Effects Of Vitamin E Isomer, Gamma Tocotrienol (GT3), At Inhibiting Cell Growth And Inducing Apoptosis In Colon Cancer Cell Line HCT-116.

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Effects Of Vitamin E Isomer, Gamma Tocotrienol (GT3), At Inhibiting Cell Growth And Inducing Apoptosis In Colon Cancer Cell Line HCT-116

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ABSTRACT

Colorectal cancer is the third most prominent cancer world-wide and it is the second leading cause of cancer deaths in the United States. Many dietary components affect the risk of developing colorectal cancer, such as Vitamin E. Of the eight isomers of Vitamin E, four have a tocotrienol structure. Tocotrienols are found at highest concentrations in palm oil, which is ingested more in areas of Asia where the incidence of colorectal cancer is the lowest, suggesting a role of tocotrienols in the prevention of colorectal cancer. The metabolism of Arachidonic acid pathway produces a host of pro-inflammatory metabolites either by the Cyclooxygenase (Cox) pathway or the Lipoxygenase (Lox) pathway. The expression of Lox is increased in various human cancer lines; this over expression has been associated with tumor cell proliferation, resistance to apoptosis, and angiogenesis. Another important pathway related to cancer involves mTOR (mammalian target of rapamycin), which is involved in cell growth and human tumorigenesis. The focus of this study included treatment of the HCT-116 colon cancer cell line with gamma tocotrienol to examine potential pathways involved in the induction of apoptosis. Also, whether the Vitamin E-mediated signaling through Arachidonic acid metabolism is necessary for the down regulation of protein translation in the mTOR pathway by using chemical inhibitors specific to Arachidonic acid in the presence and absence of vitamin E treatment was explored. The colon cancer cell line, HCT-116, was treated with gamma tocotrienol isomer and then isolated at 18 and 24 hours. Cells lysates were analyzed by Western Blotting. Our data shows that the mTOR pathway is downregulated by treatment with gamma tocotrienol at 18 hours and 14 hours for 5 µM demonstrating that protein translation is abrogated.
Phospho S6 ribosomal protein and phospho-p70 S6 kinase are both downregulated, and phospho-4EBP1 is up regulated upon treatment of gamma tocotrienol. Furthermore, at the same concentrations, Caspase 12 and Caspase 8 are cleaved indicating apoptosis. In addition, parallel up-regulation of 15-LOX-1 and down regulation of Cox-2 at 5 µM at 18 hours is observed. Upon treatment with Caffeic acid, 15-lox-1 is over expressed causing mTOR trend to reverse and down regulation of ERK. Thus, mTOR regulation is dependent on a delicate balance of fatty acid metabolites.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer death in the United States [1]. It is the third leading cause of cancer in males and fourth in females in this country. The risk of developing colorectal cancer increases with age and is related to many factors including dietary components such as Vitamin E [2]. Such cancers have great country-to-country variation in occurrence due to the changes in nutrition and environmental factors. For instance, the Americas and some of the European states have a higher risk of developing colon cancer than the Mediterranean societies, whose diets are rich with Vitamin E isomers. Vitamin E is a major fat-soluble antioxidant and it occurs naturally as eight compounds. Four of these compounds have a tocopherol structure and four have a tocotrienol structure (Figure 1). The chroman head group is common to both the isoforms of Vitamin E. However, the tocopherols have three chiral carbons on its phytlyl tail and the tocotrienols have a farnesyl tail with three unsaturated bonds. The isoforms differ at the attachment in the R position on the chroman head. The number and position of the methyl groups determine the specific tocopherol (α-,β-, γ-, or δ-tocopherol) or tocotrienol compound (α-,β-, γ-, or δ-tocotrienol) [3]. Tocotrienols are found at highest concentrations
in palm oil. Palm oil is ingested in high concentrations in areas of Asia where the incidence of colorectal cancer is the lowest, suggesting a role for tocotrienols in the prevention of colorectal cancer.

![Chemical structures of vitamin E isomers](image)

Figure 1. Vitamin E isomers [3]

The metabolism of Arachidonic acid pathway (a polysaturated fatty acid) produces a host of pro-inflammatory metabolites either by the Cyclooxygenase (Cox) pathway or the Lipoxygenase (Lox) pathway. These substances modulate various responses in the body, both physiological and pathological, including invasiveness of tumor cells. Thus far, two isozymes of Cox have been recognized: Cox-1 and Cox-2 [4]. The constitutive Cox-1 partakes in normal cell metabolic functions. Alternatively, the inducible Cox-2 is overexpressed in tumor growths. The implication that Cox is related to cancer was first demonstrated in studies of aspirin and anti-inflammatory drugs [5]. These studies showed increased levels of Cox-2 in human and animal colorectal tumors. Various other studies
point out that the loss of regulation of Cox-2 occurs early in carcinogenesis [5]. While inhibition of Cox-2 can help cure or avoid colorectal cancers, the exact mechanisms by which these inhibitors affect are believed to involve prevention of excessive inflammation a known risk factor for colorectal cancer.

