Abstract

Purpose: Vitamin E succinate (α-TOS) inhibits the growth of cancer cells without unacceptable side effects. Therefore, the mechanisms associated with the anticancer action of α-TOS, including ceramide-mediated apoptosis, were investigated using head and neck squamous cell carcinoma (HNSCC) in vitro and in vivo.

Experimental Design: Five different human HNSCC cell lines (JHU-011, JHU-013, JHU-019, JHU-022, and JHU-029) were treated with α-TOS, and its effects on cell proliferation, cell cycle progression, ceramide-mediated apoptosis, and ceramide metabolism were evaluated. The anticancer effect of α-TOS was also examined on JHU-022 solid tumor xenograft growth in immunodeficient mice.

Results: α-TOS inhibited the growth of all the HNSCC cell lines in vitro in a dose- and time-dependent manner. Thus, JHU-013 and JHU-022 cell lines were more sensitive to α-TOS than the other cell lines. Cellular levels of ceramide, sphingomyelinase activity, caspase-3, and p53 were elevated with increasing time of exposure to α-TOS. The degradation of poly(ADP-ribose) polymerase protein in JHU-022 cells treated with α-TOS provided evidence for apoptosis. The amounts of nuclear factor κB, Bcl-2, and Bcl-XL proteins were reduced in the cells treated with α-TOS for 6 hours. The levels of caspase-9, murine double minute-2, and IκB-α proteins were unchanged after α-TOS treatment. I.p. administration of α-TOS slowed tumor growth in immunodeficient mice.

Conclusions: α-TOS showed promising anticancer effects to inhibit HNSCC growth and viability in vitro and in vivo. The induction of enzymes involved in ceramide metabolism by α-TOS suggests that ceramide-mediated apoptosis may expand therapeutic strategies in the treatment of carcinoma.

Each year, ~31,000 Americans are diagnosed with head and neck cancer and ~7,400 die from the disease (1). Overall survival rates of head and neck cancers have only marginally improved over the last three decades (2). In addition, the incidence of oral cancer in African American men has been approximately twice higher than in Caucasian men, and the 5-year relative survival is lower for African Americans (1). Therefore, novel therapeutic and preventive approaches are warranted.

Accumulating evidence suggested that an esterified derivative of RRR-α-tocopherol (α-TOH), RRR-α-tocopheryl succinate (α-TOS), is a vitamin E analogue, which inhibits tumor growth (3–7). It induces apoptosis, inhibits tumor cell proliferation and differentiation, arrests DNA synthesis, and blocks cell cycle progression in various cancer cell lines and animal models of breast, colon, head and neck, prostate, and lung cancers (8–18). In addition, α-TOS selectively kills tumor cells without toxic effects on normal cells and tissues (4, 7, 18). The parent compound of vitamin E, α-TOH, is a free radical–scavenging antioxidant, which protects polyunsaturated fats from peroxidation in human body but does not induce cancer cell apoptosis (19). In contrast to α-TOH, α-TOS is a redox-inactive molecule, which has a charged side group when it exists at physiologic pH. α-TOS can be converted to α-TOH by cellular esterase (20). All the reports thus far indicate that α-TOS causes an increase in cancer cell apoptosis (3–7), but the mechanism(s) of α-TOS–induced cancer cell apoptosis and inhibition of cancer cell growth are not fully understood.

In previous studies, we found that α-TOS induced apoptosis in a hamster cheek pouch carcinoma cell line (HCPC-1) and altered sphingolipid metabolism (11). Moreover, cell viability was significantly reduced in cultures treated with α-TOS. A critical finding in our study was that α-TOS interacts with cell membrane to shift phospholipids and sphingolipid in the lipid

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bilateral of the abnormal plasma membrane. This makes sphingo-
lipids more accessible to hydrolyses and then to generate ceramide. Ceramide is a central molecule in sphingolipid metabolism, having a significant role in the apoptotic response of various cancer cells (21–27). For example, androgen ablation in LNCaP prostate cancer cells results in selective accumulation of de novo generated C16-ceramide. This accumulation of ceramide leads to G2-M arrest followed by apoptosis (24). Mice deficient in acid sphingomyelinase lose the ability to accumulate ceramide and acquire resistance to radiation-induced apoptosis (25). Interruption of sphingomyelin synthesis or hydrolysis of sphingomyelin by sphingomyelinase can increase cellular levels of ceramide. Increased levels of ceramide can damage mitochondria and induce apoptosis (27, 28). Experiments designed to reveal the relationship between α-TOS and ceramide-mediated apoptosis in cancer cells, especially in head and neck cancer cells, are warranted.

