Vitamin E Succinate Is a Potent Novel Antineoplastic Agent with High Selectivity and Cooperativity with Tumor Necrosis Factor-related Apoptosis-inducing Ligand (Apo2 Ligand) in Vivo


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ABSTRACT

α-Tocopheryl succinate (α-TOS), a redox-inactive analogue of vitamin E, is a strong inducer of apoptosis, whereas α-tocopherol (α-TOH) lacks apoptogenic activity (J. Neuzil et al., FASEB J., 15: 403–415, 2001). Here we investigated the possible antineoplastic activities of α-TOH and α-TOS and further explored the potential of α-TOS as an antitumor agent. Using nude mice with colon cancer xenografts, we found that α-TOS exerted modest antitumor activity and acted by inhibiting tumor cell proliferation. In contrast, α-TOS showed a more profound antitumor effect, at both the level of inhibition of proliferation and induction of tumor cell apoptosis. α-TOS was nontoxic to normal cells and tissues, triggered apoptosis in p53−/− and p21Waf1/Cip1−/−
cancer cells, and exerted a cooperative proapoptotic activity with tumor necrosis factor-related apoptosis-inducing ligand (Apo2 ligand) due to differences in proapoptotic signaling. Finally, α-TOS cooperated with tumor necrosis factor-related apoptosis-inducing ligand in suppression of tumor growth in vivo. Vitamin E succinate is thus a potent and highly specific anticancer agent and/or adjuvant of considerable therapeutic potential.

INTRODUCTION

Suppression of cancer growth is confounded by the clonal behavior of cancer cells with mutations in tumor suppressor or apoptosis-related genes. Thus, the development of new anticancer drugs and their combined use in cancer treatment is warranted to improve concepts for combating neoplasia. An important goal of anticancer strategies is specific induction of apoptosis in cancer cells while shielding normal cells (1).

The mechanisms of apoptosis include receptor- and mitochondria-dependent signaling pathways (2). Execution of apoptotic programs often involves the expression of genes controlled by factors, such as the tumor suppressor p53 or the cell cycle checkpoint protein p21Waf1/Cip1 (3, 4). Many apoptogens, e.g., pharmacological agents and radiation, induce p53/cell cycle-dependent death programs, thereby eliminating malignant cells (5, 6). Trolox, a water-soluble form of vitamin E, induced expression of p21Waf1/Cip1 in colon cancer cells by a p53-independent mechanism, thus triggering apoptosis and sensitizing the cells to cancerostatics (7). On the other hand, p53-dependent induction of p21Waf1/Cip1 inhibited apoptosis in DNA repair-deficient fibroblasts (8). The semisynthetic vitamin E analogue α-TOS3 inhibited proliferation of cancer cells by inhibition of cyclin A binding to the transcription factor E2F (9), suggesting an effect on cell cycle progression.

We and others have shown that α-TOS acts as a proapoptotic agent for cancer cells (10–14). The effect of α-TOS appears to be largely restricted to malignant cells (15, 16), a feature that may be associated with their high proliferation rates (9), and fast transition through the cell cycle appears to be positively correlated with cell susceptibility to apoptosis (17). However, the mechanisms of action of α-TOS on cancer cells are poorly understood. Therefore, we investigated the pathways involved in the proapoptotic/antineoplastic activity of vitamin E analogues. Here we show that α-TOS has a higher cytotoxic

3 The abbreviations used are: α-TOS, α-tocopheryl succinate; DN, dominant-negative; α-TOH, α-tocopherol; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; rhTRAIL, recombinant human TRAIL; SFU, 5-fluorouracil; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; PCNA, proliferating cell nuclear antigen; BrdUrd, bromodeoxyuridine; NF-κB, nuclear factor κB.
effect than α-TOH by both reducing proliferation and inducing apoptosis of cancer cells and that it cooperates with TRAIL (Apo2 ligand) in suppression of tumor growth.

MATERIALS AND METHODS

Cell Culture. HCT116 colon cancer cells and their p21Waf1/Cip1(−/−) and p53−/− mutants (7) were grown in DMEM with 10% FCS and antibiotics. Neonatal rat cardiomyocytes were prepared as described previously (18). The isolation and culture of human colonocytes have been described elsewhere (19).

