Gamma-Tocopherol Induces Apoptotic Cell Death in Human Adenoid Cystic Carcinoma Cells Derived from a Salivary Gland Tumor


Abstract

This study investigated both the possible antiproliferative and proapoptotic effects of, either alpha-tocopherol or gamma-tocopherol in vitro, upon a human salivary gland cancer cell line (ACC3), derived from an adenoid cystic carcinoma and M1 cells, a non-cancerous human salivary gland cell line. Prior to the use of these cell lines for treatment, they were examined histologically, to confirm their origin as human salivary gland cells. When ready for experimental use, cells were grown in a final concentration of 0, 10, 25 or 50 micromoles/liter of, either alpha-tocopherol or gamma-tocopherol, and evaluated for growth at 72 hours. Cells were analyzed for their proliferative activity by an assay, which uses 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow tetrazole which is reduced to purple formazan in living cells. The presence of apoptosis was analyzed by DNA extraction, followed by size fractionation, to look for DNA laddering, using gel electrophoresis. Apoptosis in cells was also analyzed and quantified using the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for detecting DNA fragmentation, followed by image analysis of cells using fluorescence microscopy. The proliferative assessments showed a highly significant effect of gamma-tocopherol, compared to alpha-tocopherol, upon growth suppression of the ACC3 cells. M1 cells did not appear to be affected by tocopherol treatments. Gamma-tocopherol, but not alpha-tocopherol treatment resulted in DNA fragmentation, as shown in both methods used. The results indicate that gamma-tocopherol is a potent compound that reduces growth of salivary gland cancer cells in culture, and that a possible mechanism to explain these results is that apoptosis resulting in cell death was induced by gamma-tocopherol.

Keywords: Gamma-tocopherol; Vitamin E; Apoptosis; Alpha-tocopherol

Introduction

Adenoid cystic carcinoma is a type of head and neck cancer that arises most commonly in the salivary glands. It occurs in both the major (sublingual, submandibular and parotid glands) and the minor salivary glands, but appears to be more common in the latter. Histologically similar tumors can also be found at other sites, including the trachea, external ear, lacrimal glands, esophagus, breast, prostate, cervix, ovary, Bartholin’s gland, lung, and skin [1]. Adenoid cystic carcinoma occurs over a very wide age range, from the first to the ninth decades of life, but with preponderance in the fourth to seventh decades. Typically adenoid cystic carcinoma is a slow growing, widely infiltrative tumor, with a tendency for local recurrence and metastasis. At least 5000 Americans are currently afflicted with adenoid cystic carcinoma, with about 1200 new cases being diagnosed every year, according to the Adenoid Cystic Carcinoma Research Foundation. In a recent clinical study, a high frequency of adenoid cystic carcinoma was shown in salivary glands and extra salivary sites [2]. Disease recurrence and metastasis accounts for the poor long-term prognosis of these tumors, as about 50% of patients with adenoid cystic carcinoma will eventually develop metastatic disease [1].

Gerber et al. interest is in elucidating the genetic causation of this salivary gland neoplasm and possible therapeutic modalities to control adenoid cystic carcinoma. As such, part of this effort has involved genetic analysis of adenoid cystic carcinomas from a pathology tissue bank, while an additional part of our research has been to propagate an Adenoid Cystic Carcinoma cell line (ACC3), as well as a non-tumorigenic salivary gland cell line, in order to evaluate nutrient suppression of the growth of these cells. Although it has been known for more than 80 years that vitamin E is an essential dietary component, most vitamin E research has been limited to the antioxidant properties of α-tocopherol. In fact, The Food and Nutrition Board only include Alpha-tocopherol (AT), among the 8 phytochemicals with similar structure, in determining the recommendations for vitamin E. This decision was made because AT provides the highest level of protection against fetal resorption in rat dams, the criterion used to determine vitamin E activity [3]. Several recent reviews, however, have described the numerous studies demonstrating the anticarcinogenic properties of the other vitamin E-like phytochemicals upon breast, prostate, colorectal and lung tumors in human populations, animal models and cell culture [4-8]. Other reviews of the chemistry, biosynthesis, bioavailability, metabolism, and possible role in humans of γ-tocopherol have concluded, it is likely that γ-tocopherol has a function in human health different from that of α-tocopherol [9,10], particularly upon cancerous cells. Gamma-tocopherol has been shown to induce apoptosis in tumorigenic prostate cells by interrupting sphingolipid synthesis [11]. Campbell et al. [12] attempted to explain the mechanism whereby γ-tocopherol treatment results in apoptosis, and demonstrated that colon tumor cells, but not normal colonic cells, became apoptotic when treated with γ-tocopherol. An older study showed an antiproliferative effect of α-tocopherol succinate on tumorigenic parotid acinar cells; however, a mechanism for this effect was not investigated nor was γ-tocopherol, or any of its derivatives evaluated in this system [13].