We hypothesized that α-TOS may activate the accumulation of ceramide in the cancer cells of head and neck squamous cell carcinoma (HNSCC), and that ceramide buildup triggers apoptotic events. In this report, we tested this hypothesis in five different human HNSCC cell lines, including JHU-011, JHU-013, JHU-019, JHU-022, and JHU-029 cell lines, using in vitro and in vivo systems.

Materials and Methods

Chemical reagents. Chemicals were of the highest available grade. Ceramide (N-octadecanoyl-D-erythro-sphingosine), α-TOS, and phosphatidylcholine were obtained from Sigma Chemical Company. Escherichia coli stn-1,2-diacylglycerol kinase (specific activity ≥ 2 units/mg protein) and octyl-D-β-glucopyranoside were obtained from Calbio-
chem, and [γ-32P]ATP (3,000 Ci/mmol) was obtained from Amersham.

α-TOS-liposome preparation. Two types of small unilamellar vesicles (SUV), vehicle-SUV and α-TOS encapsulated SUV, were made for in vitro experiments based on the previously reported method (11). The SUV vehicle-SUV was a suspension of phosphatidylcholine liposomes in PBS, and α-TOS-SUV consisted of phosphatidylcholine liposomes encapsulating 1 mmol/L α-TOS. In brief, 0.2 g/mL phosphatidylcholine lipid stock solution in 1:1 mixture of chloroform/methanol was dried under nitrogen and then mixed with an isotonic solution of physiologic saline (0.9% NaCl) along with the anionic detergent sodium cholate. The resulting mixed micellar solution was then dialyzed in a Mini Lipoprep dialyzer (5,000 MW cutoff; Amika Corporation) for 4 h against an 8-liter reservoir of 0.9% NaCl solution. To encapsulate α-TOS into the SUV, α-TOS was dissolved along with phosphatidylcholine using the mixture of chloroform and methanol (1:1). This was then processed as described above for the preparation of SUV.

Cell lines and culture. HNSCC cell lines including JHU-011 (larynx, p53 mutated), JHU-013 (neck node metastasis, p53 mutated), JHU-019 (tongue), JHU-022 (larynx, wild type of p53), and JHU-029 (wild type of p53) were established by Johns Hopkins University. The HNSCC cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and antibiotic-antimycotic mixture (100 IU/mL penicillin and 100 μg/mL streptomycin; Cellgro). Cells were grown in 5% CO2 at 37°C and were subcultured at an initial density of 1 × 104/mL every 3 to 4 d. Cell density was determined with a hemocytometer and a phase-contrast microscope. Trypan blue dye exclusion assay (Sigma-Aldrich) detected 0.1% of dead cells in the untreated culture. All experiments were done with cells in logarithmic phase of growth.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. HNSCC cells were seeded in flat-bottomed 96-well cell culture plate (Costar) at a density of 5,000 per well and allowed to attach overnight. The cells were then treated with α-TOS-SUV at various concentrations (5-80 μmol/L) for 12 h. Twenty microliters of 3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (Sigma) were added to each well and then the plate was incubated in a humidified CO2 incubator at 37°C for 4 h. After removing the media, 200 μL of DMSO were added to each well and mixed for 30 min at room temperature to dissolve crystals. The plate was placed inside a 37°C incubator for 5 min. Finally, the plate was transferred to a microplate reader (Bio-Rad) and absorbance at 550 nm was measured.

Colon (9) and cell assays. HNSCC cells were seeded at a density of 200 per well in 6-well tissue culture plate. The cells were allowed to attach overnight. The cultures were treated with α-TOS-SUV (20 μmol/L) for 3 to 24 h. Control cultures were treated with the same volume of medium with vehicle (SUV). The medium was removed from each well and the cells were washed with PBS. Fresh drug-free culture medium (5 mL) was added to the cultures, which were then placed back into the incubator to form colonies in 10 d. Any colony containing >50 cells was considered to represent a viable clonogenic cell. The colonies in the wells were counted after staining with 0.1% methylene blue in 50% ethanol. The experiment was done thrice for each treatment.

