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RRR- γ -tocopherol induces human breast cancer cells to undergo apoptosis via death receptor 5 (DR5)-mediated apoptotic signaling

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Abstract

Goal of this study was to investigate the pro-apoptotic properties of RRR- γ -tocopherol (γ T) in human breast cancer cells. γ T was shown to induce cancer cells but not normal cells to undergo apoptosis, sensitize cancer cells to Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL)-induced apoptosis, and increase death receptor 5 (DR5) mRNA, protein, and cell surface expression. Knockdown of DR5 attenuated γ T-induced apoptosis. Investigations of post-receptor signaling showed: caspase-8, Bid, and Bax activation, increases in mitochondria permeability, cytochrome c release and caspase-9 activation. Thus, γ T is a potent pro-apoptotic agent for human breast cancer cells inducing apoptosis via activation of DR5-mediated apoptotic pathway.

Keywords

human breast cancer cells; RRR- γ -tocopherol; DR5; apoptosis; TRAIL; vitamin E

1. Introduction

Breast cancer incidence and recurrence remain major health concerns for women [1]. Treatment limitations due to drug resistance and side effects highlight the need to explore and develop less-toxic anticancer agents, including natural products [2].

RRR- γ -tocopherol (γ T), the subject of this investigation, is the most common form of vitamin E in polyunsaturated vegetable oils and products made from them such as margarines, salad dressings and shortenings, and is the second most common form of vitamin E in human plasma [3-4]. γ T and its metabolic product [gamma-carboxyethyl hydroxychroman (γ -CEHC)] have been reported to have anticancer activity [5]. Support for anticancer actions by γ T comes primarily from the study of prostate cancer. Two human case control studies showed that subjects with high levels of plasma γ T had reduced risk of prostate cancer while no significant trend was observed for RRR- α -tocopherol (α T), the major form of vitamin E in plasma and

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tissues in humans [6]. However, other epidemiological studies reported that although prostate cancer cases had lower serum levels of γ T than matched controls, the differences were non-significant or alternatively showed no significant difference in plasma γ T levels between prostate cancer cases and controls [7].

Despite mixed results from epidemiological studies, several studies of human cancer cells in culture are supportive of anticancer actions by γ T. For example, γ T was shown to be more effective than α T in inhibiting the proliferation of human prostate carcinoma cells (DU-145, LNCaP, PC3), human colorectal adenocarcinoma cells (CaCo2), human alveolar cells (A549) and human osteosarcoma cells (SaOs-2) in cell culture [8]. γ T was shown to induce apoptosis in colon cancer and prostate cancer cells, while having no effect on normal prostate cells [9-11].

Insights into mechanisms of action of γ T's antiproliferative effects have come from studies that have shown γ T to suppress Ras p21 [12], inhibit COX-2 activity [13], downregulate cyclins D and E [8], and upregulate PPAR- γ [14]. Insights into pro-apoptotic actions by γ T include studies that have shown γ T to induce cytochrome c release, activate caspase-9, -8, -7 and -3, cleave poly-ADP-ribose polymerase (PARP) in human colon and prostate cancer cells [9,11], as well as evidence for involvement of caspase-independent pathways in LNCaP human prostate cancer cells [9]. Attenuation of γ T-induced apoptosis in human prostate cancer cells by inhibitors of key enzymes in the *de novo* synthesis of sphingolipids, suggests this pathway plays a critical role in γ T-induced apoptosis in this cell type [10].

Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL) is a member of the tumor necrosis factor superfamily [15]. TRAIL induces apoptosis in cancer cells but not normal cells by inducing apoptosis via two functional cell surface death receptors (DRs), DR4 or DR5 [15]. Molecular events downstream of DR4/DR5 activation by TRAIL include: receptor aggregation and recruitment of FADD and procaspase-8/procaspase-10 to form the death-inducing signaling complex (DISC) that promotes the proteolytic activation of caspases-8 and -10 [15-17]. Following activation of caspase 8, one or both of two downstream apoptotic signaling pathways is activated: an extrinsic death signaling pathway involving caspase-8 cleavage and activation of execution caspases-3, -6 or -7 and/or an intrinsic mitochondrial-mediated death signaling pathway involving caspase-8 processing of the pro-apoptotic Bcl-2 family member Bid to its truncated form (tBid). tBid is capable of inducing Bax conformational change. Activated Bax translocates into the mitochondria, triggering mitochondrial membrane permeability and release of cytochrome c followed by caspase-9 activation and caspase-9 activation of execution caspase-3 [17-19].

