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δ-tocotrienol Induces GGCX Expression and Inhibits HepG2 Cell Proliferation

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***Abstract*—*Des-gamma-carboxy-prothrombin (DCP) is an autologous growth factor as well as a well-known tumor marker of hepatocellular carcinoma. DCP is converted to prothrombin by γ-glutamyl carboxylase (GGCX), the expression of which is low in hepatocellular cancer (HCC) tissue. An alternatively spliced variant of GGCX, lacking exon 2, shows expression patterns correlating with DCP level in specific HCC cell lines such as HepG2. In order to seek for chemical compounds that may correct the exon 2 skipping of GGCX, we tested three well-known splice-modulating compounds that induce exon-inclusion: kinetin; EGCG; and δ-tocotrienol. Among the three, δ-tocotrienol decreased cell viability of HepG2 cells in MTS assays. By RT-PCR and Western blot analyses, δ-tocotrienol induced GGCX mRNA expression strongly at 3 μM that was inhibited by sulforaphane but with no effect on alternative splicing. Though δ-tocotrienol did not significantly affect DCP production, it had a strong effect on HepG2 cell viability, evidenced by PARP cleavage and scratch assays.*

Keywords*— *δ -tocotrienol; Gamma-glutamyl carboxylase; HepG2.

I. INTRODUCTION

Gamma-glutamyl carboxylase (GGCX), with its cofactor vitamin K, adds a carboxyl group to glutamate residues (γ-carboxylation) of substrates such as vitamin K-dependent coagulation factors (II, VII, IX, X)[1,2]. Human *GGCX* has fifteen exons and is a membrane protein that functions in the microsome[3,4]. A report shows that GGCX overexpression decreases apoptosis in osteoarthritis chondrocytes of model rabbits[5]. In the human promyelocytic cell line HL60, the intracellular metabolite of Vitamin K2, VK2-O (γ-carboxylated by GGCX), interacts with Bak (Bcl-2 antagonist killer) to induce apoptosis[6]. The involvement of GGCX in apoptosis is also reported with human prostate cancer LNCap cells where VK2-induced anticancer effects are abrogated by GGCX knockdown[7]. In the liver, GGCX converts the glutamate residue of DCP (Des-gamma-carboxy-prothrombin) or known as PIVKAII (protein induced by vitamin K absence or antagonist II) into the γ-carboxyglutamate residue and metabolize it to prothrombin[8]. Functional analyses of *GGCX* by a knockout mouse study[9] have shown massive intra-abdominal hemorrhage shortly after birth. Liver-specific knockout mice[10] live after birth but also have bleeding diasthesis with shorter life span. Mutations of *GGCX* have been reported in severe bleeding disorder and comorbid Keutel syndrome[11]. Decreased carboxylation of extrahepatic vitamin K-dependent proteins such as matrix γ-carboxyglutamate protein result in vascular calcification and PXE-like syndrome with mutations in the *GGCX* gene[12].

A recent report shows that GGCX exon 2 is skipped in DCP-positive liver cancer cells[13]. We sought to correct this aberrant splicing event by splice-modifying compounds[14].

II. MATERIALS AND METHODS

HepG2 cell, cell viability assay

HepG2 cells were cultured with DMEM (low glucose) with non-essential amino acids and 10% FBS with antibiotics included. For cell viability assays, HepG2 cells were plated at $10^4/cm^2$ and cultured by the conditions indicated. After 24 h, viable cells were detected by CellQuanti-MTT Cell Viability Assay Kit (BioAssay Systems).