Flow cytometry assay. HNSCC cells (5 × 104) were seeded in six-
well, flat-bottomed plates and were exposed to 20 μmol/L α-TOS-SUV for 6, 12, and 24 h. The cells were collected, washed, and suspended in cold PBS. The cells were fixed in chilled 75% methanol and then incubated in the dark for 15 min at room temperature in a solution containing Annexin V-FITC (Clontech Laboratories, Inc.) and propi-
dium iodide (5 μg/mL). The expression of Annexin V and cell cycle status were analyzed by FACStar flow cytometer (Becton Dickinson & Co.). Ten thousand cells per sample were analyzed.

Human tumor xenograft in mice. Four-week-old, female, athymic nude mice (nu/nu) were obtained from Harlan Sprague-Dawley, Inc. They were given Harlan Teklad #2018 Global 18% Protein Rodent Diet containing 101 mg/kg α-tocopherol and water ad libitum in the animal facility for 3 wk before use. Mice were housed in temperature-controlled rooms (74 ± 2°F) with a 12-h alternating light-dark cycle. The mice were separated into three groups (three to five per group): α-TOS treatment group, DMSO vehicle control group, and untreated control group. The body weight and food/water intake were measured twice a week. The mice in the α-TOS group received a 3-wk pretreatment with α-TOS (1.0 mg dissolved in 0.1 mL DMSO/mouse by i.p. injection) every other day before injecting JHU-022 cells. The mice in the DMSO group received 0.1 mL DMSO by i.p. injection. JHU-022 cancer cells (2 × 106/100 μL/mouse) were injected s.c. into the lower back of the mice using a 25-gauge needle on day 21. I.p. injections of α-TOS were continued on alternate days till day 55. Thus, each mouse in the α-TOS group received a total of 25 mg of α-TOS via i.p. injection for the 55-d treatment period. Tumor volume was determined from either caliper measurements (Tvol = length × width × depth × 0.5236) or directly by weighing the tumor. Guidelines for the humane treatment of animals were followed as approved by the Howard University Animal Care and Use Committee.

Immunohistochemistry. Tumor tissues were fixed in 10% formalin, embedded in paraffin, and cut into 5-μm-thick sections. The immuno-

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reverse-phase high-performance liquid chromatography. Each plasma sample (100 μL) was extracted thrice in hexane/ethanol 3:1 with SDS. The lipid fraction was evaporated to dryness under a nitrogen stream. The samples were then resuspended in methanol and injected into Agilent 1100 high-performance liquid chromatography. The mobile phase consisted of 99.4% methanol and 0.6% glacial acetic acid. Samples were separated on an Elite hypersil ODS2 5 μm (250 × 4.6 mm) column (Elite). HPLC detector was set at 284 nm for all detection. Quantitation of the separated compounds was done based on α-TOS standard pattern and Agilent software for data analysis.

Diacylglycerol kinase assay. Ceramide levels were determined with the diacylglycerol kinase assay as described in the previous study (11). In brief, monolayer cultures of HNSCC cells (10⁶) were treated with α-TOS-SUV (20 μmol/L) or SUV for 30 min to 6 h. Cellular lipids were extracted from the treated cells using a mixture of chloroform/methanol/1 N HCl at a ratio of 100:100:1 (v/v), and then hydrolyzed with 0.1 N methanolic KOH for 1 h at 37 °C to remove glycerophospholipids. Ceramide containing samples were resuspended in 100 μL of reaction mixture containing 150 μg cardiolipin, 280 μmol/L diethylenetriaminepentaacetic acid, 51 μmol/L octyl-β-D-glucopyranoside (Calbiochem), 1 mmol/L ATP, 10 μCi [γ-32P]ATP (DuPont New England Nuclear), and 35 μg/ml E. coli diacylglycerol kinase, pH 6.5 (Calbiochem). After 60 min at room temperature, the reaction was stopped by extraction of lipids with 1 mL of solvent mixture of chloroform/methanol/1 N HCl (100:100:1, v/v). Ceramide 1-phosphate was separated on TLC plate using a solvent system of chloroform/methanol/acetate (65:15:5, v/v) and detected by autoradiography. The incorporated 32P was quantified by liquid scintillation counting. Ceramide was determined by comparison with standard samples containing known amounts of ceramide.