Despite advances in knowledge about anticancer properties of γ T, the mechanisms of γ T-induced apoptosis are not well defined. A better understanding of the mechanisms by which γ T induces apoptosis is necessary for its further development as a clinically useful anticancer agent. Data presented here show that γ T induces apoptosis in human MDA-MB-435 and MCF-7 breast cancer cells in a dose- and time-dependent manner, and sensitizes MDA-MB-435 cells to TRAIL-induced apoptosis. In contrast to its effects on cancer cells, no pro-apoptotic effect was observed on normal human mammary epithelial cells (HMECs). γ T treatment enhanced DR5 (also called TRAIL-R2) mRNA and protein levels, and increased cell surface expression of DR5. Combinations of γ T and TRAIL significantly increased apoptosis in comparison to single treatments. siRNA to DR5 or FADD significantly blocked γ T-induced apoptosis. γ T-treated breast cancer cells underwent intrinsic mitochondrial-mediated death signaling events including: activation of caspase 8, processing of Bid, induction of Bax conformational change, increases in mitochondria permeability and cytochrome c release and activation of caspase-9. Thus, γ T is a potent pro-apoptotic agent for human breast cancer cells mediating apoptosis, at least in part, via activation of DR5 apoptotic pathway.

2. Material and Methods

2.1. Chemicals

Reagents for morphological analyses of apoptosis were purchased from Boehringer Mannheim (Indianapolis, IN). α T and γ T were purchased from TAMA Biochemical Company (LTD, Tokyo, Japan). TRAIL was purchased from Alexis Biochemicals (San Diego, CA).

2.2. Cell culture

Sources and culture conditions for maintenance of MDA-MB-435 and MCF-7 human breast cancer cells have been described previously [20]. Human mammary epithelial cells (HMEC; Cooperative Human Tissue Network, Birmingham, AL) were primary cultures of human mammary cells derived from normal mammaplasty specimens [20]. For experiments, FBS was reduced to 2%. Cells were plated at 3×10^5 cells/100 mm dish or 6×10^5 /150 mm dish for apoptosis and western blot analyses, respectively. γ T and α T (200 mM) were dissolved in DMSO and further diluted in ethanol to 40 μ M. Equivalent levels of DMSO/ethanol (1:4) were used as vehicle control (VEH).

2.3. Quantification of apoptosis

Apoptosis was quantified based on nuclear morphology following staining of DNA with DAPI as described previously [20], or an Annexin V-FITC/PI assay (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Annexin V-FITC/PI assay measures amount of phosphatidylserine on the outer surface of the plasma membrane (a biochemical alteration unique to membranes of apoptotic cells) and amount of propidium iodide (PI), a dye that does not cross the plasma membrane of viable cells but readily enters dead cells or cells in the late stages of apoptosis and binds DNA. Fluorescence was measured using Fluorescence Activated Cell Sorter (FACS) analyses with a FACSCalibur flow cytometer and data were analyzed using CellQuest software (BD Biosciences, San Jose, CA). Cells displaying phosphatidylserine on their surface (positive for annexin-V fluorescence) were considered to be apoptotic.

2.5. Western blot analyses

Preparation of whole cell protein extracts and western blot analyses were conducted as described previously [20]. Protein bands were imaged and quantified after correcting for GAPDH loading control using Scion Image Software (Scion Corporation, Frederick, MD). Primary antibodies used and their sources were as follows: PARP (Santa Cruz Biotechnology, Santa Cruz, CA); Bid and Bax (PharMingen, Rockville, MD); caspase-8 and caspase-9 (Cell Signaling Technology, Beverly, MA); DR5 (Cell Sciences, Canton, MA); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, made in house). Secondary antibodies used included: horseradish peroxidase conjugated goat-anti-rabbit and rabbit-anti-mouse (Jackson ImmunoResearch, Rockford, IL); and bovine-anti-goat (Santa Cruz Biotechnology).

2.6. FACS analyses of cell surface expression of DR4 and DR5 and their ligand TRAIL

Cell surface expression of DR4, DR5 and TRAIL was determined using FACS analyses. Treated and control cells were detached and washed three times with 0.5% BSA in PBS. Next, cells (25 μ l of 8×10^6 cells/ml in 0.5% BSA in PBS) were incubated with 1 μ g normal mouse IgG₁ or IgG_{2b} for 15 min at room temperature followed by incubation with phycoerythrin-conjugated antibodies to DR4, DR5 or TRAIL or phycoerythrin-conjugated isotype immunoglobulins (R&D Systems, Minneapolis, MN) for 45 min. Intensity of red fluorescence was detected using a FACSCalibur flow cytometer and data were analyzed using CellQuest software.