Apoptosis Induction and Assessment. Apoptosis was induced with α-TOS, α-TOH, Adriamycin (Sigma Chemical Co.), sphingosine (Calbiochem), 5FU, Trolox (Aldrich), or rhTRAIL prepared as follows. The extracellular part of human TRAIL (amino acids 95–281) obtained by PCR from the HPB T-cell line cDNA library was subcloned into pBSK, sequenced, and further subcloned into the His-tagged reading frame of pET15b. The protein was expressed in Escherichia coli and purified using the TALON (Clontech) and SP-Sepharose columns. Apoptosis was assessed by staining with FITC-conjugated TUNEL (In Situ Cell Detection Kit; Roche) or annexin V (PharMingen) according to the manufacturer’s protocols and quantified by flow cytometry (Becton Dickinson). FITC-conjugated annexin V was used for flow cytometry measurement, and phycoerythrin-labeled annexin V was used for fluorescence microscopy of cells transfected with pEGFP-F. Cardiomyocytes were fixed and stained with BODIPY-phallacidin (Molecular Probes) combined with nuclear counterstaining (Adriamycin) and evaluated by fluorescence microscopy. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed as described elsewhere (20).

Vitamin E Analysis. Levels of α-TOH and α-TOS in plasma and tissue (tumor, kidney, and liver) from mice, α-TOS- and α-TOH-treated mice were assessed using high-performance liquid chromatography (21, 22).

Colony-forming and Invasion Assays. HCT116 cells were seeded in semisolid agar and exposed to increasing concentrations of α-TOS, and the number of colonies was counted after 7 days (7). Invasive capacity of the cells was assessed as described previously (23).

Transfections. HCT116 cells were transfected (12), with an efficacy of 30–50%. The vectors used carried the following genes: (a) DN caspase-9 (24); (b) cFLIP (25); (c) CrmA (26); and (d) green fluorescent protein with a p21Waf1/Cip1 farnesylation motif (pEGFP-F; Clontech). For visualization or assessment of apoptosis, cells were transfected with a 5-fold higher amount of the functional gene compared to pEGFP-F. After induction of apoptosis, green cells with apoptotic morphology were scored (27) or analyzed by the annexin V method.

Assessment of Cardiomyocyte Beating Rate. Cells were plated at near confluence, and after 3 days, cells were treated as indicated. At given intervals, coverslips with cells were placed on the stage of a light microscope and kept at 37°C. After a 5-min adaptation, the frequency of contractions was scored in three randomly selected fields, and the average value was calculated. Three independent experiments were carried out for each condition.

Animal Studies. HCT116 cells were used for xenografts as described previously (7). Once tumors were established, animals received an i.p. dose of 50 μl of 200 mm α-TOS or α-TOH in DMSO or the vehicle every third day. For assessment of combined effects of α-TOS and TRAIL, mice were injected every second day with 50 μl of 100 mm α-TOS and 20 μg of rhTRAIL, separately or together. Tumor volumes were estimated as described previously (7). At the end of the treatment, mice were sacrificed, and sections of organs and tumors were prepared after fixation and paraffin embedding. Mouse organ sections were stained with H&E and inspected by microscopy. Intestinal sections were also stained for cell proliferation using anti-PCNA IgG (Santa Cruz Biotechnology; Ref. 28). Tumor sections were assessed for apoptosis using the TUNEL kit and for proliferation using the BrdUrd kit (Zymed) and PCNA IgG (Santa Cruz Biotechnology). Sections were evaluated in randomly selected fields close to the edges of tumors, and three sections from different animals were used for calculating mean values.