In vivo sphingomyelinase assay. Sphingomyelinase assay was modified based on Gifone's method (29). In brief, the monolayer culture of HNSCC cells with 5% serum was labeled for 48 h with [N-methyl-14C]-choline (NEN Life Science Products, Inc.). The cells were washed thrice with Ca- and Mg-free PBS and then treated with α-TOS-SUV (20 μmol/L) or SUV for 30 min to 6 h. The cells were harvested from cultures by trypsinization and kept at 4 °C to avoid protein degradation. Cellular lipids were extracted with a mixture of ice-cold methanol/chloroform/water at a ratio of 250:125:100 (v/v), and the samples corresponding to equal amounts of proteins were loaded onto individual lanes on the TLC plate. The lipids were separated by developing the thin layer chromatogram using a mixed solvent system composed of chloroform/methanol/acetate acid/water at a ratio of 100:60:20:5 (v/v). The radioactive spots were visualized by autoradiography and scraped along with silica gel from the plate and transferred to a scintillation vial containing 1 mL of scintillation cocktail of water and aquasol for determination of radioactivity.

Apopain assay. Caspase-3 activity measurement was done with FluorAce Apopain Kit (Bio-Rad Laboratories) according to the manufacturer's instructions. In brief, the α-TOS-SUV–treated (20 μmol/L) or SUV-treated cells (1 × 10⁶) were lysed with apopain cell lysis buffer and the supernatant was incubated with fluorogenic peptide substrate Ac-DEVD-AFC, 55 μmol/L, and reaction buffer at 37 °C. At the same time, to distinguish caspase-3 activity from nonspecific protease activity, the sample reactions were carried out after adding relatively nonspecific caspase inhibitor z-VAD-fmk (50 μmol/L) or caspase-3 inhibitor z-DEVD-fmk (50 μmol/L; Clontech). The fluorescence readings were recorded after 30 and 60 min of incubation using a VersaFluor fluorometer (Bio-Rad), with excitation and emission wavelengths of 390 and 520 nm, respectively. Results were expressed as units of Apopain per 0.1 milligram of protein, according to the formula provided with the FluorAce Apopain Assay Kit. Protein concentration was measured using a DC Protein Assay Kit (Bio-Rad Laboratories).

Western immunoblotting. The treated and untreated HNSCC cells were harvested and washed twice in PBS and then suspended in lysis buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, phenylmethylsulfonyl fluoride at 100 μmol/L, aprotinin at 2 μg/mL, pepstatin at 1 μg/mL, and leupeptin at 10 μg/mL]. This mixture was placed on ice for 30 min. After centrifugation at 15,000 × g for 15 min at 4 °C, the supernatant was collected. Protein concentrations were quantitated by using the Bio-Rad protein assay (Bio-Rad Laboratories). Whole-cell lysates (50 μg) were separated by 8% SDS-PAGE gel, transferred onto a polyvinylidene difluoride membrane (Immobilon; Amersham Corp.), and then probed sequentially with antibodies against the following proteins: caspase-3, caspase-9, poly(ADP-ribose) polymerase (PARP), Bcl-2, Bcl-xL, and β-actin (Sigma). Blots were washed thrice for 10 min with PBS + 0.1% Tween 20 and incubated with horseradish peroxidase–conjugated antirabbit, antimouse, or antigoat antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Blots were developed by a peroxidase reaction using the enhanced chemiluminescence detection system (Bio-Rad).

Statistical analyses. Results are presented relative to untreated controls. Values represent mean ± SD of three or more replicate tests.
Data were analyzed by Duncan test following the ANOVA procedure when multiple comparisons were made. \( P < 0.05 \) was considered significant.