2.7. Detection of active (conformationally changed) Bax

Active (conformationally changed) Bax was detected as described previously [21]. Briefly, 500 µg protein from whole cell extracts/0.5 ml was incubated overnight at 4°C with 2 µg of anti-Bax 6A7 (Sigma-Aldrich, St. Louis, MO), an antibody that specifically recognizes conformationally changed Bax, followed by reaction with 20 µl of protein G-agarose beads (Calbiochem, San Diego, CA) for 2 hours at 4°C. The conformationally changed Bax protein was analyzed by SDS-PAGE and detected using regular anti-Bax antibody.

2.8. Detection of mitochondrial permeability transition

The effect of γ T on mitochondrial membrane potential was measured using the dye JC-1 (5,5', 6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide/chloride; Molecular Probes, Carlsbad, CA). The JC-1 dye bearing a positive charge enters intact mitochondrial membrane due to the negative charge established by the intact mitochondrial membrane potential, accumulates as aggregates producing a red fluorescence [22]. In the absence of intact mitochondrial membrane, JC-1 accumulates in the cytoplasm in monomeric form characterized by green fluorescence [22]. Cells were collected, incubated with JC-1 for 30 min at 37°C followed by one wash with PBS. Red and green fluorescence intensity was measured using a FACSCalibur flow cytometer and data were analyzed using CellQuest software.

2.9. Detection of cytochrome c release from mitochondria into cytosol

A rapid and reliable technique to detect cytochrome c release during drug-induced apoptosis by FACS analyses was used [23]. Briefly, cells were treated, collected (2×10^5) and incubated with 100 µl digitonin (50 µg/ml in PBS with 100 µM KCl) for 5 min on ice followed by fixing the cells with 4% paraformaldehyde in PBS for 20 min at room temperature, and incubating the cells in blocking buffer (3% BSA and 0.05% saponin in PBS) for 1 h. Cells were incubated with anti-cytochrome c antibody (clone 6H2.B4, BD Pharmingen, San Diego, CA) at 1:200 in blocking buffer overnight at 4°C, washed three times with PBS, incubated with FITC-labeled secondary antibody in blocking buffer for 1 h at room temperature. Cytochrome c fluorescence intensity was determined using a FACSCalibur flow cytometer and data were analyzed using CellQuest software.

2.10. Semi-quantitative reverse transcription PCR analysis

Semi-quantitative analyses were conducted (following company instructions) to detect DR5 mRNA expression by reverse transcriptase-polymerase chain reaction (RT-PCR) with Superscript RTase (250 U, Invitrogen) and Duplex PCR with Taq PCR Master Mix Kit (Qiagen Inc), respectively. Primers for DR5 (478 bp) were: forward GCC TCA TGG ACA ATG AGA TAA AGG TGG CT and reverse CCA AAT CTC AAA GTA CGC ACA AAC GG, and for β -actin (202 bp) were: forward GGC GGC ACC ACC ATG TAC CCT and reverse AGG GGC CGG ACT CGT CAT ACT [24]. PCR conditions employed were: 94°C for 3 min for initial denaturation, 35 cycles at 94°C for 1 min for denaturation, 61°C for 1 min for annealing and 72°C for 2 min for elongation [24]. PCR fragments were separated by electrophoresis on a 1.5% agarose gel and visualized by staining with ethidium bromide.

2.11. Transfection with DR5 and FADD siRNA

Cells were transiently transfected with siRNAs to DR5 or FADD (Ambion, Austin, TX) using a reverse transfection protocol following company instructions. A negative control siRNA (Ambion, Austin, TX), which has no significant sequence similarity to any mouse, rat or human gene sequences, was used to control for any nonspecific effects on gene expression. Optimal conditions were determined using Silencer CellReady siRNA Transfection Optimization and GAPDH Kit provided by Ambion. Briefly, for each well of a 6 well plate, 9 µl transfection agent; siPORT NeoFX (Ambion, Austin, TX) was mixed with 0.5 ml serum free media (OPTI-

MEM-I) and incubated at room temperature for 10 min followed by addition of 2.3 μ l siRNA (20 μ M). The mixture of transfection agent and siRNA was incubated at room temperature for 10 min followed by mixing with 1 ml cells at 3.75×10^5 /ml in culture media. Cells were cultured for 1 day followed by replacement of the transfection media with culture media and incubating for an additional day. Transfected cells were re-cultured in 100 mm dish at 3×10^5 cells for 8 hrs prior to γ T treatments for 2 days.