Reverse-transcription PCR (RT-PCR), immunoblot assay

HepG2 cells cultured by the indicated conditions were washed twice with PBS and resuspended in 0.5 ml TRIzol reagent (Invitrogen). After passing through a 25G needle five times, the sample was centrifuged. For RT-PCR, the aqueous phase was precipitated by isopropanol, treated by RQ1 DNase (Promega, M6101) and total RNA was precipitated. One µg of total RNA was subjected to reverse transcription by ReverTra Ace (TRT-101) and 0.5 µl of the reverse transcribed product was subjected to subquantitative PCR (25 to 30 cycles) by TaKaRa Ex Taq (TaKaRa Bio, RR001A). For immunoblot assays, protein was purified from the interphase and organic phase by precipitating with 6V of a solution for precipitating protein (50% Ethanol, 24.5% Acetone, 24.5% Methanol, 1% distilled water). Twenty micrograms of protein were boiled in sample buffer and separated by 10% sodium dodecyl sulfate-PAGE (SDS-PAGE), transferred to a PVDF membrane and analyzed by primary antibodies: GGCX antibody (BioSS, bs-7759R) or PARP antibody (Sigma Aldrich, P7605), and secondary antibodies conjugated to peroxidase (rabbit: Vector Laboratories, mouse: Cell Signaling) and detected by Chemi-Lumi One L (Nacalai Tesque).

ELISA

HepG2 cells cultured by the indicated conditions. The secreted DCP (PIVKAII) was measured by Human PIVKA-II (Protein Induced by Vitamin K Absence or Antagonist-II) ELISA Kit (MyBioSource.Inc., MBS2516006). ELISA was performed following the manufacturer's instructions.

In vitro wound healing assay

HepG2 cells cultured by the indicated conditions were scratched at the center of the well with a 2-mm-wide silicone tip of a CELL Scratcher (AGC Techno Glass, Shizuoka, Japan), washed with PBS and replaced with fresh medium. At 0 h and after 24 h, the area of the cells were evaluated by subtracting the scratched area from the whole by the wound healing tool of the Image J software (1.47v, NIH).

III. Results

δ-tocotrienol Inhibited HepG2 Cell Proliferation

GGCX has a splicing isoform that skips exon 2 (*GGCXΔex2*) and is expressed in DCP-positive liver cancer cells 13. Restoring *GGCX* exon 2 by preventing exon skipping would restore GGCX activity and reduce DCP expression, a hallmark of DCP-positive liver cancer. We sought to test whether known splice-modifying compounds that prevent exon-skipping have an effect on HepG2 cell proliferation, a cell line that expresses *GGCXΔex2* and is DCP-positive. We tested three splice-modifying compounds that are known to induce exon-inclusion: a plant cytokinin, kinetin[15]; a polyphenol, EGCG ((-)-epigallocatechin gallate)[16]; and a vitamin E family member, δ-tocotrienol[17]. Two conditions were tested for 24 h, compounds were added before adhered to possibly check their effect on metastasis (Fig. 1abc, left panels) and after they adhered to check their effect on growth (Fig. 1abc, right panels). Kinetin had the tendency to stimulate HepG2 cell growth when added before cells were adhered but was effective at 25 µM for adhered cells (Fig. 1a). EGCG also had the tendency to stimulate HepG2 cell growth (Fig. 1b). δ-tocotrienol significantly decreased HepG2 cell viability in a dose dependent manner before cells were adhered and had a tendency to suppress adhered cell viability (Fig. 1c). Herein, δ-tocotrienol treatment is designated as addition of δ-tocotrienol before HepG2 cell attachment.