**Results**

**Effect of \( \alpha \)-TOS on the proliferation and viability of HNSCC cell lines in vitro.** We first confirmed the inhibitory effects of \( \alpha \)-TOS on cell proliferation and viability in five different human HNSCC cell lines (JHU-011, JHU-013, JHU-019, JHU-022, and JHU-029) in vitro by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and colony formation assays. \( \alpha \)-TOS inhibited cell proliferation in all five cell lines in a dose-dependent manner when the cells were exposed to graded doses (5-80 \( \mu \)mol/L) for 12 hours. This inhibition was most pronounced in JHU-013 and JHU-022 cell lines (Fig. 1A). Because 20 \( \mu \)mol/L \( \alpha \)-TOS-SUV was the half maximal inhibitory concentration (IC\(_{50}\)) in JHU-013 and JHU-022 cell lines, this dose of \( \alpha \)-TOS-SUV was used for further in vitro studies. The potency of \( \alpha \)-TOS was determined by using colony formation assay to measure the reproductive integrity of JHU-022 and JHU-029 cells treated with 20 \( \mu \)mol/L \( \alpha \)-TOS-SUV from 0 to 24 hours (Fig. 1B). As expected, clonogenic capacities of JHU-022 and JHU-029 cell lines decreased with increasing exposure time to 20 \( \mu \)mol/L \( \alpha \)-TOS-SUV (Fig. 1B). As compared with untreated cancer cells, the relative survival of JHU-022 cells decreased to 31.9% and 8.7%, whereas survival of JHU-029 cells decreased to 43.3% and 20.4% after treatment with \( \alpha \)-TOS-SUV for 12 and 24 hours, respectively (Fig. 1B). The effects of \( \alpha \)-TOS-SUV treatment of the different cell lines were explored further by flow cytometry analysis for cell cycle distribution and estimation of apoptosis. As summarized in Table 1, flow cytometry data indicated that a 24-hour exposure to \( \alpha \)-TOS induced S-phase arrest and reduced the proportion of cells in \( G_1 \) phase in all five HNSCC cell lines after treatment. Moreover, \( \alpha \)-TOS induced a significant proportion of apoptosis in JHU-011 (57.87%), JHU-013 (58.02%), JHU-019 (54.87%), JHU-022 (49.39%), and JHU-029 (48.23%) cell lines after a 24-hour exposure (Table 1). However, there was <1% apoptotic cells in JHU-011 cells treated for 12 hours with \( \alpha \)-TOS-SUV, vehicle (SUV), and untreated control cells (Fig. 1C). There were no significant differences in the cell cycle distribution patterns of untreated cultures and control cultures treated with SUV alone.

**Antitumor effect of \( \alpha \)-TOS against JHU-022 human and head neck cancer cells in vivo.** We further examined the antitumor effects of \( \alpha \)-TOS on JHU-022 cell growth as solid tumor xenografts in immunodeficient mice. The experiment is outlined in Fig. 2A. Tumor growth occurred in all JHU-022 inoculated mice. All mice were euthanized on day 55 and tumors were dissected and weighed. The mice in the \( \alpha \)-TOS–treated group showed suppression of tumor growth as compared with untreated and DMSO control groups (Fig. 2B and C). Average tumor weight was significantly lower in the \( \alpha \)-TOS–treated group (0.24 g) than in the untreated (0.39 g) and DMSO-treated (0.36 g) groups (Fig. 2C). The body weight increased