2.12. Statistical Analyses

Apoptosis data were analyzed using a one-way analysis of variance followed by ANOVA Tukey test or a two-tailed student *t*-test to determine statistical differences among treatments and control values. Differences were considered statistical significant at $p < 0.05$.

3. Results

3.1. γ T, but not α T, induced human breast cancer cells to undergo apoptosis

The ability of γ T to induce apoptosis in MDA-MB-435 and MCF-7 human breast cancer cells was evaluated based on enumeration of cells containing condensed and/or fragmented DNA (DAPI assay), western blot assessment of PARP cleavage, or monitoring of the phosphatidylserine (PS) content of the outer leaflet of the plasma cell membrane (PS externalization) with FITC-conjugated annexin V.

Photomicrographs of DAPI stained cells showing bright condensed or fragmented chromatin in nuclei from apoptotic cells in comparison to non-apoptotic nuclei exhibiting an overall dull staining pattern are provided in Fig. 1A. Condensed/fragmented DNA in MDA-MB-435 cells and condensed DNA in MCF-7 cells were observed following treatment with 40 μ M γ T for 3 days. Quantification of cells undergoing apoptosis as determined by the DAPI assay, showed that γ T treatments (40 μ M) for 2 and 3 days produced significant increases in the percentage of apoptosis in comparison to vehicle controls in both cell types (Fig. 1B). A dose-dependent increase in apoptosis, as quantified by the DAPI assay, was observed in both cell types (Fig. 1C); namely, 12%, 22% and 43% apoptosis in MDA-MB-435 cells and 10%, 21% and 39% apoptosis in MCF-7 cells after 3 day treatment with 10, 20 or 40 μ M γ T, respectively. In contrast, no γ T-induced apoptosis was seen in HMECs (Fig. 1C); thus showing specificity of γ T-induced apoptosis for cancer cells. Interestingly, α T, the most abundant vitamin E form in human plasma and tissues, at equivalent concentrations (namely, 10-40 μ M) did not induce apoptosis in any of the three cell types (Fig. 1C); thus showing that pro-apoptotic effects by γ T are not a general property of vitamin E compounds. Cleavage of PARP into 89 kDa fragments, a well established biomarker for apoptosis, provides further evidence for the dose-dependent induction of apoptosis by γ T in both cell types (Fig. 1D). γ T treatments (40 μ M) for 2 and 3 days induced 26% and 31% annexin V positive cells in MDA-MB-435 cells versus 7% annexin V positive cells in VEH treated cells, and 28% and 48% annexin V positive cells in MCF-7 cells versus 16.4% annexin V positive cells in VEH treated cells, respectively (Fig. 1E & F).

3.2. γ T sensitized human breast cancer cells to TRAIL-induced apoptosis

In preliminary experiments, both MCF-7 and MDA-MB-435 cells were shown to be insensitive to TRAIL treatment in comparison to MDA-MB-231 cells which have been reported previously to be responsive [25] (data not shown). Interestingly, pre-treatment of MDA-MB-435 cells with 40 μ M γ T for 1 day followed by removing the treatment media and culturing the cells with 10 ng/ml TRAIL for 2 days significantly enhanced TRAIL-induced apoptosis to 42% from 13% by pre- γ T treated alone (Fig. 2A). Treatment of cells with 10 ng/ml of TRAIL alone did not induce apoptosis (Fig. 2A). Employing the same treatments to HMECs did not induce any apoptosis (Fig. 2A), showing TRAIL specificity for cancer cells, as predicted based on the

literature [15]. Additional proof of the ability of γ T pre-treatment to sensitize TRAIL-refractory MDA-MB-435 cells to TRAIL-induced apoptosis came from analyses of PARP cleavage (Fig. 2B). These analyses showed higher levels of cleaved PARP (89 kDa) in cells receiving pre- γ T + TRAIL than in cells receiving TRAIL alone (VEH + TRAIL) or pre- γ T treatment alone (Fig. 2B). Furthermore, γ T exhibited a dose-dependent increase in ability to sensitize the TRAIL-refractory MDA-MB-435 cells to TRAIL-induced apoptosis (Fig. 2C) which was again confirmed by analyses of PARP cleavage (Fig. 2D). Quantification of apoptotic cells using the Annexin V-FITC/PI assay confirmed that pretreatment (40 μ M) or co-treatment (20 μ M) of γ T significantly enhanced TRAIL-induced apoptosis in comparison to γ T or TRAIL alone treated MDA-MB-435 cells (Fig. 2E & F).