There are three major isoforms indentified for Lox thus far in human colorectal tissues: 5-lox, 12-lox, and 15-lox. The expression of Lox is increased in various human cancer lines; this over expression has been associated with tumor cell proliferation, resistance to apoptosis, and angiogenesis. However, Lox expression is not clearly defined in human colorectal cancers. Recent findings reveal that 5-Lox is up-regulated in colorectal cancer. Thus, inhibition of the 5-Lox isoform could be valuable in the treatment of colorectal cancer. On the other hand, little is known about 12-Lox and varying debates exist regarding the effects of 15-Lox [4]. One finding suggest that 15-Lox is not only down regulated but also acts as a potential tumor suppressor [4]. Activation of 15-Lox-1 in colorectal cancer cells stimulates apoptosis through a PPAR-delta-mediated pathway [6].

Another important pathway related to cancer involves TOR (target of rapamycin) and mTOR (mammalian target of rapamycin). TOR effects cell growth and proliferation through protein synthesis regulation. It controls protein synthesis via number of downstream targets either directly or indirectly phosphorylated by TOR. One of the very important regulatory proteins of TOR is Raptor. Raptor serves as an adaptor that recruits mTOR substrates [7] (Figure 2). mTOR has recently attracted many researchers due to its involvement in cell growth and human tumorigenesis [8]. A serine/threonine kinase, mTOR controls various aspects of cellular physiology, including transcription and translation. Basically, mTOR exists in two complexes, mTORC1 [9], mTOR bound to Raptor [10], and
mTORC2 [9], mTOR bound to Rictor (Rapamycin-insensitive) [10]. mTORC1 is regulated by many signals, including growth factors, insulin, nutrients, and cellular stressors [9]. Alternatively, mTORC2 phosphorylates and activates AKT, a serine/threonine protein kinase, also known as protein kinase B (PKB). This activation of AKT by mTOR could be a pathway by which mTOR effects tumorigenesis because AKT promotes cell proliferation and survival and inhibits apoptosis. AKT also activates mTORC1 indirectly by direct phosphorylation of tumor suppressors, TSC1 and TCS2. Activation of mTORC1 results from inhibition of TSC2 by AKT. Collectively, these studies reveal that the AKT pathway affects the mTOR pathway by TSC2-dependent and independent mechanisms. mTOR is deactivated via signals through activation of the Ras/MEK/ERK pathway [10].

Regulation of protein synthesis occurs via mTOR substrates: inactivation of eukaryotic initiation factor 4E-binding protein (4E-BP1) and activation S6 kinase (S6K1). While Raptor binds to both downstream effectors of mTOR, Rapamycin disrupts the mTOR-Raptor interaction, preventing mTOR from phosphorylating S6K1 and 4E-BP1. On the other hand, growth factors, such as insulin, increase the phosphorylation of S6K1 and 4E-BP1 in a rapamycin-sensitive manner [7]. As discussed above, activation of the mTOR pathway promotes tumorigenesis, and, thus, makes mTOR a likely target in the treatment of cancer. Currently, inhibitors of mTOR, such as rapamycin, are being tested for possible cancer treatments [10]. Still, many details relating to mTOR regulation remain unanswered [7].
Caspases are a family of proteases that are cleaved when cells undergo apoptosis. There are twelve total caspases available, from which caspases 8 and 12 were used in HCT-116 cells to indicate apoptosis. Caspase 8 is cleaved in response to a death receptor pathway or the Fas Ligand pathway. Fas activates pro-caspase 8 which activates caspase 8, eventually leading to apoptosis. This is a well understood pathway and has been seen before in this particular cell line with the vitamin E isomers, gamma tocopherol [13]. However, unlike caspase 8, caspase 12 cleavage by Vitamin E has not been found in this particular cell line. Caspase 12 induces apoptosis when the Endoplasmic Reticulum is stresses, indicating a decrease in translation.
The focus of this study included the treatment of the HCT-116 colon cancer cell line with gamma tocotrienol to examine potential pathways involved in the induction of apoptosis. Once the pathways were identified, cells were examined for the caspase pathway to determine if the cells actually did undergo apoptosis. Next, the mTOR pathway was examined of vitamin E’s effects of mTOR’s substrates. Finally, to combine all this, HCT-116 cells were treated with an inhibitor of Arachidonic acid pathway (Caffeic acid) to see whether over expression of 15-Lox-1 enhanced the mTOR pathway decease or not.