RESULTS AND DISCUSSION

α-TOS Is a Potent Antitumor Agent. Vitamin E analogues can inhibit proliferation (29) and trigger apoptosis of malignant cells (11–15). To explore effects of α-TOH and α-TOS on tumor growth, mice with HCT116 cell-derived xenografts were treated with equimolar doses of the agents. Treatment with α-TOS and α-TOH resulted in inhibition of tumor volume growth by 80% and 35%, respectively (Fig. 1 A). Whereas both α-TOS and α-TOH decreased the percentage of BrdUrd- and PCNA-positive cells by 30–50%, tumors from α-TOS-treated mice showed a >2-fold increase in TUNEL-positive cells as compared with those from control or α-TOH-treated mice (Fig. 1, B–D). The dual mode of antiproliferative and proapoptotic activity exerted by α-TOS may explain why it was more efficient than α-TOH and is consistent with the effects of the two vitamin E analogues in vitro (12, 29). The efficacy of α-TOS against colon cancer is further supported by an inhibitory effect on the colony-forming activity of HCT116 cells in agarose (Fig. 1E) and inhibition of their invasive capacity through Matrigel-coated filters (Fig. 1F).

Selective Toxicity of α-TOS for Malignant Cells. Histological analysis did not reveal any signs of toxicity of α-TOS in the heart, liver, kidney, jejenum, or colon. This refers to general organization of these tissues (data not shown) as well as the proliferative activity of intestinal crypts determined by staining of jejunum (Fig. 2A) and colon sections with anti-PCNA IgG (data not shown). These data suggest that the in vivo cytotoxic effect of α-TOS is selective for tumor cells, without adverse effects on organs or proliferating cells in the intestinal crypts, which are frequently attacked by established antitumor drugs. Consistent with this, we found that cultured colonocytes (19), in contrast to the HCT116 cells, were resistant to α-TOS-induced apoptosis (data not shown). To further illustrate this, we compared the effect of α-TOS on cardiomyocyte viability with that of the antitumor agent Adriamycin and the lipid second messenger sphingosine. Whereas Adriamycin and sphingosine were toxic to the cells, inducing myosin destabilization and a decrease in beating rate, a tumoricidal concentration of α-TOS proved...
nontoxic (Fig. 2, B and C). Similar tumor cell selectivity for α-TOS was observed in studies demonstrating α-TOS-mediated toxicity toward several murine leukemia cell lines but not toward normal bone marrow cells (10). In fact, α-TOS has been shown to protect normal cells from the effects of toxic challenges, including Adriamycin (10, 16, 30), and even to increase the number of erythroid colony-forming unit-derived colonies (31) at concentrations comparable with those used in our studies (11, 12).

α-TOS Turnover Is Rapid in Vivo. Previous studies have shown that the antitumor activity of α-TOS is related to the action of the intact molecule (10). To develop a dosing regimen for its retention in vivo, we examined the pharmacokinetics of α-TOS in mice injected with 50 μl of 200 mM α-TOS (as used in the tumor inhibition studies) by analyzing plasma levels of α-TOS and its hydrolysis product, α-TOH. We observed that α-TOS was rapidly absorbed; its level reached 47.2 ± 6.7 μM on day 1 and then declined, presumably due to liver clearance and/or esterase activity. Concomitantly, the plasma α-TOH level increased from 4.8 ± 2.1 to 8.3 ± 2.9 μM on day 7. These data suggest that α-TOS would have to be administered every 2 or 3 days to maintain high plasma α-TOS levels and that it is converted, at least in part, into the redox-active α-TOH.

To test the hypothesis that the high sensitivity of tumor cells to α-TOS in vivo may be related to its high accumulation in these cells, we analyzed tissue levels of α-TOH and α-TOS in mice after their treatment with the two vitamin E analogues. Surprisingly, administration of α-TOH and α-TOS resulted in liver and kidney levels of both vitamin E analogues that were 10-fold higher than those found in the tumors (Table 1). Thus, the accumulation of vitamin E in a tumor placed s.c. appears to be less efficient than that in well-vascularized organs. This indicates that the selective tumoricidal activity of α-TOS is not related to differences in tissue accumulation but may rather be due to differences in apoptotic pathways between colon cancer cells and normal tissue or to other effects of α-TOS, such as inhibition of tumor angiogenesis. Indeed, it has been reported that α-TOS inhibited expression of vascular endothelial growth factor, an angiogenic cytokine, in breast cancer cells (32), and accordingly, we have observed lower vascularity in tumors from α-TOS-treated mice.3 Although the long-term administration of α-TOS in this study resulted in high plasma levels of α-TOS, additional studies are needed to determine the importance of angiogenesis in α-TOS treatment of colon cancer. It is also noteworthy that the tissue accumulation of α-TOS, after its long-term administration, was 2 times higher that that of α-TOH (after α-TOH administration; Table 1). The enhanced ability of α-TOS to accumulate in tissue is in agreement with previous studies on α-TOS disposition (16) and supports the premise that the intact α-TOS molecule is the cytotoxic agent (10).

