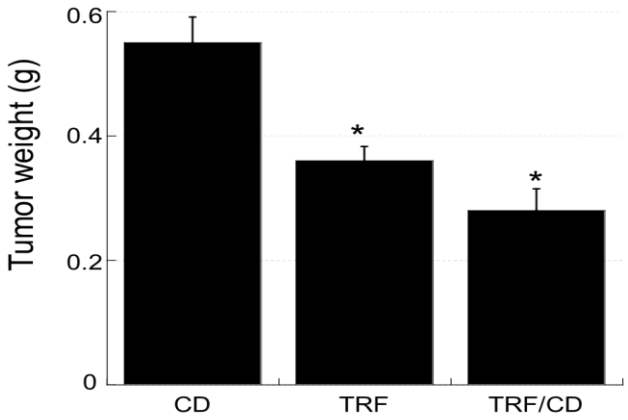

Figure 1. Effect of TRF and TRF/CD on the development of MSTO-211H tumors in nude mice. Tumor weight was measured on day 33 after inoculation. Each value is the mean of three determinants; vertical lines indicate SD. *P<0.05 compared to CD treatment.

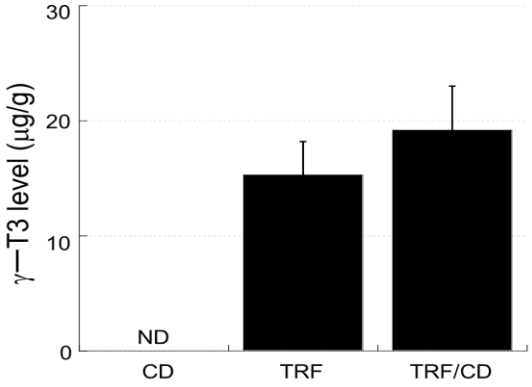

Figure 2. The accumulation of T3 in tumor tissues in mice treated with TRF and TRF/CD. Each value is the mean of three determinants; vertical lines indicate SD. ND, not detected.

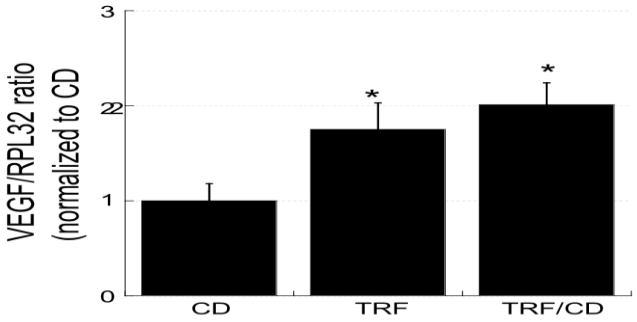

Figure 3. Effects of TRF and TRF/CD treatment on the expression of VEGF in MSTO-211H tumors in nude mice. VEGF mRNA level was determined by RT-realtime PCR as described above. Each value is the mean of three determinants; vertical lines indicate SD. *P<0.05 compared to CD treatment.

DISCUSSION:

In our previous study [16], we compared the bioavailability of γ-T3 when given as a TRF inclusion complex with CD with that administered as a free form dissolved in corn oil. The Cmax and AUC values for plasma γ-T3 levels were markedly greater in mice administered TRF/CD orally than those given TRF alone. Moreover, TRF/CD-administered mice tended to be more resistant against the lethal effects of LPS-induced endotoxin shock than those given

TRF alone. Form these results we concluded that these effects could be attributed, at least in part, to the elevated level of plasma γ-T3 level. Similarly, our present study indicated that TRF/CD treatment reinforced anti-mesothelioma effect in TRF treatment and that the reinforcement was associated with γ-T3 level in tumors and plasma. The present observation completely supports a notion that the inclusion system with γ-CD improves the bioavailability of γ-T3, and that the improvement leads to enhancement of γ-T3 potential as an anti-mesothelioma agent. Thus, TRF/CD may be a promising procedure to utilize γ-T3 in complementary medicine against mesothelioma.

With respect to anti-cancer mechanism of γ-T3, several types of targets have been reported [9]. Of the determined target molecules, c-Src is one of the promising target molecules in mesothelioma [17]. The expression of activated c-Src correlates with advanced-stage and metastasis of mesothelioma [18]. It suggests the involvement of c-Src in driving oncogenesis. Furthermore, inhibition of Src expression and activation in mesothelioma leads to apoptosis, cell cycle arrest, and suppression of invasion [19]. It has been reported that T3 inhibited c-Src kinase activity or regulated one or more events upstream of c-Src kinase activation in brain tissues [20]. Additionally, we observed that the expression of VEGF, a main molecule located in the downstream of c-Src signaling [21], was significantly suppressed by TRF and TRF/CD treatment. This observation strongly supports a hypothesis that the inactivation of c-Src and its related signaling closely relate to γ-T3-dependent negative growth control of mesothelioma cells *in vivo*. Also, we can speculate which molecule in c-Src signaling contributes to the suppression of VEGF expression in TRF and TRF/CD-treated mice, based on the following reports. (1) A signal transducer and activator of transcription 3 (Stat3) is determined as a major positive transcription factor for VEGF [22]; (2) the inhibition of c-Src leads to the inactivation of Stat3 as a transcription factor [22]; (3) consecutive activation of Stat3 has been observed in human mesothelioma cells [23]; (4) a specific inhibitor towards Stat3 effectively has negative growth control against mesothelioma cells [18]. These reports suggest that Stat3 is a main molecule in c-Src signaling to be necessary for anti-mesothelioma effect of γ-T3. Overall, it seems to be possible that c-Src/Stat3 signaling contribute to γ-T3-dependent negative growth control of mesothelioma cells *in vivo*.

CONCLUSION:

γ-T3 inclusion complex with CD is a promising procedure to improve human mesothelioma treatment.

Abbreviations: Cyclodextrins (CDs), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tocopherols (Ts), tocotrienols (T3s), T3-rich fraction (TRF), vascular endothelial growth factor (VEGF).

Competing interests: The authors declare that they have no competing interests.

Authors' contributions: TY designed the study; TY and KK collected and analyzed the data; all authors contributed to interpretation of data and reviewed the manuscript.

Acknowledgements: Supported by a research grant for Health Sciences Focusing on Drug Innovation from the Japan Health Sciences and a grant by Toyo University to T. Yano.

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