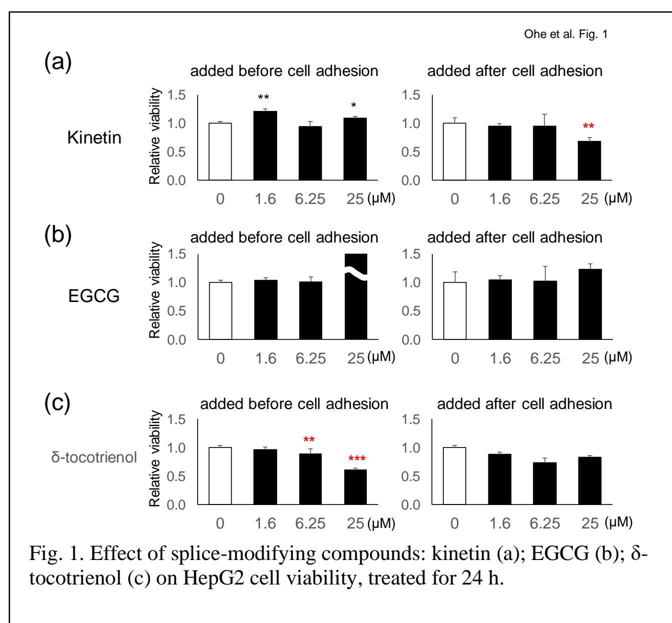

Fig. 1. Effect of splice-modifying compounds: kinetin (a); EGCG (b); δ-tocotrienol (c) on HepG2 cell viability, treated for 24 h.

δ-tocotrienol Induced GGCX Expression

GGCX has a splicing isoform lacking exon 2 13. We hypothesized that δ-tocotrienol would alter the ratio of Δexon2 to full length of *GGCX* in HepG2 cells. However, there was no drastic change of alternative splicing in δ-tocotrienol-24h-treated HepG2 cells; both mRNA isoforms were induced at 1.6 and 3.1 µM; increasing the concentration decreased both mRNA levels, simultaneously (Fig. 2a). Next we checked GGCX protein level in δ-tocotrienol-treated HepG2 cells. A dose-dependent increase of GGCX protein was observed (Fig. 2b). Thus, δ-tocotrienol did not change the alternative spicing pattern of *GGCX*, but induced considerable increase of full length expression up to 3.1 µM at mRNA and protein level.

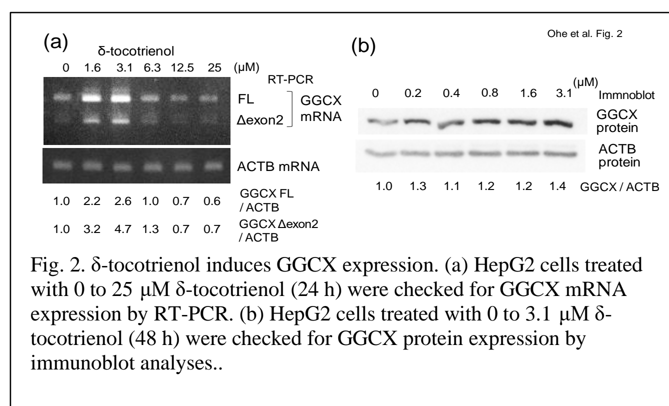

Fig. 2. δ-tocotrienol induces GGCX expression. (a) HepG2 cells treated with 0 to 25 µM δ-tocotrienol (24 h) were checked for GGCX mRNA expression by RT-PCR. (b) HepG2 cells treated with 0 to 3.1 µM δ-tocotrienol (48 h) were checked for GGCX protein expression by immunoblot analyses..

δ-tocotrienol Enhanced Vacuole Formation and Induced Apoptosis

When δ-tocotrienol was added to HepG2 cells, enhanced vacuole formation was observed after 24 h at 1.6 µM (Fig. 3a). HepG2 cells were badly damaged by 25 µM and PARP cleavage was detected (Fig. 3b).

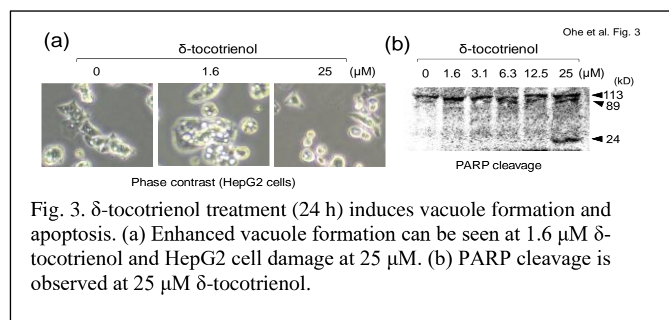

Fig. 3. δ-tocotrienol treatment (24 h) induces vacuole formation and apoptosis. (a) Enhanced vacuole formation can be seen at 1.6 µM δ-tocotrienol and HepG2 cell damage at 25 µM. (b) PARP cleavage is observed at 25 µM δ-tocotrienol.