3.3. γ T induced increased levels of cell surface DR5

To understand how γ T might overcome the human breast cancer cell's resistance to the apoptosis-inducing effects of TRAIL, membrane expression of the TRAIL receptors DR4 & DR5 as well as the ligand TRAIL were examined by indirect immunofluorescence staining/FACS analyses. Data show that following γ T treatment (40 μ M for 2 days), MDA-MB-435 cells and MCF-7 exhibited increased membrane-bound DR5 expression in comparison to VEH control treated cells (Fig. 3A). No change in DR4 expression was observed following γ T treatment in either cell type (Fig. 3A). Expression of TRAIL on the cell surface was also examined and revealed no change in either cell type following γ T treatment (Fig. 3A). These data suggest that γ T sensitizes the TRAIL-refractory cancer cells to TRAIL-induced apoptosis via increased expression of membrane-bound DR5.

3.4. γ T induced increased levels of DR5 mRNA and protein

To investigate the molecular basis for the increase in membrane expression of DR5, the effects of γ T on DR5 mRNA and protein levels were examined. γ T increased DR5 mRNA levels by 4.6 and 4.7 fold after 2 and 3 days of treatment in MDA-MB-435 cells, and 2.1, 3.6 and 4.1 fold after 1, 2 and 3 days of treatment in MCF-7 cells, respectively (Fig. 3B, panels 1 & 2). DR5 protein levels also increased in both cell lines after 2 and 3 days of treatment with γ T (Fig. 3B, panels 3 & 4).

3.5. siRNA knockdown of DR5 or FADD block γ T-induced apoptosis

Functional knockdown of DR5 using siRNA was performed to address the necessity of these cellular components in γ T-induced apoptosis. DR5 siRNA significantly blocked γ T-induced apoptosis (36%) in comparison to γ T treated cells transfected with negative control siRNA (Fig. 4A). Validation of the success of the DR5 siRNA knockdown by western immunoblotting showed a markedly reduction in DR5 (DR5L/S) protein expression (Fig. 4B). To confirm if FADD, a death receptor associated protein that triggers death receptor mediated caspase-8 dependent apoptosis is involved in γ T-induced apoptosis we also blocked FADD protein expression using siRNA to FADD. Likewise, data show a significant attenuation of γ T-induced apoptosis (46%) by FADD siRNA (Fig. 4A) and a marked decrease in FADD protein expression (Fig. 4B). These data document a role for DR5 and FADD in γ T-induced apoptosis in MDA-MB-435 cells, suggesting that γ T induces apoptosis via DR5/FADD death receptor mediated events. Knockdown of DR5 using siRNA to DR5 also attenuated pre- γ T + TRAIL-induced apoptosis by 60% in comparison to negative control siRNA (Fig. 4C), demonstrating that γ T upregulation of DR5 plays a critical role in sensitizing breast cancer cells to TRAIL-induced apoptosis.

3.6. γ T induced apoptosis is mitochondria dependent

Downstream events involved in prototypical DR5 apoptotic signaling were investigated to establish what occurs in human breast cancer cells following γ T-triggered apoptosis including:

activation of caspases, increase in mitochondrial permeability, cytochrome c release, and activation of two pro-apoptotic members of the Bcl-2 family that can contribute to increased mitochondrial permeability (truncation of Bid and conformational change of Bax).

The activation status (i.e. cleavage) of two initiation caspases; caspase-8 (involved in DR apoptotic signaling) and caspase-9 (involved in intrinsic mitochondrial-mediated apoptotic signaling) were measured by western blot analyses. Cleavage of both caspases occurred after γ T treatments of 1, 2, or 3 days in MDA-MB-435 cells and after 2 or 3 days in MCF-7 cells (Fig. 5A).

Mitochondrial permeability was examined using JC-1 dye. As described in the Materials and Methods, in the absence of intact mitochondria this dye accumulates in the cytoplasm as a green fluorescing monomeric form thus serving as an indicator of mitochondria permeability [22]. γ T (40 μ M) induced a higher percentage of green stained MDA-MB-435 cells after 2 and 3 days treatments (40% and 55%, respectively) in comparison to VEH control (8% and 9%, respectively), indicating that γ T induces increased mitochondrial permeability in breast cancer cells (Fig. 5B).