Epidemiological studies looking at the relationship between forms of vitamin E in the diet and cancer development have shown contradictory findings. Whereas one case-control study showed an inverse relationship between γ-tocopherol, but not α-tocopherol, and
prostate cancer incidence [14], another study failed to demonstrate that γ-tocopherol intake was inversely related to prostate cancer incidence [15]. More recently, higher serum α-tocopherol was associated with decreased risk of developing prostate cancer [16]. In the case of aerodigestive tract cancers, one group of investigators showed that serum γ-tocopherol, but not α-tocopherol, was lower in patients with these cancers [17], while another group looking at oral and pharyngeal cancer found that high serum α-tocopherol correlated with reduced risk of cancer development at these sites, but that increased γ-tocopherol serum levels correlated with greater risks of oral and pharyngeal cancer development [18].

In the current study, the authors investigated both the possible antiproliferative and proapoptotic effects, of either α-tocopherol or γ-tocopherol, upon a tumorogenic human salivary gland cell line (ACC3), as well as a non-tumorogenic salivary gland cell line (M1). The choice of these compounds was based upon the knowledge that vitamin E and γ-tocopherol are consumed at levels higher than any of the other tocopherols and tocotrienols consumed by people in the United States [19]. The authors found that at physiological levels, γ-tocopherol resulted in dramatic inhibition of the proliferation of the ACC3 cells, and that γ-tocopherol induced apoptosis in the propagated ACC3 cells. Our work agrees strongly with some of the studies identified above, which showed that γ-tocopherol suppressed proliferation of cultured tumorogenic prostate, colon, breast and lung cells. It also demonstrates that there appears to be a similar mechanism involved in this suppression of proliferation, via an increase in apoptosis subsequent to γ-tocopherol treatment.

Materials and Methods

Chemicals

All-rac-α-Tocopherol (>98% pure) was purchased from Fluka (Basel, Switzerland), while γ-Tocopherol (99.1% pure was purchased from Sigma (St. Louis, MO)). Tissue culture reagents were purchased from either BioWhittaker (Walkersville, MD), or Fisher Healthcare (Pittsburg, PA). The In Situ Cell Death Detection Kit and DNAase I, grade I was purchased from Roche Diagnostics (Basel, Switzerland). Triton X-100 was purchased from Fluka (Basel, Switzerland), while γ-Tocopherol (99.1% pure was purchased from Sigma (St. Louis, MO)). Tissue culture reagents were purchased from either BioWhittaker (Walkersville, MD), or Fisher Healthcare (Pittsburg, PA). The In Situ Cell Death Detection Kit and DNAase I, grade I was purchased from Roche Diagnostics (Basel, Switzerland). Triton X-100 was purchased from Fluka (Basel, Switzerland). Hematoxylin and Eosin were purchased from Poly Scientific (Bay Shore, NY), to be used for histological analysis. The following immunohistochemical markers were utilized: cytokeratin7 (clone OV-TL12/30, Cell Marque, Rocklin, CA), CAM 5.2 (clone CAM 5.2, BD Biosciences, San Jose, CA), cytokeratin 34βE12 (clone 34Be12, Enzo LifeSciences, Farmingdale, NY), AE1/AE3 (clone AE1-AE3, Dako, Carpenteria, CA), cytokeratin 5/6 (clone D5/16B4, Cell Marque, Rocklin, CA), cytokeratin 19 (clone RCK108, Dako, Carpenteria, CA), cytokeratin 20 (clone KS20.8, Cell Marque, Rocklin, CA), vimentin (clone V9, Biogenex, Fremont, CA), S100 (polyclonal, Dako, Carpenteria, CA), p63 (clone 4A4, Biogenex, Fremont, CA), smooth muscle actin (clone 1A4, Dako, Carpenteria, CA), S100 (polyclonal, Dako, Carpenteria, CA), p63 (clone 4A4, Biogenex, Fremont, CA), smooth muscle myosin (clone SMMS-1, Cell Marque, Rocklin, CA), and carcinoembryonic antigen (polyclonal, Cell Marque, Rocklin, CA).