An SXR Inhibitor blocked δ-tocotrienol Induced GGCX Expression

To elucidate the mechanism of δ-tocotrienol-induce expression of *GGCX* in HepG2 cells, we sought to test sulforaphane. Sulforaphane can significantly down-regulate cytochrome P450 3A4 expression in human primary hepatocytes and has inhibitory activity for the steroid and xenobiotic receptor (SXR)[18]. It is known that δ-tocotrienol binds to and activates SXR (Landes et al., 2003; Zhou et al., 2004). Sulforaphane suppressed δ-tocotrienol-induced *GGCX* expression and completely blocked it at 3.1 µM (Fig. 4a). GGCX protein was suppressed at 6.3 µM and higher concentrations of sulforaphane (Fig. 4b).

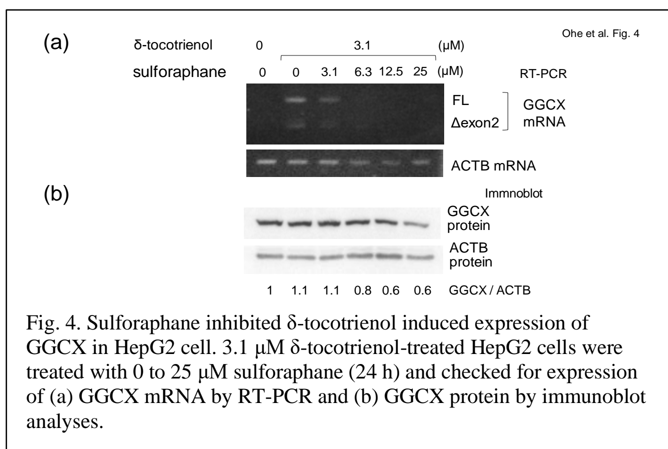

Fig. 4. Sulforaphane inhibited δ-tocotrienol induced expression of GGCX in HepG2 cell. 3.1 µM δ-tocotrienol-treated HepG2 cells were treated with 0 to 25 µM sulforaphane (24 h) and checked for expression of (a) GGCX mRNA by RT-PCR and (b) GGCX protein by immunoblot analyses.

δ-tocotrienol Suppressed DCP(PIVKA II) Expression

Since δ-tocotrienol suppressed GGCX expression, we next checked DCP protein secretion of δ-tocotrienol-treated-HepG2 cells. The medium of HepG2 cells treated with 0 to 25 µM δ-tocotrienol were collected and DCP was measured by ELISA. DCP secretion was suppressed by δ-tocotrienol up to 12.5 µM but not 25 µM (Fig. 5).

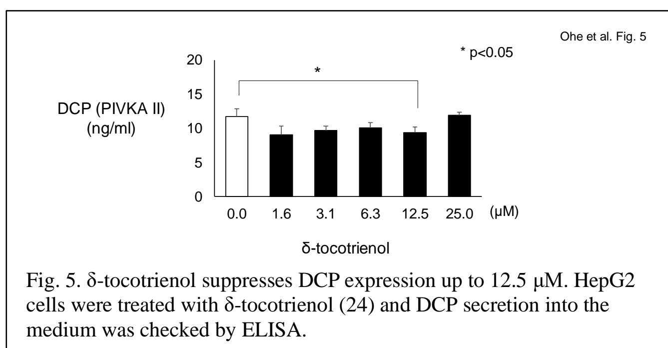

Fig. 5. δ-tocotrienol suppresses DCP expression up to 12.5 µM. HepG2 cells were treated with δ-tocotrienol (24) and DCP secretion into the medium was checked by ELISA.