Indirect immunofluorescence staining of cytochrome c in permeabilized cells, to permit cytosolic cytochrome c diffuse from the cells was used as an indicator of cytochrome c release from mitochondria into the cytosol [23]. During this procedure cytoplasmic but not mitochondrial cytochrome c is lost, thus a decrease in staining intensity is correlated with release of cytochrome c from the mitochondria. Three day γ T (40 μ M) treatments produced marked reductions in cytochrome c fluorescence in MDA-MB-435 and MCF-7 cells (60% and 36%, respectively) in comparison to VEH treated control cells (5% and 4%, respectively; Fig. 5C). These data support the involvement of intrinsic mitochondria-mediated apoptosis in γ T-induced apoptosis.

Activation of Bax and Bid, upstream contributors to mitochondria-mediated apoptotic signaling, was analyzed. Active Bax was detected using a two-step procedure of immunoprecipitating conformationally changed Bax followed by detection by western immunoblotting. γ T at 40 μ M increased protein levels of active Bax by 3.9 and 3.6 fold after 2 and 3 days treatment in MDA-MB-435 cells, and 1.6, 5.2, and 3.6 fold after 1, 2 and 3 days treatments in MCF-7 cells in comparison to cells treated with VEH for 3 days (Fig. 5D). Western immunoblotting was used to detect cleavage of intact Bid, a caspase-8 substrate, to truncated Bid (tBid), a pro-apoptotic active form of Bid that promotes Bax conformational change. Data show that γ T treatment induced Bid cleavage from its intact 21 kDa to truncated 15 kDa (tBid) form (Fig. 5D).

4. Discussion

Although γ T is the predominate form of vitamin E in corn, soybean, canola, and peanut vegetable oils and despite recent research suggesting anticancer potential for colon and prostate cancer, its potential as an anticancer agent for human breast cancer cells has not been investigated. Results from these studies showed that γ T: (i) induced human MDA-MB-435 and MCF-7 breast cancer cells to undergo apoptosis in a time- and dose- dependent manner; whereas, normal breast epithelial cells (HMECs) were refractive; (ii) sensitized MDA-MB-435 breast cancer cells to TRAIL-induced apoptosis; (iii) increased death receptor DR5 mRNA, protein and plasma membrane levels; (iv) induced-apoptosis was attenuated by functional knockdown of either DR5 or FADD; (v) sensitization of breast cancer cells to TRAIL-induced apoptosis was attenuated by functional knockdown of DR5, and (vi) signaled apoptosis in human breast cancer cells via DR5 mediated intrinsic apoptotic pathways involving tBid and Bax.

The data that γ T activated DR5-mediated apoptotic pathways in human breast cancer cells and sensitized breast cancer cells to TRAIL-induced apoptosis add this compound to a list of clinically relevant agents including radiation, anticancer drugs, resveratrol, vitamin E succinate, and synthetic triterpenoids that increase the expression of TRAIL receptors (DR4 and/or DR5) and thereby induce apoptosis or enhance TRAIL-induced apoptosis [16,25-34]. Agents that can up-regulate the cell surface expression of DR4 or DR5 have the potential for clinical application in combination with TRAIL; however, many of the agents that promote TRAIL-induced apoptosis also produce significant systemic toxicity [25-27]; thus, it is of special interest that γ T is effective on sensitizing cancer cells to TRAIL-induced apoptosis without any overt toxicity. Additional studies in different animal models of human cancers will need to be conducted to address this important issue.

Previously, we evaluated the pro-apoptotic property of six naturally occurring vitamin E compounds: α -, γ -, δ -tocopherols and α -, γ -, δ -tocotrienols; as well as, succinate and acetate derivatives of RRR- α -tocopherol [RRR- α -tocopheryl succinate (VES) and RRR- α -tocopheryl acetate] in MDA-MB-435 and MCF-7 cells [35]. Only γ -tocotrienol, δ -tocotrienol, and VES exhibited pro-apoptotic properties at concentrations with physiological relevance [35]. In the studies reported here, we modified the test conditions by reducing cell density in culture and found that γ T, but not α T at 10-40 μ M possesses pro-apoptotic property in both MDA-MB-435 and MCF-7 cells. This data is in agreement with data reported by Dr. Jiang and co-workers showing that γ T at low cell density induced apoptosis in prostate cancer cells [10]. Additionally, γ T has been shown by other investigators to be effective in inhibiting cell proliferation in human cancer cell lines [8] and γ T, but not α T, has been shown to exhibit pro-apoptotic properties in human colon and prostate cancer cells [9-11]. Thus, studies reported here add to the literature supportive of anticancer actions by γ T in human cancer cells in culture.