Cell culture

The ACC3 human salivary gland cancer cell line was obtained from Drs. Takashi Saku (Division of Oral Pathology, Department of Tissue Regeneration and Reconstruction, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan) and Christopher Moskaluk (Department of Pathology, University of Virginia Medical School, Charlottesville, Virginia). These cells were originally propagated from cells removed from an adenoid cystic carcinoma of the parotid salivary gland of a 49-year old male patient in China [20]. The choice of the ACC3 cells as a model system for γ-tocopherol activity is predicated upon a number of factors. We realize that only using one cell line limits our ability to generalize; however, this line has very attractive attributes, which outweigh this deficit. In a recent report from the Moskaluk laboratory, Pramoonjago et al. [20] clearly state that “ACC3 is the only available cell line derived from an ACC tumor, that seems to maintain phenotypic characteristics of adenoid cystic carcinoma.” They conclude, after citing numerous studies demonstrating that the phenotype and gene expression of these cells closely resembles primary ACC tumors, that the ACC3 cells are an appropriate model for primary ACC tumors. In addition, our immunohistochemical studies (see below) with strong cytokeratin 7, vimentin, smooth muscle actin, and AE1/AE3 staining support origin from a salivary gland tumor. Cells from passages 20-25 were maintained routinely in RPMI Medium 1640 with 10% Fetal Bovine Serum, 1% glutamine, and 100 µg/ml streptomycin. In the presence of media only, the doubling time of the cells was about 24 hours. The control non-tumorogenic salivary gland cell line, M1, was obtained from Dr. Ruy Jaeger (University of Sao Paulo, Brazil), and propagated using DMEM media, containing the same additives, as used in the media for the ACC3 cells. For the growth experiments, 6-well plates were seeded initially at 5x10^4 cells/well. Cells were seeded at a density of 7.5x10^4 cells in 6-well chamber slides (Tissue-tek, Sakura Finetek, Torrance, CA), as needed for the apoptotic analysis and DNA fractionation evaluation.

For tissue culture experiments, either α-tocopherol or γ-tocopherol was dissolved in DMSO. Exposure to light was avoided and the tocopherol stocks were sealed under Argon between uses. Final concentrations of DMSO in all experimental cultures were less than 0.15% [7]. Control cultures were used without tocopherol, but contained equivalent levels of DMSO in media. α-tocopherol and γ-tocopherol final concentrations were either 0, 10, 25, or 50 µmoles/litre.