RESULTS

To begin with, it was determined that the Linoleic acid pathway does influence the Arachidonic acid pathway. 15-lox-1 converts Linoleic acid to 13-SHODE (13-hydroxyoctadecadienoic acid) which blocks translation, promoting cells to undergo apoptosis. Additionally, Cox-2 uses arachadonic acid converting to ligands used by the PPAR pathway. According to the data, in 18 hour Vitamin E treated cells, 15-lox-1 is upregulated compared to vehicle treated cells (Figure 3). This shows that gamma tocotrienol had an effect of fatty acid metabolism. Furthermore, gamma tocotrienol down regulates Cox-2 compared to vehicle treated cells (Figure 4). This is important because an upregulation of Cox-2 is a possible risk factor in colon cancer cells. So, thus far, our data demonstrates that gamma tocotrienol downregulates Cox-2, a risk factor for colon cancer, and upregulates 15-Lox-1 at 18 hours at 5μM, which has been shown to cause apoptosis in colon cancer cell lines. So next, we decided to examine the caspase pathway to see if the cells were actually undergoing apoptosis.
Caspases are family of proteases that play essential role in apoptosis. They will be cleaved if the cells are undergoing apoptosis. Out of the 12, we looked at 2 caspases: caspase 8, which acts via Fas ligand pathway, and caspase 12, which is cleaved under ER stress. According to the data, caspase 8 and 12 are both cleaved indicating apoptotic induction upon gamma tocotrienol treatment (Figure 5). Caspase 8 is cleaved in response to death receptor pathway or the Fas ligand pathway. Fas activates pro-caspase 8 which activates caspase 8, eventually leading to apoptosis. Our data shows that gamma tocotrienol induces caspase 12 cleavage and since caspase 12 cleavage occurs during ER stress we postulated that gamma tocotrienol is affecting the mTOR pathway which is located in the ER and is the site of mTOR signaling.
Next, we probed the blots with the different mTOR antibodies to look at the effects of gamma tocotrienol. We observe down regulation of the phospho S6 ribosomal protein and phospho-p70 S6 kinase, and up regulation of phospho- 4EBP1 (Figure 6). These data demonstrate that protein translation is abrogated in the presence of gamma tocotrienol at 5 μM at 18 hours.

Following this find, we decided to examine whether or not the over expression of 15-Lox-1 would enhance the decrease in the mTOR pathway using an inhibitor of the
Arachidonic acid pathway, Caffeic acid (CA). When HCT-116 cells were treated with gamma tocotrienol for 24 hours, expression of 15-lox-1 was increased and treatment with CA increased it even further (Figure 7). Furthermore, gamma tocotrienol down regulates mTOR by increasing 15-lox-1 in cells treated without CA. However, too much expression of 15-lox-1 upon treatment with CA reverses this trend. There is no significant decrease or increase of mTOR substrates following gamma tocotrienol and caffeic acid treatment. This data demonstrates that mTOR is dependent upon a delicate balance of 15-lox-1 expression and that too little up regulation of 15-lox-1 decreases the mTOR pathway, shutting off translation causing the cells to undergo apoptosis. On the other hand, when 15-lox-1 is up regulated too much, mTOR stays on (Figure 7). This demonstrates that having too much fatty acid in the diet can be harmful because mTOR cannot be controlled. Additionally, ERK, a deactivator of mTOR, is downregulated upon too much expression of 15-Lox-1 adding to the effect of the inhibition of mTOR shutting off (Figure 7).

![Image](https://via.placeholder.com/150)

Figure 7: Gamma tocotrienol up regulates 4EBP-1 and down regulates Phospho-p70 and Phospho-S6 after 24 hours of treatment without Caffaic acid treatment. With Caffeic acid treatment, 15-lox-1 is over expressed, reversing mTOR trend.
DISCUSSION

In the presence of gamma tocotrienol, caspase 8 and 12 are cleaved indicating apoptosis and ER stress. In response to the ER stress, the mTOR pathway is downregulated at 18 and 24 hours at 5μM. In the presence of an inhibitor of Arachidonic acid metabolism, mTOR pathway substrates stay turned on and gamma tocotrienol cannot turn the pathway off. Additionally, the ability of Vitamin E to modulate ERK (mTOR pathway’s deactivator) is reversed. Finally, mTOR signaling is dependent on a delicate balance of fatty acid pathway.