Only limited mechanistic data are available on the pro-apoptotic properties of γ T. Activation of both caspases-8 and -9 have been reported to be associated with γ T-induced apoptosis in human colon and human prostate cancer cell lines [9,11], and γ T modulation of sphingolipid synthesis has been reported in human prostate cancer cells [10]. Studies reported here demonstrate, for the first time, that γ T induces apoptosis in human breast cancer cells via activation of DR5 apoptotic pathway. γ T upregulation of DR5 is not only involved in γ T-induced apoptosis but also involved in γ T sensitization of breast cancer cells to TRAIL-induced apoptosis. γ T activation of DR5 is, in part, via upregulation of DR5 messenger RNA and total cell protein levels; as well as, increased cell surface membrane levels. DR5 mRNA levels have been reported to be regulated by multiple factors, including JNK [34], p53 [36], NF- κ B [37], and Myc [38]. Preliminary data showed that γ T treatment of human breast cancer cells induces increased levels of phospho-JNK and phospho-c-Jun, suggesting JNK-mediated induction of DR5, perhaps in a sequence similar to that reported for the up-regulation of DR5 by a novel triterpenoid in human lung cancer cells [34]. On-going studies will determine if JNK/c-Jun is involved in γ T-induced apoptosis and in the activation of DR5 signaling.

Caspase-8 activation is required for two downstream DR apoptotic signaling pathways; namely, extrinsic, mitochondrial-independent (type I) and/or intrinsic, mitochondrial-dependent (type II) apoptotic signaling which involve DISC formation via adaptor protein FADD [17,39]. In type I apoptosis, high levels of activated caspase-8 can rapidly activate execution caspase-3 directly, leading to mitochondrial independent apoptosis. In type II mitochondrial dependent apoptosis, production of lower levels of activated caspase-8 requires involvement of the mitochondria apoptotic cascade to activate execution caspases. In this latter case, caspase-8 activates its substrate Bid by cleaving intact Bid into its active form (tBid) that is capable of inducing Bax conformational change [17-18]. Activated Bax translocates into the mitochondria, triggering the mitochondria-dependent apoptotic cascade involving mitochondria permeability transition, cytochrome c release from mitochondria into cytosol,

caspase-9 activation, and execution caspase activation (i.e. caspases-3,-6,-7) [17,19]. Finding that γ T-induced apoptosis involves DR5, FADD, caspase-8 and Bid, as well as Bax, increased mitochondrial permeability, cytochrome c release and caspase-9 suggests that γ T induces death receptor-mediated type II/intrinsic apoptosis.

In summary, γ T triggers apoptosis in human breast cancer cells via up-regulation of DR5 expression thereby enhancing TRAIL-induced apoptosis; as well as, triggering mitochondria-dependent apoptosis. The ability of γ T to sensitize breast cancer cells, but not HMECs, to TRAIL-induced apoptosis, suggests the potential application of γ T as an adjuvant for DR5 targeted therapeutics.

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Abbreviations

VEH, Vehicle control
 γ T, RRR- γ -tocopherol
 α T, RRR- α -tocopherol
HMECs, Human mammary epithelial cells
FACS, Fluorescence Activated Cell Sorter
 γ -CEHC, gamma-carboxyethyl hydroxymethoxychroman
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
TRAIL-R2, death receptor 5 (DR5)/Tumor necrosis factor-Related Apoptosis-Inducing Ligand-Receptor 2
FADD, Fas-associated protein with death domain
PARP, poly (ADP-ribose) polymerase
DR, death receptor
DR5, death receptor 5
JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide/chloride

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Fig.1.

γ T induces apoptosis in human breast cancer cells but not in HMECs. (a) Cells were treated with 40 μ M γ T for 3 days, stained with DAPI, and analyzed for apoptotic morphological changes (nuclear shrinking, condensation & apoptotic bodies). (b) Percentage of cells judged to be apoptotic using morphological criteria of DAPI stained DNA following treatment with 40 μ M γ T for 1-3 days. * indicates significantly different from the vehicle control and 1 day treatment groups ($p < 0.05$). ** indicates significantly different from the 2 day treatments ($p < 0.05$). (c and d) Cells were treated with 10, 20, or 40 μ M α T or γ T for 3 days, and percentage of apoptotic cells determined by DAPI assay or western immunoblot analyses of PARP cleavage, respectively. * indicates significantly different from the vehicle control ($p < 0.05$). ** indicates significantly different from γ T 10 μ M treatments ($p < 0.05$). *** indicates significantly different from γ T 20 μ M treatments ($p < 0.05$). (e) γ T-induced apoptosis was measured by FACS analyses of Annexin V and PI stained cells. (f) Quantification of apoptosis as measured by FACS analyses of Annexin V positive cells from (e). * indicates significantly different from the vehicle control ($p < 0.05$). Data in (a) and (d) are representative of 2 or more experiments. Data in (e) are representative of three experiments. Data in (b), (c), and (f) are presented as mean $\pm$ S.D. of three independent experiments.