Experimental design

For Experiment 1, ACC3 cells were seeded at a density of 5x10^4 cells/well in seven 6-well plates, and each of the 6 wells per plate was treated with, either α- or γ-tocopherol at 10, 25 and 50 µmoles/litre of media, or with an equivalent concentration of DMSO, used in the solubilization of tocopherols (0 µmoles treatment). Cells were removed after 72 hours and assayed by the MTT assay described below. For Experiment 2, M1 cells were seeded at a density of 5x10^4 cells/well in seven 6-well plates, and each of the 6 wells per plate was treated with, either α- or γ-tocopherol at 10, 25 and 50 µmoles/litre of media, or with an equivalent concentration of DMSO, used in the solubilization of tocopherols. Cells were removed after 72 hours and assayed by the MTT assay described below. For Experiment 3, ACC3 cells were grown under similar conditions to that of Experiment 2 in chamber slides for apoptosis and DNA fragmentation analysis. Cells were treated with either α-tocopherol or γ-tocopherol, at a final concentration of 25 µmoles/litre, or with equivalent levels of DMSO, as used in the tocopherol treatments of Experiments 1 and 2. The decision to use 25 µmoles/litre of α− and γ− tocopherol in these studies was based upon physiological considerations, since a 50 µmoles/litre treatment is in excess of the levels that would be normally found in human serum, while 25 µmoles/litre more closely approximates physiological conditions [19], and this level of γ−tocopherol was
effective in reducing the growth of ACC3 cells in our study. In addition, apoptosis in prostate cancer cells was induced by this level, and not by the 10 µmole/litre level of γ-tocopherol in a similar study [11]. The amount of cells used was commensurate with the number of cells needed for the analyses to be accomplished on these cells. These cells were treated for 6-7 days with a media plus treatment change after 3 days, and then were either assayed for apoptosis by TUNEL assay, as described below, or their DNA was extracted for gel electrophoresis to evaluate the possibility of DNA fragmentation at 6-7 days.

Cell growth and viability

MTT assays were used to evaluate cell viability by assessing the number of viable cells at a given time point, after treatment with the tocopherols. This assay, which uses 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow tetrazole, is reduced by living cells to purple formazan. The MTT assays were accomplished using MTT from Sigma (St. Louis, MO), and evaluating the samples colometrically at 590 nm using a Biotek Plate Reader [21]. Cells were also manually counted using a hemocytometer and light microscope for validation of the MTT assays (data not shown).

Evaluation of apoptosis

Detection and quantification of apoptosis was accomplished on cells grown on chamber slides fixed with paraformaldehyde in PBS, after tocopherol treatments for 6-7 days. Cells were permeabilised with a freshly prepared solution of 0.1% Triton X-100 in a 0.1% sodium citrate solution. Cells were then labeled with a TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) reaction mixture purchased from Roche Diagnostics (Basel, Switzerland). Negative controls were produced using non-tocopherol treated fixed and permeabilised cells with label solution only, while positive controls used non-tocopherol treated fixed and permeabilised cells, treated with DNase I (Grade I) to induced DNA strand breakage, prior to labeling and TUNEL reaction procedures.

Image analysis of treated cells

Images were captured of cells from experiment 2 and 3, using a digital camera (Sony, Japan) attached to a fluorescent microscope (Olympus BX41). The DAPI and FITC stained cells were highlighted by thresholding and segmentation, and counted using the Image Pro Plus 5.1 software (Media Cybernetics). In each group, 16 microscopic fields were evaluated with a total average of 1400 cells counted per treatment.

DNA analysis

DNA size analysis was used to detect the presence of internucleosomal DNA cleavage, which is characteristic of apoptosis. DNA was extracted using the method of De Paep (clone OV-TL12/30), CAM 5.2 (clone CAM 5.2, cytokeratin 34βe12) (clone 34Be12), AE1/AE3 (clone AE1-AE3), cytokeratin 5/6 (clone D5/16B4), cytokeratin 19 (clone RCK108) cytokeratin 20 (clone KS20.8) vimentin (clone V9), S100 (polyclonal), p63 (clone 4A4), smooth muscle actin (clone 1A4), smooth muscle myosin (clone SMMS-1), and carcinomaembryonic antigen (polyclonal).

Statistics and calculations

The results in Experiments 1 and 2 were evaluated using ANOVA, followed by “t” tests. For experiment 3, the study groups were analyzed for their distribution, using the Kolmogorov-Smirnov test [23], followed by Bonferonni post-hoc tests [24], to determine differences in apoptotic ratios.

Results

Cell viability

Experiment 1 demonstrated that α-tocopherol did not have an effect upon the growth of ACC3 cells at 72 hours, when cells were treated with either 10, 25 and 50 µmole/litre. The results of Experiment 1 also showed that γ-tocopherol resulted in a reduction in growth of ACC3 cells at 72 hours, when treated with either 25 or 50 µmole/litre. These growth reductions were significant at a p<.0001 level (Figure 1). Experiment 2 demonstrated that neither α-tocopherol nor γ-tocopherol had an effect upon the growth of M1 cells at 72 hours, when cells were treated with either 10, 25 and 50 µmole/litre (Figure 2).