This is only a small piece of the bigger puzzle. Many more studies still need to be conducted in order to, first, confirm these results, and then to further the study. Along with the appropriate vitamin E concentration, a delicate balance of 15-Lox-1 also needs to be found.

MATERIALS AND METHODS

Cell culture

HCT-116 cell line growth media was prepared using the McCoy (from ATCC) Media (500ml) with 5ml (100 IU) of PenStep along with 55ml of 10% FBS. HCT-116 colon cancer cells were seeded at half million cells/flask with 10ml of McCoy’s media in an incubator at 37 °C. To split cells, the cells were first removed from the existing media from flasks with a pipet into a 50 ml tube. Typsin-EDTA was added to the flasks to remove the attached cells. The cells were collected by centrifuge for 10 minutes at 1200 rpm. The cell pellet was re-suspended into the media. Once the cells became fully re-suspended, they were then counted using a hemocytometer with trypan blue staining to
measure the number of cells in the flasks. Finally, the cells were seeded accordingly to more flasks or 100X20mm petri dishes (from VWR International).

Treatment of cells with appropriate vitamin E concentrations

Vitamin E isomer gamma tocotrienol were diluted 1 to 10 with ethyl alcohol. HCT-116 colon cancer cells were seeded at 2.5 million cells/plate with 5ml of media. The concentration of Vitamin E was determined by using an HP diode array spectrophotometer with an E=at 292. Cells were treated with 0, 5 and 25 μM Gamma tocotrienol for 18 and 24 hours.

Isolation of cells

Cells were removed from glass plates using a cell scraper. The media plus the cells were removed from the plates and added to appropriately labeled 15ml tubes. Cells were collected by centrifugation in Sorvall refrigerated centrifuge. The cells were rinsed three times with PBS. The cells were collected into labeled 1.5 mL centrifuge tubes and counted via Hemocytometer. Cells were lysed with mammalian Cell Lysis Buffer (0.1% SDS, 0.1% Triton X-100, PBS) and 1μL of Protease Inhibitor cocktail (Sigma, Product Number 1860932) were added and stored at -80°C fridge for later use.

BCA Protein Assay (bicinchoninic acid)/Western blot/Incubates WB with various antibodies to distinguish the pathway the cancer cells affect

The protein concentration of the cells lysates was determined by the BCA protein assay. For the BCA Assay, a working media was prepared by combining 50 parts Reagent A with 1 part Reagent B (Pierce Biotechnology, Rockford, IL). Serial Dilutions were prepared at
2000, 1000, 500, 250, 125, 62.5, 31.25, and 15.6 μg/μL to obtain a protein standard curve. 10 μL of each standard was pipeted into the microtiter plate in the first 2 columns. Then, 1μL of sample + 9μL of Cell Lysis Buffer were pipeted into the appropriate wells with 200 μL of Working Reagent was added into each well. The samples were then mixed for 30 seconds on a microtiter plate shaker. After covering the microtiter plate, the samples were incubated at 37°C for 30 minutes. The absorbance of each well was 562 nm with a microtiter plate reader (96 well cell culture, Corning Incorporated).

The cell lysates were subjected to SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE) on a 10% Polyacrylamide gel. The gels were run using 1X Running Buffer (1g SDS + 100ml of 10X Tris Glycine Buffer Base and bring it up to 1 liter with dionized water) for hour and a half minutes at 100V. They were then transferred onto a Nitrocellulose membrane using the Transfer Buffer (200ml Methanol + 100ml of 10X Tris Glycine Buffer Base and bring it up to 1 liter with dionized water) for 1 hour at 100V. The gels were then stained in Coomassie Blue stain and the membrane with Ponceau S stain. After rinsing the gel and membrane, observations were made and recorded. Blots were rinsed in TBST for 15min and then blocked overnight in 5% skimmed milk. Blotted membranes were incubated with the primary antibodies overnight. Primary antibody (Caspase 12 from Cell Signaling #2202, Caspase 8 from Cell Signaling #9746, 15-LOX-1 from Santa Cruz #32940, ERK from Cell Signaling #4695, Phospho P70 from Cell Signaling #9234, Phospho S6 from Cell Signaling #2211, Cox-2 from Santa Cruz #1745, and Phospho 4EBP1 from Cell Signaling #9451) was probed with the appropriate secondary antibody (Anti Goat from R&D systems, Anti Rabbit from Cell signaling #7074, and Anti Mouse from Cell Signaling # 7076). It was
then conjugated with horseradish peroxidase and exposed. To control for consistent loading, membranes were probed with the β-actin antibody (Cell Signaling #3700).
References