### Table 1. Comparison of cell cycle in \( \alpha \)-TOS–treated cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>( G_1 ) (%)</th>
<th>( G_2 ) (%)</th>
<th>( S ) (%)</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JHU-011 untreated</td>
<td>61.04</td>
<td>12.18</td>
<td>26.78</td>
<td>0.12</td>
</tr>
<tr>
<td>JHU-011 vehicle</td>
<td>62.31</td>
<td>11.62</td>
<td>25.05</td>
<td>0.16</td>
</tr>
<tr>
<td>JHU-011 ( \alpha )-TOS</td>
<td>51.83</td>
<td>8.97</td>
<td>39.19</td>
<td>57.87</td>
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<tr>
<td>JHU-013 untreated</td>
<td>62.09</td>
<td>11.15</td>
<td>26.76</td>
<td>0.08</td>
</tr>
<tr>
<td>JHU-013 vehicle</td>
<td>63.31</td>
<td>12.15</td>
<td>24.54</td>
<td>0.10</td>
</tr>
<tr>
<td>JHU-013 ( \alpha )-TOS</td>
<td>54.26</td>
<td>9.09</td>
<td>36.65</td>
<td>58.02</td>
</tr>
<tr>
<td>JHU-019 untreated</td>
<td>63.03</td>
<td>12.63</td>
<td>24.34</td>
<td>0.15</td>
</tr>
<tr>
<td>JHU-019 vehicle</td>
<td>61.07</td>
<td>13.56</td>
<td>25.37</td>
<td>0.07</td>
</tr>
<tr>
<td>JHU-019 ( \alpha )-TOS</td>
<td>57.5</td>
<td>13.17</td>
<td>29.33</td>
<td>54.87</td>
</tr>
<tr>
<td>JHU-022 untreated</td>
<td>61.27</td>
<td>11.08</td>
<td>27.65</td>
<td>0.16</td>
</tr>
<tr>
<td>JHU-022 vehicle</td>
<td>60.68</td>
<td>15.56</td>
<td>23.76</td>
<td>0.08</td>
</tr>
<tr>
<td>JHU-022 ( \alpha )-TOS</td>
<td>54.68</td>
<td>8.06</td>
<td>37.26</td>
<td>49.39</td>
</tr>
<tr>
<td>JHU-022 untreated</td>
<td>60.02</td>
<td>10.98</td>
<td>27</td>
<td>0.09</td>
</tr>
<tr>
<td>JHU-022 vehicle</td>
<td>58.34</td>
<td>10.61</td>
<td>31.05</td>
<td>0.06</td>
</tr>
<tr>
<td>JHU-022 ( \alpha )-TOS</td>
<td>52.79</td>
<td>11.11</td>
<td>36.10</td>
<td>48.23</td>
</tr>
</tbody>
</table>
slightly in all mice during the 55-day experiment, with no significant differences among α-TOS–treated group and the two control groups (Fig. 2D). In addition, there were no differences in food and water intake among the groups. The blood samples were also collected on the last day of the experiment and the plasma α-TOS levels were determined by high-performance liquid chromatography. Plasma α-TOS was only detected in the α-TOS–treated group and was not detectable in the control and DMSO-treated groups. The plasma α-TOS level in the α-TOS–treated group was in an average range of 5 μg α-TOS/100 μL plasma (P < 0.001).

Effect of α-TOS on ceramide metabolism in JHU-022 cancer cells. The effects of α-TOS on the ceramide expression and sphingomyelinase activity in JHU-022 cells were studied. The amount of ceramide product gradually increased with time in JHU-022 cell cultures treated with 20 μmol/L α-TOS-SUV for up to 6 hours. The levels of ceramide were maintained nearly at this elevated level in cultures treated with α-TOS-SUV for 6 and 9 hours (Fig. 3A). There was an ∼2.5-fold increase in the ceramide level of cells treated with α-TOS-SUV for 6 hours compared with control cells treated with vehicle (SUV) alone (Fig. 3A). The level of sphingomyelinase activity was significantly increased in the first hour of treatment of cells with α-TOS-SUV. There was a 3.3-fold and a 3.7-fold increase of the sphingomyelinase activity in cells after treatment with α-TOS-SUV for 30 minutes and 1 hour, respectively. After that, the enzyme activity gradually diminished to ∼2.5-fold higher level relative to DMSO-treated cells and remained at this level throughout the treatment period (Fig. 3B). The ceramide level in tumor specimens was examined by immunohistologic methods. Tumors from mice treated with α-TOS exhibited a significant degree of immune staining of ceramide within the cellular membranous regions, whereas the untreated group or DMSO-vehicle control group displayed little staining with ceramide antibody (Fig. 3C).

Effects of α-TOS on caspase-3, caspase-9, and PARP proteins. Caspase-3 activities were nearly 8-fold higher in JHU-022 cells treated with α-TOS for 12 hours and JHU-029 treated similarly for 6 hours, compared with control cells treated with the vehicle (DMSO) alone (Fig. 4A). Caspase-3 activities were lower in cultures treated with α-TOS for longer periods. Coadministration of the selective caspase-3 inhibitor z-DEVD-fmk or nonselective caspase inhibitor z-VAD-fmk significantly reduced α-TOS induced caspase-3 activities with a range of 70% to 84% inhibition in both JHU-022 (Fig. 4A) and JHU-029 (Fig. 4B) cell lines. In addition, both caspase inhibitors showed an ability to partially protect against α-TOS–induced cell death in JHU-22 cell cultures (Fig. 4C). The expression