Apoptosis analysis using TUNEL assay

As can be seen from figures 3A-C, compared to DMSO control-treated cells, there was no evidence of apoptotic activity in the ACC3 cells due to the α-tocopherol treatment, but the γ-tocopherol treatment resulted in a highly visible pattern of apoptotic activity upon the treated cells. This was highly significant statistically as shown in figure 4.

Figure 1: ACC3 cells were grown in media containing 10, 25 or 50 micromoles/litre of alpha- or gamma-tocopherol for 72 hours. The control group did not contain these compounds, and all growth was compared to DMSO control-
DNA analysis

The cells treated with either 25 µmole/l γ-tocopherol or α-tocopherol were subjected to DNA electrophoresis, as described previously. As can be seen from figure 5, there was evidence of internucleosomal DNA cleavage due to γ-tocopherol treatment, as shown in a visible “laddering” of DNA, indicative of apoptotic activity, but the α-tocopherol treatment did not show this cleavage pattern.

Histological analysis

Staining results from the ACC3 and M1 cell lines are summarized in table 1 and illustrated in figure 6. Cytokeratin 7, AE1/AE3, Vimentin and Smooth Muscle Actin stained diffusely (3 to 4+ positive) in the ACC3 cell line (Figure 6), while CAM 5.2 and MAK 6 stained weakly. All other stains were negative. The M1 cell line stained only for Vimentin and Smooth Muscle Actin.

Discussion

Our current study demonstrates that γ-tocopherol had an inhibitory effect upon the growth of ACC3 cells. The use of a non-tumorogenic salivary gland cell line, which does not respond to γ-tocopherol, confirms that the inhibitory impact occurs in tumor cells, but not non-tumor cells. With additional studies designed to determine the possible mechanism, we were able to tentatively identify the mechanism of γ-tocopherol’s inhibitory effect upon salivary gland tumor cells. Specifically, we have shown that γ-tocopherol but not α-tocopherol, induces apoptosis in ACC3 cells. Since γ-tocopherol lacks the methyl group present on position 5 of the chroman ring, unlike α-tocopherol, which has the methyl group on position 5, it is not unexpected to have different metabolic effects. Although studies have shown that γ-tocopherol is a less potent antioxidant against the typical reactive oxygen species that α-tocopherol is able to quench, other studies indicate that this difference may confer γ-tocopherol with the ability to quench other types of reactive oxygen species [10].
Recent studies have shown a putative role for γ-tocopherol in cancer prevention. Epidemiological evidence has suggested that γ-tocopherol may prevent prostate [14] and breast cancer [25] development. γ-tocopherol has been shown on a cellular level to inhibit prostate cancer cell growth [26] and to inhibit growth of cancerous colonocytes [12]. Our study adds adenoid cystic carcinoma cells to the growing list of cancerous cells that can be inhibited by γ-tocopherol and suggests a mechanism for this inhibition.

Although we have demonstrated a generalized mechanism for the growth repression of adenoid cystic carcinoma cells by γ-tocopherol via apoptosis, the specific mechanism(s) which result in the apoptosis have not been elucidated. Other laboratories have looked at putative mechanisms of apoptosis for different tumorigenic cell lines. In a recent study [11] γ–tocopherol treatment at similar concentrations to our study resulted in apoptosis of LNCaP prostate cancer cells. These results suggest that the apoptotic pathways that are activated by γ–tocopherol are both caspase-dependent and caspase-independent. Using a specific caspase inhibitor in their system, Z-VAD-fmk, did not prevent PARP cleavage or DNA fragmentation, indicating that apoptosis was still occurring, despite caspase blockade. The investigators in this study present data that suggests that the caspase-independent apoptosis is mediated by a block in lipid biosynthesis.

Speciﬁcally, they demonstrated that by adding inhibitors of enzymes in the ceramide synthetic pathway, myriocin and fumonisin B1, a signiﬁcant amount of cell death was prevented, as evidenced by reduced cytochrome c release, PARP cleavage and formation of active caspase 3.