Fig.2.

γT sensitizes human MDA-MB-435 breast cancer cells but not HMECs to TRAIL-induced apoptosis. (a) Cells were pre-treated with 40 μM γT for 1 day followed by removing the treatment media and treating with 10 ng/ml TRAIL for 2 days. Apoptosis was determined using the DAPI assay. * indicates significantly different in comparison to cells pre-treated with vehicle control. ** indicates significantly different in comparison to cells pre-treated with γT alone or TRAIL alone ($p < 0.05$). (b) MDA-MB-435 cells treated as described in (a) were analyzed for PARP cleavage by western immunoblotting. (c) MDA-MB-435 cells were treated with γT or with combination of γT + TRAIL at concentrations indicated for 2 days. Apoptosis was quantified using the DAPI assay. * indicates significantly different in comparison to control. ** indicates significantly different in comparison to cells treated with γT or TRAIL alone ($p < 0.05$). (d) Samples from (c) were analyzed for PARP cleavage by western immunoblotting. (e) MDA-MB-435 cells were pre-treated with 40 μM γT for 1 day, washed and then treated with 10 ng/ml TRAIL for 2 days, or co-cultured with 20 μM γT + 10 ng/ml TRAIL for 2 days. Annexin V positive and PI stained cells were determined by FACS analyses. (f) Percentage of Annexin V positive cells (from d data). * indicates significantly different in comparison to vehicle control, pre-treated with γT alone, or treated with TRAIL alone ($p < 0.05$). ** indicates significantly different in comparison to vehicle control, treated with γT alone or treated with TRAIL alone ($p < 0.05$). Data in (a), (c) and (f) are presented as mean $\pm$ S.D. of three independent experiments. Data in (b), (d), and (e) are representative of 2 independent experiments.

Fig. 3.

γ T induced increases in levels of DR5 mRNA, protein, and cell surface membrane expression. (a) Cells were treated with 40 μ M γ T for 2 days. Cell membrane expression of TRAIL, DR4 and DR5 was determined by FACS analyses. (b) DR5 mRNA was detected by RT-PCR using β -actin as internal control (upper two panels) and DR5 protein level was detected by western immunoblot analyses with GAPDH serving as loading control (lower two panels). All data in Fig. 3 are representative of 2 or more independent experiments.

Fig.4.

Knockdown of DR5 or FADD attenuates γ T-induced apoptosis. (a) MDA-MB-435 cells were transiently transfected with siRNA to DR5, FADD or negative control (NC) siRNA followed by treatment with 40 μ M γ T for 2 days. Apoptosis was evaluated by Annexin V assay. Data are depicted as mean $\pm$ S.D. of three independent experiments. (b) Western blot analyses were performed on whole cell extracts from (a) to verify the ability of siRNA to DR5 or FADD to reduce DR5 or FADD protein levels, respectively, and to monitor the blockage of γ T-induced apoptosis as determined by PARP cleavage. Data are representative of 2 experiments. (c) MDA-MB-435 cells were transiently transfected with siRNA to DR5 and pre-treated with 40 μ M γ T for 1 day followed by removing the treatment media and treating with 10 ng/ml TRAIL for 1 day. Apoptosis was evaluated by Annexin V assay. Data are depicted as mean $\pm$ S.D. of

2 independent experiments.*indicates significant reduction in comparison to cells treated with negative control siRNA prior to treatment with γ T ($p<0.05$).

Fig.5.

γT induced apoptosis is mitochondria dependent. (a) Western blot analyses were performed to detect pro- and cleaved caspase-8 and -9. (b) Mitochondrial permeability was detected by FACS analyses after MDA-MB-435 cells were stained with JC-1. (c) Release of cytochrome c from mitochondria to cytosol was determined by FACS analyses of cells treated with γT (40 μM) for 2 days. (d) MDA-MB-435 and MCF-7 cells were treated with 40 μM γT for 1-3 days and evaluated for levels of Bid, tBid, active Bax and total Bax by western immunoblot analyses. All data in Fig 5 are representative of 2 independent experiments.