![Fig. 3. Increased levels of ceramide in JHU-022 cells treated with α-TOS. A, ceramide levels in JHU-022 cells treated with α-TOS-SUV (20 μmol/L) for 0 to 9 h. Top right inset, TLC bands of ceramide from cells treated with α-TOS-SUV (20 μmol/L) for 3, 6, and 9 h. B, acid-sphingomyelinase (SMase) activity relative to untreated control in JHU-022 cells treated with α-TOS-SUV (20 μmol/L) or vehicle (SUV) for various exposure times from 0 to 6 h. Points, mean ceramide levels and relative sphingomyelinase activity of two independent experiments with triplicate dishes; bars, SD. C, immunohistochemical staining of ceramide in JHU-022 tumor specimens from mice, which were untreated or treated with DMSO (vehicle) or α-TOS as described in Materials and Methods. At the end of the experiment, JHU-022 tumors were removed, fixed in formalin, and stained with H&E and ceramide monoclonal antibody. Arrows, ceramide specific staining. Magnification, ×400.](cancertherapypreclinical.org)
levels of caspase-3 protein and related molecules such as caspase-9 and PARP proteins were also analyzed by Western blot (Fig. 4A and C). A qualitative assessment of apoptotic markers in JHU-022 cell cultures treated with α-TOS for 3 to 24 hours showed an increase in caspase-3, but not caspase-9, protein (Fig. 4A). Treatment of JHU-022 cells with α-TOS for 3 hours resulted in apoptosis as indicated by PARP cleavage to produce 85-kDa fragments (4 D).

**Effects of α-TOS on the expression of ceramide-related apoptotic markers.** The role of ceramide-mediated apoptosis was examined in α-TOS–treated JHU-022 and JHU-029 cells. First, Annexin V-FITC antibody was applied to track the cells through apoptotic markers in JHU-022 cell cultures treated with α-TOS–SUV (20 μmol/L) for 30 min and 1, 2, 3, 4, and 5 h. PARP expression and degradation were analyzed by Western blotting with antibodies directed against PARP protein. The intact form of PARP is 116 kDa and the cleaved form is 85 kDa. The amount of protein was normalized to β-actin content.

The expression levels of Bcl-2 and Bcl-XL were dramatically decreased in JHU-022 cells treated with α-TOS for 6 and 12 hours as compared with control groups (Fig. 5B). The level of p53 increased with prolonged exposure of JHU-022 cells to α-TOS. In contrast, the level of nuclear factor κB was lower in JHU-022 cells treated with α-TOS for 12 hours. There was a very slight change in murine double minute-2 protein level of cells treated with α-TOS. There was no change in the level of IκB-α in the α-TOS–treated and control groups (Fig. 5C).

**Discussion**

The key findings from the present study are that α-TOS inhibits the growth and viability of human HNSCC, and that this effect may be a consequence of ceramide-mediated apoptosis. In vitro studies showed dose- and time-dependent cytotoxicity of α-TOS. In vivo studies showed that i.p. injection of low-dose α-TOS on alternating days significantly decreased primary tumor burden. Regarding the safety of α-TOS, our limited studies in mice suggest that it is relatively nontoxic for up to 55 experimental days. The α-TOS treatment did not produce any overt signs of toxicity such as weight loss or observable changes in behavior. Clearly, more studies are needed for establishing the safety of α-TOS for potential use in cancer chemoprevention or treatment.
Our data showed that α-TOS induced sphingomyelinase activity and enhanced ceramide levels in cultured cells and tumor specimens. The importance of ceramide lies in its ability to modulate the biochemical and cellular processes that lead to apoptosis (30, 31). The accumulation of ceramide in cells could be influenced through one or more routes of ceramide generation and/or clearance. Sphingomyelinase catalyzes the hydrolysis of sphingomyelin to ceramide and phosphocholine. Mechanism for generation of ceramide may also involve the activation of acid sphingomyelinase, which participates in regulating apoptotic responses. Neutral sphingomyelinase is also implicated in the regulation of apoptosis in response to a range of stimuli, including tumor necrosis factor α in breast cancer cells, ethanol in HepG2 hepatoma cells, and the Alzheimer proapoptotic amyloid-β peptide in neuronal cells (24, 32, 33). Our analysis of sphingomyelinase activity included neutral and acidic contributions toward ceramide formation.