Additionally, other investigators using different cell lines have demonstrated that γ-tocopherol-quinone, an oxidation product of γ-tocopherol, induces apoptosis in colon adenocarcinoma cells in culture [27], leukemic cells in culture [27,28], and breast cancer cells in culture [28].

Gerber et al. results using ACC3 cells strongly demonstrate that γ-tocopherol induces apoptosis as evidenced by the results of the TUNEL assay and DNA fragmentation. Experiments will be carried out to determine whether γ-tocopherol is involved in enhancing proapoptotic pathways in ACC3 cells through caspase dependent or independent pathways. These experiments will be accomplished using western blots of gamma-tocopherol treated or untreated extracts of ACC3 cells, to evaluate the possible cleavage of caspases (cysteine aspartyl proteases) 3, 7, 8 and 9, as well as PARP (nuclear poly ADP-ribose polymerase). The results of these experiments will enable us to understand if γ-tocopherol is enhancing apoptosis via a “death receptor” (external pathway) and/or a mitochondrial (internal pathway) by analysis of the caspase and PARP cleavage pattern. Cleavage of these proteins is generally associated with apoptotic activity; however, differences in cleavage patterns are associated with different modes of apoptosis. For instance, Campbell et al. [12] have identified cleavage of PARP and caspases 3, 7, and 8 in colon cells treated with γ-tocopherol, indicating a “death receptor” pathway. Alternatively, Jiang et al. [11] have identified that γ-tocopherol treatment of prostate cancer cells resulted in cleavage of PARP and caspases 3 and 9, but did not observe cleavage of caspase 8, indicating a mitochondrial mechanism of apoptosis.

In summary, our study demonstrates that γ-tocopherol decreases growth of the salivary gland tumor cell line, ACC3, by increasing apoptotic activity. Since there was no evidence of a decrease in growth of these cells due to α-tocopherol, this compares well with studies [29], which suggest that quenching of some types of oxygen radicals by antioxidants, such as α-tocopherol could block apoptosis; therefore, high-dose antioxidant therapy to decrease tumor proliferation with α-tocopherol may be counterproductive. Studies using animal models and well-designed human intervention trials are needed to better understand the independent value of γ-tocopherol, as compared with α-tocopherol, in order to better establish human nutrition needs.

Summary

Although the Food and Nutrition Board only considers alpha-tocopherol in determining the recommendations for vitamin E, numerous studies have demonstrated the anticarcinogenic value of other phytochemicals similar to vitamin E. In particular, anticarcinogenic activities have been evaluated primarily for breast, prostate, colorectal and lung tumors. The majority of these studies have focused upon the effects of Gamma-tocopherol (GT) primarily because it is generally consumed in quantities higher than alpha-tocopherol in the U.S. diet, and dietary consumption results in a reasonably high level of GT. Observations of cultured tumorigenic cells, supplemented with various tocopherols, have enabled researchers to evaluate possible mechanisms by which these phytochemicals exert their anticarcinogenic effects. These studies...
have resulted in some understanding of how GT and possibly other tocopherols may impact the progress of colon, breast, prostate and lung cancer. The overall findings are that GT has been demonstrated to be an extremely potent inducer of apoptosis in these cell types.

This submitted research manuscript highlights recent findings from our laboratories which demonstrate that salivary gland tumors (adenoid cystic carcinoma cells) are also affected by gamma-tocopherol treatments, similarly to published research on the effects of this vitamin E-like phytochemical upon breast, colon, prostate and lung tumors. Specifically, GT but not Alpha-tocopherol (AT) results in decreased proliferation of adenoid cystic carcinoma cells, and GT also results in cell death (apoptosis) of these cells.

These findings are significant because they demonstrate that GT has biological activity different from AT, which is currently the only one of 8 similar compounds that is recognized as vitamin E. In addition, numerous studies have shown the correlation of higher fruit and vegetable consumption, and a decrease in numerous types of cancers. It is significant that GT, currently only recognized as a phytochemical, may be one of the possible phytochemicals contributing to this effect.

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