δ-tocotrienol Suppressed Wound Healing Activity of HepG2 cells

Since δ-tocotrienol suppressed DCP secretion and related GGCX expression, we next checked its effect on wound healing of HepG2 cells. Control and solvent-treated HepG2 cells showed increase of HepG2 cell area. 1.6 µM of δ-tocotrienol inhibited the extent of wound healing and a tendency or significant decrease of HepG2 cell area was observed for 3.2 µM or higher (Fig. 6).

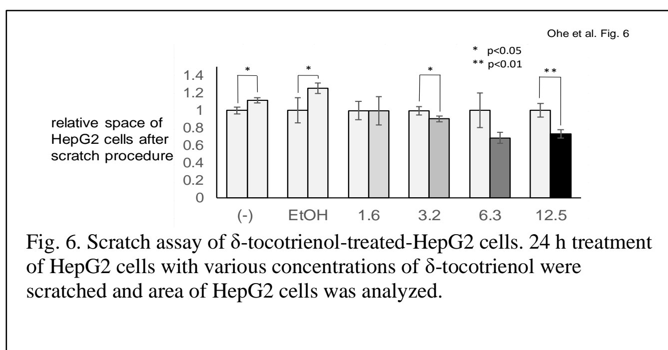

Fig. 6. Scratch assay of δ-tocotrienol-treated-HepG2 cells. 24 h treatment of HepG2 cells with various concentrations of δ-tocotrienol were scratched and area of HepG2 cells was analyzed.

IV. DISCUSSION

The anti-cancer effects of tocotrienols have been earning great attention. One of the four members, δ-tocotrienol, is presently undergoing many clinical trials for treating cancer[19,20]. The tocotrienol-rich fraction of palm oil is known for its beneficial effects on anti-proliferation of breast cancer cells[21] as well as its antioxidant and lipid-lowering properties[22]. It is also effective on prostate cancer cells[23], colon cancer cells[24,25], glioma cancer cells[26], and pancreatic cancer cells[27]. Among these cancers, clinical trials for breast cancer, metastatic colon cancer, and pancreas cancer are on their way for new applications (Table 1). There are also trials using tocotrienol as a nutritional supplement in advanced non-small cell lung cancer and ovarian cancer. For the liver, there is a clinical trial on NAFLD, NASH, and chronic hepatitis C liver disease (ClinicalTrials.gov identifier (NCT number): NCT02581085).

TABLE 1. Recent clinical trials for tocotrienol in cancer patients.

cancer	ClinicalTrials.gov identifier (NCT number)	start year	description
breast	NCT02909751	2016	phase 2 : combination with neoadjuvant chemotherapy
colon	NCT02705300	2016	alleviation of FOLFOXIRI side effects
lung	NCT02644252	2015	nutritional supplement on top of chemotherapy
ovary	NCT02399592	2015	nutritional supplement on top of chemotherapy
pancreas	NCT00985777 NCT01450046	2009 2011	Phase 1: pharmacokinetics of δ-tocotrienol on resectable pancreas tumors

Among the spice-modifying (exon including) compounds tested here, δ-tocotrienol was the most effective in inducing *GGCX* expression. The mechanism was not through alternative splicing regulation but through transcriptional regulation via the steroid and xenobiotic receptor, SXL. δ-tocotrienol is a member of the vitamin E family, comprised of four members (alpha, beta, gamma, delta) of tocopherol and tocotrienol[20]. The tocopherols lack interaction with SXR as tocotrienols do, with δ-tocotrienol having the strongest interacting capacity[28]. The closest SXR binding site (XRE core motif; 5'-GCGTG-3' [29]) to the translation start site can be found from -1413 to -1408 relative of the *GGCX* ATG. Since the transcription start site has not been determined, this XRE core motif may be located closer to the core promoter. Additionally, δ-tocotrienol has been shown to induce apoptosis in HepG2 cells[30].

Thus, we found for the first time that δ-tocotrienol can induce *GGCX* expression in HepG2 cells resulting in reduced DCP levels and inducing apoptosis. We hope that δ-tocotrienol could reach clinical trials for liver cancer as it is undergoing for other cancers.

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