The results also show that ceramide-mediated apoptotic signaling seems to affect activation of caspase and Bcl-2 family proteins. One effect of α-TOS is the activation of caspase-3 activity and expression. Caspase-3 is a member of the caspase family of aspartate-specific cysteine proteases that play a central role in the execution of the apoptotic program, and is primarily responsible for the cleavage of PARP during cell death. The sequence at which caspase-3 cleaves PARP is very well conserved in the PARP protein, indicating the potential importance of PARP cleavage in apoptosis (34–36). In α-TOS–treated JHU-022 cells, caspase-3–like activity, measured with a specific apopain enzymatic assay in vitro, peaks at 12 hours after exposure to α-TOS, concomitant with the onset

Fig. 5. Effect of α-TOS on the induction of apoptosis in JHU-022 cells. A, JHU-022 and JHU-029 cells were cultured either with α-TOS-SUV (20 μmol/L) or vehicle for 0, 3, 6, 12, and 24 h, followed by measurement of Annexin V protein in the cell membrane by flow cytometry using FITC-conjugated Annexin V antibody. The cells were also stained with propidium iodide. Vehicle-treated cells served as controls. The pixels in the bottom right quadrant of each panel display V-FITC-positive and propidium iodide (PI)–negative cells, indicating that the cells were in an early stage of apoptosis. Pixels in the top right quadrant represent cells stained with both propidium iodide and Annexin V, indicating that the cells were in late apoptosis or no longer viable. B, JHU-022 cells were treated with α-TOS-SUV (20 μmol/L) for 0, 3, 6, 12, and 24 h, and the expression levels of apoptotic markers such as Bcl-2, Bcl-XL, p53, murine double minute-2 (MDM-2), nuclear factor (NF)-κB, and IκB-α were evaluated by Western blot with appropriate antibody probes. The amount of protein was normalized to β-actin.
of apoptosis. The expression level of caspase-3 was significantly increased after exposure to α-TOS, whereas the amount of caspase-9 remained constant. In addition, pretreatment with caspase-3 inhibitor or nonselective caspase inhibitor blocks α-TOS–mediated cell death, suggesting that caspase-3 activity is involved in ceramide-mediated apoptosis in HNSCC cells. We have also examined the time course of PARP protein cleavage during apoptosis in these cells by Western blot analysis. PARP cleavage occurred at an early stage of induced apoptosis in JHU-022 cells. Further work will be required to discern whether α-TOS–induced ceramide-mediated apoptosis is truly caspase-9 independent or involves other regulation such as phosphorylation of caspase-9 protein. We should note that the immunoblotting experiments reflect a limited assessment of relative differences of apoptotic markers within and between samples, and more detailed quantitation of the apoptotic profiles under α-TOS treatment is warranted. Furthermore, the variability observed when using specific and nonspecific caspase inhibitors may be a consequence of using a single concentration of inhibitor, culture conditions, or the degree of sensitivity of the cell lines. Caspase-9 has been linked to ceramide-induced neuronal death (37). The regulation of alternative processing of pre-mRNA of both caspase-9 and Bcl-XL was reported in response to the proapoptotic action of ceramide (38). A collective interpretation of our data suggests that the rapid response of sphingomyelinase activity and ceramide accumulation in cells exposed to α-TOS (Fig. 3A and B) precede the maximal caspase expression profiles and earliest detection of PARP cleavage (Fig. 4A and C). This is consistent with the hypothesis that caspase-mediated apoptosis is a consequence of ceramide accumulation within HNSCC treated with α-TOS.

This study also provides evidence that inhibition of Bcl-XL/Bcl-2 function represents a major pathway whereby α-TOS induces ceramide-mediated apoptosis in head and neck cancer cells. The data indicate that α-TOS can markedly decrease the levels of Bcl-XL and Bcl-2 in the first 12 hours of treatment. The tendency of Bcl-2 and Bcl-XL to recover during later time points could reflect metabolic degradation of α-TOS on prolonged incubation with cells. Bcl-2 and Bcl-XL are anti-apoptotic paralogs that inhibit apoptosis elicited by a wide variety of stimuli, and play critical roles in cancer development and resistance to treatment. Many clinical studies have indicated that expression of these antiapoptotic proteins in tumors is associated with poor prognosis (39). In addition, the nuclear factor-κB expression level seemed to decrease after the exposure of cells to α-TOS. Nuclear factor-κB is an important molecular target that has a role in carcinogenesis and cancer progression (40).

In summary, our data show that the vitamin E analogue α-TOS decreases primary tumor burden in a human tumor xenograft model. The findings presented here strongly suggest that antitumor activity of α-TOS is initiated through a ceramide-mediated apoptotic pathway without any overt toxic effects in vivo.

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References


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