α-TOS Synergizes with TRAIL in Vitro. A common approach for more efficient treatment of neoplasia is the combination of agents with synergistic/additive activity to enable the use of lower doses and thereby minimize their toxic side effects. Alternatively, biochemical agents can sensitize cells to killing by immunological apoptogens, as shown for α-TOS, rendering Fas-resistant cells sensitive to Fas-dependent killing (35). In contrast to the Fas ligand, which is toxic to normal cells,
TRAIL, which is produced by cells of the immune system such as the natural killer cells (36), appears to be largely selective for malignant cells (37, 38), likely due to differential expression of components of the TRAIL signaling pathway (39). Cooperative antitumor effects of TRAIL have been shown in conjunction with chemotherapy (40, 41) or radiation (42). Moreover, chemotherapeutic agents may sensitize resistant cells to TRAIL killing (41), and resistance to TRAIL has been found for certain types of cancer (43).

Because the mechanisms of α-TOS- and TRAIL-induced apoptosis appear to differ substantially (12, 44, 45), we hypothesized that these agents may synergize. Indeed, exposure to subapoptotic concentrations of rhTRAIL and α-TOS (Fig. 4A) resulted in efficient apoptosis of HCT116 cells, suggesting synergistic effects and/or sensitization of the cells toward TRAIL by α-TOS (Fig. 4B). Such synergism could be due to differences in proapoptotic signaling of the two agents because α-TOS can trigger apoptosis via mitochondrial destabilization (12, 46), whereas such a mechanism appears secondary to proximal caspase activation in the TRAIL pathway (44–46).

To dissect differences in distal and proximal signaling of α-TOS- and TRAIL-induced apoptosis, HCT116 cells were cotransfected with DN caspase-9 because caspase-9 mediates activation of effector caspases after mitochondrial destabilization (47) or with the proximal caspase inhibitor cFLIP (25) and pEGFP-F to visualize their effects on susceptibility to apoptosis (27). As evaluated by annexin V binding, DN caspase-9-transfected cells were more resistant to α-TOS but not to TRAIL (Fig. 4C). This was confirmed by pretreatment of the cells with pharmacological inhibitors of caspase-9, which strongly suppressed α-TOS apoptosis but exerted only an insignificant effect in the case of TRAIL (data not shown). Conversely, HCT116 cells cotransfected with DN caspase-9 were more resistant to α-TOS but not to TRAIL (Fig. 4C). This was confirmed by pretreatment of the cells with pharmacological inhibitors of caspase-9, which strongly suppressed α-TOS apoptosis but exerted only an insignificant effect in the case of TRAIL (data not shown). Conversely, HCT116 cells cotransfected with DN caspase-9 were more resistant to α-TOS but not to TRAIL (Fig. 4C). This was confirmed by pretreatment of the cells with pharmacological inhibitors of caspase-9, which strongly suppressed α-TOS apoptosis but exerted only an insignificant effect in the case of TRAIL (data not shown).

**Table 1** Levels of α-TOH and α-TOS in mouse organs and plasma after 10-day supplementation

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* Mice were injected i.p. with 50 μl of DMSO or 100 mM α-TOH or α-TOS every second day for 10 days. Fifteen h after the last injection, mice were sacrificed, and plasma and organs were assessed for vitamin E levels.

* Values shown are mean ± SD (n = 5).

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**Fig. 2** α-TOS is not toxic to normal tissues and cells. Mice were injected every second day for 10 days with 50 μl of DMSO or 200 mM α-TOS, and their jejunum was sectioned, stained with anti-PCNA IgG, and counterstained with hematoxylin (A). Cardiomyocytes were treated with DMSO, α-TOS, sphingosine, or Adriamycin and assessed for their beating rate (B) or morphological integrity (BODIPY-phallacidin; C).

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HCT116 cells were treated for 12 h with DMSO (control), 10 or 50 μg/ml rhTRAIL, and evaluated for apoptosis. Cells were protected from TRAIL (25) but not from α-TOS, exposed for 12 h to 50 μg/ml rhTRAIL, or 5 ng/ml rhTRAIL plus 10 μM α-TOS, and evaluated for apoptosis. Note that the combined effect of the subapoptotic doses of α-TOS, 5 or 40 ng/ml rhTRAIL, or 5 ng/ml rhTRAIL plus 10 μM α-TOS and evaluated for apoptosis. Whereas this appears to be a plausible explanation for the synergism observed, it should be noted that there are other options. For example, α-TOS is an inhibitor of NF-κB (48, 49) and protein kinase C (12), and both NF-κB and protein kinase C are activated in TRAIL-treated cells (50–53). Interestingly, a correlation between the level of NF-κB activation and resistance to TRAIL has been shown in breast cancer cell lines (54). We are currently investigating whether this could be another mechanism involved in the synergism of α-TOS and TRAIL in killing colon cancer cells as well as other malignant cell types.

α-TOS and TRAIL Show Combined Effects against Tumor Growth. We asked whether α-TOS and TRAIL cooperate in vivo. Inhibition of colon cancer xenografts has been shown by two daily 100-μg doses of TRAIL (37). We observed similar effects of α-TOS (50 μl, 200 mM) injected every third day (compare Fig. 1A). To detect a potential combined effect between α-TOS and TRAIL, mice with HCT116 xenografts were treated every second day with 50 μl of 100 mM α-TOS, 20 μg of rhTRAIL, or α-TOS and TRAIL together. At the indicated time points, tumor volume was measured. Data shown are mean ± SE (n = 8); asterisks denote significant differences between tumor volumes of mice cotreated with TRAIL and α-TOS and control or single-agent-treated mice (P < 0.01).

rhTRAIL was confirmed by visual evaluation of the cells after their cotransfection with pEGFP-F (data not shown). These data strongly support differences in proapoptotic signaling of α-TOS and TRAIL and suggest an explanation for the synergism between the two unrelated agents, whereby they may maximize the apoptotic potential of the cell. Whereas this appears to be a plausible explanation for the synergism observed, it should be noted that there are other options. For example, α-TOS is an inhibitor of NF-κB (48, 49) and protein kinase C (12), and both NF-κB and protein kinase C are activated in TRAIL-treated cells (50–53). Interestingly, a correlation between the level of NF-κB activation and resistance to TRAIL has been shown in breast cancer cell lines (54). We are currently investigating whether this could be another mechanism involved in the synergism of α-TOS and TRAIL in killing colon cancer cells as well as other malignant cell types.
gestive of toxicity of rhTRAIL to human hepatocytes (55, 56), lowering the effective dose of TRAIL by coadministration with drugs like α-TOS may minimize its potential deleterious side effects.

Conclusions. In this study, we show that (a) α-TOS inhibited growth of colon carcinoma by almost 80%, and α-TOH inhibited growth of colon carcinoma by ~35%; (b) α-TOS-treated mice showed increased apoptosis and decreased proliferation, whereas α-TOH inhibited proliferation only; (c) α-TOS efficiently killed cancer cells deficient in p53 and p21WAF1/CIP1; (d) α-TOS cooperated with TRAIL in apoptosis induction and inhibition of tumor growth; (e) α-TOS accumulated in tissues and was partially hydrolyzed to α-TOH; and (f) α-TOS was nontoxic to normal murine organs and to normal cells.

Our data strongly suggest that α-TOS is a nontoxic antineoplastic agent that cooperates with TRAIL in vivo and suggest an explanation why α-TOH is often found to be inefficient in cancer treatment (57). We propose that this semisynthetic vitamin E analog is a promising anticancer agent or adjuvant, which warrants its testing in other models of cancer with a realistic prospect of its use in human therapy.

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